Glucose supplementation impacts lifespan and immunity in *Drosophila melanogaster*

by

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Abstract

Interactions between diet, lifespan, metabolism, and immunity are complex but can have profound impact on human health. The *Drosophila* model is useful to study these interactions as many pathways and processes are highly conserved between flies and humans. In this thesis, I used the defined holidic diet to ask precisely how manipulation of a single nutrient affected aspects of health. I found that simple modifications to the defined holidic diet have a significant impact on *Drosophila* health, immunity, and lifespan. I found that glucose-supplemented holidic food extended median lifespan in male flies by 31%, but it also increased diversity in the intestinal microbiota composition. With antibiotic treatment, I found that glucose supplementation to the holidic diet extends lifespan independent of the intestinal microbiota. As glucose-supplemented food extended lifespan independent of the microbiota, I next sought the host-intrinsic mechanism of diet-dependent lifespan extension.

Glucose-supplementation did not appear to extend lifespan through differences in caloric intake or altered insulin activity. Through RNA-Seq analysis, I found that glucose-supplemented food increased the expression of cell junction proteins. I used immunofluorescence to show that glucose supplementation increased localization of the septate junction protein, Coracle, to the junction by about 1.31-fold compared to the unmodified holidic food. Through a smurf assay, I found that flies raised on glucose-supplemented food had improved barrier function with age, and their lifespan advantage could be removed by chemically induced barrier disruption. Combined, I found that glucose-supplementation may extend lifespan through improved intestinal barrier integrity.

Finally, I studied interactions between immunity and metabolism. I found that glucose-supplemented food improved survival to infection against *V. cholerae*. I also found that *imd* is required for metabolic homeostasis. Flies that lack *imd* have higher weight, glucose, and triglycerides. As well, *imd* flies have increased levels and slower clearance of glucose in a glucose tolerance test. Through RNA-Seq analysis, I found that *imd* may be involved in regulation of lipid metabolism. Combined, my major findings were that glucose supplementation may extend lifespan through regulation of the intestinal barrier and that the IMD pathway has a role in the metabolic regulation of lipids.

Preface

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All data presented in chapter 3 was produced by Anthony Galenza with the exception of Figures 3-1 and 3-2 which were produced from data generated by Jaclyn Hutchinson. Dr. Bart Hazes assisted with analysis of data in Figure 3-5. Parts of chapter 3 were published as Galenza A, Hutchinson J, Campbell SD, Hazes B, and Foley E. (2016) Glucose modulates *Drosophila* longevity and immunity independent of the microbiota. *Biol. Open.* 6:165-173.

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List of Recurring Abbreviations

Ab	Antibiotic
Akh	Adipokinetic hormone
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BDSC	Bloomington *Drosophila* Stock Center
CAFE	Capillary feeding
CFU	Colony forming units
Cora	Coracle
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
Egfr	Epidermal growth factor receptor
esg	Escargot
flyPAD	Fly proboscis and activity detector
GFP	Green fluorescent protein
GO	Gene ontology
GS	GeneSwitch
GSF	Glucose (50 g/L) supplemented food
HF	Holidic food
Htl	Heartless
IIS	Insulin/insulin-like growth factor signaling
Ilp	Insulin-like peptide
ILP2-FH	Flag and HA epitope-tagged Ilp2
IMD	Immune deficiency
InR	Insulin receptor
IPC	Insulin-producing cell
LB	Luria-Bertani
MAPK	Mitogen-activated protein kinase
MRS	De Man, Rogosa, and Sharpe

myo	myoglianin
N	Notch
PC	Principal component
PGRP	Peptidoglycan receptor protein
pSJ	Pleared septate junction
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
RTK	Receptor tyrosine kinase
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
SJ	Septate junction
sSJ	Smooth septate junction
TCJ	Tricellular junction
TNF	Tumor necrosis factor
TOR	Target of rapamycin
ts	Temperature sensitive
w	White

Chapter 1
Introduction

Portions of this chapter have been published as:

Galenza A and Foley E. (2019) Immunometabolism: Insights from the *Drosophila* model. *Dev. Comp. Immunol.* 94:22-34.

1. Introduction

1.1. Nutrition and aging

Increasing life expectancy and declining fertility rates drive population aging throughout the world, intensifying the burden of age-related challenges to global economic and health systems (WHO, 2015). Minimizing the detrimental aspects of aging is essential to address these concerns. As the impact of nutrition on health is well-established, optimizing nutritional regimes to promote healthy aging has received considerable attention as a preventative approach (Kalache et al., 2019). Nutritional deficiencies increase risk of a range of age-related chronic diseases, but we have limited knowledge of what dietary interventions are effective at improving human aging (Shlisky et al., 2017). Studying the relationship between nutrition and aging in humans is challenging due to genetic variance, diverse nutritional sources, imperfect markers of healthy aging, and the ethics of long-term restriction of intake. For these reasons, model organisms, including *Drosophila melanogaster*, have been critical in determining interactions between nutrition and aging (Fontana and Partridge, 2015; Lee et al., 2015; Piper and Partridge, 2017).

1.1.1. *Drosophila* as a model for dietary studies

Drosophila melanogaster, known as the fruitfly or vinegar fly, has been used to uncover many fundamental aspects of metabolism and physiology as key metabolic pathways are highly conserved between flies and mammals (Rajan and Perrimon, 2013). Further details on these conserved metabolic pathways are provided later in the introduction. The main metabolic organs in flies perform functionally analogous roles to those of mammals (Musselman and Kühnlein, 2018). For example, insulin-producing cells (IPCs) perform an analogous function to mammalian β-islet cells, the *Drosophila* intestinal tract processes nutrients and faces challenges similar to the human gastrointestinal tract, while the fly fat body, which has an analogous function to both the human liver and white adipose tissue, integrates and regulates both metabolic and immune pathways **(Figure 1-1)**.

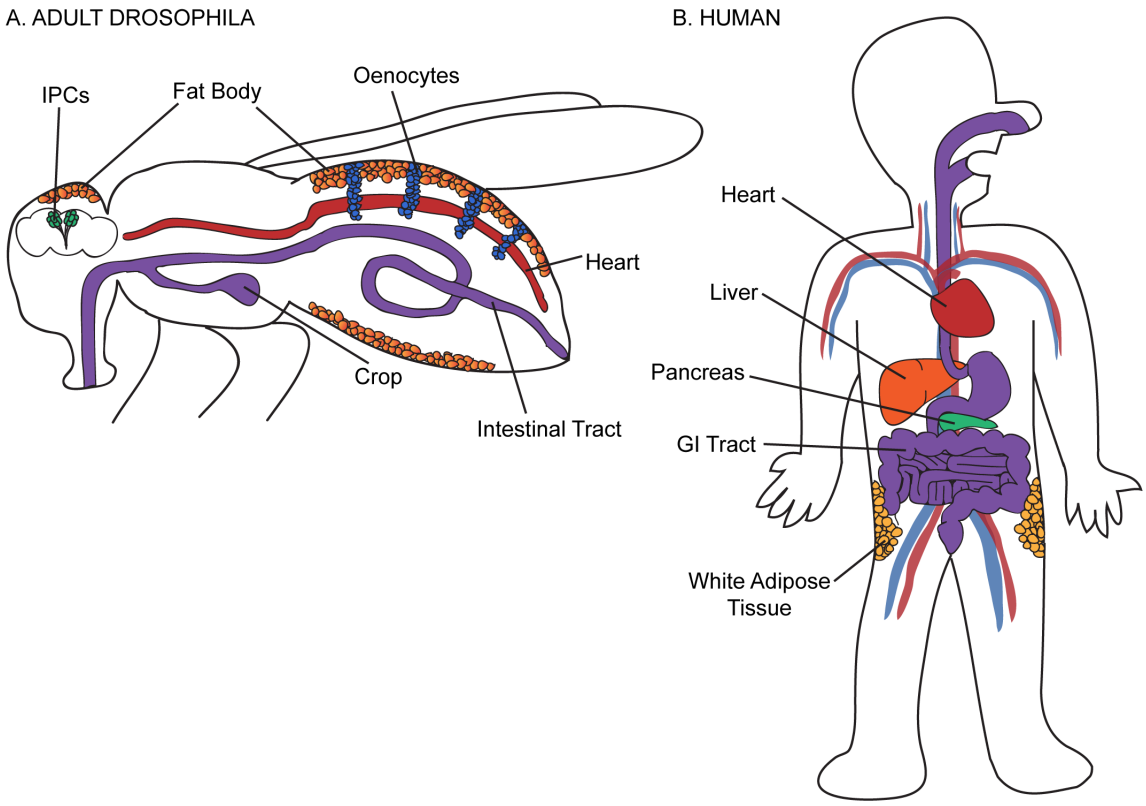


Figure 1-1 Comparison of metabolic organs between flies and humans

Metabolic tissue comparison between (A) adult *Drosophila* and (B) humans. Identical colours indicate tissues with similar or analogous functions. Abbreviations used: IPCs, insulin-producing cells; GI Tract, gastrointestinal tract.

One of the leading advantages of the *Drosophila* model is its genetic tractability (Rodriguez et al., 2013). Over a century of research with *Drosophila* has provided a massive repertoire of genetic tools that allow for simple and precise manipulation. This includes large collections of readily available fly stocks, including transgenics and mutants, to suit diverse experimental needs (Dietzl et al., 2007; Rodriguez et al., 2013). *Drosophila* is also a useful model for its relatively short lifespan and the availability of large numbers of organisms for an experiment.

One of the biggest problems facing dietary studies using the *Drosophila* model is variance between recipes and ingredients used by different labs (Piper and Partridge, 2007). The most common recipe is the cornmeal food in use at the Bloomington Drosophila Stock Center (BDSC) at Indiana University; however, even the BDSC provides recipes for nine different variations of food. This makes comparison of results difficult from one lab to the next. A recent publication designed an online calculator to allow for comparison between diets used between different groups (Lesperance and Broderick, 2020). In an attempt to standardize the food used in *Drosophila* research, a recent paper described a holidic diet for flies, in which the composition and concentrations of all ingredients is completely defined (Piper et al., 2014). Though it is labor-intensive to prepare and uses purified ingredients that are less representative than what a fly might naturally encounter, the holidic diet allows for precise manipulation of individual dietary components.

1.1.2. Dietary interventions

Nutritional optimization to promote healthy aging has received considerable attention (Kalache et al., 2019). Nutritional deficiencies increase risk of a range of age-related chronic diseases, but we have limited knowledge of dietary interventions that extend life and healthspan (Shlisky et al., 2017). The most thoroughly studied dietary intervention, caloric restriction, appears to extend lifespan in several vertebrate and invertebrate models, though the implications for humans are unclear (Most et al., 2017). Further studies generated interest in protein restriction or intermittent fasting to extend lifespan (Fontana and Partridge, 2015). Recent focus has shifted to nutritional geometry, or the effect of mixtures of dietary components rather than isolated nutrients (Simpson and Raubenheimer, 2009; Solon-Biet et al., 2015b). These studies revealed that low protein to carbohydrate

ratios extend longevity of mice and flies, with maximal benefits for 1:16 protein:carbohydrate ratio in flies (Lee et al., 2008; Solon-Biet et al., 2014). When considered as a whole, these advances point to a remarkably nuanced relationship between the uptake of dietary nutrients and animal wellbeing.

1.1.2.1. Caloric restriction

Caloric restriction is the reduction of caloric intake, while maintaining sufficient levels of required nutrients to avoid malnutrition. An early study on dietary impact on longevity in rats indicated that caloric restriction could extend lifespan (McCay et al., 1935). Since this initial study, research in models from *C. elegans* to primates have provided support for this hypothesis that organisms fed a calorically restricted diet exhibit longer lifespans (Dilova et al., 2007; Fontana et al., 2010). Several studies have even shown that caloric restriction has health benefits for humans (Fontana et al., 2010; Fontana and Klein, 2007). Recent long-term experiments yielded mixed observations on the benefits of caloric restriction for primates (Colman et al., 2014; Mattison et al., 2012). However, the two studies in question differed considerably in their experimental protocols, making comparisons difficult. Research in *Drosophila melanogaster* found that the longevity extension from caloric restriction is attributed to protein restriction, a form of dietary restriction, rather than the reduction in calories (Mair et al., 2005; Piper and Partridge, 2007). However, conflicting reports in mice found that this may not be the case for mammals (Speakman et al., 2016). Practical differences in methodology of feeding in these two models may account for the different results (Piper and Partridge, 2007). Specifically, flies are fed *ad libitum* whereas there can be direct control over the amount of food rodents are fed. Further studies in mice and rats suggest that the effects of caloric restriction are dependent on context of sex, genotype, and type of diet used (Mitchell et al., 2016; Swindell, 2012). While the effect of caloric restriction on aging is under debate, it appears that caloric restriction can reduce risk of age-associated disease (Fontana et al., 2018, 2010).

1.1.2.2. Protein restriction

Research on the mechanism of how caloric restriction extends lifespan found that the reduction of dietary protein, or protein restriction, appeared to be largely responsible for the observed improvement on longevity (Mair et al., 2005; Piper and Partridge, 2007). Work with yeast, *C. elegans*, and *Drosophila* found that dietary restriction lifespan extension may be mediated in part through reduction in Target of Rapamycin (TOR) activity (Fontana et al., 2010; Kapahi et al., 2010). The nutrient-sensing TOR pathway is highly conserved and is a major regulator for coordination of metabolism and growth (Saxton and Sabatini, 2017). While mutation of components of the TOR pathway can cause developmental arrest in *C. elegans* (Long et al., 2002) and *Drosophila*, the reduction of TOR signaling extends lifespan in both models (Partridge et al., 2011). In *Drosophila* and *C. elegans*, deprivation of amino acids appears to phenocopy the loss of TOR mutations (Kapahi et al., 2010). In *Drosophila*, supplementation with essential amino acids during protein restriction reduces lifespan, while the supplementation of non-essential amino acids has little effect, suggesting that the level of specific amino acids may be more important for aging than overall protein (Grandison et al., 2009). This study and others challenged the traditional perspective that simply reducing protein or calories was sufficient to explain lifespan extension (Piper et al., 2011).

It is also worth noting that many animals, including humans, appear to regulate feeding by having a threshold of protein intake, rather than calories or carbohydrates (Simpson and Raubenheimer, 2005). On a low protein diet, this can lead to overconsumption in what has been termed "protein leverage". In *ad libitum* studies, if food intake is not precisely quantified, experimental animals may be consuming more food on a low protein diet than controls to reach this protein threshold. It is important to consider experimental factors such as food intake in interpretation and comparison of studies. As higher protein diets tend to benefit reproduction in model organisms, standard laboratory diets have generally been optimized to balance the effects on reproduction and lifespan (Le Couteur et al., 2016). Consequently, what is considered a standard lab diet may lack physiological relevance. As studies of caloric or protein restriction are in comparison to these standard control diets, the initial level and type of protein also influences the outcome of protein restriction.

1.1.2.3. Nutritional geometry

To address the multidimensional nature of diet-lifespan studies, several studies investigated how macronutrient balance affects lifespan through a Geometric Framework for nutrition (Simpson and Raubenheimer, 2009). These studies of nutritional geometry investigated the interactions of macronutrients through comprehensive multi-diet experiments with the advantage of not comparing to an artificially defined control diet. Recent studies of nutritional geometry have demonstrated that the ratio of macronutrients in a diet may be the most important characteristic of diet that impacts lifespan (Lee et al., 2008; Simpson and Raubenheimer, 2009; Solon-Biet et al., 2015b, 2014). In both mice and flies, a diet with a low protein to carbohydrate ratio was found to have the greatest benefits for lifespan, with a macronutrient ratio of about 1:10 protein to carbohydrates (Lee et al., 2008; Solon-Biet et al., 2014).

An early study with Canton-S *Drosophila* females raised on 28 diets of varying yeast and sucrose levels was one of the first to employ nutritional geometry to study how diet impacts lifespan (Lee et al., 2008). The authors found that flies raised on a diet with a protein to carbohydrate ratio of 1:16 was optimal for lifespan, though the higher protein diets at ratios of 1:2 and 1:4 were optimal for egg-laying rate and lifetime egg production, respectively. Interestingly, when given a choice, flies would choose a diet that optimized reproduction over lifespan. Similar to *Drosophila*, both male and female crickets raised on 24 diets maximized lifespan on diets with a low protein to carbohydrate ratio (Maklakov et al., 2008), and female Queensland fruit flies maximized lifespans on a diet with a protein to carbohydrate ratio of 1:21 (Fanson et al., 2009).

These early studies approximated protein to carbohydrate ratios from the dietary yeast to sucrose ratios. It was later confirmed through the use of a defined diet that lower protein to carbohydrate ratios maximized lifespan in Queensland fruit flies (Fanson and Taylor, 2012). Further nutritional geometry experiments with *Drosophila* (Jensen et al., 2015), crickets (Harrison et al., 2014), and even ants (Dussutour and Simpson, 2012) showed that a low protein to carbohydrate ratio was beneficial for lifespan. While low protein to carbohydrate ratios appeared to benefit lifespan of insect models, a similar result was found in mice raised on 25 diets with varying protein to carbohydrate to fat ratios (Solon-Biet et al., 2014). As with previous studies on insects, mice raised on diets with low protein and

high carbohydrate lived the longest. Combined, these studies demonstrate the importance of macronutrient ratios for lifespan. While labor-intensive, it will be of interest to determine if similar results are found in other model organisms and with defined diets, such as the holidic diet. Notably, the holidic diet, and other similar defined diets, use individual amino acids as the source of protein so it is more accurate to refer to the macronutrient ratio as "amino acids to carbohydrates". However, I will use the term "protein to carbohydrate" in this thesis to make comparisons to other types of diets clear.

1.1.2.4. Amino acid restriction

As nutritional geometry studies found that a low protein to carbohydrate ratio benefits lifespan, interest was generated on the impact of individual amino acids. The restriction of the amino acid methionine, in particular, has received considerable attention as reducing methionine intake extends lifespan in yeast, *C. elegans*, *Drosophila*, mice, and rats (Mcisaac et al., 2016). Beyond methionine, the restriction of the branched chain amino acids, leucine, isoleucine, and valine has been considered, though supplementation of branched chain amino acids was found to extend lifespan in mice (D'Antona et al., 2010).

To understand the mechanism of how a low protein to carbohydrate ratio extends lifespan, research has largely focused on the restriction of protein. The alternative approach of studying how increased carbohydrates impact lifespan has received less attention.

1.2. Sugar and lifespan

In this thesis, sugar is defined as a simple carbohydrate, primarily referring to monosaccharides and disaccharides. High sugar intake has long been considered a health hazard (Kroemer et al., 2018), particularly in association with obesity, Type 2 diabetes, and cardiometabolic risk (Prinz, 2019). Model systems are commonly used to study effects of sugar on health and longevity. For example, providing *C. elegans* 5-50 mM glucose shortens lifespan (Schlotterer et al., 2009; Schulz et al., 2007). Interestingly, high glucose (2% or 111 mM) treatment in young worms (1-3 days old) reduces lifespan, but beginning glucose treatment after worms are at a post-reproductive age (7 days old) extends lifespan (Lei et al., 2018).

1.2.1. Sugar and *Drosophila* health

The *Drosophila* model has often been used to study the consequences of being raised on a high-sugar diet (Graham and Pick, 2017). Flies raised on a high-sucrose diet (1.0M compared to 0.15M controls) have increased weight alongside increased triglyceride stores, and develop insulin resistance (Musselman et al., 2011). However, less is known about how sugar impacts longevity. In part, this is due to the difficulty in comparing studies between labs as the type of diet used, genotype and sex of flies, and type and concentration of sugar supplementation can have complex effects on lifespan. I have summarized the outcomes of several recent studies on the effect of sugar on fly lifespan **(Table 1-1)**.

Table 1-1 Summary of *Drosophila* sugar-lifespan studies

Summary of selected studies investigating the effect of supplemented sugar on *Drosophila* lifespan. Percent change color heatmap ranging from -30% (dark blue) to +30% (dark red). Abbreviations used: BSDM, Bloomington semi-defined medium; F, female; M, male.

Sugar	Genotype	Type of Food	Sex	Control Sugar	Median (d)	Experimental Sugar	Median (d)	Percent Change (%)	Reference
Sucrose	*yw*	2.5% Yeast + Sugar	F	0.15M (5%)	50.3	0.07M (2.5%)	51.7	2.8	Skorupa *et al.* (2008)
			F			0.29M (10%)	49.9	-0.8	
			F			0.58M (20%)	49.9	-0.8	
			F			1.17M (40%)	36.1	-28.2	
		5% Yeast + Sugar	F	0.15M (5%)	54.3	0.07M (2.5%)	51.8	-4.6	
			F			0.29M (10%)	55.4	2	
			F			0.58M (20%)	55.4	2	
			F			1.17M (40%)	40.9	-24.7	
		10% Yeast + Sugar	F	0.15M (5%)	52.6	0.07M (2.5%)	51	-3	
			F			0.29M (10%)	54.7	4	
			F			0.58M (20%)	54.7	4	
			F			1.17M (40%)	43.8	-16.7	
		20% Yeast + Sugar	F	0.15M (5%)	49.1	0.07M (2.5%)	50.1	2	
			F			0.29M (10%)	53.3	8.6	
			F			0.58M (20%)	55.7	13.4	
			F			1.17M (40%)	44.3	-9.8	
		40% Yeast + Sugar	F	0.15M (5%)	48.4	0.07M (2.5%)	43	-11.2	
			F			0.29M (10%)	42.2	-12.8	
			F			0.58M (20%)	43.4	-10.3	
			F			1.17M (40%)	35.9	-25.8	
Sucrose	w^{1118}	BSDM	M	0.15M (5%)	40	1.0M (34%)	34.7	-13.3	Na *et al.* (2013)
Sucrose	Canton S	BSDM	M	0.15M (5%)	45.9	1.0M (34%)	34.3	-25.3	
Sucrose	$w^{Dahomey}$	10% Yeast + Sugar	F	0.15M (5%)		1.17M (40%)		-43	Al Saud *et al.* (2015)
Sucrose	Oregon-R	Synthetic	F	1.33%	63	5.32%	69	9.5	Reis (2016)
Sucrose	$w^{Dahomey}$	10% Yeast + Sugar	F	0.15M (5%)	79	0.07M (2.5%)	74	-6.3	Chandegra *et al.* (2017)
			F			0.29M (10%)	77	-2.5	
			F			0.58M (20%)	67	-15.2	
			F			1.17M (40%)	49	-38	
			M	0.15M (5%)	64	0.07M (2.5%)	57	-10.9	
			M			0.29M (10%)	67	4.7	
			M			0.58M (20%)	67	4.7	
			M			1.17M (40%)	56	-12.5	
Sucrose	$w^{Dahomey}$	10% Yeast + Sugar	F	0.15M (5%)		1.17M (40%) *		-7	Dobson *et al.* (2017)
Glucose	Oregon-R	Defined (0.135% Met)	F	(15%)	71.7	(5%)	71.9	0.3	Troen *et al.* (2007)
		Defined (0.045% Met)	F			(15%)	70.3	-2	
		Defined (0.405% Met)	F			(15%)	64.9	-9.5	

1.2.2. Sugar and *Drosophila* lifespan

In the *Drosophila* model, most studies on sugar and lifespan have been performed with sucrose supplementation. High-sucrose treatment (1.0 M compared to 0.15 M controls) reduced lifespan of both w^{1118} and *Canton-S* wild-type male flies raised on a semi-defined diet (Na et al., 2013). Similarly, a high-sucrose diet (1.2 M compared to 0.15 M controls) reduced lifespan of female flies from an outbred *Dahomey* population (w^{Dah}) raised on 10%

yeast food (Al Saud et al., 2014). Even transient high-sucrose exposure (1.2 M compared to 0.15 M controls) for the first 3-weeks of adulthood, was sufficient to reduce lifespan of female w^{Dah} flies (Dobson et al., 2017). In contrast, lower sucrose supplementation (0-5 mM compared to 50 mM controls) reduced median lifespan in female *Dahomey* flies raised on a holidic diet, while higher levels of sucrose (75-100 mM) had no effect (Wu et al., 2020). On a synthetic diet, higher sucrose supplementation (5.32% compared to 1.33%) extended median lifespan of *Oregon-R* females (Reis, 2016).

As suggested by research on nutritional geometry, the amount of protein provided can also affect how sugar-supplementation impacts lifespan. A comprehensive study of female *yw* flies raised on 20 diets where both yeast and sucrose were varied between 2.5% and 40% found optimal lifespan when both yeast and sucrose were between 10-20% (Skorupa et al., 2008). Importantly, increasing amounts of sucrose had a different effect on lifespan depending on the amount of yeast in the diet. However, the effect of sucrose supplementation on lifespan has also been found to be sexually-dimorphic (Chandegra et al., 2017). In w^{Dah} flies raised on a 10% yeast diet, females appeared to respond worse than males to increasing amounts of sucrose supplementation. Lower supplemented sucrose (2.5% compared to 5% controls) decreased lifespan of both males and females. In contrast, increasing sucrose supplementation to 10%, 20%, or 40% decreased female lifespan, whereas 10% and 20% sucrose extended male lifespan.

The type of sugar is important, as sucrose may be more detrimental to lifespan than glucose or fructose (Lushchak et al., 2014). When supplementing a 0.25% yeast diet with variable concentrations (ranging from 0.25% to 20%) of sucrose, glucose, fructose, or a 1:1 mixture of glucose and fructose, female flies of an in-house generated wild-type genotype survived significantly worse on the sucrose-supplemented diets compared to the other three diets tested. However, glucose supplementation can have a differential effect on lifespan depending on the amount of dietary methionine provided (Troen et al., 2007). While lower glucose (5% compared to 15%) slightly increased median lifespan, the amount of methionine provided had a greater impact on lifespan in female Oregon-R flies. Higher methionine supplementation (0.405% compared to 0.135%) significantly reduced lifespan, whereas lower methionine supplementation (0.045% Met, -2% median lifespan) only had a slight effect on lifespan, while glucose was kept constant at 15%.

Combined, these studies establish a clear effect of sugar intake on lifespan, where moderate sugar supplementation appears to extend lifespan, but larger amounts are detrimental. However, much remains to be determined about the effect of sugar on *Drosophila* lifespan. Several factors can affect lifespan and further study is required to improve our understanding both of what aspects of sugar influence as well as what mechanisms that sugar acts through to impact lifespan.

1.3. Carbohydrate metabolism in *Drosophila*

The metabolism of carbohydrates is essential for all life and involves complex regulatory mechanisms. *Drosophila* has become a useful model for studying the regulation of carbohydrate metabolism **(Figure 1-2)** (Mattila and Hietakangas, 2017). Much of the regulatory signaling of carbohydrate metabolism is highly conserved between flies and mammals, from intracellular sensing to systemic signaling pathways. As in mammals, glycogen is an important form of carbohydrate storage in flies, stored mainly in the fat body and muscle of adults (Baker and Thummel, 2007). Prominent regulatory pathways of growth and metabolism, such as the insulin and insulin-like peptide (IIS), and Target of Rapamycin (TOR) pathways are highly conserved between flies and vertebrates (Nässel et al., 2015; Teleman, 2010).

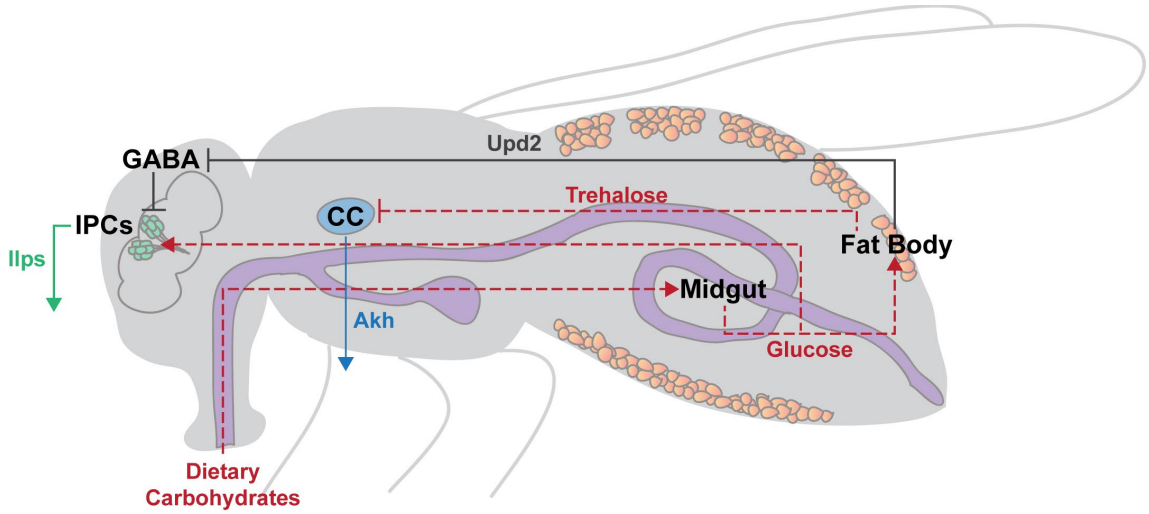


Figure 1-2 Carbohydrate metabolism in *Drosophila*

Mechanisms that regulate insulin-like peptide (Ilp) and adipokinetic hormone (Akh) release in response to dietary carbohydrates. Ingested dietary carbohydrates are digested to glucose in the midgut and absorbed by enterocytes. Glucose is converted to trehalose in the fat body. Circulating glucose can directly stimulate Ilp release from insulin-producing cells. Circulating trehalose inhibits release of Akh from corpora cardiaca (CC) cells. Ilp release is also stimulated by fat body derived Upd2 inhibition of inhibitory GABAergic neurons. Abbreviations used: IPCs, insulin-producing cells; CC, corpora cardiaca; Ilp, insulin-like peptide; Akh, adipokinetic hormone; Upd2, unpaired 2; GABA, γ-aminobutyric acid. Figure based on Fig.4B (Mattila and Hietakangas, 2017).

1.3.1. Intracellular carbohydrate sensing

With periodic feeding, fluctuation of carbohydrate levels requires tightly controlled regulation to maintain homeostasis. Intracellularly, glucose is sensed by the heterodimer of the transcription factors Mondo and Max-like protein X (Mlx) (Havula and Hietakangas, 2012). The highly conserved Mondo-Mlx heterodimer regulates expression of target genes with the Carbohydrate Response Element (Shih et al., 1995). Mondo-Mlx also control expression of the transcription factors Cabut and Sugarbabe (Bartok et al., 2015; Mattila et al., 2015). Cabut and Sugarbabe, amongst other sugar regulatory genes, are important in sugar tolerance of both low and high sugar environments (Mattila et al., 2015).

1.3.2. Trehalose metabolism

Trehalose, a disaccharide of two glucose molecules, is the primary circulating sugar in adult *Drosophila*. As a non-reducing sugar, it is non-toxic and can be tolerated circulating at high concentration (Ugrankar et al., 2015). It has been suggested that high levels of circulating trehalose are critical to provide energy required for flight (Becker et al., 1996). Trehalose is also essential for brain function, as the glial cells that form the blood-brain barrier metabolize trehalose into lactate and alanine as energy for the neurons (Volkenhoff et al., 2015). Trehalose is synthesized in the fat body, secreted into circulation, and taken up into other tissues (Kanamori et al., 2010). Interestingly, unlike glucose, circulating trehalose levels are not strongly affected by dietary sugars, and circulating glucose and trehalose levels appear to be regulated independently (Ugrankar et al., 2015).

1.3.3. Hormonal control of carbohydrate metabolism

The *Drosophila* response to glucose follows a very similar pathway as seen in mammals from initial detection by taste-sensing neurons, through intestinal absorption, and into allocation and storage of circulating sugars (Baker and Thummel, 2007; Fujii et al., 2015; Miguel-Aliaga et al., 2018). The maintenance of glucose homeostasis is tightly regulated and involves several metabolic tissues and signaling pathways (Baker and Thummel, 2007).

1.3.3.1. Insulin-like peptide expression

In *Drosophila*, a group of 14 median neurosecretory cells, referred to as the insulin-producing cells (IPCs), perform an analogous function to pancreatic β-islet cells in mammals (Rulifson et al., 2002). Eight Insulin-like peptides (Ilp) have been identified in *Drosophila*, and four have well characterized roles in metabolism (Garelli et al., 2012; Grönke et al., 2010). In adults, the IPCs in the brain secrete Ilp2, Ilp3, and Ilp5. The fat body secretes Ilp6, which has an important role in lipid and carbohydrate metabolism. There is less known about the roles of the other Ilps, at least in regard to metabolism (Nässel et al., 2015, 2013). It is important to note that while Ilp2, Ilp3 and Ilp5 are all secreted by the IPCs, they do exhibit different expression patterns, particularly during the development of the fly (Broughton et al., 2005). In larvae, Ilp2 is expressed in the IPCs, but also in the imaginal discs, salivary glands, and glial cells. In adults, Ilp2 is restricted to the IPCs. In larvae, Ilp3 is restricted to the IPCs but in the adult it is expressed in both the IPCs and the muscle cells of the midgut (Veenstra et al., 2008). Despite having eight different Ilps, there is only one insulin receptor (InR) for Ilp1-7 as well as the neuronal relaxin receptor Lgr3 for the more recently characterized and distantly related Ilp8 (Garelli et al., 2015). This has led to much debate about what the role of the different Ilps may be since they are all recognized by the same receptor and activate the same pathway. The current understanding is that the differences in spatial and temporal expression of the *ilp* genes contribute to their different physiological roles (Nässel et al., 2015, 2013). Unlike mammals, insulin mutants are viable in flies, and in fact, live longer than their wild-type counterparts (Clancy et al., 2001; Tatar et al., 2001). This is partially explained by flies having the non-reducing disaccharide, trehalose, as their primary circulating sugar, thereby avoiding the glucose toxicity seen in mammals.

There are multiple mechanisms of regulation of Ilp secretion in adult flies. The first is direct sensing of circulating glucose in the hemolymph by IPCs. Similar to mammalian β-islet cells, glucose uptake through the Glut1 transporter on IPCs stimulates mitochondrial ATP-production, shutting down K^{ATP} channels. This leads to cell depolarization, potassium influx, and the exocytosis of vesicles containing Ilps (Kréneisz et al., 2010; Park et al., 2014). Beyond direct sensing of glucose, the IPCs are also regulated through signals from the fat body and midgut. The fat body signals to the IPCs through multiple mechanisms

including the secretion of the cytokine Unpaired 2 (Upd2) (Rajan and Perrimon, 2012), the Activin-like ligand Daw (Ghosh and O'Connor, 2014), or CCHamide-2 (Sano et al., 2015).

Following high-sugar or high-fat feeding, *upd2* expression is upregulated in the fat body. Upd2 acts on the IPCs indirectly by blocking a group of intermediate γ-aminobutyric acid (GABA)ergic neurons (Rajan and Perrimon, 2012). The GABAergic neurons block Ilp2 secretion by IPCs, and Upd2 removes this inhibition, allowing Ilp2 secretion. Upd2 may perform a similar function as the mammalian leptin, as expression of human leptin in the fat body can rescue *upd2* mutants (Rajan and Perrimon, 2012). Dietary sugars activate *daw* expression in the fat body in a Mondo-Mlx-dependent mechanism (Ghosh and O'Connor, 2014). Secreted Daw binds the Activin-like receptor Baboon on IPCs to regulate secretion of Ilp2 and Ilp5. Finally, CCHamide-2 can be released by the fat body and midgut enteroendocrine cells through a sugar-inducible mechanism, to promote insulin secretion from the IPCs (Sano et al., 2015).

1.3.3.2. Intracellular insulin pathway

In insulin target cells, the IIS pathway is activated when a circulating Ilp binds the transmembrane InR **(Figure 1-3)** (Fernandez et al., 1995). The signal cascade downstream of the InR is highly conserved with mammals. After binding to an Ilp, the InR autophosphorylates, then interacts with the insulin receptor substrate-like homolog, Chico (Böhni et al., 1999). As in mammals, the signal progresses through PI3K/PDK1 to the phosphorylation of the kinase Akt (Rintelen et al., 2001). Akt is essential in implementing the growth and metabolic effects of the IIS pathway, and has numerous targets (Buttrick et al., 2008; Potter et al., 2002; Sieber et al., 2016), including the phosphorylation of the forkhead transcription factor, FoxO (Puig et al., 2003). Phosphorylated FoxO is retained in the cytoplasm repressing its activity. During low IIS activity, nuclear FoxO regulates expression of catabolism and growth, as well as stress and immunity (Gershman et al., 2007). The IIS pathway is critical in orchestrating the response to nutrients and maintaining homeostasis and intersects with several other important metabolic pathways, including the target of rapamycin (TOR) pathway.

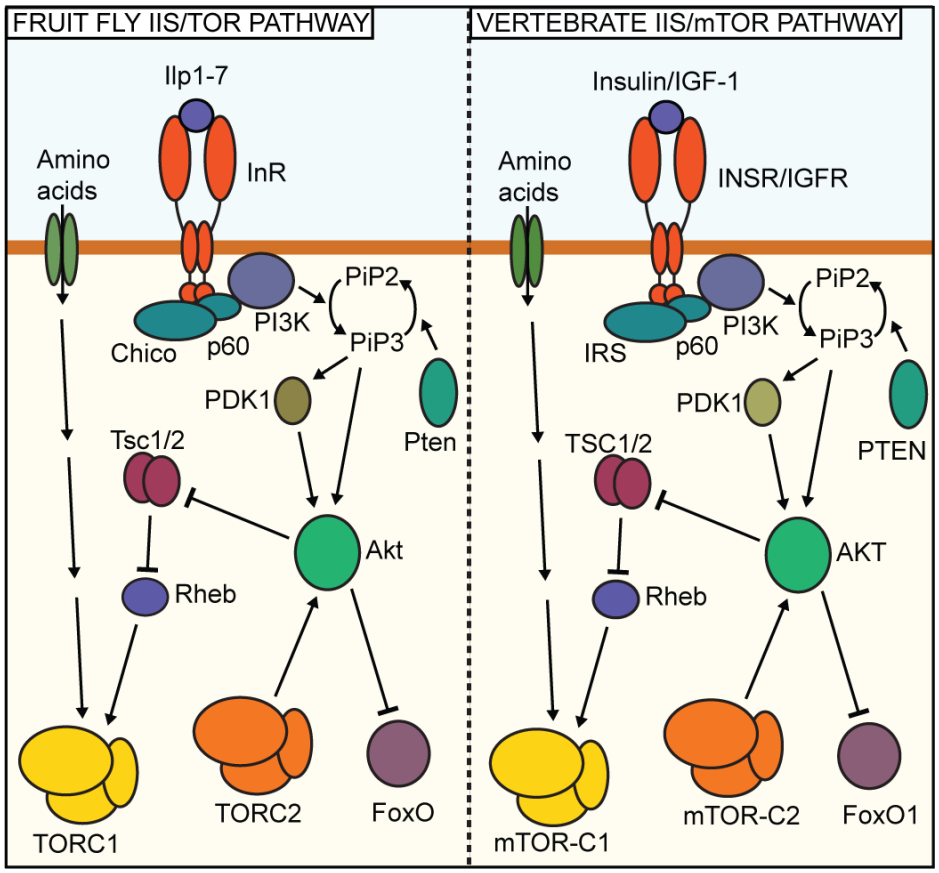


Figure 1-3 Comparison of the *Drosophila* and vertebrate IIS/TOR pathways

Identical colors indicate orthologous proteins between fly and vertebrate pathways. Abbreviations used: IlP, *Drosophila* insulin-like peptide; InR, insulin-like receptor; PI3K, phosphatidylinositol 3 kinase; PiP2, phosphatidylinositol 4,5-bisphosphate; PiP3, phosphatidylinositol (3,4,5)-trisphosphate; Pten, phosphatase and tensin homolog; Pdk1, phosphoinositide-dependent kinase-1; Akt, protein kinase B; FoxO, forkhead box, sub-group O; Tsc1/2, tuberous sclerosis protein 1/2; Rheb, Ras homolog enriched in brain; TORC1, target of rapamycin complex 1; TORC2, target of rapamycin complex 2; INSR, insulin receptor; IGFR, insulin-like growth factor receptor; FoxO1, forkhead box protein O1; mTOR-C1, mechanistic/mammalian target of rapamycin complex 1; mTOR-C2, mechanistic/mammalian target of rapamycin complex 2.

1.3.3.3. TOR signaling pathway

TOR is homologous to the vertebrate mechanistic target of rapamycin (mTOR) and, as in other eukaryotes, functions as part of two separate complexes TORC1 and TORC2 (Wullschleger et al., 2006). The IIS pathway is highly integrated with the TOR signaling pathway. Activation of Akt involves phosphorylation from TORC2 in addition to PDK1 (Hietakangas and Cohen, 2007; Yang et al., 2006). Conversely, one mechanism of negative regulation of the insulin signaling cascade is through TORC1 inhibition of Akt (Kockel et al., 2010). While TORC2 responds to growth cues, TORC1 appears to be regulated in part by amino acid levels (Dann and Thomas, 2006). While both complexes are involved in the regulation of cell growth and size, TORC1, which is sensitive to rapamycin, was traditionally thought to control the temporal aspects, while TORC2, which is insensitive to rapamycin, was thought to control the spatial aspects (Gonzalez and Rallis, 2017). However, their roles are likely more complex as growing evidence suggests that TORC1 controls spatial arrangement of the cytoskeleton, while TORC2 is implicated in control of the timing of cell growth and division (Gonzalez and Rallis, 2017).

1.3.3.4. Adipokinetic hormone signaling

In a simplification of the mammalian regulation of glucose homeostasis, increased circulating glucose leads to secretion of insulin from pancreatic β-islet cells, while low glucose concentration triggers glucagon release from pancreatic α-cells (Aronoff et al., 2004). *Drosophila* regulates glucose homeostasis through a similar insulin/glucagon axis with the glucagon-like protein, adipokinetic hormone (Akh) (Schaffer et al., 1990). In adults, Akh is expressed and secreted from corpora cardiaca (CC) neuroendocrine cells, which are functionally analogous to the pancreatic α-cells in mammals (Isabel et al., 2005). Although the regulation of Akh in flies is not fully understood, it appears to be secreted in response to both low and high concentrations of circulating trehalose (Kim and Neufeld, 2015; Kim and Rulifson, 2004). The intracellular signaling of Akh also appears to be well conserved with the glucagon-responsive pathway in mammals (Song et al., 2017).

1.3.4. Lipid metabolism in *Drosophila*

Excess dietary sugar intake can be stored as glycogen or converted into triglyceride lipids. Triglyceride lipids (also referred to as triacylglycerides) are one of the main forms of energy storage in *Drosophila*, and act as carbon storage for excess dietary sugar (Garrido et al., 2015; Musselman et al., 2013). Each triglyceride molecule consists of a glycerol backbone with three fatty acids attached through an ester bond. Triglycerides are stored in lipid droplets in cells, primarily in the fat body (Arrese and Soulages, 2010; Thiam et al., 2013).

As a central nutrient-sensing pathway, the insulin/Akh axis plays an important role in regulation of triglyceride metabolism. Foxo, the insulin pathway responsive transcription factor, regulates expression of a broad range of metabolic genes including expression of several lipid metabolism genes. During starvation, Foxo is nuclear and controls expression of genes involved in lipolysis or the breakdown of triglycerides (Vihervaara and Puig, 2008; Wang et al., 2011). Conversely, Akh, named for its promotion of lipid mobilization, signaling stimulates the formation of triglycerides (Baumbach et al., 2014). However, regulation of triglyceride metabolism is further complicated by regulation from juvenile hormone and ecdysone signaling (Francis et al., 2010; Kang et al., 2017).

1.4. *Drosophila* intestinal physiology

1.4.1. The *Drosophila* intestine

Drosophila is a popular model for the characterization of intestinal homeostasis. The *Drosophila* intestine is divided into three sections: the foregut, which is subdivided into the esophagus, cardia, and crop; the midgut, the main site of digestion and absorption; and the hindgut, which is subdivided into the pylorus, ileum, and rectum (Miguel-Aliaga et al., 2018). Though the midgut was previously divided into anterior, mid, and posterior, recent work suggests the midgut should be further subdivided into 14 distinct subregions (Buchon et al., 2013b). In this project, I will focus on the midgut, and particularly the posterior midgut, or R4/R5 region of the midgut based on new categorization. The posterior midgut is well characterized with functional roles in nutrient absorption and immunity. The *Drosophila* intestinal epithelium is a single cell layer that largely consists of the absorptive

enterocytes, as well secretory enteroendocrine cells. These differentiated cells are renewed by a pool of intestinal stem cells, transient enteroblasts, and enteroendocrine mother cells (Guo and Ohlstein, 2015; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

As the developmental pathways for the establishment and maintenance of intestinal functions are conserved across large evolutionary distances, discoveries made with flies have the potential to provide valuable insights into fundamental aspects of gut function in vertebrates. For example, the midgut and vertebrate intestine arise from embryonic endoderm, and are surrounded by a sheath of visceral muscle with extensive neuronal innervations (Miguel-Aliaga et al., 2018; Spence et al., 2011; Tepass and Hartenstein, 1994a). In flies and vertebrates, intestinal stem cells occupy a niche created by muscle, differentiated epithelium, and basement membrane, where they divide and differentiate to generate the entire epithelium (Jiang and Edgar, 2012, 2011; Losick et al., 2011; Resende and Jones, 2012; Takashima and Hartenstein, 2012). Unlike mammals, the intestines of many insects, including *Drosophila* and mosquitoes, have a layer of chitin and glycoproteins called the peritrophic matrix that lines the midgut lumen (Hegedus et al., 2009; Lehane, 1997). The peritrophic matrix is produced in a region of the anterior midgut called the cardia, and extends continuously to the posterior (King, 1988). The peritrophic matrix has an analogous function to mammalian mucosal layers as a protective barrier between the intestinal lumen and epithelium (Kuraishi et al., 2011), and *drop-dead* mutants, which lack a peritrophic matrix, have impaired gut function with reduced food movement (Conway et al., 2018). The synthesis and maintenance of the peritrophic matrix depends on the microbiota in mosquitoes (Rodgers et al., 2017), and its permeability is under neuronal control in *Drosophila* (Kenmoku et al., 2016).

1.4.2. *Drosophila* intestinal microbiota

Microbes growing on the food consumed by both wild and lab-reared *Drosophila* serve as a seed for the fly intestinal microbiota as well as, in the case of yeast, an important source of dietary protein (Blum et al., 2013; Broderick and Lemaitre, 2012). While the mammalian gut contains 500-1000 separate bacterial taxa, the fly gut is far simpler to study with 5-30 aerotolerant, cultivable commensal taxa (Broderick and Lemaitre, 2012; Buchon et al., 2013a). Lab-reared flies typically contain a handful of bacterial operational taxonomic

units, frequently dominated by *Lactobacillus* and *Acetobacter* taxa, which have been shown to modulate host metabolism (Broderick and Lemaitre, 2012). Some strains of *Lactobacillus plantarum* improve host growth through secretion of the by-product N-acetyl-glutamine (Martino et al., 2018), while the fly host reciprocally improves the fitness of its symbiont (Storelli et al., 2018). Similarly, larval development depends on the essential vitamin thiamine provided by the symbiont *Acetobacter pomorum* (Sannino et al., 2018). Germ-free flies have higher stores of triglyceride than conventionally-reared counterparts (Wong et al., 2014), and mutualistic interactions between *Acetobacter fabarum* and *Lactobacillus brevis* lower triglyceride storage in the fly (Sommer and Newell, 2018).

It is clear that the microbiota contributes to *Drosophila* health. For example, a number of recent publications established clear mechanistic relationships between the intestinal microbiota of flies and events as diverse as nutritional regulation (Newell and Douglas, 2014; Storelli et al., 2011; Wong et al., 2014), activation of pro-growth pathways (Shin et al., 2011; Storelli et al., 2011), control of immune pathways (Broderick et al., 2014; Erkosar et al., 2014), and defense against microbial challenge. In combination, these studies point to a deep-rooted dependency of *Drosophila* on microbial factors for development and viability.

Interestingly, there is little evidence to suggest that flies house a core microbiome (Wong et al., 2013). Instead, the microbiome appears to vary in size and composition from fly to fly, and from lab to lab. External factors such as food composition, handling, and passage frequency affect the density and composition of the microbiome (Blum et al., 2013; Chaston et al., 2015; Obadia et al., 2017). In lab-raised flies, gut bacteria shuttle between food substrates and the lumen of the gut, and it is not clear if bacteria take up permanent residence within the lumen. As is often the case, the experimental malleability of *Drosophila* makes it an attractive model for the characterization of such animal-microbe interactions (Erkosar et al., 2013; Trinder et al., 2017). Scientists have access to an impressive collection of transgenic fly lines for the manipulation of genetic activity in the intestine. In addition, protocols exist for the culture and genetic modification of common fly symbionts, allowing investigators to identify bacterial factors that modify host physiology. Investigators also have simple procedures to generate and culture axenic or

gnotobiotic flies (Koyle et al., 2016). These advantages allow extensive characterization of interactions between the fly and their commensal microbes.

1.5. *Drosophila* epithelial barriers

1.5.1. Types of *Drosophila* epithelial barriers

Barrier maintenance is critical to maintaining proper physiology. As in vertebrates, *Drosophila* use occluding junctions to tightly connect adjacent cells and restrict movement across epithelial barriers. In vertebrates, occluding junctions are called tight junctions (Zihni et al., 2016). In invertebrates, septate junctions (SJ) perform an analogous role to tight junctions (Izumi and Furuse, 2014). In *Drosophila*, there are two morphologically distinct types of SJ **(Figure 1-4)**. The first, pleated SJ (pSJ), are found in ectodermally-derived epithelial cells, such as glial cells which form the blood-brain barrier (BBB), and localize basally to adherens junctions, which has inverted positioning compared to vertebrates (Hall and Ward, 2016). The other type, the smooth SJ (sSJ), is found in endodermally-derived epithelial cells, such as the enterocytes of the midgut (Tepass and Hartenstein, 1994b). The sSJ localizes apically to adherens junctions, similar to vertebrate tight junctions. Extensive work has uncovered many of the protein components of the pSJ (Izumi and Furuse, 2014; Tepass et al., 2001). Conversely, less is known about the components, function, and structure of the sSJ.

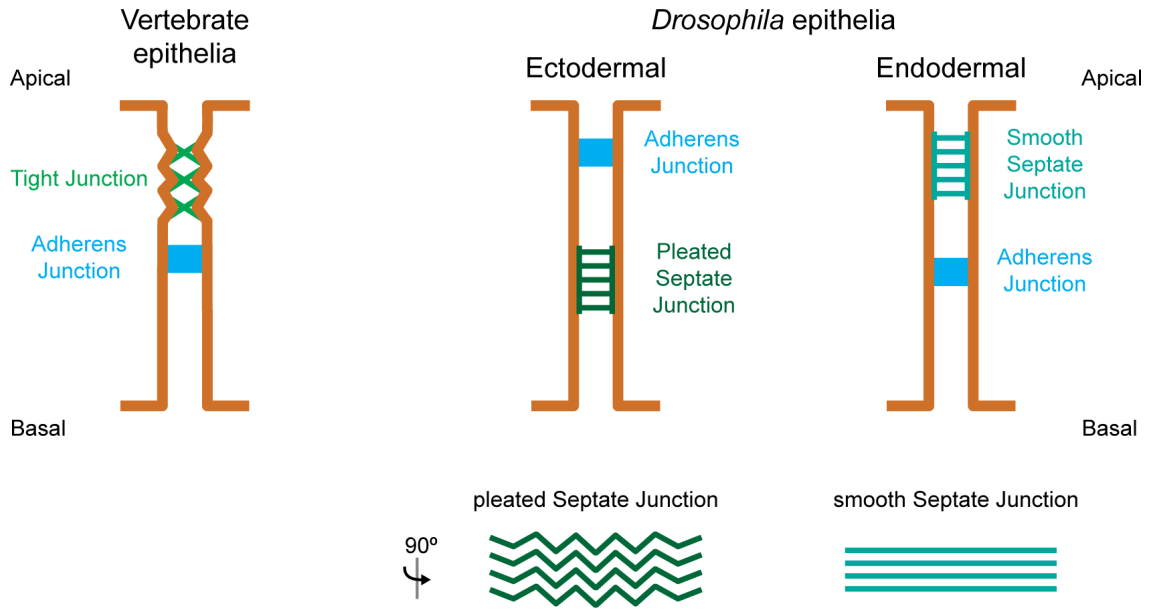


Figure 1-4 Comparison of the vertebrate and *Drosophila* occluding junctions

The vertebrate occluding junction is the tight junction and is located apically to the adherens junction. The *Drosophila* occluding junction is the septate junction, and *Drosophila* have two types of septate junctions: the pleated septate junction in ectodermally derived epithelia and the smooth septate junction in endodermally derived epithelia. Pleated septate junctions are named for their zigzagging appearance and are located basal to the adherens junction. Smooth septate junctions are named for their relatively parallel appearance and are located apical to the adherens junction.

1.5.2. *Drosophila* blood-brain barrier

As in vertebrates, the BBB is essential in *Drosophila* to protect the vulnerable brain tissue, though as flies possess an open circulatory system, the BBB must extend over the entire brain (Hindle and Bainton, 2014). The BBB is formed by two layers of glial cells, where the subperineurial glial cells in the lower layer form a tight barrier through their pSJ (Love and Dauwalder, 2019). Over 20 proteins have been identified to be associated with pSJs, either as transmembrane or cytoplasmic components, or involved in the formation and assembly of pSJs (Izumi and Furuse, 2014). The core structure of the pSJ appears to consist of the membrane proteins Neurexin IV (Nrx-IV), Neuroglian (Nrg), Na pump α subunit (ATPα), Nervana2 (Nrv2), Sinuous (Sinu), and Megatrachea (Mega, also known as Pickel), as well as the intracellular proteins Coracle (Cora) and Varicose (Vari) (Oshima and Fehon, 2011). The loss of any of these proteins is sufficient to disrupt the function of pSJs. However, several other proteins have also been found to be important for the function of pSJ including Gliotactin (Gli), Contactin (Cont), Discs large 1 (Dlg1), Fasciclin 3 (Fas3), and Moody (Hindle and Bainton, 2014). This list is not exhaustive as several other proteins have been found to be required for formation or maintenance of pSJs (Izumi and Furuse, 2014).

1.5.3. *Drosophila* intestinal barrier

To simultaneously allow the absorption of nutrients and protect against pathogenic bacteria, the intestinal epithelium must act as a selectively permeable barrier. sSJs form between adjacent enterocytes and between enterocytes and enteroendocrine cells (Resnik-Docampo et al., 2017). While more is known about the structure of pSJ, recent work has revealed several core components of sSJs including Snakeskin (Ssk), Mesh, and Tetraspanin 2A (Tsp2A) (Furuse and Izumi, 2017). Depletion of the sSJ proteins Ssk, Mesh, and Tsp2A in enterocytes leads to barrier dysfunction and reduces lifespan in flies (Izumi et al., 2019). Conversely, the increased expression of Ssk can extend lifespan (Salazar et al., 2018). Likewise, Big bang (Bbg) is also required for sSJ function, and *bbg* mutants have significantly decreased lifespan (Bonnay et al., 2013). Other proteins, such

as the adhesion molecule Fas3, Cora, Dlg1, and Lgl have also been observed to be localized to sSJs (Izumi et al., 2012).

Tricellular junctions (TCJ), where three cells join together, have a molecularly distinct role compared to the bicellular junctions between two adjacent cells. Two proteins, Gli and Bark beetle (Bark, also known as Anakonda) specifically associate with the TCJ (Byri et al., 2015; Hildebrandt et al., 2015; Schulte et al., 2003). Depletion of the TCJ-specific protein, Gli, leads to barrier dysfunction and increased intestinal stem cell proliferation, similar to what is observed in older flies (Resnik-Docampo et al., 2017).

As flies age, organization of the intestinal epithelium breaks down, and the intestine starts to fail as a barrier to extrinsic factors (Biteau et al., 2008; Choi et al., 2008; Park et al., 2009). Age-related phenotypes, such as the increase of antimicrobial peptide gene expression, are linked to this intestinal barrier dysfunction (Rera et al., 2012). Age-dependent intestinal epithelial breakdown is a consistent characteristic across a diversity of aging organisms, including *C. elegans*, zebrafish (Dambroise et al., 2016), mice (Thevaranjan et al., 2017), and even primates (Mitchell et al., 2017). Evidence suggests that the human intestinal barrier appears to weaken with age as well (Mabbott, 2015). Loss of barrier integrity is hallmarked by changes in microbiome composition, increases in total bacterial numbers resident in the gut, elevated immune activity, and metabolic shifts (Clark et al., 2015; Li et al., 2016; Ren et al., 2007). Many diseases; including inflammatory bowel disease, celiac disease, and ulcerative colitis; are associated with intestinal barrier dysfunction (Choi et al., 2017).

1.6. *Drosophila* innate immunity

The *Drosophila* immune response involves several cellular and humoral processes, many of which share an evolutionary relationship with those in vertebrates (Buchon et al., 2014; Lemaitre and Hoffmann, 2007). The immune response has often been separated into either innate immunity, or the first-line defensive responses, and acquired/adaptive immunity, or the long-term antigen-specific response. Unlike vertebrates, *Drosophila* lack the somatic rearrangement or hypermutation of immune receptors associated with an acquired immune response (Hoffmann et al., 1999). With the initial discovery of the pattern recognition receptor, Toll, *Drosophila* was established as a critical model for expanding

our knowledge of innate immunity (Lemaitre et al., 1996; Medzhitov et al., 1997). Innate immunity includes physical barriers, specialized immune cells, and inducible release of antimicrobial effectors (Hoffmann et al., 1999). In *Drosophila*, bacterial-sensing and subsequent effector release is accomplished largely through two pathways, the Toll pathway and Immune Deficiency (IMD) pathway (Buchon et al., 2014; Myllymäki et al., 2014; Valanne et al., 2011).

The fly antibacterial Toll pathway and IMD pathway share remarkable similarities with the mammalian Toll-like Receptor (TLR) pathway and the Tumor Necrosis Factor Receptor (TNFR) cellular pathway, respectively (Buchon et al., 2014; Myllymäki et al., 2014; Valanne et al., 2011). In flies, the Toll and IMD pathways direct complex physiological responses to the detection of microbe-associate molecular patterns. The Toll pathway mainly operates in the fat body and hemocytes, a macrophage-like immune cell (El Chamy et al., 2008), while the IMD pathway is active in immune tissues throughout the body. With these highly conserved pathways and vast genetic tools, *Drosophila* is an ideal model to explore the mechanisms of innate immunity (Lemaitre and Hoffmann, 2007).

1.6.1. Antimicrobial peptides

The discovery of antimicrobial peptides (AMPs) in *Drosophila* was an important advancement in the field of innate immunity (Boman et al., 1972). AMPs are small cationic peptides released to target and disrupt the negatively-charge membranes of microbes (Lai and Gallo, 2009). Seven families of AMPs have been characterized in *Drosophila* comprised of 21 AMP and AMP-like genes (Hanson and Lemaitre, 2020). Both Toll and IMD signaling pathways lead to the production of AMPs in response to microbial challenge, in a pathogen-specific manner, though a majority appear to be IMD-responsive. Recently, a new group of immune effector peptides was identified and named Bomanins (Clemmons et al., 2015). Bomanins appear to be responsive to Toll activation and may be more important to the Toll immune response than AMPs, as mutation of 10 of 12 Bomanins mimics the infection response of Toll mutants. The fat body is the primary source of AMP production, particularly during systemic infection, though other tissues such as intestinal

epithelium or circulating hemocytes can also produce AMPs (Lemaitre and Hoffmann, 2007).

1.6.2. Immune deficiency (IMD) signaling pathway

Imd was discovered as a recessive mutation that impaired the inducible expression of AMPs (Lemaitre et al., 1995). The IMD pathway, which is active in immune tissues throughout the body, recognizes diaminopimelic acid (DAP)-type peptidoglycan associated with the cell wall of most Gram-negative and some Gram-positive bacteria, such as *Bacillus* spp. **(Figure 1-5)** (Kaneko et al., 2005). The IMD pathway regulates a p105/110 NF-κB ortholog, Relish (Dushay et al., 1996). As the Toll pathway appears to be inactive in the intestine, the IMD pathway is the main bacterial-sensing intestinal response in *Drosophila* (Buchon et al., 2009).

IMD signaling is initiated by the binding of DAP-type peptidoglycan to the membrane-bound PGRP-LC receptor, or the cytoplasmic PGRP-LE (Gottar et al., 2002; Leulier et al., 2003). Following binding to DAP-type peptidoglycan, signal transduction from the PGRP-LC receptor recruits a complex of Imd, a death domain containing protein which is similar to the human RIP-1; the adaptor protein Fadd; and Dredd, the ortholog of mammalian Caspase-8 (Georgel et al., 2001; Leulier et al., 2002). Dredd is subsequently activated through ubiquitination by Iap2 (Meinander et al., 2012). An activated Dredd then cleaves Imd, allowing for Iap2 to bind and ubiquitinate Imd (Paquette et al., 2010). This leads to recruitment of the Tab2/Tak1 complex, which activates IKK through phosphorylation (Kleino et al., 2005; Rutschmann et al., 2000). The IKK complex then phosphorylates Relish (Silverman et al., 2000). Interestingly, in *Drosophila* the C-terminal domain of Relish functions like the I-κB inhibitor of NF-κB in mammals, and is likely cleaved by Dredd (Stöven et al., 2003). Following phosphorylation, the C-terminal domain of Relish remains in the cytoplasm while the N-terminal domain translocates to the nucleus, where it induces expression of immune response genes, such as the AMPs *attacin* and *diptericin* (Stöven et al., 2000). As in the mammalian TNFR pathway, the IMD pathway bifurcates into two branches, signaling through the caspase c-Jun N-terminal Kinase (JNK) or the NF-κB-like transcription factor Relish, respectively (Boutros et al., 2002; Dai et al., 2012).

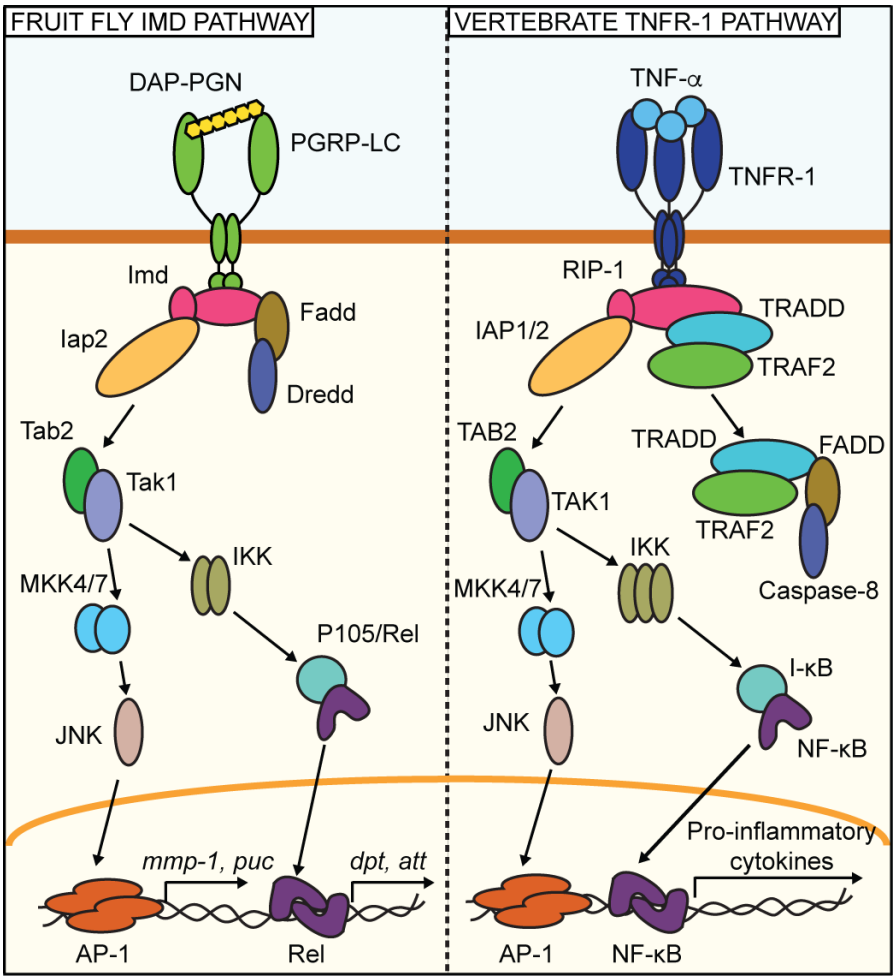


Figure 1-5 Comparison of the *Drosophila* IMD pathway and vertebrate TNFR-1 pathway

Identical colors indicate orthologous proteins between fly and vertebrate pathways. Abbreviations used in the IMD pathway: DAP-PGN, diaminopimelic acid-type peptidoglycan; PGRP-LC, peptidoglycan recognition protein LC; Imd, immune deficiency; Fadd, Fas-associated death domain; Dredd, death related ced-3/Nedd2-like caspase; Iap2, inhibitor of apoptosis 2; Tab2, Tak1-associated binding protein 2; Tak1, TGF-beta activated kinase 1; IKK, I-kappaB kinase; MKK4/7, MAP kinase kinase 4/7; JNK, c-Jun N-terminal kinase; AP-1, adaptor protein complex 1; Rel, Relish. Additional abbreviations in the TNFR-1 pathway: TNF, tumor necrosis factor; TNFR-1, tumour necrosis factor receptor 1; RIP-1, receptor interacting protein 1; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF2, TNF receptor-associated factor 2; I-kB, inhibitor of kappa B; NF-kB, nuclear factor-kB.

1.7. Bacterial Pathogenesis

While few natural pathogens of *Drosophila* have been identified in the wild, *Drosophila* has been an exceptional model for exploring the host-pathogen interactions of numerous human relevant microbes (Panayidou et al., 2014). *Drosophila* can be used as a model for both systemic and oral infection, and several tools and assays are also available to evaluate the infection response (Troha and Buchon, 2019). For many pathogens, similar mechanisms of infection are employed in both mammalian and fly hosts (Alarco et al., 2004; Fauvarque, 2014), including the bacterial pathogen *V. cholerae* (Blow et al., 2005).

1.7.1. *V. cholerae* infection in the *Drosophila* model

Drosophila was established as a model for *Vibrio cholerae* infection, and found to mimic the human disease cholera (Blow et al., 2005). Cholera toxin, a multimer composed of CtxA and CtxB components, increases lethality of infection, but it is not required to induce host mortality in flies. Cholera toxin (CtxA) secreted by *V. cholerae* disrupts intestinal barrier integrity in flies through blocking the trafficking of junction proteins (Guichard et al., 2013). However, CtxA is not solely responsible for lethality in flies as infection with CtxA-deficient *V. cholerae* still causes host death (Blow et al., 2005). Invading *V. cholerae* consume microbiota-derived short chain fatty acids, disrupting host insulin activity and lipid homeostasis (Hang et al., 2014a). Suppression of host intestinal stem cell proliferation also appears to contribute to pathogenesis (Wang et al., 2013). Interactions between *V. cholerae* and host microbiota also appear to impact the host survival response (Fast et al., 2020, 2018b). Combined, *V. cholerae* has emerged as an interesting pathogenic model in flies, particularly for its effect on host metabolism.

1.8. Immunometabolism

The field of immunometabolism, or the study of immunity-metabolism interactions, can be traced back as far as Elie Metchnikoff, often considered a founder of immunology, who first noticed metabolic changes associated with inflammation (Tauber, 2003). Later studies uncovered direct links between immunity and metabolism. For example, adipocytes produced the Tumor Necrosis Factor-alpha (TNF-α) cytokine in the *fa/fa* rat obesity

models, and blocking TNF-α signaling increased glucose uptake (Hotamisligil et al., 1993). These data implicated a canonical immune cytokine, TNF-α, in the control of host responses to a metabolic hormone – insulin. Additionally, the discovery of infiltrating macrophages in obese adipose tissue established a critical relationship between obesity and inflammation (Weisberg et al., 2003; Xu et al., 2003). Chronic metabolic disorders, such as Type 2 diabetes, have emerged as one of the greatest health burdens globally and these metabolic diseases are often associated with an inflammatory component (Esser et al., 2014; Gregor and Hotamisligil, 2011; Hotamisligil and Erbay, 2008).

In vertebrates, immunometabolic homeostasis requires efficient communication between organs that primarily store energy, such as the liver and white adipose tissue, and those that require it, such as muscles and the brain. This is further complicated by rapid changes in the demands and numbers of lymphocyte populations during an infection. In *Drosophila*, humoral and immune pathways are integrated in the fat body, an organ that controls energy stores and circulating antimicrobial peptide levels simultaneously (Arrese and Soulages, 2010; Dionne, 2014; Zhang and Xi, 2014). While the fat body is unique to insects, it offers a simple tractable system to study the molecular integration of immune and metabolic pathways upon detection of microbial patterns. For example, one of the first characterized immunometabolic switches, the Mef2 transcription factor, was described in the fly fat body (Clark et al., 2013). When nutrients are abundant, Mef2 is phosphorylated and increases transcription of anabolic enzymes to support animal growth. However, upon infection, Mef2 is dephosphorylated and shifts the fat body to production of antimicrobial peptides, a response that temporarily limits growth in favour of the elimination of microbial invaders.

Due to the integration of immune and metabolic functions, the fat body offers a unique model to investigate interactions in immunometabolism. The fat body is mainly composed of lipid-rich trophocytes, and associated with a class of cells separate from the fat body called oenocytes that are involved in lipid metabolism **(Figure 1-1)** (Arrese and Soulages, 2010; Makki et al., 2014). In *Drosophila*, the fat body has distinct anatomical differences between larval and adult stages. In the larval stage, the fat body exists as a compact organ that extends through most of the animal, whereas the adult fat body is a loose tissue of trophocytes on the interior of the abdomen wall, as well as a smaller population in the head

(Arrese and Soulages, 2010). One of the roles of fat body cells, as in vertebrate adipocytes, is the regulation, storage, and release of nutrients (Boulan et al., 2015; Zhang and Xi, 2014). This is accomplished in part through the IIS pathway. The fat body signals to IPCs in the brain to promote release of Ilps into circulation (Nässel et al., 2015). Ilps coordinate growth and energy homeostasis in peripheral tissues such as flight muscle (Boulan et al., 2015). In addition to fat storage and metabolism, fat body cells perform several functions analogous to vertebrate hepatocytes including the *de novo* synthesis of fat, secretion of serum proteins, and storage of glycogen. Alongside its metabolic functions, activation of the IMD or Toll pathways alerts the fat body to the detection of molecular patterns of bacterial, fungal, or viral invasion (Myllymäki et al., 2014; Valanne et al., 2011). In return, the fat body releases high titres of antimicrobial peptides into circulation. Thus, the fat body is a central node in the establishment of immunometabolic homeostasis in *Drosophila*.

1.8.1. Diet and immunity

An important area of immunometabolism research is studying how diet influences immunity. Proper nutrition is essential to maintain an adequate immune response, especially with the high energy demand of the vertebrate acquired immune responses, and much research is devoted to improving immunity through dietary intervention (Childs et al., 2019; Gombart et al., 2020). For example, increased intake of polyunsaturated fatty acids, such as in fish oil, can reduce production of inflammatory cytokines (Calder, 2006). Undernutrition is well known to inhibit immune function (Calder and Jackson, 2000). Conversely, obesity is associated with an increase in susceptibility to infections (Genoni et al., 2014). In mice, diet-induced obesity disrupts the immune system and inhibits the response to infection with *P. gingivalis* (Amar et al., 2007). Invertebrate immune function is also impacted by diet. For example, feeding different grape extracts alters antimicrobial activity and hemocyte count in European grape berry moth larvae (Vogelweith et al., 2015), while protein restriction decreases immune gene expression in bumblebees (Brunner et al., 2014).

Diet can also have a profound impact on immunity and survival to infection in *Drosophila*. Starvation in flies leads to higher expression of AMPs (Becker et al., 2010). As with lifespan studies, sex, genotype, and type of diet can all influence the effect of diet

on the immune response (Howick and Lazzaro, 2014). The complexity is further increased by the specific pathogen used. Dietary restriction, through 0.5x dilution of food, reduced survival of male flies against *L. monocytogenes*, improved survival against *S. typhimurium*, and had no effect on survival against *E. faecalis* (Ayres and Schneider, 2009). Similarly, protein restriction altered tolerance to *E. coli* but did not affect the response to *L. lactis* (Kutzer and Armitage, 2016). Lower protein to carbohydrate diets increased expression of AMP genes and improved survival against infection with *M. luteus* (Ponton et al., 2020). Combined, these studies illustrate that diet has a clear effect on immunity and the outcome of infection survival, however as with diet-lifespan studies, the impact of diet on immunity encompasses several factors and requires more research to determine both what dietary components influence immunity as well as what the underlying mechanisms of how diet impacts immunity.

1.9. Summary

In this thesis, I investigated interactions between diet, lifespan, and immunity **(Figure 1-6)**. With the defined holidic diet, I was able to ask precisely how manipulation of a single nutrient affected aspects of health. I found that simple modifications to the defined holidic diet can have significant impact on *Drosophila* health, immunity, and lifespan. In particular, I noticed that glucose-supplemented holidic food had a dramatic impact on lifespan, particularly in male flies, but it also affected the composition of the microbiota. Following antibiotic treatment, I found that glucose supplementation to the holidic diet extends lifespan independent of the intestinal microbiota. As glucose-supplemented food extended lifespan independent of the microbiota, I next sought the host-intrinsic mechanism of diet-dependent lifespan extension.

I found that glucose-supplementation did not appear to extend lifespan through caloric intake or altered insulin activity. Rather, through RNA-Seq analysis, I found that glucose-supplemented food increased expression of cell junction proteins. I used immunofluorescence to show that flies raised on glucose-supplemented food had greater junction localization of the septate junction protein, Coracle, than those raised on unmodified holidic food. I found that flies raised on glucose-supplemented food had improved barrier function with age, and their lifespan advantage could be removed by

chemically induced barrier disruption. Combined, I found that glucose-supplementation may extend lifespan through improved intestinal barrier integrity.

Finally, I studied interactions between immunity and metabolism. Specifically, I found that glucose-supplemented food improved survival to infection. I also found that *imd* is required for metabolic homeostasis. Flies that lack *imd* have increased weight, glucose, and triglycerides. *imd* flies respond poorly in a glucose tolerance test. Through RNA-Seq analysis, I found that Imd may be involved in regulation of lipid metabolism.

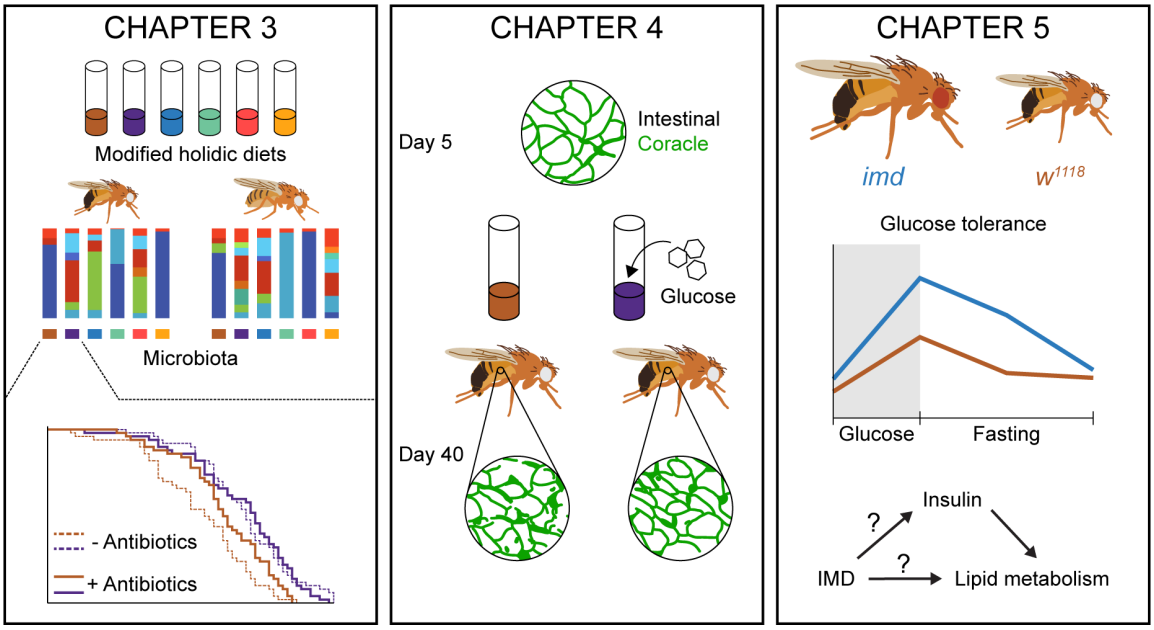


Figure 1-6 Summary of major findings in data chapters 3-5

In chapter 3, w^{1118} male and female flies were raised on either an unmodified (brown) or modified holidic diet supplemented with glucose (purple), starch (blue), casein (green), palmitic acid (red), or ethanol (yellow). Modified holidic diets resulted in large changes to intestinal bacterial composition. Glucose-supplemented food extended lifespan independent of antibiotic elimination of the intestinal microbiota. In chapter 4, glucose-supplemented lifespan extension in w^{1118} males was found to be associated with enhanced intestinal barrier integrity and improved localization of Coracle to septate junctions. In chapter 5, *imd* males were found to weigh more than w^{1118} males. The lack of *imd* impaired the response to glucose in an oral glucose tolerance test. The IMD pathway appears to be involved in regulation of insulin signaling and lipid metabolism.

1.10. Aims of research project and hypotheses tested

In my project, I sought to take advantage of the precision of the holidic diet to ask how a series of simple dietary modifications affected health and lifespan in the *Drosophila* model. Collectively, my results emphasize the important interconnected relationships between diet, lifespan, metabolism, and immunity.

1.10.1. Chapter one aims

The primary aim of chapter one was to determine how a series of simple modifications to a defined food affected health, lifespan, and immunity. Initially, I was interested in how diet affected microbiota composition, and how diet-dependent changes to microbiota would affect host health and longevity. I hypothesized that simple dietary supplementations with simple or complex carbohydrates, protein, fat, or ethanol to the holidic diet would have a measurable impact on fly health and microbiota.

1.10.2. Chapter two aims

In chapter two, I sought the mechanism of how glucose-supplemented food extended lifespan in male *Drosophila*. My findings in chapter 1 revealed that flies fed glucose-supplemented food had a remarkable increase in longevity compared to flies fed an unmodified holidic diet. As my findings suggested that this lifespan extension was independent of an intact intestinal microbiota, I hypothesized that glucose-supplemented food extended lifespan through a host-intrinsic mechanism.

1.10.3. Chapter three aims

The primary aim of chapter 3 was to explore interactions between immunity and metabolism in flies raised on the holidic diet. In my project, I had uncovered several links between diet and immunity including diet-dependent changes in infection survival response and immune gene expression. During this work, I noticed that *imd* mutants had increased weight and energy stores and I decided to explore how the lack of *imd* impacted metabolism. I hypothesized that IMD signaling was required for regulation of metabolic homeostasis.

Chapter 2: Materials and Methods

2.1. List of buffers and solutions

LB broth

1 %	Tryptone
0.5%	Yeast extract
0.5%	NaCl
(100 µg/mL)	(Streptomycin)

LB agar

1 %	Tryptone
0.5%	Yeast extract
0.5%	NaCl
1.5%	Agar
(100 µg/mL)	(Streptomycin)

MRS plate

63 g/L	MRS agar base

GYC plate

50 g/L	glucose
10 g/L	yeast extract
30 g/L	calcium carbonate
25 g/L	agar

PBS (1X)

140 mM	NaCl
10 mM	Na_2HPO_4-$7H_2O$
2.7 mM	KCl
1.4 mM	KH_2PO_4

PBT (PBS + 0.3% Triton-X)

140 mM	NaCl
10 mM	Na_2HPO_4-$7H_2O$
2.7 mM	KCl
1.4 mM	KH_2PO_4
0.3%	Triton-X

TET

10 mM	Tris pH 7.4
1 mM	EDTA
0.1%	Trition X-100

Trehalase buffer

5 mM	Tris pH 6.6
137 mM	NaCl
2.7 mM	KCl

Sodium carbonate/bicarbonate buffer

4.24 g/L	Na_2CO_3
13.44 g/L	$NaHCO_3$

2.2. *Drosophila* handling

2.2.1. *Drosophila* maintenance

Unless otherwise specific, virgin w^{1118} flies were used in all experiments. Other fly stocks used in this project are listed in the table below **(Table 2-1)**. Wild-caught flies were maintained in lab culture on Bloomington stock food for 2-3 months prior to experiment. *imd* mutants were backcrossed with the lab control w^{1118} for 8 generations.

Fly stocks were all maintained on Bloomington cornmeal food. Upon eclosion, freshly emerged adults were transferred to their respective experimental diets. For all experiments, flies were maintained on an unmodified or modified variation of the holidic medium using the original amino acid solution (Oaa) at 100 mM biologically available nitrogen **(Table 2-2 and Table 2-3)** (Piper et al., 2014). Flies were maintained at 30 flies/vial and raised at 25˚C in a humidified incubator with a 12-hour light: 12-hour dark cycle.

For experiments with gene-switch (GS) flies, mifepristone (RU486) was dissolved in 80% ethanol at 4 mg/mL. 100 µL of 4 mg/mL RU486, or 80% ethanol in controls, was added to the top of the food in each vial and left overnight to evaporate the ethanol.

Table 2-1. List of *Drosophila* stocks used

Name	Genotype	Source
w^{1118}	*w[1118]*	BDSC (Stock #5905)
esg-GAL4^{ts}	*w; esg-GAL4, tub-GAL80[ts], UAS-GFP*	Bruce Edgar
UAS-Ras^{V12}	*w; P{w[+mC]=UAS-Ras85D.V12}TL1*	BDSC (Stock #4847)
Wild-caught	unknown	wild
UAS-InR	*w[1118]; P{w[+mC]=UAS-InR}3*	Seung K. Kim
Ilp3-GAL4	*w[1118]; P{w[+mc]=Ilp3-GAL4.C}2/Cyo*	BDSC (Stock #52660)
r4-GAL4	*y[1] w[*]; P{w[+mC]=r4-GAL4}3*	BDSC (Stock #33832)
UAS-Egfr^{DN}	*y[1]w[*]; P{w[+mC]=UAS-Egfr.DN.B}29-77-1; P{w[+mC]=UAS-Egfr.DN.B}29-8-1*	BDSC (Stock #5364)
UAS-htl^{DN}	*y[1] w[*]; P{w[+mC]=UAS-htl.DN.M}33-B40; P{w[+mC]=UAS-htl.DN.M}33-B61*	BDSC (Stock #5366)
UAS-TOR^{DN}	*y[1] w[*]; P{w[+mC]=UAS-Tor.TED}II*	BDSC (Stock #7013)
UAS-N^{RNAi}	*w[*]; P{w[+mC]=UAS-N.dsRNA.P}9G*	BDSC (Stock #7077)
imd	*w[1118]; P{EPgy2}imd*^{EY08573}	Bruno Lemaitre
Ilp2-FH	*y[1] w[1118]; Ilp2[1] P{y[+t7.7] w[+mC]=gd2HF}attP2*	Seung K. Kim
ilp2-3,5	*w[1118]; Df(3L)Ilp2–3, Ilp5[3]*	Seung K. Kim
GS-5966	*P{Switch2}GSG5966*	Heinrich Jasper
UAS-cora^{RNAi}	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03418}attP40*	BDSC (Stock #51845)
ΔAMPs	*w[1118]; Def*^{SK3}*, Dro*^{SK4}*, Dro-AttAB*^{SK2}*, Dpt*^{SK1}*, Drs*^{R1}*, Mtk*^{R1}*; AttD*^{SK1}	Bruno Lemaitre
WT	*w[1118]*	Bruno Lemaitre

Table 2-2 Holidic medium recipe

	Ingredient	Amount per litre (g or ml)
sugar		
	sucrose	17.12
amino acids		
	L-arginine	0.242
	L-alanine	1.059
	L-asparagine	0.514
	L-aspartic acid	0.514
	L-cysteine	0.015
	L-glutamic acid	0.757
	L-glutamine	0.757
	glycine	0.968
	L-histidine	0.303
	L-isoleucine	0.91
	L-leucine	0.605
	L-lysine	0.575
	L-methionine	0.242
	L-phenylalanine	0.393
	L-proline	0.454
	L-serine	0.575
	L-threonine	0.605
	L-tryptophan	0.151
	L-tyrosine	0.21
	L-valine	0.847
lipid components		
	cholesterol	0.3
	choline chloride	0.05
	myo-inositol	0.005
nucleic acids		
	insoine	0.065
	uridine	0.06
salts		
	KH2PO4	3
	NaHCO3	1
	CaCl2	0.25
	CuSO4	0.0025
	FeSO4	0.025
	MgSO4	0.25
	MnCl2	0.001
	ZnSO4	0.025
vitamins		
	thiamine	0.0014
	riboflavin	0.0007
	nicotinic acid	0.0084
	Ca pantothenate	0.0108
	pyridoxine	0.0017
	biotin	0.0001
	folic acid	0.0005
other		
	acetic acid	3
	abs. ethanol	15
	propionic acid	6
	nipagin	15
	agar	20

Table 2-3 Modifications to holidic recipe

	Diet Description	Supplementation (per L)	Calories (kcal/L)	P:C
Chapter 3				
	Unmodified	N/A	216.3	1 : 1.6
	Glucose	glucose (100 g)	616.3	1 : 10.9
	Starch	starch (50 g)	416.3	1 : 6.3
	Casein	casein (70 g)	496.3	1 : 0.2
	Palmitic Acid	palmitic acid (50 g)	666.3	1 : 1.6
	Ethanol	ethanol (10 mL)	286.3	1 : 1.6
Chapter 4				
	Unmodified (HF)	N/A	216.3	1 : 1.6
	Glucose (GSF)	glucose (50 g)	416.3	1 : 6.3
	Glucose	glucose (20 g)	296.3	1 : 3.5
	Glucose	glucose (100 g)	616.3	1 : 10.9
	Glucose	glucose (200 g)	1016.3	1 : 20.3
	Casein	casein (50 g)	416.3	1 : 0.3
	Lard	lard (22.2)	416.3	1 : 6.3
Chapter 5				
	Unmodified (HF)	N/A	216.3	1 : 1.6
	Glucose (GSF)	glucose (50)	416.3	1 : 6.3

2.2.2. Lifespan analysis

Lifespan studies were performed with 30 flies/vial. Flies are raised at 25˚C, unless otherwise specified, in a humidified incubator. Flies were flipped to fresh food every 2-3 days. Deaths were recorded daily.

2.2.3. Starvation analysis

To test starvation, flies were transferred to vials prepared with 1% agar in water. Flies were maintained in a 25˚C humidified incubator. Flies were flipped to fresh vials daily and deaths were recorded every 2-3 hours during the day.

2.2.4. Generation of germ-free flies

To generate germ-free flies, an antibiotic cocktail (100 µg/mL ampicillin, 100 µg/mL neomycin, 100 µg/mL metronidazole, 50 µg/mL vancomycin) was added to the food. Flies were fed this antibiotic-supplemented food for the duration of the experiment. To verify that antibiotic-treatment successfully eliminated the intestinal microbiota, treated flies were homogenized and plated on both MRS (to select for *Lactobacillus*) and GYC (to select for *Acetobacter*) plates.

2.3. *Drosophila* nutrient assays

2.3.1. Nutritional assays

Samples of 5 flies were weighed and then mashed in 125 µL TET buffer (10mM Tris, 1mM EDTA, 0.1% Triton X-100, pH 7.4). Macronutrient measurements were performed in 96-well plates using commercial kits: DC Protein Assay kit (Bio-Rad, 500-0116), Triglyceride Assay kit (Sigma, TG-5-RB), and Glucose (GO) Assay kit (Sigma, GAGO20). Colorimetric readings were obtained using a microplate spectrophotometer (Molecular Devices, SpectraMax M5).

2.3.2. Hemolymph extraction

To measure circulating sugars, hemolymph was extracted from samples of 15-20 flies. Flies were carefully pierced in the thorax with a 26G needle and placed in a filter collection tube. Tubes were centrifuged at 9000g for 5 min at 4˚C yielding at least 1 µL of hemolymph. Then, 1 µL of hemolymph was diluted 1:100 in trehalase buffer (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl), and placed in a 70˚C water bath for 5 min. Each sample was split into two 50 µL aliquots, one to measure glucose and one to measure trehalose. Trehalase was prepared by diluting 3 µL porcine trehalase (1 UN) in 1 mL trehalase buffer. Then, 50 µL of this trehalase solution was added to one aliquot of each sample while 50 µL trehalase buffer was added to the other, then samples were incubated at 37˚C for 24 hours. Next, 30 µL of samples and standards were added to a 96-well plate and glucose was measured using the Glucose Oxidase (GO) Assay kits (Sigma, GAGO20). Total circulating sugars was measured from the trehalase-treated sample, free glucose was measured from the untreated sample, and trehalose was calculated as the difference between treated and untreated samples.

2.4. Insulin signaling analysis

2.4.1. Enzyme-linked immunosorbent assay (ELISA)

To measure total and circulating ILP2 levels, the *ilp2*^{1} *gd2HF* fly stock and protocols were acquired from Dr. Seung K. Kim (Park et al., 2014). To prepare each sample, the black posterior was removed from 10 males, then remaining bodies were transferred to 60 µL of PBS, followed by a 10 min vortex at maximum speed. Tubes were centrifuged at 1000 g for 1 min, then 50 µL of the supernatant was transferred to a PCR tube to be the circulating ILP2-FH sample. To the tubes with the remaining flies, 500 µL of PBS with 1% Triton X-100 was added, homogenized with a pestle and cordless motor (VWR 47747-370), and followed by a 5 min vortex at maximum speed. These tubes were centrifuged at maximum speed for 5 min, then 50 µL of the supernatant was transferred to a PCR tube, to be the total ILP2-FH sample.

For the ELISA, I used FLAG(GS)HA peptide standards (DYKDDDDKGGGGSYPYDVPDYA amide, 2412 Da: LifeTein LLC). 1 µL of the stock

peptide standards (0-10 ng/ml) was added to 50 µL of PBS or PBS with 1% Triton X-100. Wells of a Nunc Maxisorp plate (Thermo Scientific 44-2404-21) were coated with 100 µL of anti-FLAG antibody diluted in 0.2M sodium carbonate/bicarbonate buffer (pH 9.4) to 2.5 µg/mL, then the plate was incubated at 4°C overnight. The plate was washed twice with PBS with 0.2% Tween 20, then blocked with 350 µL of 2% bovine serum albumin in PBS at 4°C overnight. Anti-HA-Peroxidase, High Affinity (clone 3F10) (Roche 12013819001, 25 µg/mL) was diluted in PBS with 2% Tween at a 1:500 dilution. Then, 5 µL of the diluted anti-HA-peroxidase was added to the PCR tubes containing 50 µL of either samples or standards, vortexed, and centrifuged briefly. Following blocking, the plate was washed three times with PBS with 0.2% Tween 20. Samples and standards were transferred to wells of the plate, the plate was sealed with adhesive sealer (BIO-RAD, MSB-1001), and then placed in a humid chamber at 4°C overnight. Samples were removed with an aspirator and the plate was washed with PBS with 0.2% Tween 20 six times. 100 µL 1-Step Ultra TMB – ELISA Substrate (Thermo Scientific 34028) was added to each well and incubated at room temperature for 30 mins. The reaction was stopped by adding 100 µL 2M sulfuric acid and absorbance was measured at 450 nm on a Spectramax M5 (Molecular Devices).

2.4.2. Oral glucose tolerance test (OGTT)

Flies were starved overnight for 16 hours on 1% agar, switched to vials containing 10% glucose and 1% agar for 2 hours, and then re-starved on vials of 1% agar. Samples of 5 flies were obtained after initial starvation, after 2 hours on 10% glucose, and then at both 2 hours and 4 hours following re-starvation. Samples of 5 flies were weighed and then mashed in 125 µL TET buffer (10mM Tris, 1mM EDTA, 0.1% Triton X-100, pH 7.4). Glucose was measured using the Glucose Oxidase (GO) Assay kits (Sigma, GAGO20).

2.5 Consumption assays

2.5.1. flyPAD

The fly Proboscis and Activity Detector (flyPAD) instrument was acquired from Dr. Pavel M. Itskov and Dr. Carlos Ribeiro (Itskov et al., 2014). The flyPAD records changes in capacitance that occur when a fly comes in contact with a droplet of food in the center

of an arena. Data obtained from the flyPAD can be used to analyze different aspects of feeding behavior based on the timing of changes in capacitance.

Flies were starved for 2 hours prior to the assay. HF and GSF was prepared as described, with the exception of the substitution of agarose for agar, based on a recommendation from Dr. Itskov. Prepared food was melted at 95°C and then maintained at 60°C to facilitate pouring. Individual flies were placed in each flyPAD arena using a mouth aspirator at n=32 for each sample. Eating behaviour was recorded for 1 hour.

2.5.2. CAFE

For the capillary feeding (CAFE) assay, flies were maintained in empty vials at 10 flies/vial and fed liquid food through capillary tubes. To prepare liquid food for this assay, HF and GSF were prepared as described, but without the addition of agar. Vials were prepared with three capillaries each with approximately 7.5 µL of liquid food per capillary, and the height of the food was marked with a Sharpie. Control vials were left without flies to monitor for evaporation of food. Flies were transferred in the CAFE setup to a 25˚C humidified incubator. Flies were fed the liquid version of their respective diets for a period of 3 days. Food consumed was measured every 24 hours, and fresh food was provided each day. A ruler was used to measure the displacement of food in each capillary and converted to volume of food consumed.

2.6. Bacterial assays

2.6.1. Microbiome analysis

Samples of 10 adult male or female flies were raised for 10 days at 29°C. This nonstandard temperature was used to enable comparison to fly lines using the temperature sensitive GAL80. Intestinal tracts were dissected as described in 2.8.1. and bacterial genomic DNA was isolated with the Ultraclean Microbial DNA Isolation Kit (MO- BIO, 12224). Bacterial 16S DNA was amplified with primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-GGCTACCTTGTTACGACTT-3′ (reverse). Samples were purified with a QIAquick PCR Purification Kit (Qiagen, 28104). Samples were prepared for sequencing using a Nextera XT DNA Library Preparation Kit

(Illumina, FC-131-1024), and DNA libraries were sequenced using a MiSeq Desktop Sequencer (Illumina). Taxonomy assignment was based on the SILVA SSU Ref NR99 database release 115, using software developed by Dr. Bart Hazes. Diversity between samples was analysed with Shannon and Gini-Simpson indexes.

2.6.2. Bacterial culture

The day prior to an infection assay, streak a colony of *V. cholerae* C6706 from glycerol stocks onto an agar plate of LB with 100µg/mL Streptomycin and grow overnight at 37˚C. On the day of infection, prepare a liquid culture of *V. cholerae* in LB broth with 100µg/mL Streptomycin. Liquid culture was prepared to an absorbance of OD_{600} = 0.125 for oral infection or OD_{600} = 1.0 for systemic infection.

2.6.3. *Vibrio cholerae* infection protocol

For oral infection, flies are starved in empty vials for 2 hours prior to infection. Vials are prepared with cotton plugs at the bottom and saturated with the prepared liquid *V. cholerae* culture (OD_{600}=0.125). Mock vials are prepared with sterile LB broth. Following starvation, flies are added to experimental vials at 10 flies/vial then transferred to a humidified incubator at 25˚C. Dead flies are counted every 2-4 hours as often as possible.

For systemic infection, flies are anesthetized on a CO_2 pad. A 0.15 mm diameter minuten pin is dipped into prepared liquid *V. cholerae* culture (OD_{600}=1.0), then gently inserted into the abdomen of the fly. Mock infected flies are stabbed with a pin dipped into sterile LB broth. Flies are maintained in vials unmodified or modified holidic food in a humidified incubator at 25˚C. Dead flies are counted every 2-4 hours as often as possible.

2.6.4. Colony forming unit (CFU) measurement

Prior to CFU analysis, live infected flies are selected and sacrificed for 20 min at -20˚C. Flies are then surface sterilized through serial treatment with 10% bleach, 75% ethanol, and sterile water. 5 flies/sample are then homogenized in 500 µL sterile LB broth. A dilution series is prepared for each sample ranging from 10^0 to 10^{-7} in a 96-well plate. Next, 10 µL of each dilution is streaked onto a LB with 100µg/mL Streptomycin agar plate.

Plates are incubated overnight at 37˚C. CFU are counted the following day, and the CFU per fly can be calculated.

2.7. RNA isolation and Transcriptomics

2.7.1. RNA isolation

To isolate RNA for RT-qPCR, samples of 5 whole flies were homogenized in 250 µL TRIzol, then incubated at room temperature for 5 min. Samples were centrifuged at 12000 g for 10 min at 4˚C. Clear homogenate was transferred to a 1.5 mL Eppendorf tube, then 50 µL of chloroform was added, shaken vigorously for 15 seconds, and incubated at room temperature for 3 min. Samples were centrifuged at 12000 g for 15 min at 4˚C. The upper aqueous layer was transferred to a 1.5 mL Eppendorf tube, 125 µL isopropanol was added, then left at -20˚C overnight. Samples were centrifuged at 12000 g for 10 min at 4˚C. The RNA pellet was washed with 500 µL 75% ethanol, centrifuged at 7500 g for 5 min at 4˚C, then allowed to air dry. RNA pellet was dissolved in RNAse free water, then incubated at 37˚C for 30 min with 1 µL DNAse.

2.7.2. RT-qPCR

For RT-qPCR, the following primers were used in this study. Relative expression was calculated as a ΔΔCT value. All transcripts were normalized to expression of *rp49*.

rp49 F: 5'-AAGAAGCGCACCAAGCACTTCATC-3'
R: 5'-TCTGTTGTCGATACCCTTGGGCTT-3'
ilp2 F: 5'-TCCACAGTGAAGTTGGCCC-3'
R: 5'-AGATAATCGCGTCGACCAGG-3'
ilp3 F: 5'-AGAGAACTTTGGACCCCGTGAA-3'
R: 5'-TGAACCGAACTATCACTCAACAGTCT-3'
ilp5 F: 5'-GAGGCACCTTGGGCCTATTC-3'
R: 5'-CATGTGGTGAGATTCGGAGCTA-3'
CecC F: 5'- TGTAAGCTAGTTTATTTCTATGG-3'
R: 5'- GATGAGCCTTTAATGTCC-3'
Att F: 5'-AGTCACAACTGGCGGAC-3'

R: 5’-TGTTGAATAAATTGGCATGG-3’

Dpt F: 5’- ACCGCAGTACCCACTCAATC-3’

R: 5’- ACTTTCCAGCTCGGTTCTGA-3’

bbg F: 5’-GCCAGGATTTGGTCATCAACG-3’

R: 5’-CTAGGCTTCCGGGGAGTACC-3’

Mesh F: 5’-AGCCCGATCAATACTCAGGA-3’

R: 5’-CCATATACCAGGCCAGAGGA-3’

Ssk F: 5’-CACTGGATGCCACACCATT-3’

R: 5’-TGGTGTCGCACAGCTCTC-3’

2.7.3. Microarray

Female w^{1118} flies were raised for 10 days on glucose-supplemented (100 g/L) or unmodified holidic food. Microarrays were performed using the GeneChip *Drosophila* Genome 2.0 Array (Affymetrix) in triplicate. RNA was extracted from both whole fly samples and dissected gut samples (dissection described in 2.8.1.), at 10 flies per sample. Next, 100 ng of purified RNA was used to make labeled cRNA using the GeneChip 3’IVT Plus Reagent Kit (Affymetrix). Preliminary analysis was performed with the Transcriptome Analysis Console software (Affymetrix). Panther was used to determine Gene Ontology (GO) term enrichment of downregulated and upregulated gene sets. Microarray data have been submitted to the NCBI GEO database (GSE147237).

2.7.4. RNA-Sequencing

To isolate RNA for RNA-seq, samples of 5 whole flies were homogenized in 250 µL TRIzol, then incubated at room temperature for 5 min. Samples were centrifuged at 12000 g for 10 min at 4˚C. Clear homogenate was transferred to a 1.5 mL Eppendorf tube, then 50 µL of chloroform was added, shaken vigorously for 15 seconds, and incubated at room temperature for 3 min. Samples were centrifuged at 12000 g for 15 min at 4˚C. The upper aqueous layer was transferred to a 1.5 mL Eppendorf tube, 125 µL isopropanol was added, then left at -20˚C overnight. Samples were centrifuged at 12000 g for 10 min at 4˚C. The RNA pellet was washed with 500 µL 75% ethanol, centrifuged at 7500 g for 5 min at 4˚C,

then allowed to air dry. RNA pellet was dissolved in RNAse free water, then incubated at 37˚C for 30 min with 1 µL DNAse.

An average of 60 million reads were obtained per biological replicate. Quality check was performed with FastQC to evaluate the quality of raw, paired-end reads. Adaptors and reads of less than 36 base pairs in length were trimmed from the raw reads using Trimmomatic (version 0.36). HISAT2 ((version 2.1.0) was used to align reads to the *Drosophila* transcriptome-bdgp6, and the resulting BAM files were converted to SAM files using SAMtools (version 1.8). Converted files were counted with Rsubread (version 1.24.2) and loaded into EdgeR. In EdgeR, genes with counts less than 1 count per million were filtered and libraries normalized for size. Normalized libraries were used to identify genes that were differentially expressed between treatments. Genes with P value < 0.01 and FDR < 0.05 were defined as differentially expressed genes. Panther was used to determine Gene Ontology (GO) term enrichment of downregulated and upregulated gene sets. FlyAtlas2 was used for tissue enrichment analysis of genes of interest. RNA-seq data have been submitted to the NCBI GEO database (GSE147222).

2.8. Immunofluorescence and microscopy of midgut samples

2.8.1. Sample preparation

For dissection of midguts, flies were briefly washed with 95% ethanol then dissected in PBS to isolate midguts. Samples were fixed for 30 min at room temperature in 4% paraformaldehyde. Samples were quickly washed in PBS + 0.3% Triton-X (PBT), followed by 3x 10 min washes in PBT. Samples were blocked for 1 hour in PBT + 3% bovine serum albumin (BSA) at room temperature, then incubated overnight at 4˚C in PBT + 3% BSA with 1˚ anti-Cora 1:100 (DSHB, C615.16). Samples were washed 3x for 10 min in PBT, then incubated for 1 hour at room temperature with 2˚ Alexa anti-mouse 1:500. Samples were briefly washed with PBT, followed by 3x 10 min washes in PBT. Hoechst DNA stain 1:500 was added to the second 10 min wash. Samples were washed in PBS, then mounted on slides in Fluoromount (Sigma-Aldrich F4680).

2.8.2. Confocal microscopy

Slides were visualized on a spinning disk confocal microscope (Quorum WaveFX; Quorum Technologies Inc). The R4/R5 region of the posterior midgut of each sample was located by identifying the midgut-hindgut transition and moving 1-2 frames anterior from the attachment site of the Malpighian tubules. Images were acquired using Volocity Software (Quorum Technologies). 3D reconstruction was performed with Icy.

2.8.3. Quantification of Coracle

Quantification of localization of coracle in images was performed in FIJI. Three representative cells were selected per 40X image. For each cell, a transverse line was drawn across the bicellular junction into the cell to measure coracle expression. Peak expression was recorded as the junction value and 2.24 μm (10 px) into the cell from this peak level was recorded as the cytosol value. The junction/cytosol ratio was calculated from these two values. This was performed in triplicate for each cell, and the average of these three measurements was recorded as the value for the cell. Sample sizes for flies raised on HF (n = 7 guts, 66 cells) and GSF (n = 8 guts, 84 cells).

2.9. Barrier integrity analysis

2.9.1. Blood-brain barrier assay

To test the integrity of the blood-brain barrier (BBB), I used a dye injection assay, where 10 kDa-Dextran conjugated to fluorescent Texas Red dye is effectively excluded from a brain with an intact BBB. Glass injection needles were prepared with a Flaming/Brown Micropipette Puller (Model P-97) using the specifications Heat=515, Pull=30, Velocity=40, and Time=165. Flies were anesthetized on a CO_2 pad, then injected with a microinjector. Approximately 50 nL of dye was injected into the fly abdomen. Following injection, the dye rapidly circulates throughout the fly hemolymph. Fly brains were dissected 4 hours after injection.

For dissection of brains, flies were briefly washed with 95% ethanol then dissected in PBS to isolate brains. Samples were fixed for 30 min at room temperature in 4% paraformaldehyde. Samples were quickly washed in PBS, followed by 3x 20 min washes

in PBS. DNA stain (Hoechst) was added to the third wash (1:500). Samples were briefly washed once more in PBS, then mounted on slides in Fluoromount (Sigma-Aldrich F4680). Slides were visualized on a spinning disk confocal microscope (Quorum WaveFX; Quorum Technologies Inc). Images were acquired using Velocity Software (Quorum Technologies).

2.9.2. Smurf assay

For the smurf assay, unmodified holidic and 50 g/L glucose-supplemented food were prepared as described with the addition of 1% erioglaucine disodium salt (Brilliant Blue FCF). Flies were raised on their respective diets and monitored daily for extraintestinal leakage of dye or 'smurfing'.

2.9.3. Dextran sulphate sodium (DSS) challenge

For the dextran sulphate sodium (DSS) challenge, flies were raised on either unmodified holidic or 50 g/L glucose-supplemented food for 20 days, then transferred to either unmodified holidic with 5% DSS added or 50 g/L glucose-supplemented food with 5% DSS added, respectively. Deaths were recorded daily and flies were transferred to fresh food every 2-3 days.

2.10. Statistical analysis

Statistical analysis was performed using Graphpad Prism (Version 8). Statistical significance was defined as $p < 0.05$. Significance between two samples was determined by Student's T-tests. Significance in experiments with two or three independent variables were determined by two-way or three-way analysis of variance (ANOVA), respectively. For lifespan and survival analysis, significance was determined using log-rank (Mantel-Cox) test. Hazard function for lifespan was calculated with bins of 5 days.

Chapter 3

Glucose extends lifespan independent of the intestinal microbiota

Portions of this chapter have been published as:

Galenza A, Hutchinson J, Campbell SD, Hazes B, and Foley E. (2016) Glucose modulates *Drosophila* longevity and immunity independent of the microbiota. *Biol. Open.* 6:165-173.

Jaclyn Hutchinson performed macronutrient assays shown in Figure 3-1 and longevity assays shown in Figure 3-2. Bart Hazes helped analyze 16S sequencing data shown in Figure 3-5.

3. Glucose extends lifespan independent of the intestinal microbiota

3.1. Introduction

Many studies of the interplay between nutrition and health overlook microbial contributions. In particular, we know very little about the relationship between the intestinal microbiota, host diet, and host intestinal immunity. I consider this a particularly relevant aspect of health and lifespan, as diet and health are intimately linked by the intestinal microbiota (Flint et al., 2012). Diet shapes the composition of the intestinal microbiota, which, in turn, influences events as diverse as nutrient allocation, intestinal physiology, immune responses, and the onset of chronic diseases (Hooper et al., 2012; Round and Mazmanian, 2009; Wlodarska et al., 2015).

The genetically accessible model system *Drosophila melanogaster* is a particularly valuable tool to reveal key aspects of relationships between diet, the microbiota, and the host (Erkosar and Leulier, 2014; Ma et al., 2015). The fly gut shares numerous similarities with mammalian counterparts that include developmental origin, cellular composition, and metabolic pathways (Miguel-Aliaga et al., 2018). Previous studies with *Drosophila* as a tool to explore host-diet-microbiota relationships relied on partially defined diets. Recently, Piper et al. established a protocol to prepare a holidic diet for *Drosophila*, in which the exact composition and concentration of every ingredient is known (Piper et al., 2014). This allows for precise manipulation of nutrient availability in dietary studies, as individual components can be modified to a specified quantity and effects on the organism can be observed.

In this chapter, I investigated how dietary modifications affect the health of a fly. I designed five separate modifications that represent different macronutrient categories relevant to human diets. Specifically, I made five separate modifications to the original holidic recipe that include the addition of supplementary glucose, starch, casein, palmitic acid, or ethanol. Respectively, these additions represent diets with increased levels of simple carbohydrates, complex carbohydrates, protein, saturated fatty acids, or alcohol. I investigated how these dietary modifications affect lifespan, immune response, and microbial composition of the host.

3.2. Results

3.2.1. Modifications to the holidic medium impact health and lifespan

3.2.1.1. Modified holidic medium variants alter fly macronutrients

The experiments in 3.2.1.1 were performed alongside an undergraduate student, Jaclyn Hutchinson, whom I was working with for this part of my project. We initially measured the relationship between age, diet, and metabolism in w^{1118} wild-type flies. For these assays, we raised flies on an unmodified holidic diet, or a holidic diet supplemented with glucose (100 g/L), starch (50 g/L), casein (70 g/L), palmitic acid (50 g/L), or ethanol (1%). These supplementary regimes allowed us to interrogate the impacts of increased levels of simple or complex carbohydrates, protein, saturated fatty acids, or moderate amounts of alcohol on a common experimental model. As preparation of each holidic variation is labor-intensive, we decided to initially focus on a single concentration for each modification, and the concentrations of each modification were chosen based on studies investigating the effects of similar dietary modifications. If we found that a particular dietary modification had a marked effect on *Drosophila* health, then we could focus on that modification of interest and test a range of concentrations in later studies.

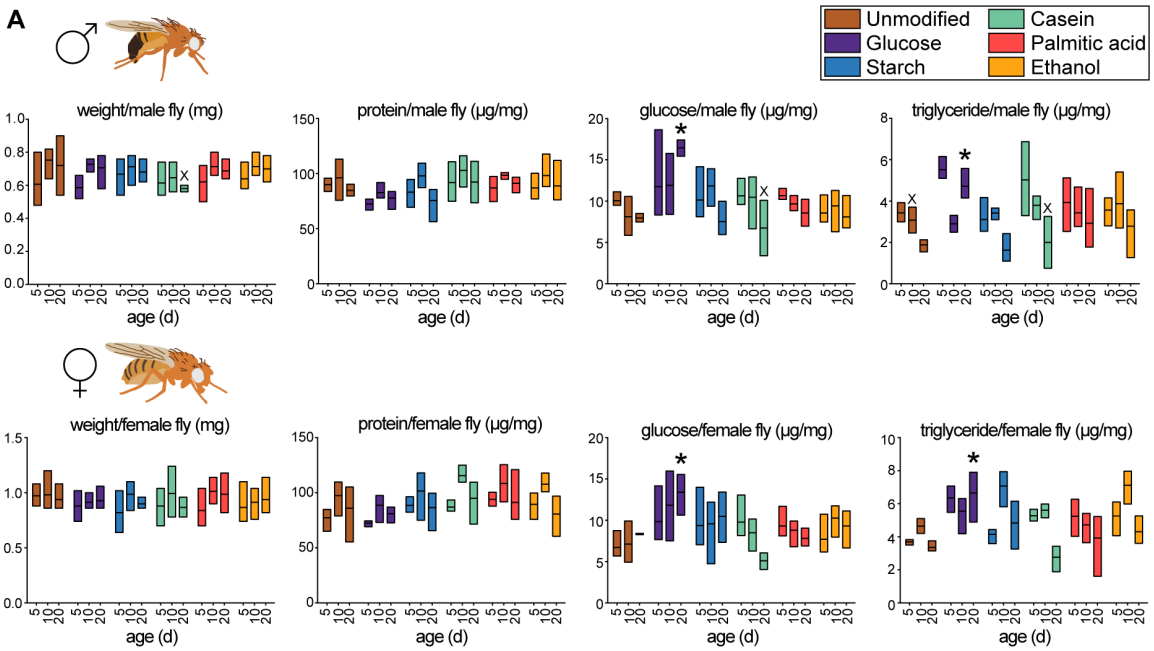
Initially, we asked how our experimental dietary modifications affected weight and macronutrient levels in flies. We raised freshly emerged adult male and female w^{1118} flies on either the unmodified holidic diet or one of our five modified diets. Flies were maintained on their respective diets for 5 days, 10 days, or 20 days. Here, we wanted to explore the impact of diet at relatively young age points, in part to avoid survivorship bias if any particular diet impaired survival. We measured the weight, protein content, triglyceride levels, and glucose levels of male and female flies raised on the respective diets **(Figure 3-1A)**. With both age and diet as variables, we used a 2-way ANOVA to test for a significant impact on variation in weight or macronutrient levels **(Figure 3-1B)**.

For weight, we found that neither diet nor age impacted weight of females, though age affected variation of male weight ($p=0.036$), as males appear to have increased weight with age. To analyze the impact of each diet specifically, we performed multiple comparison analysis with a Dunnett's test within each age, which allows for comparison of each

modified diet treatment to the unmodified control. We found no statistical change in weight in flies raised on any of the modified diets.

To investigate if the modified diets affected fly energy storage, we next asked if our modified holidic diets altered macronutrient levels of male and female flies. Specifically, we measured protein, glucose, and triglycerides in 5-, 10-, and 20-day old flies. For protein levels, 2-way ANOVA showed no effect in male flies or from diet on females, but we found significant age-dependent variation in female flies. Multiple comparison analysis found no significant change in protein content from any of the modified diets. When we measured glucose content of flies raised on modified diets, we found that glucose decreased with age in males. However, diet appeared to have a significant effect on variation of glucose levels in both males and females. When we performed multiple comparison analysis, we found that glucose-supplemented food increased glucose levels in 20-day old males, about a 2.2-fold increase, and 20-day old females, about a 1.7-fold increase. Finally, for triglyceride levels, 2-way ANOVA showed that age significantly affected triglyceride in males, where triglyceride levels seemed to decrease with age. For females, 2-way ANOVA found significant effects from diet, age, and the interaction of both variables on triglyceride levels. Upon multiple comparison analysis, we found that glucose-supplemented food significantly increased triglyceride levels in 20-day old males, about a 2.2-fold increase, and 20-day old females, about a 2-fold increase.

Combined, these data suggest that increased availability of dietary glucose elevates energy stores, particularly in 20-day old flies, without significant effects on weight or protein content.



B

2-way ANOVA	Weight		Protein		Glucose		Triglyceride	
	Male	Female	Male	Female	Male	Female	Male	Female
Interaction	0.976	0.983	0.994	0.983	0.301	0.419	0.107	**0.044**
Age	**0.036**	0.221	**0.023**	**0.002**	**0.009**	0.081	**0.008**	**0.001**
Diet	0.581	0.933	0.083	0.141	**0.001**	**0.002**	0.094	**0.017**

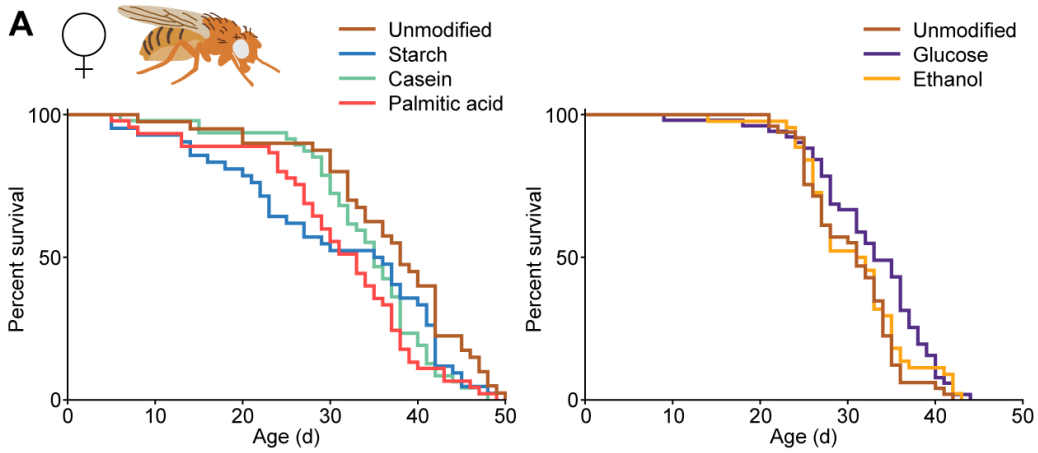
Figure 3-1 Diet and age impact adult *Drosophila* metabolic profile

(A) Longitudinal analysis of weight, protein, glucose, and triglyceride content in male and female flies raised on unmodified or modified holidic diets. Each column shows the result of three replicate measurements at the indicated time point, except for columns indicated with an 'X', which show the values of two replicate measurements. Mean values for each diet and time point were compared with the means of unmodified diets at the same time with a Bonferroni correction for multiple comparisons. Significance of $p<0.05$ indicated by asterisk. (B) Two-way ANOVA analysis of data from A. Significant P values are highlighted in bold typeface.

3.2.1.2. Modified holidic medium variants alter fly lifespan

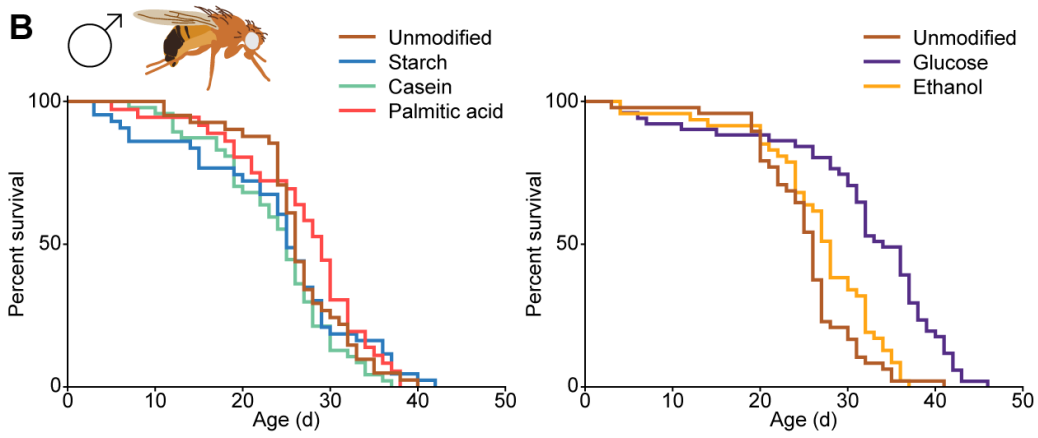
Our data overlap with previous suggestions that dietary modifications have considerable impacts on the metabolic profile of flies (Wong et al., 2014). Numerous studies describe the availability of nutrients and calories in the control of animal longevity, with a frequent implication that caloric or dietary restriction extends life (Fontana and Partridge, 2015;

Simpson et al., 2017; Tatar et al., 2014). However, recent studies also suggest that relative amounts of macronutrients in the diet are important determinants of *Drosophila* lifespan (Lee et al., 2008). Importantly, this hypothesis has not been tested with a defined diet in *Drosophila*. To address this issue, we determined the lifespans of adult male and female w^{1118} flies raised on our defined, modified holidic diets. We found that dietary modifications had slightly different effects on the longevity of male and female flies **(Figure 3-2)**. In general, dietary modifications that diminished lifespans, such as supplementation with palmitic acid or protein, had more pronounced effects on female flies than male flies, while dietary modifications that extended lifespans, such as addition of ethanol or glucose had more pronounced effects on male flies than females **(Figure 3-2)**. We found that the addition of glucose had a particularly marked impact on longevity in male flies, with a median lifespan extension of 31%. For this reason, I decided to focus on glucose-dependent lifespan extension mainly in males. A recent meta-analysis suggested that the longevity benefits of dietary restriction are adaptations to laboratory culture, not a physiological response observed in the wild (Nakagawa et al., 2012). To test if the benefits of glucose addition are restricted to lab-raised w^{1118} flies, I fed adult males from a wild-derived population of *Drosophila melanogaster* an unmodified diet or one supplemented with glucose. As with our lab strains, I found that elevated levels of dietary glucose significantly increased the lifespan of wild flies **(Figure 3-3A)**.



Diet	Median (d)	χ^2	P
Unmodified	38		
Palmitic acid	33	8.796	**0.003**
Starch	35.5	3.685	0.055
Casein	35	6.332	**0.012**

Diet	Median (d)	χ^2	P
Unmodified	31		
Ethanol	31.5	0.856	0.355
Glucose	33	6.832	**0.009**



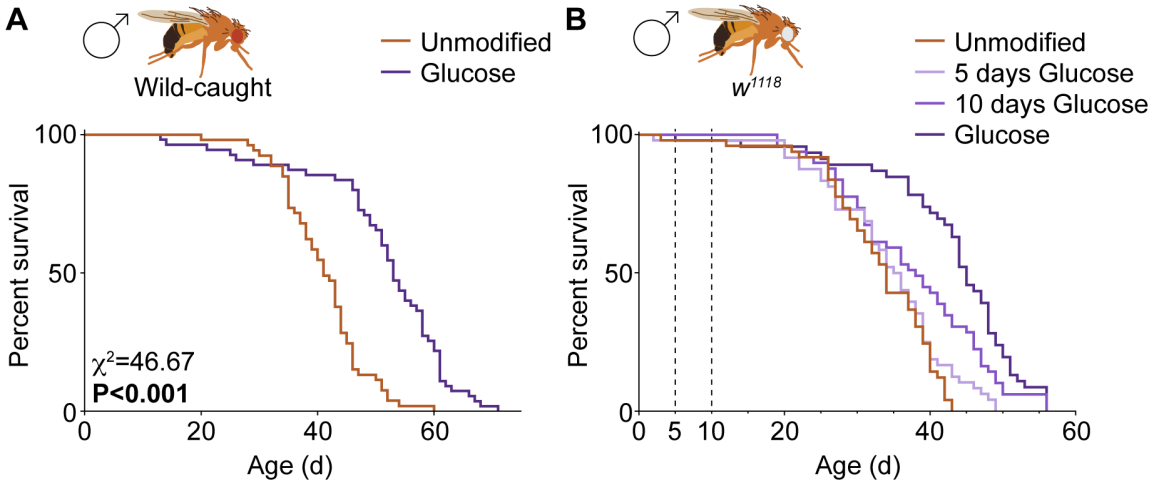
Diet	Median (d)	χ^2	P
Unmodified	26		
Palmitic acid	25	0.803	0.307
Starch	29	0.001	0.981
Casein	25	2.279	0.131

Diet	Median (d)	χ^2	P
Unmodified	26		
Ethanol	28	3.908	**0.048**
Glucose	34	34.35	**<0.001**

Figure 3-2 Longevities on different diets

(A) Survival curves of female w^{1118} flies raised on unmodified or modified holidic diets. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test. (B) Survival curves of male w^{1118} flies raised on unmodified or modified holidic diets. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test.

Restoration of a complete diet reverts lifespan-extension benefits of dietary restriction in *Drosophila* (Mair et al., 2003). To determine if the benefits of glucose were permanent or transient, I measured the longevity of male w^{1118} flies raised on an unmodified holidic diet, or male flies raised on a holidic diet supplemented with glucose for the first five days, the first ten days, or the duration of adult life. My results show that longer periods of dietary supplementation with glucose have more significant effects on lifespan **(Figure 3-3B)**. For example, supplementation of the adult diet with glucose for the first ten days of life extended median survival rates by 12%, while permanent addition of extra glucose extended median survival rates by 32%. These data suggest that early, transient exposure to glucose supplementation is beneficial to lifespan, but continuous feeding is required for optimal lifespan extension.



Group	Median (d)	χ^2	P
Unmodified	34		
5 days glucose	35.5	1.583	0.208
10 days glucose	38	11.65	**0.001**
Permanent glucose	45	47.45	**<0.001**

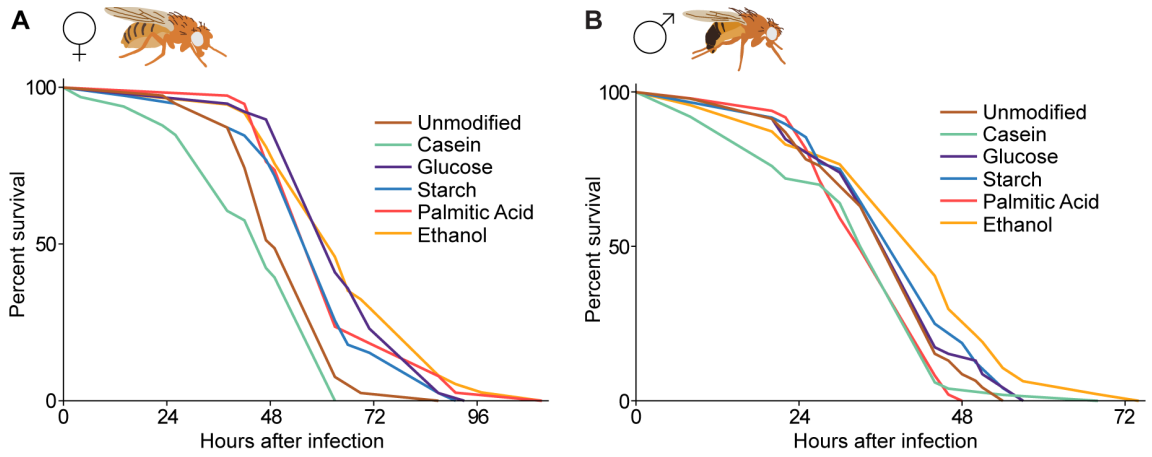
Figure 3-3 Supplemental dietary glucose extends longevity

(A) Survival curves of a wild strain of male flies raised on an unmodified diet or on a diet supplemented with glucose. Significance determined by log-rank (Mantel–Cox) test. (B) Survival curves of male w^{1118} flies raised on an unmodified diet, or on a diet supplemented with glucose for 5 days, 10 days, or permanently. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test.

3.2.1.3. Modified holidic medium variants affect survival to oral infection with *V. cholerae*

As malnutrition impairs immune functions in *Drosophila* (Vijendravarma et al., 2015), I asked if defined dietary modifications influence host responses to challenges with an intestinal pathogen. *Drosophila* is an established model for infection with the enteric pathogen *Vibrio cholerae* (Blow et al., 2005). As *V. cholerae* appears to disrupt host metabolism and insulin signaling (Hang et al., 2014b), I believed that investigating how dietary modifications impact infection survival would be particularly intriguing in the *V. cholerae* infection model.

To determine if diet altered survival time during a *V. cholerae* infection, I raised adult female or male flies on defined diets for ten days and measured survival after delivering an oral lethal infectious dose of *V. cholerae*. I found that female flies on an unmodified holidic diet had a median survival of 49 hours after infection **(Figure 3-4A)**. Supplementation with casein, an increase in dietary protein level, led to a slight decrease in median survival, consistent with studies that found a high P:C ratio is detrimental to infection survival (Ponton et al., 2020). Conversely, the other dietary modifications all showed an increase in median survival. Female flies that were raised on diets supplemented with starch, palmitic acid, or ethanol had improved survival, while those raised on glucose-supplemented food showed the most significant extension in survival during infection (χ^2=19.82, P<0.0001). In male flies, different modified diets had less of an impact on survival to infection than for females **(Figure 3-4B)**. Supplementation with ethanol led to an increase in survival for males, while the other dietary modifications did not have a significant impact on survival. These data establish that defined nutritional regimes influence the ability of *Drosophila* to combat an enteric infection, and in particular, that supplementation with glucose or ethanol significantly elevate the survival times for female adult flies.



Diet	Median (h)	χ^2	P
Unmodified	49		
Casein	47	2.604	0.107
Glucose	63	19.820	**<0.001**
Starch	63	6.788	**0.009**
Palmitic Acid	63	10.320	**0.001**
Ethanol	63	15.560	**<0.001**

Diet	Median (h)	χ^2	P
Unmodified	44		
Casein	38.5	1.441	0.230
Glucose	44	0.587	0.444
Starch	44	1.507	0.220
Palmitic Acid	33	3.529	0.060
Ethanol	44	6.897	**0.009**

Figure 3-4 Diet impacts survival after *Vibrio cholerae* infection

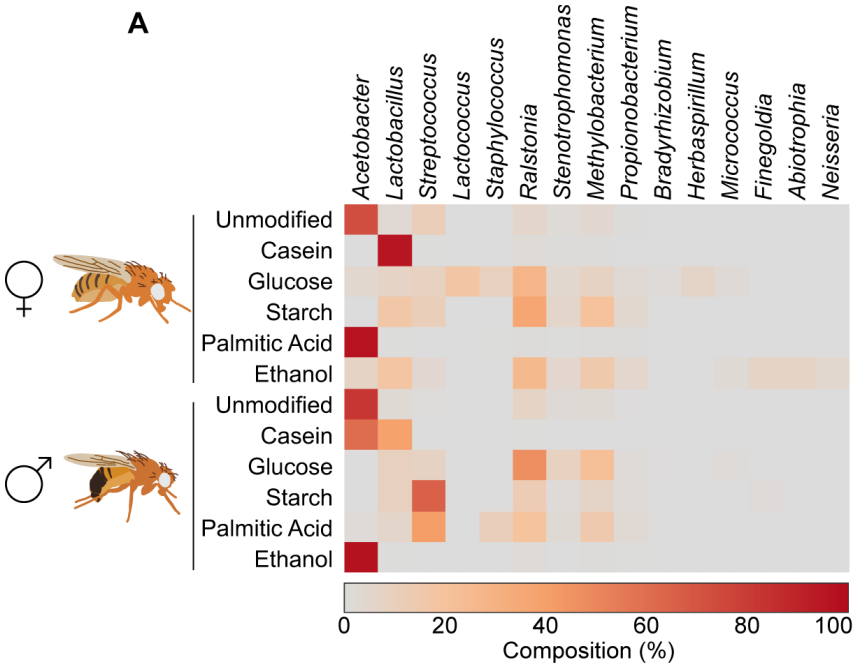
(A) Survival curves of female w^{1118} flies raised on unmodified or modified holidic diets for ten days and then challenged with an oral infection with *V. cholerae*. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test. (B) Survival curves of male w^{1118} flies raised on unmodified or modified holidic diets for ten days and then challenged with an oral infection with *V. cholerae*. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test.

3.2.1.4. Modified holidic medium variants alter intestinal microbial composition

The studies detailed above uncover a number of effects of defined dietary modifications on the health of adult flies. As the microbiota of the host is known to affect these factors, I assessed the impact of defined diets on the intestinal microbiota. For these assays, I raised adults on either an unmodified holidic diet or one of the five modified diets for ten days, then performed 16S DNA sequencing on bacterial DNA isolated from their intestinal tracts. Males and females raised on an unmodified holidic diet had similar microbiota that were dominated by the *Acetobacter* genus **(Figure 3-5A)**. I found that simple alterations to this holidic diet resulted in profound changes in microbiota composition and diversity **(Figure**

3-5). For example, when flies were raised on a diet supplemented with casein, the microbiota shifted to predominantly *Lactobacillus* species. In contrast, supplementation with glucose resulted in the largest increase in microbiota diversity (Shannon: females=2.387, males=1.789). I also noticed a different response between males and females to the same dietary modification, as seen for a diet supplemented with ethanol (Shannon: females=2.383, males=0.193). My data suggest that both host diet and sex markedly impact the composition of intestinal microbiota, with supplementary glucose contributing to the greatest increase in species diversity.

A



B

Sample			Species Diversity Indices	
Sex	Diet	# of reads	Shannon	Gini-Simpson
Female ♀	Unmodified	175940	1.119	0.454
	Casein	208045	0.431	0.156
	Glucose	154324	2.387	0.874
	Starch	76009	1.966	0.802
	Palmitic Acid	229722	0.220	0.064
	Ethanol	136261	2.383	0.882
Male ♂	Unmodified	204249	0.819	0.320
	Casein	192392	0.796	0.495
	Glucose	84795	1.789	0.731
	Starch	117962	1.589	0.628
	Palmitic Acid	113260	2.062	0.813
	Ethanol	171197	0.193	0.061

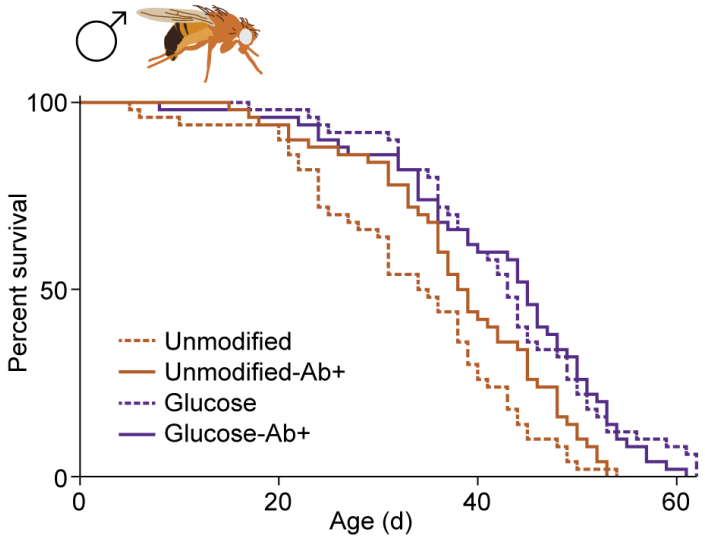
Figure 3-5 Diet alters intestinal microbial composition

(A) Heat map summary showing abundance of bacterial genera present (with >1% abundance) in midguts of male and female w^{1118} flies raised on different diets for 10 days. Each sample consists of 5 flies. Abundance of each bacterial genus in a sample ranges from 0% (grey) to 100% (dark red) as indicated by the scale. (B) Summary showing the number of reads from 16S sequencing and the results from both Shannon and Gini–Simpson diversity values of each sample.

3.2.2. Initial investigation of the mechanism of glucose-supplemented food lifespan extension

3.2.2.1. Antibiotic treatment does not impact lifespan or infection survival advantages of glucose-supplemented food

My data reveal wide-ranging impacts of dietary glucose supplementation on adult flies, with significant effects on longevity, energy stores, microbiota composition, and infection survival. Given the established links between intestinal microflora diversity and host health (Mosca et al., 2016), I asked if the microbiota is required for the beneficial effects of glucose supplementation on longevity. For these assays, I fed adult male flies an unmodified holidic diet or one supplemented with glucose and raised the flies under conventional conditions or made germ-free through antibiotic treatment. Germ-free status was verified by plating out samples of antibiotic treated flies on both LB and MRS plates. Consistent with recent reports (Clark et al., 2015; Petkau et al., 2014), I found that flies raised under germ-free conditions outlived their conventionally-reared counterparts **(Figure 3-6)**. Similar to my earlier experiments, I found that elevated dietary glucose increased the median lifespan of adult flies by 25% compared to an unmodified diet. Strikingly, I found that elimination of the microbiota did not affect the lifespan of flies raised on diets with added glucose, suggesting that glucose levels influence host longevity independently of the microbiota.



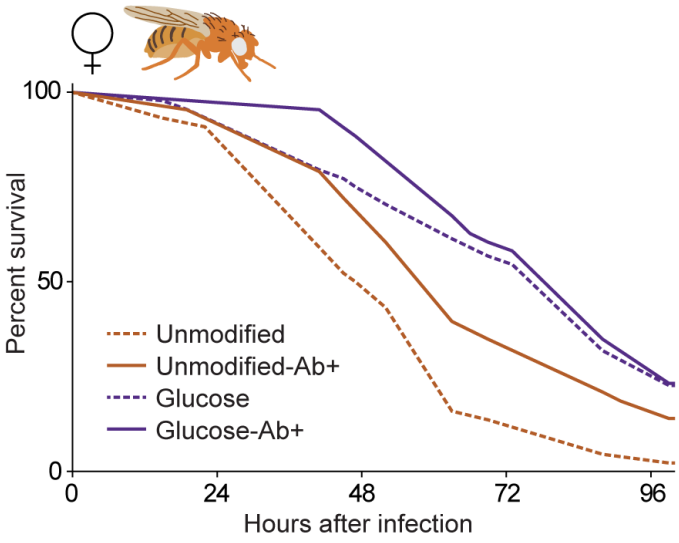
Comparison	χ^2	P
Unmodified vs Unmodified-Ab+	4.26	**0.039**
Unmodified vs Glucose	17.76	**<0.001**
Glucose vs Glucose-Ab+	<0.01	0.993
Unmodified-Ab+ vs Glucose-Ab+	7.30	**0.007**

Figure 3-6 Glucose extends longevity independent of the microbiota

Survival curves of male w^{1118} flies fed a glucose-supplemented or unmodified holidic diet and raised under conventional or germ-free (Ab+) conditions. Significance determined by log-rank (Mantel–Cox) test.

These unexpected observations prompted me to ask if the glucose-mediated improvements to survival after challenges with *V. cholerae* require a microbiota. To assess this, I fed adult female flies an unmodified holidic diet or one supplemented with glucose and raised the flies under conventional or germ-free conditions for ten days. I then measured survival following oral infection with *V. cholerae*. Conventional flies on an unmodified diet had a median survival of 49.5 hours **(Figure 3-7)**. Removal of the microbiota significantly improved survival after infection with *V. cholerae* **(Figure 3-7)**. As before, I found that added dietary glucose significantly improved survival compared to an unmodified diet ($\chi2=17.390$, $P<0.0001$). Remarkably, elimination of the microbiota did not alter the survival rates of flies raised on a glucose-supplemented diet and challenged

with *V. cholerae*. Combined, these data establish that the microbiota shifts associated with glucose-supplemented food are not essential for the immunological and lifespan benefits of such a diet.



Comparison	χ^2	P
Unmodified vs Unmodified-Ab+	7.17	**0.007**
Unmodified vs Glucose	17.39	**<0.001**
Glucose vs Glucose-Ab+	0.28	0.598
Unmodified-Ab+ vs Glucose-Ab+	5.12	**0.024**

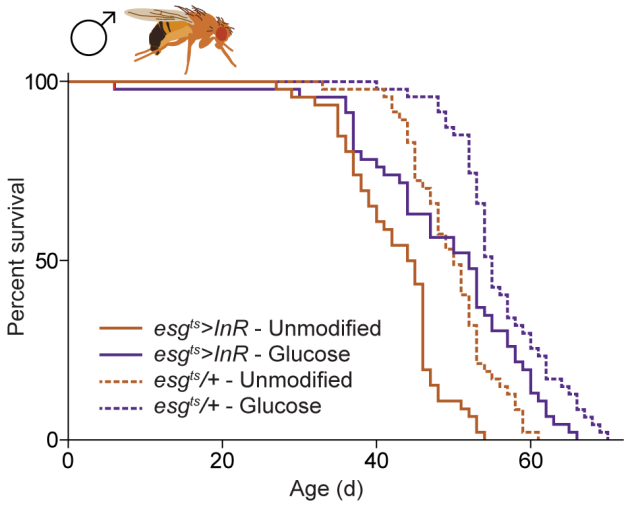
Figure 3-7 Glucose improves infection response independent of the microbiota

Survival curves of female w^{1118} flies fed a glucose-supplemented or unmodified holidic diet and raised under conventional or germ-free (Ab+) conditions for 10 days, then orally infected with *V. cholerae*. Significance determined by log-rank (Mantel–Cox) test.

3.2.2.2. Insulin signaling in intestinal stem cells is not required for glucose-dependent lifespan extension

My observation that glucose improves infection survival independently of an intact intestinal microbiota suggests direct effects of glucose on host intestinal physiology. Previous studies showed that high-sucrose diets cause insulin resistance in *Drosophila* (Musselman et al., 2011), insulin mutants live longer (Clancy et al., 2001; Tatar et al.,

2001), and inhibition of insulin signaling specifically in the gut promotes longevity (Biteau et al., 2010). Thus, I hypothesized that added dietary glucose leads to insulin insensitivity in the intestine of adult flies, thereby extending the lifespans of the fly. To test this hypothesis, I generated a temperature sensitive *esg-GAL4, GAL80^{ts}/+; UAS-InR/+* (*esg^{ts}>InR*) *Drosophila* strain to control insulin receptor activity in midgut progenitors. In this strain, the combination of *esg-GAL4* and *GAL80^{ts}* transgenic elements induce insulin receptor (InR) activity in midgut progenitors of adult flies at the restrictive temperature of 29°C. As described in a previous study (Biteau et al., 2010), I found that activation of the insulin receptor decreased the lifespans of adult flies compared to *esgGAL4, GAL80^{ts}/+* (*esg^{ts}/+*) control flies **(Figure 3-8)**. Contrary to my hypothesis, I found that *esg^{ts}>InR* flies raised on a diet with added glucose significantly outlived *esg^{ts}>InR* counterparts raised on an unmodified diet. In fact, the lifespan extensions observed upon addition of glucose were comparable for *esg^{ts}>InR* and *esg^{ts}/+* controls **(Figure 3-8)**. These data suggest that a glucose-supplemented diet extends adult *Drosophila* lifespan independent of insulin receptor activity in intestinal progenitor cells.



	Median (d)		Diet Comparison	
	Unmodified	Glucose	χ^2	P
esg^{ts}/+	50.0	55.0	23.63	**<0.001**
esg^{ts}>InR	44.5	52.0	22.03	**<0.001**

Figure 3-8 Glucose extends longevity independent of intestinal insulin activity

Survival curves of male *esg^{ts}>InR* or *esg^{ts}/+* flies fed a glucose-supplemented or unmodified holidic diet. Significance determined by log-rank (Mantel–Cox) test.

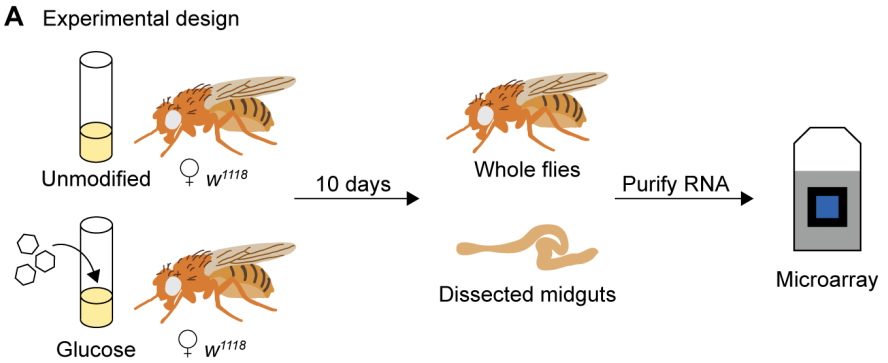
3.2.2.3. Microarray comparison suggests glucose supplementation decreases growth pathway and increases immune gene expression

As I found that glucose extends lifespan and improves infection survival independent of the microbiota, I sought an unbiased method to investigate how glucose-supplemented food alters host biology. To address this, I used microarray analysis to compare gene expression between flies raised on either glucose-supplemented or unmodified holidic food. I raised female w^{1118} flies on either diet for 10 days, then isolated RNA from whole fly samples and dissected intestines **(Figure 3-9A)**. Whole fly samples allowed me to investigate global effects while dissected intestines were chosen as the midgut is an important region of diet-host interactions. I used female flies as glucose-supplemented food improved both their lifespan and infection response, and their larger size allows for easier dissection.

For dissected intestinal samples, comparison of differentially expressed genes showed 86 genes were upregulated, and 55 genes were downregulated in flies raised on glucose-supplemented food (fold-change > 1.5). While most of the glucose-responsive genes in intestinal samples were uncharacterized CG genes, I found that glucose supplementation altered expression of several metabolic genes **(Figure 3-9B)**. Specifically, many of the downregulated genes were digestive enzymes such as *MalA-4*, a maltase, and *Amyrel*, an amylase. This suggests that glucose supplementation leads to reduced expression of genes involved in digestion of more complex carbohydrates, such as maltose or starch, respectively. Gene ontology (GO) term analysis did not identify statistically significant enrichment of differentially expressed genes for any biological processes. Combined, dietary glucose does not appear to have a large effect on gene expression at the intestinal level, but there is a diet-dependent shift in production of digestive enzymes.

For whole fly samples, comparison of gene expression revealed a larger glucose-dependent effect than in midgut samples. I observed that 527 genes were upregulated, and 454 genes were downregulated (fold-change > 1.5). The top differentially expressed genes showed a diverse effect of glucose that includes increased expression of immune effectors, including *LysX* and *Mtk*, altered metabolic gene expression, with increased expression of *AkhR*, and possible effects on oogenesis, represented by decreased expression of *Cp15*

(Figure 3-9C). GO term analysis of upregulated differentially expressed genes supports the finding of glucose-dependent upregulation of immune gene expression, alongside increased phototransduction and fatty acid synthesis **(Figure 3-10A)**. Notably, analysis of downregulated genes found that glucose lowers expression of many cell cycle genes, and affects several growth and differentiation pathways including the mitogen-activated protein kinase (MAPK) pathway, the target of rapamycin (TOR) and insulin/insulin-like growth factor signaling (IIS) pathways, and Notch signaling **(Figure 3-10B)**. Combined, supplemented glucose drives a systemic downregulation in the expression of growth-related genes, with a concomitant upregulation in immune gene expression. As previous studies suggest that the downregulation of growth promotes longevity, I predict that glucose-supplemented food extends lifespan through the reduction of growth pathway signaling.



B Dissected midguts - Glucose vs Unmodified

TOP UPREGULATED GENES

Gene	Fold-Change	Function
CG17325	4.73	unknown
CG4650	4.05	serine protease
CG13215	3.72	unknown
TwdlT	3.58	cuticle development
CG32369	2.70	unknown
CG13616	2.63	unknown
CG1648	2.56	unknown
CG30281	2.39	unknown
CG34227	2.38	unknown
CG4998	2.35	serine protease
CG15394	2.32	unknown
CG30334	2.25	unknown
CG32751	2.24	unknown
Zip42C.2	2.13	zinc transporter
Gnmt	2.11	glycine N-methyltransferase

TOP DOWNREGULATED GENES

Gene	Fold-Change	Function
Tsf1	-4.43	iron binding
CG8093	-4.32	unknown
Amyrel	-3.76	amylase
CG15533	-2.76	sphingomyelin catabolism
CG14957	-2.68	unknown
Mal-A4	-2.61	maltase
CG6283	-2.50	phospholipase
MtnB	-2.43	metal binding
CG18179	-2.43	chymotrypsin-like
CG14456	-2.43	unknown
CG8997	-2.39	unknown
Jon66Cii	-2.30	chymotrypsin-like
Lip3	-2.25	lipase
GstD10	-2.20	glutathione transferase
MtnD	-2.01	metal binding

C Whole fly - Glucose vs Unmodified

TOP UPREGULATED GENES

Gene	Fold-Change	Function
LysX	19.63	lysozyme
Mtk	13.92	antifungal peptide
CG32368	8.38	unknown
CG13947	7.14	unknown
CG42825	4.66	unknown
CG17224	4.55	uridine phosphorylase
CG7567	4.39	unknown
CG8353	3.92	unknown
Mur29B	3.76	mucin
CG14499	3.70	C-type lectin
CG1648	3.57	unknown
CG3088	3.51	serine protease
AkhR	3.33	Akh receptor
Act88F	3.12	actin
CG7829	3.10	trypsin-like

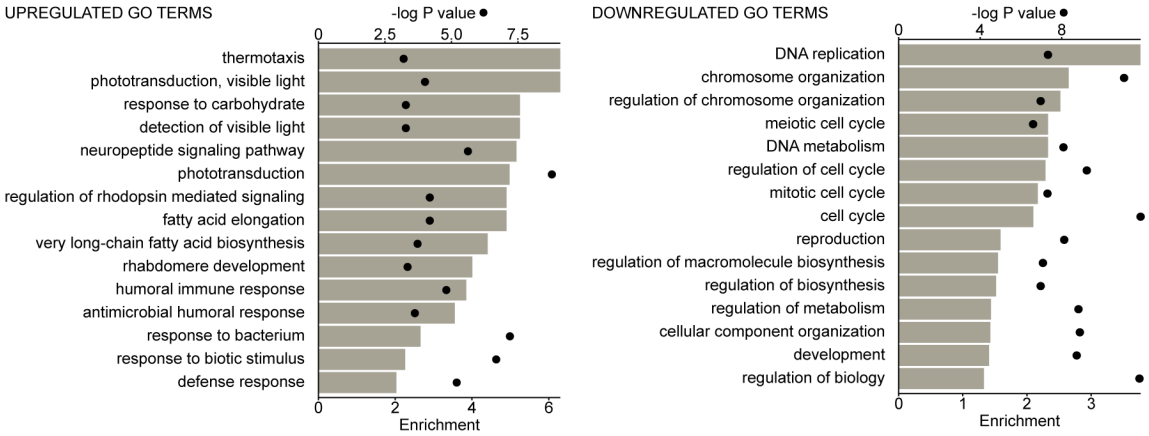
TOP DOWNREGULATED GENES

Gene	Fold-Change	Function
Cp15	-29.37	chorion membrane
Lsp2	-4.01	unknown
CG8093	-3.13	unknown
Hsp70Aa	-3.04	heat shock protein
CG6508	-2.82	unknown
Zdhhc8	-2.60	palmitoyltransferase
hb	-2.59	transcription factor
CG12708	-2.56	unknown
Pcp	-2.51	pupal cuticle
CG9993	-2.44	acyl-CoA synthetase
Rfx	-2.41	transcription factor
Spps	-2.41	transcription factor
Phf7	-2.39	histone binding
lov	-2.38	gravitaxis
how	-2.35	development

Figure 3-9 Microarray comparison of flies raised on glucose-supplemented or unmodified food

(A) Experimental design of microarray experiment. (B) Top differentially expressed genes between intestinal samples comparing flies fed glucose-supplemented or unmodified holidic food. (C) Top differentially expressed genes between whole fly samples comparing flies fed glucose-supplemented or unmodified holidic food.

A Whole fly - Glucose vs Unmodified



B

MAPK SIGNALING	
Gene	Fold-Change
cno	-2.27
ttk	-2.02
S	-1.78
cic	-1.75
jing	-1.75
ksr	-1.69
fs(1)N	-1.67
Src64B	-1.63
dlp	-1.62
Sos	-1.61
Dab	-1.59
slpr	-1.56
PTP-ER	-1.55
fs(1)M3	-1.54
cnk	-1.52
clos	-1.51
Ptp10D	-1.51
htl	1.66
boss	1.69
sty	1.70
grh	1.76
Socs36E	1.88
trol	1.91
Ptth	1.92

TOR/IIS SIGNALING	
Gene	Fold-Change
unk	-1.59
Cul1	-1.55
Tsc1	-1.52
Slob	1.68
Akh	1.73
Ilp6	1.87
Thor	2.08
sug	2.12
AkhR	3.33

NOTCH SIGNALING	
Gene	Fold-Change
dx	-1.87
H	-1.79
Dl	-1.72
RN-tre	-1.66
numb	-1.64
h	1.55
pigs	1.67
bab2	1.69

CELL CYCLE	
Gene	Fold-Change
shtd	-2.00
dup	-1.79
CycE	-1.78
borr	-1.78
stg	-1.67
Wee1	-1.66
CycB	-1.55
CycA	-1.52

Figure 3-10 Glucose decreases growth pathway gene expression

(A) Gene Ontology (GO) analysis from down- or up-regulated differentially expressed genes from comparison of flies raised on glucose supplemented or unmodified holidic food. Bars (bottom x axis) represent enrichment scores and black circles (top x axis) represent -logP values for each enriched GO term. (B) Differentially expressed genes of interest in important growth pathways.

3.2.2.4. Role of growth pathway signaling in glucose-dependent lifespan extension

Blocking or reducing the activity in several growth pathways, including MAPK and TOR/IIS, extends lifespan in *Drosophila* (Clancy et al., 2001; Kapahi et al., 2004; Piper and Partridge, 2017; Slack et al., 2015; Tatar et al., 2001). As microarray analysis showed that flies raised on glucose-supplemented food have decreased expression of many genes associated with cell cycle regulation and growth signaling pathways, I hypothesized that glucose supplementation extends lifespan through reducing growth pathway activity. However, as many growth pathways were affected by glucose-treatment, it was unclear if glucose extends lifespan through a specific pathway or a more global effect on regulation of growth. To address this, I designed a screen in which I activated, or blocked affected growth pathways in important metabolic tissues, and measured lifespan of flies raised on either glucose-supplemented or unmodified holidic food. As MAPK signaling encompasses several pathways that all converge on the Ras protein, including the IIS signaling pathway, I initially expressed a constitutively active form of Ras (Ras^{V12}) in important metabolic regulatory tissues. Due to availability, I first used an insulin-producing cell (IPC)-specific driver (*Ilp3-GAL4*) and a fat body-specific driver (*r4-Gal4*). Strikingly, I found that expression of Ras^{V12} in insulin-producing cells removed the glucose-dependent lifespan extension (**Figure 3-11A)**. Conversely, flies with fat body expression of Ras^{V12} still lived longer on glucose-supplemented food, though the control line *UAS-*Ras^{V12}*/+* failed to live significantly longer on glucose-supplemented food **(Figure 3-11B)**. The result that the control *UAS-*Ras^{V12}*/+* flies did not live significantly longer on glucose-supplemented food is concerning and may indicate a genetic background effect in this fly stock (Chandler et al., 2013). As there may be potential background effects in the *UAS-*Ras^{V12} line, I was curious if activating or blocking MAPK signaling in IPCs through other components would also remove the glucose-dependent lifespan extension.

In *Drosophila* MAPK signaling, at least eight receptor tyrosine kinases (RTK) signal through Ras, including Epidermal growth factor receptor (Egfr) and Heartless (Htl). Based on reagent availability, I initially blocked either Egfr or Htl signaling in IPCs through expression of dominant-negative versions of these RTKs. I found that blocking either Egfr **(Figure 3-11C)** or Htl removed the glucose-dependent lifespan extension. This was an unexpected result as the activation of Ras and inhibition of two RTKs in IPCs all appeared

to remove the glucose-dependent lifespan extension. As glucose affected expression of other growth and differentiation pathways, I also blocked the TOR pathway and Notch signaling in IPCs. I found that both the expression of a dominant-negative Tor or Notch RNAi also removed the glucose-dependent lifespan extension. Combined, these data reveal a potential technical limitation in this genetic screen as both the *UAS-Ras*^{V12} and the *Ilp3-GAL4* fly stocks may have a genetic background that affects the outcome of these lifespan studies, such as the transgene possibly being inserted into a gene that affects lifespan, for example. While glucose supplementation may impact lifespan through decreased expression of growth pathway genes, a future genetic screen for lifespan effects will require crossing transgenic genes into the same genetic background to avoid potential confounding background effects (Chandler et al., 2013).

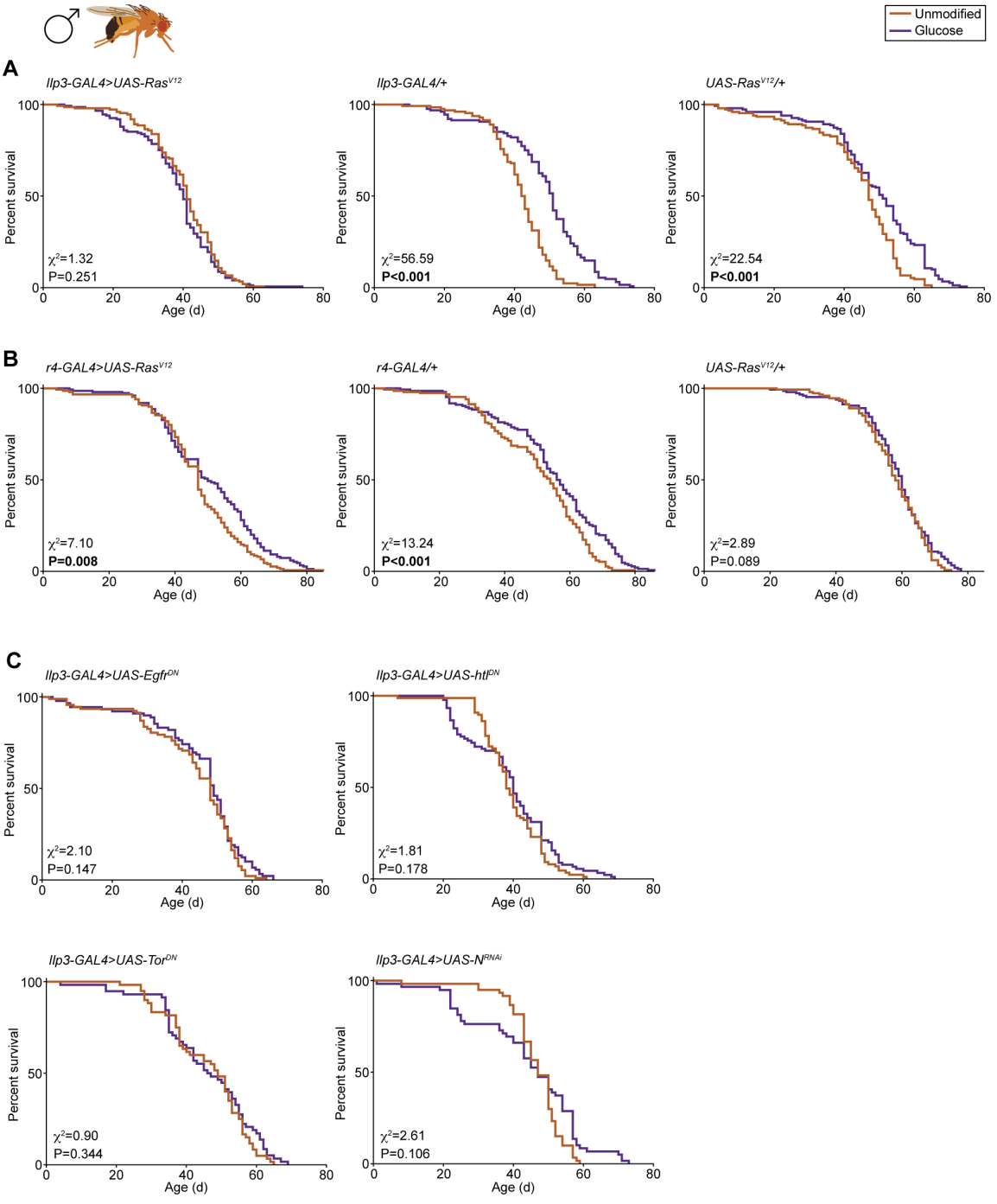


Figure 3-11 Role of growth pathway signaling in glucose lifespan extension

(A) Survival curves of male *Ilp3-GAL4>UAS-Ras^{V12}*, *Ilp3-GAL4/+*, or *UAS-Ras^{V12}/+* fed glucose-supplemented or unmodified holidic food. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test. (B) Survival curves of male *r4-GAL4>UAS-Ras^{V12}*, *r4-GAL4/+*, or *UAS-Ras^{V12}/+* fed a glucose-supplemented or unmodified holidic diet. Significance compared to unmodified diet determined by log-rank

(Mantel–Cox) test. (C) Survival curves of male *Ilp3-GAL4>UAS-Egfr*^{DN}, *Ilp3-GAL4>UAS-htl*^{DN}, *Ilp3-GAL4>UAS-Tor*^{DN}, and *Ilp3-GAL4>UAS-N*^{RNAi} flies fed a glucose-supplemented or unmodified holidic diet. Significance compared to unmodified diet determined by log-rank (Mantel–Cox) test.

3.3. Discussion

In this chapter, I investigated several aspects of overall health and nutrition and found that relatively modest dietary modifications exert profound impacts on the lifespan, immune response, and microbial composition of the host. Of the five dietary modifications tested, I found that the supplementation with glucose emerged as the most beneficial manipulation, with effects that included an extended lifespan and enhanced immunity against an enteric pathogen. I was particularly intrigued by the relationship between diet, the microbiota, longevity and immunity, as this issue has not been tackled in a systematic study to date. I found that dietary supplementation of glucose greatly increased the diversity of the intestinal microbiota. However, when I eliminated the microbiota from flies, I found that the health benefits of increased glucose were largely independent of the microbiota. Combined, my observations established that elevated levels of dietary glucose provide numerous benefits to fly health and immunity, and that these benefits do not require an intestinal microbiota.

Since I observed benefits from glucose independent of the microbiota, I was interested in the host response to glucose that extends lifespan. Microarray analysis suggested that glucose may extend lifespan through reduction of growth pathway expression. As reduced activity of growth pathways is an established regulator of *Drosophila* lifespan, I hypothesized that flies raised on glucose-supplemented food live longer through a similar mechanism. However, I was unable to establish a concrete connection between growth pathways and glucose-dependent lifespan extension. A more extensive analysis is required to determine the involvement of growth pathway regulation.

In summary, this chapter showed that glucose supplementation significantly affected immunity and microbiota diversity. Despite the links between the intestinal microbiota and animal health, I established that glucose acts independent of the microbiota to increase lifespan and responses to *V. cholerae* infections. As physiological responses to diet are extensively conserved throughout the animal kingdom, I believe my findings may be of relevance to a general appreciation of the relationship between glucose consumption and animal health.

Chapter 4

Glucose extends lifespan through enhanced intestinal barrier integrity

Portions of this chapter have been submitted for review as:

Galenza A and Foley E. (2020) Glucose extends lifespan through enhanced intestinal barrier integrity in *Drosophila*. Under review at *Experimental Gerontology*.

4. Glucose extends lifespan through enhanced intestinal barrier integrity

4.1. Introduction

In the previous chapter, I investigated how different dietary modifications to the defined holidic diet impacted *Drosophila* health, lifespan, and survival from infection. Notably, I found that glucose-supplemented holidic food extends lifespan, particularly in males, through a host-intrinsic mechanism independent of the intestinal microbiota. My findings suggest that this mechanism may involve downregulation of growth and cell cycle signaling. However, due to limitations in my experimental approach, I was unable to precisely address this hypothesis.

Prior to this point, my studies involved both males and females raised on several modified holidic diets and examining several aspects of healthspan and longevity. Here, I decided to focus my efforts specifically on asking how glucose-supplemented food extends lifespan in males. Previous research on how sugar impacts lifespan has been mixed and studies are difficult to compare due to different types of diets, variable amounts of sugar, and different types of sugar being added. As the holidic diet was designed to facilitate comparison between labs, I aimed to address this question through comparison of male flies raised on either glucose-supplemented or unmodified holidic food.

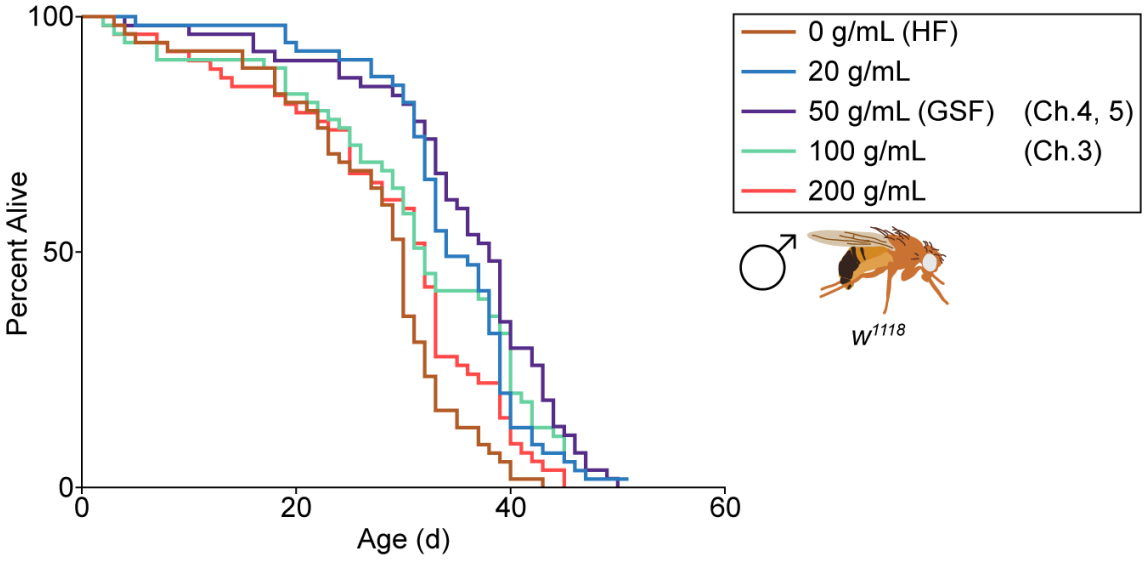
In this chapter, I investigated how glucose extends lifespan in male w^{1118} flies. I initially optimized the amount of supplemental glucose for lifespan extension, then tested potential mechanisms of lifespan extension. I found that glucose-supplemented food extends lifespan independent of calorie or insulin activity. Instead, I found that flies raised on glucose-supplemented food have increased expression of intestinal-associated cell junction proteins and improved intestinal barrier integrity with age. Chemical disruption of the intestinal barrier removed the glucose-dependent lifespan extension, suggesting that glucose-supplemented food extends lifespan through enhanced intestinal barrier integrity.

4.2. Results

4.2.1. Macronutrient comparison of flies raised on glucose-supplemented or unmodified holidic food

4.2.1.1. Optimization of glucose dependent longevity extension

In chapter 3, I found that glucose-supplemented (100 g/L) holidic food extends the lifespan of adult *Drosophila* compared to unmodified holidic food, particularly in males. As prolonged consumption of sugar-rich food has often been associated with diminished fly health and lifespan outcomes, I asked how the addition of glucose extends longevity of flies. Before addressing this question, I first tested a range of glucose concentrations to identify the optimal amount required for increased longevity. Specifically, I measured longevity of wild-type (w^{1118}) male flies raised on holidic food that I supplemented with 0 g/L (unmodified), 20 g/L, 50 g/L, 100 g/L, or 200 g/L glucose. I found that the addition of 50 g/L glucose had the greatest effect, leading to a 27% increase in median lifespan compared to unmodified food **(Figure 4-1)**. Thus, for the remainder of this chapter, I compared the effects of **50 g/L glucose-supplemented holidic food (GSF)** to **unmodified holidic food (HF)** on health and longevity.



Glucose (g/L)	n	Median	P	χ^2
0	55	30	-	-
20	55	34	**<0.001**	23.19
50	54	38	**<0.001**	34.00
100	55	32	**<0.001**	11.90
200	54	32	**0.034**	4.48

Figure 4-1 Longevities on glucose-supplemented holidic food

Survival curve of w^{1118} male flies raised on holidic food supplemented with glucose ranging from 0 g/L to 200 g/L. (HF: unmodified holidic food, GSF: 50 g/L glucose-supplemented holidic food). Significance compared to 0 g/L glucose (HF) diet determined by log-rank (Mantel-Cox) test.

4.2.1.2. Macronutrient comparison between flies raised on GSF or HF

With the change from 100 g/L to 50 g/L of supplemental glucose in my experimental diet, I initially asked how this new level of added glucose affects metabolism by comparing weight and macronutrient content in w^{1118} male flies raised on HF or GSF for 20 or 40 days. I found no difference in weight at day 20 or 40 **(Figure 4-2A)**. Likewise, protein levels remained comparable between flies raised on GSF or HF at both time points **(Figure 4-2B)**. When I measured total glucose, I found that flies raised on GSF had higher levels of glucose at day 40 **(Figure 4-2C)**. Similarly, I found that triglyceride levels were significantly higher at day 40 in GSF-treated flies than in HF-treated flies **(Figure 4-2D)**.

With no corresponding weight change, flies raised on GSF appear to maintain higher energy stores with age. These data for flies raised on GSF parallel what we previously found in flies raised on 100 g/L supplemental glucose.

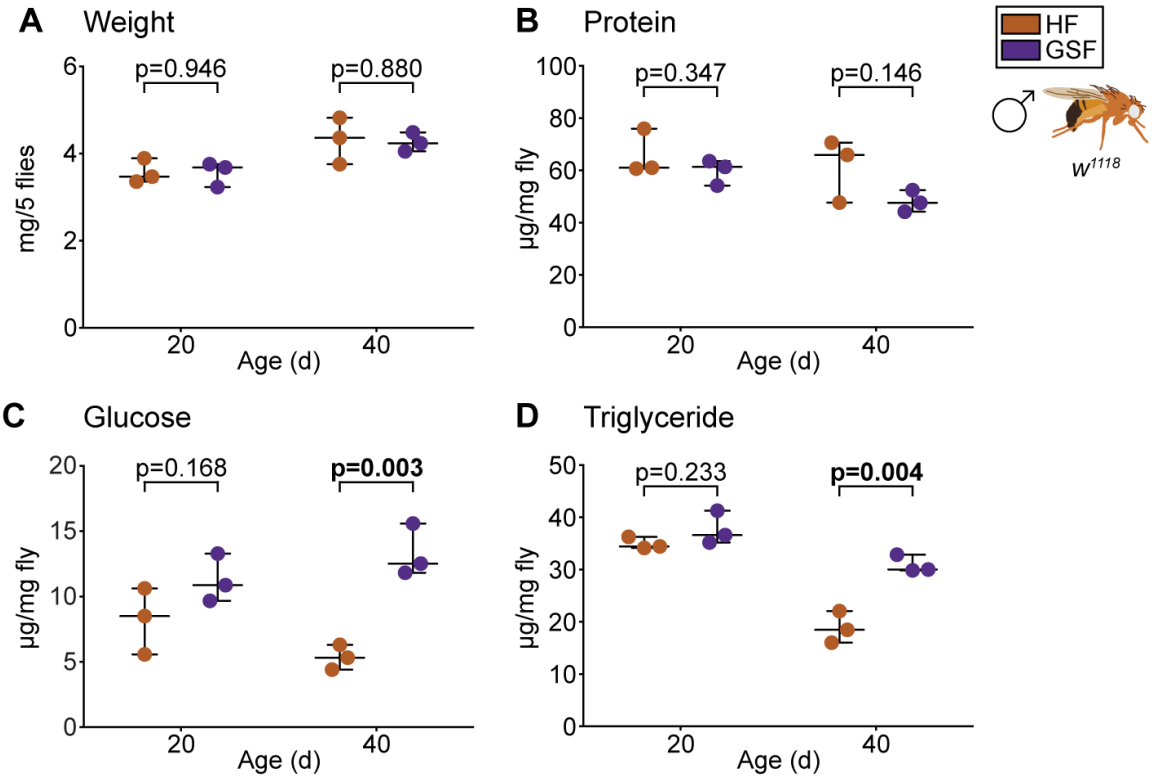


Figure 4-2 Macronutrient comparison between flies raised on GSF or HF

(A-D) Quantification of (A) weight, (B) protein, (C) glucose, and (D) triglycerides in w^{1118} flies raised on glucose-supplemented food (GSF) versus unmodified holidic food (HF) for 20 or 40 days (n = 3). Each dot represents 5 flies. Significance compared to HF determined by Student's T-test.

As GSF elevated total glucose content, I asked if GSF also impacted levels of circulating glucose and trehalose, the primary blood sugar in adult flies. I raised male flies on either HF or GSF for 20 or 40 days, extracted hemolymph, and measured circulating sugars. I found that flies raised on GSF had elevated total circulating sugars at day 40 compared to flies raised on HF **(Figure 4-3A)**. Looking at the component circulating sugars, this difference is likely attributable to increased free glucose, a 5.9-fold increase **(Figure 4-**

3B), with limited effect on trehalose levels, a 1.3-fold increase **(Figure 4-3C)**. Similarly, larvae fed a high-sucrose diet (20% compared to 5% control) have increased circulating glucose but not trehalose (Ugrankar et al., 2015). These data suggest that trehalose levels may not be responsive to dietary change. Alternatively, a recent study linked feeding behavior to levels of circulating glucose but not trehalose (Ugrankar et al., 2018). Specifically, aversion to feeding in larvae elevated circulating glucose, but did not affect trehalose. Perhaps flies raised on GSF have different feeding behavior than those raised on HF.

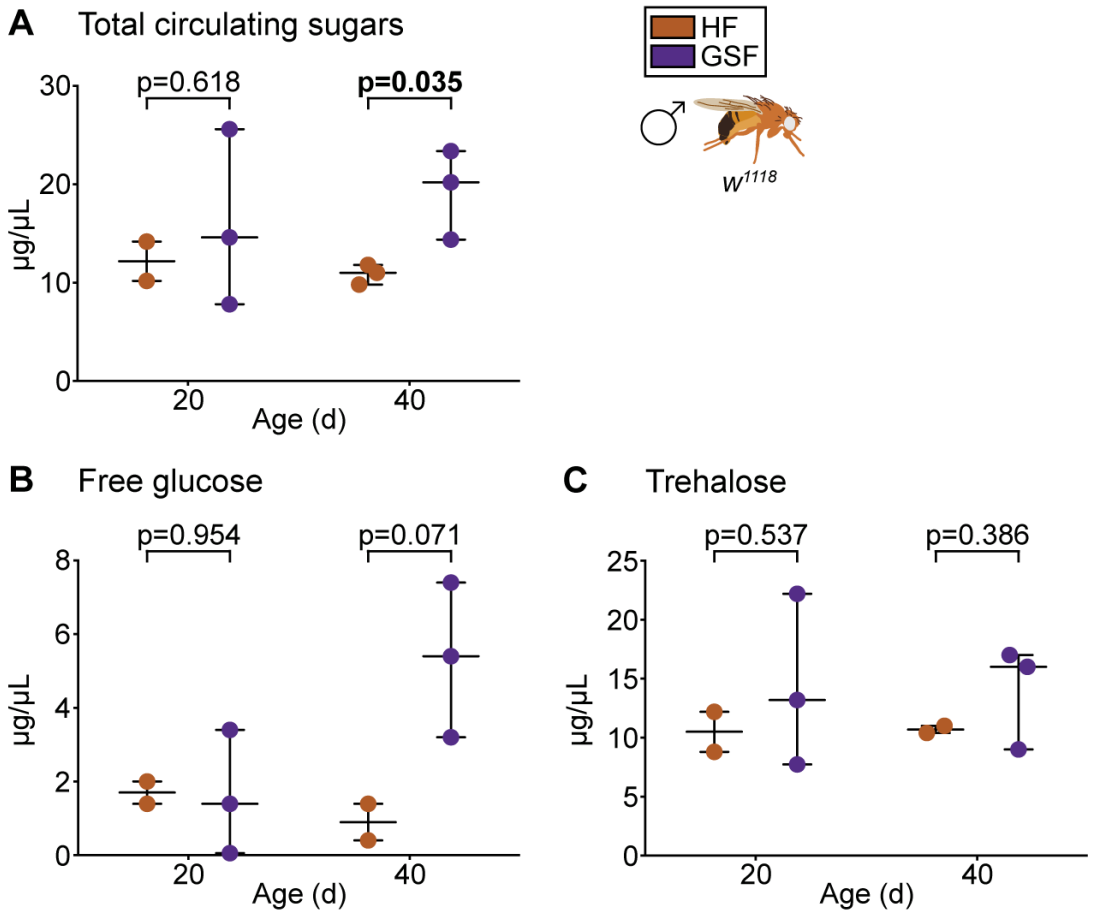


Figure 4-3 Circulating sugar comparison between flies raised on GSF or HF

(A-C) Quantification of (A) total circulating sugars, (B) free glucose, and (C) trehalose in w^{1118} flies raised on GSF versus HF for 20 or 40 days (n = 2-3). Statistical significance compared to HF determined by Student's T-test.

4.2.2. Comparison of feeding behavior between flies raised on GSF or HF

4.2.2.1. No difference in feeding behavior over a short time interval

As my flies are fed *ad libitum*, I did not know if GSF-dependent effects on macronutrients and circulating sugars were an indirect result of changes in feeding. I consider this an important question to address, as calorie intake and feeding frequency are linked to lifespan in several experimental organisms, including flies (Fontana and Partridge, 2015).

To measure feeding frequency, I used the fly Proboscis and Activity Detector (flyPAD), an automated monitoring device for feeding behavior analysis (Itskov et al., 2014). The flyPAD records changes in capacitance that occur when a fly's proboscis makes contact with food in the center of an arena and can be used to count individual sips; bursts, which are clusters of sips; and bouts, which are clusters of bursts **(Figure 4-4A)**. The flyPAD uses solid food and allows precise recording of a fly's interaction with food. For this assay, I raised male flies on either HF or GSF for 20 days, then starved them for 2 hours prior to feeding in a flyPAD arena for 1 hour with their respective diet. I saw no difference in sips, bursts, or bouts **(Figure 4-4B)** between flies raised on HF or GSF, suggesting that GSF does not significantly alter feeding behavior over short periods.

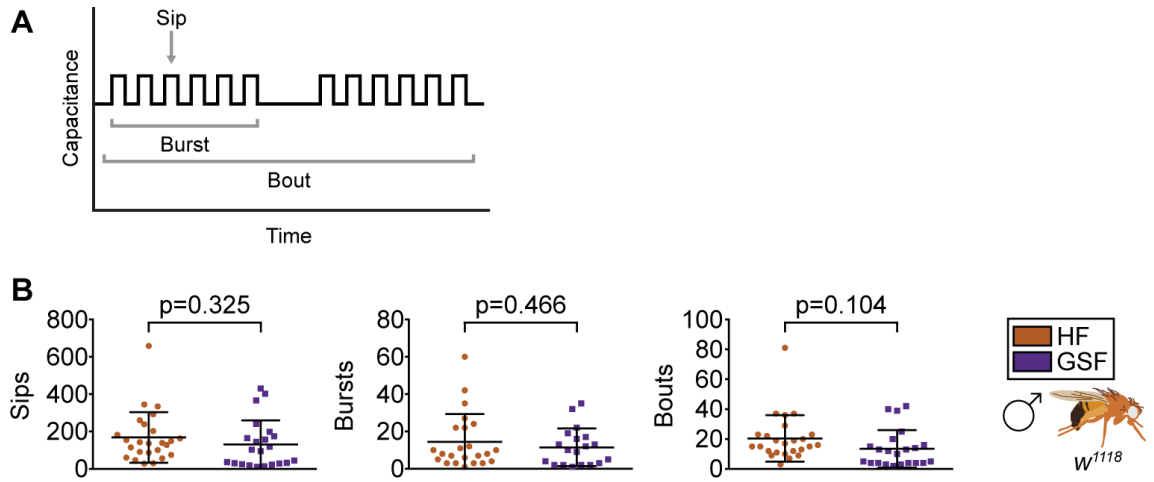


Figure 4-4 Comparison of feeding behavior of flies raised on GSF or HF by flyPAD

(A) Illustration of temporal capacitance measurement with the flyPAD device. of feeding terms sips, bursts, and bouts. (B) Quantification of number of sips, duration of feeding bursts, and total feeding bouts in 20-day old w^{1118} flies raised on glucose-supplemented food (GSF) versus unmodified holidic food (HF) using a flyPAD. Statistical significance compared to HF determined by Student's T-test.

4.2.2.2. Flies fed GSF consume more calories than flies fed HF

To determine if GSF impacts feeding behavior over longer timeframes, I used the capillary feeding (CAFE) assay, to calculate food consumption across three days (Ja et al., 2007). In the CAFE assay, flies are fed through capillary tubes that allow me to quantify liquid food consumption. I raised male flies on HF or GSF for 20 days before transfer to the CAFE setup, with 10 vials of 10 flies/vial, for a 3-day period, where flies were fed a liquid version of their respective food. I found that flies raised on HF consumed a greater volume than those raised on GSF, about a 1.2-fold daily increase **(Figure 4-5A)**. As I knew the precise composition of the liquid HF (P:C of 1:1.6) and GSF (P:C of 1:6.3), I could calculate the total calories consumed, as well as the contribution to total calories from either carbohydrates or protein sources. I found that GSF-treated flies had a 2.3-fold increase in calorie intake compared to HF-treated flies **(Figure 4-5B)**. The increased calorie intake is a result of elevated carbohydrate consumption, as flies raised on GSF consumed approximately 3.2-fold more calories from carbohydrates per day than their counterparts

raised on HF **(Figure 4-5C)**. Conversely, amino acids provided approximately 20% fewer calories to flies raised on GSF than on HF **(Figure 4-5D)**. Together, these data indicate that flies raised on GSF are not caloricially restricted, in fact, they consume significantly more calories in the form of carbohydrate. However, as diets with a low protein to carbohydrate ratio have been found to extend lifespan (Fontana and Partridge, 2015; Lee et al., 2008; Simpson and Raubenheimer, 2009; Solon-Biet et al., 2015b, 2014), the lower protein intake of flies raised on GSF may contribute to their longer lifespan .

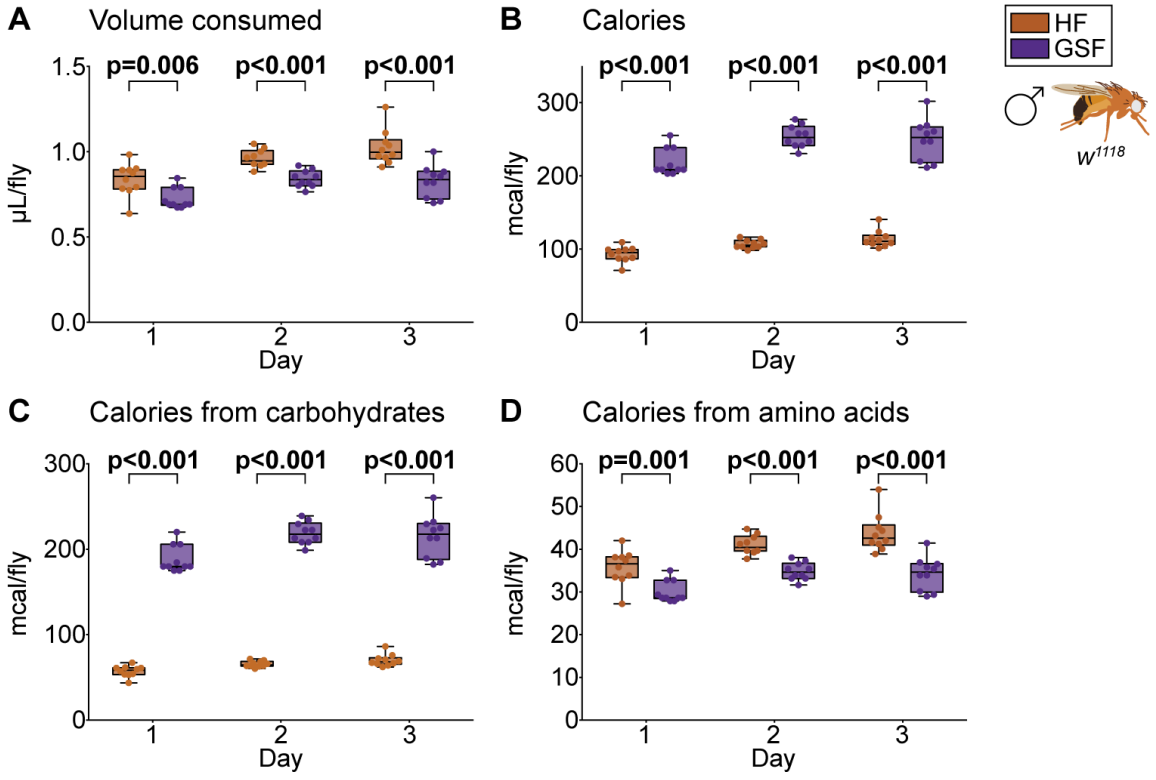
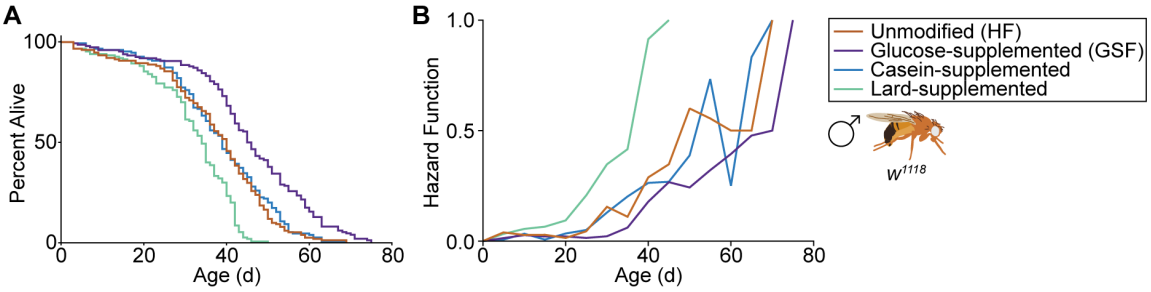


Figure 4-5 Comparison of feeding behavior of flies raised on GSF or HF by CAFE

(A-D) Quantification of liquid food consumption in 20-day old w^{1118} flies raised on GSF versus HF using a CAFE measuring (A) volume consumed, (B) total calories, (C) calories from carbohydrates, and (D) calories from amino acids (AA). Statistical significance compared to HF determined by Student's T-test.

4.2.2.3. Higher calories are not sufficient to extend lifespan on holidic food

To test if the lifespan extension observed for flies raised on GSF is simply a consequence of feeding adults a higher calorie food, I measured the lifespans of male flies raised on modified holidic food isocaloric to GSF, where extra energy was provided either from lard, or casein. These additions provide extra energy from either a fat source or protein source respectively. As expected, flies raised on GSF lived significantly longer than their counterparts on HF **(Figure 4-6A)**. In contrast, casein-supplemented holidic food had no detectable effects on lifespan, whereas lard-supplemented holidic food shortened lifespan, and significantly increased the risk of early death **(Figure 4-6B)**. Thus, simply adding extra calories to HF does not extend longevity, indicating that GSF extends lifespan through a more specific mechanism.



Diet	Cal./L	Med.	P	χ^2
Unmodified (HF)	111.3	40	-	-
Glucose-supplemented (GSF)	311.8	45.5	**<0.001**	32.62
Casein-supplemented	311.8	39	0.475	0.51
Lard-supplemented	311.8	34	**<0.001**	39.25

Figure 4-6 Longevities on isocaloric modified holidic diets

(A) Survival curve and (B) hazard function of w^{1118} male flies raised on HF, GSF, casein-supplemented food, or lard-supplemented food. Med.: Median lifespan. Statistical significance compared to HF determined by log-rank (Mantel-Cox) test shown in table.

4.2.3. GSF extends lifespan independent of insulin activity

4.2.3.1. GSF suppresses insulin production

As I observed increased total and circulating glucose in flies that I raised on GSF, I wondered what effects GSF has on the insulin pathway, a known modifier of longevity (Clancy et al., 2001; Tatar et al., 2003) . To answer this question, I quantified transcription of the insulin-like peptides (Ilp) *ilp2*, *ilp3*, and *ilp5*, in male flies raised on HF or GSF for 20 or 40 days. These three Ilps have a similar function to mammalian insulin in the adult fly, and are primarily expressed in insulin-producing cells in adult flies (Nässel and Broeck, 2015). I found that expression of *ilp2* and *ilp5* was lower in 40-day old flies raised on GSF compared to flies raised on HF **(Figure 4-7A, C)**, while the expression of *ilp3* was unaffected **(Figure 4-7B)**. Though the Ilps share some functional redundancy, they display diverse functions that are not fully understood (Grönke et al., 2010; Kannan and Fridell, 2013). Interestingly, feeding triggers expression of *ilp2* and *ilp5*, but not *ilp3* (O'Brien et al., 2011). However, *ilp* gene expression is complex, and does not necessarily reflect amount of peptide in storage, or in circulation (Nässel and Broeck, 2015). Ilps are stored in vesicles within insulin-producing cells and released into the hemolymph in response to regulatory signals. Thus, I used an ELISA to quantify total, and circulating amounts of FLAG and HA epitope-tagged Ilp2 (Ilp2-FH) in flies raised on HF or GSF. In this fly line, Ilp2-FH expression is controlled by the *ilp2* promoter, and accurately reports Ilp2 protein levels (Park et al., 2014). I raised male *Ilp2-FH* flies on either HF or GSF for 40 days, extracted hemolymph, and measured both total and circulating levels of ILP2-FH. I observed significantly lower total amounts of Ilp2-FH in GSF-treated flies compared to age-matched HF-treated controls **(Figure 4-7D)**. However, I did not detect food-specific effects on levels of circulating Ilp2-FH **(Figure 4-7E)**. Combined, these data suggest that Ilp production is slightly suppressed by GSF-treatment. However, it is unclear if lowered Ilp production in flies raised on GSF impacts the functional insulin response or insulin sensitivity.

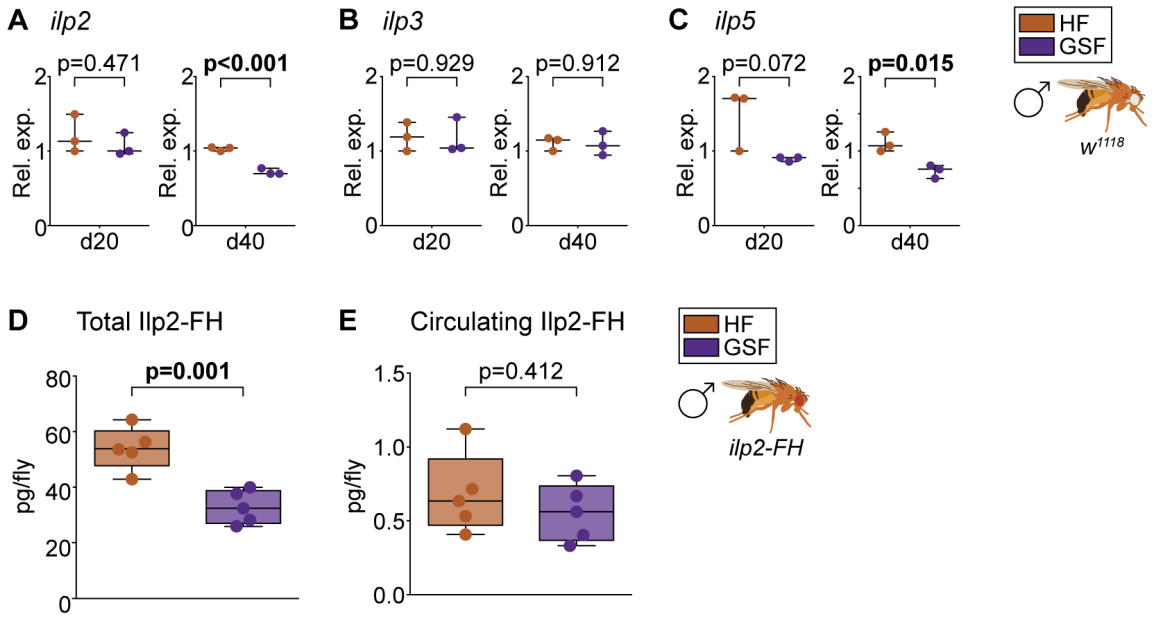


Figure 4-7 GSF suppresses insulin production

(A-C) Quantification of the relative expression of (A) *ilp2*, (B) *ilp3*, and (C) *ilp5* in w^{1118} flies raised on glucose-supplemented food (GSF) versus unmodified holidic food (HF) for 20 or 40 days. (D-E) Quantification of (D) total and (E) circulating Ilp2-FH in *ilp2-FH* flies raised on GSF versus HF for 20 days. Statistical significance compared to HF determined by Student's T-test.

4.2.3.2. GSF does not impair insulin function

To determine if GSF-dependent shifts in insulin peptide expression translate into effects on insulin activity, I measured starvation resistance and oral glucose tolerance in flies raised on HF and GSF. In flies, insulin impairs starvation resistance (Oldham et al., 2002; Post et al., 2018), and improves glucose tolerance (Haselton et al., 2010). Thus, I expect that any effects of GSF on insulin signaling will have measurable impacts on starvation resistance or glucose tolerance. Specifically, if GSF decreases insulin activity, then I expect to observe improved survival in a starvation assay, and a delayed response in an OGTT. For starvation assays, I raised flies on HF or GSF for 15 or 30 days, and measured survival after switching to nutrient-deficient medium (1% agar in water). For both time points, I did not detect food-dependent effects on starvation resistance **(Figure 4-8)**.

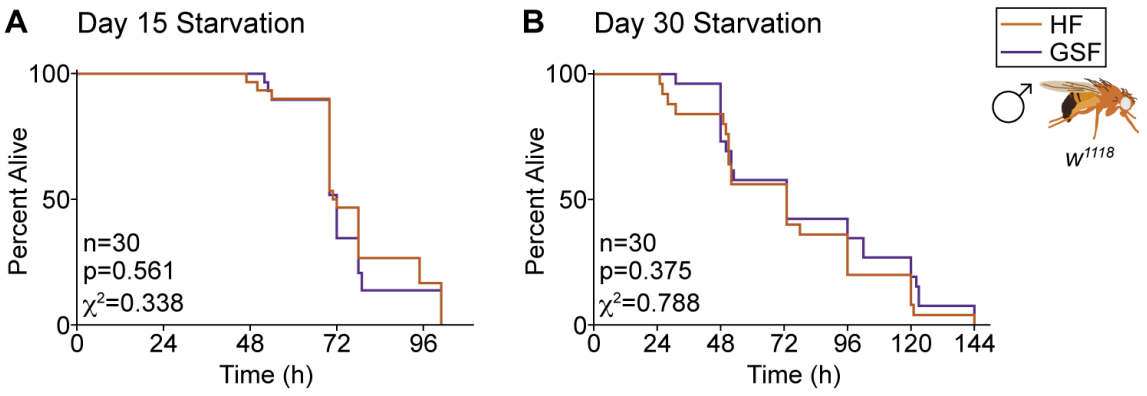
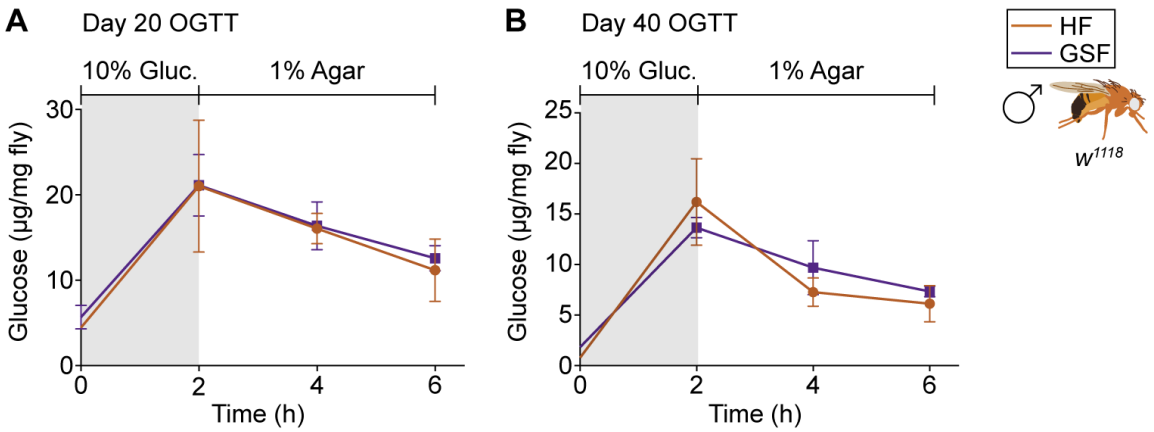


Figure 4-8 Starvation of flies raised on GSF or HF

(A-B) Survival curve upon starvation of w^{1118} flies raised on GSF versus HF for (A) 15 or (B) 30 days. Statistical significance compared to HF determined by log-rank (Mantel-Cox) test.

For the oral glucose tolerance test (OGTT) I raised flies on HF or GSF for 20 or 40 days, followed by a 16h fast, prior to a 2h *ad libitum* feed on a 10% glucose medium, followed by a period of re-fasting. I quantified total glucose in flies following the initial fast (0h), after feeding on 10% glucose (2h), and twice during the re-fast period (4h, 6h). In insulin-sensitive flies, glucose levels rise during feeding, and drop during the fast, due to insulin-dependent stimulation of glucose uptake. Excluding the initial 0h fasted measurement of 40-day old flies, I found that flies raised on either GSF or HF processed glucose with equal efficiency at all time points in both ages, **(Figure 4-9)**, arguing that GSF does not significantly impair insulin sensitivity as the flies age. Despite lower insulin production, the functional insulin response of flies raised on GSF appears similar to their counterparts raised on HF, even in older flies. These data suggest that GSF does not reduce insulin activity to an extent that may account for the large GSF-dependent lifespan extension.



Time Point	P
0h (Fasted)	0.130
2h (Fed)	0.979
4h (Re-fasted)	0.839
6h (Re-fasted)	0.450

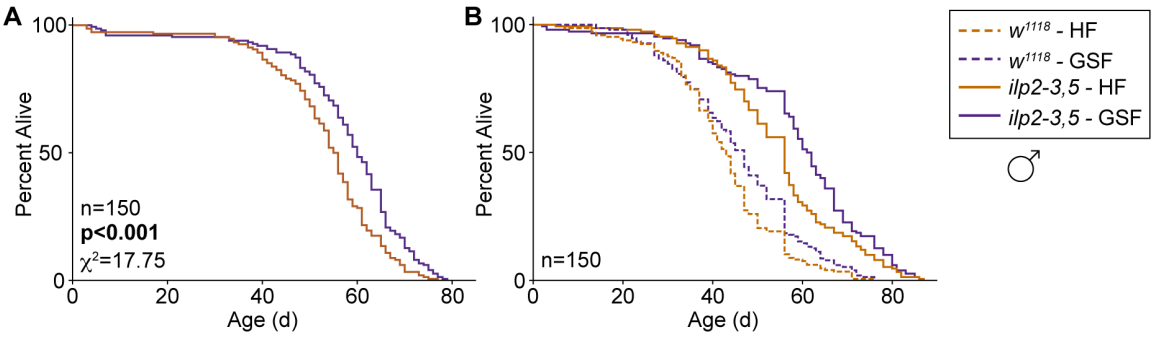
Time Point	P
0h (Fasted)	**0.018**
2h (Fed)	0.290
4h (Re-fasted)	0.161
6h (Re-fasted)	0.248

Figure 4-9 Oral glucose tolerance of flies raised on GSF or HF

(A-B) Oral glucose tolerance test (OGTT) performed on w^{1118} flies raised on GSF versus HF for (A) 20 or (B) 40 days. Statistical significance compared to HF determined by Student's T-test at each time point.

4.2.3.3. Insulin is not required for GSF-dependent lifespan extension

As GSF did not appear to impair the functional insulin response, I asked if insulin is required for the GSF-dependent lifespan extension. To test this, I measured the lifespans of HF and GSF-treated *ilp2-3,5* mutant flies. These triple mutants are deficient for insulin signaling and outlive wild-type controls. Thus, if insulin signaling is required for GSF-mediated extension of lifespan, I predict that *ilp2-3,5* mutants will not benefit from being raised on GSF. Contrary to my hypothesis, *ilp2-3,5* mutants raised on GSF significantly outlived *ilp2-3,5* mutants raised on HF **(Figure 4-10A)**. I confirmed this finding in a replicate assay alongside w^{1118} controls **(Figure 4-10B)**. Thus, although GSF has effects on the expression of two insulin-like peptide genes, I did not detect GSF-dependent effects on insulin activity, or on the survival of insulin-deficient flies, suggesting that GSF extends life through insulin-independent means.



Group	Median	χ^2	P
w^{1118} - HF	43		
w^{1118} - GSF	47	5.889	**0.015**
ilp2-3,5 - HF	56		
ilp2-3,5 - GSF	61.5	8.127	**0.004**

Figure 4-10 Longevity of insulin mutants on GSF or HF

(A) Survival curve of male *ilp2-3,5* mutants raised on GSF or HF. (B) Survival curve of male *ilp2-3,5* mutants and w^{1118} flies raised on GSF or HF. Statistical significance compared to HF determined by log-rank (Mantel-Cox) test.

4.2.4. RNA-Seq comparison of flies raised on GSF or HF

4.2.4.1. GSF-treated flies have increased expression of cell junction genes

As I found that GSF extends lifespan independent of caloric intake or insulin activity, I sought an unbiased method to investigate how GSF extends longevity. To address this, I used RNA sequencing (RNA-Seq) to compare transcription in whole flies raised on GSF to flies raised on HF. Whole flies were chosen as GSF may have a systemic effect and I did not want to make assumptions about where the effects of GSF are realized. I raised w^{1118} male flies on either HF or GSF for 20 days, then isolated total RNA from whole fly samples for RNA-Seq. Principal component (PC) analysis showed that samples grouped with their replicates **(Figure 4-11A).** When I looked at differential gene expression, I found 488 upregulated genes and 555 downregulated genes in GSF-fed flies compared to HF-fed controls **(Figure 4-11B)**. Gene ontology (GO) analysis of downregulated processes showed that GSF primarily leads to a decline in the expression of genes required for metabolism, and energy use **(Figure 4-11C)**. In particular, I noticed significant decreases

in the expression of genes involved in gluconeogenesis and lipid catabolism **(Figure 4-11C)**, likely a result of the increased availability of glucose as an energy source, and consistent with my observation that flies raised on GSF have elevated triglyceride stores relative to HF-treated counterparts **(Figure 4-2D)**.

In contrast to the dominance of metabolic terms among downregulated GO terms, I found that GSF enhanced the expression of genes involved in a number of distinct cellular processes, including immunity, cell adhesion, and cell mobility **(Figure 4-11C)**. In fact, many of the genes with the highest GSF-dependent changes in gene expression encode antimicrobial peptides such as *attacins* and *diptericins* **(Figure 4-11B** and **Figure 4-12A)**. Interestingly, I observed a similar phenomenon in microarray analysis comparing 10-day old female flies raised on 100 g/L glucose-supplemented holidic food compared to females raised on unmodified holidic food **(Figure 3-10)**. Within the list of enriched GO terms for upregulated genes, I was struck by increased expression of several genes associated with cell-cell junctions **(Figure 4-11C** and **Figure 4-12B)**. Cell-cell junctions are critical for maintenance of epithelial structures, particularly in the intestinal tract, where barrier damage is linked to mortality (Rera et al., 2012). As I used whole fly samples, I was interested in where these differentially expressed genes are typically expressed in an adult fly. To investigate this, I used the online resource, FlyAtlas 2, which reports tissue-specific enrichment of *Drosophila* genes based on RNA-Seq data (Leader et al., 2018). When I used FlyAtlas 2 to identify tissues that prominently express GSF-responsive immunity genes, I found high expression in the intestinal tract and nervous system, with immune effectors particularly highly expressed in the fly head **(Figure 4-12A)**. Similarly, for GSF-responsive cell-cell junction genes I noted that a substantial number of these genes are highly expressed in the intestinal tract, particularly septate junction genes, as well as in the neuronal system **(Figure 4-12B)**.

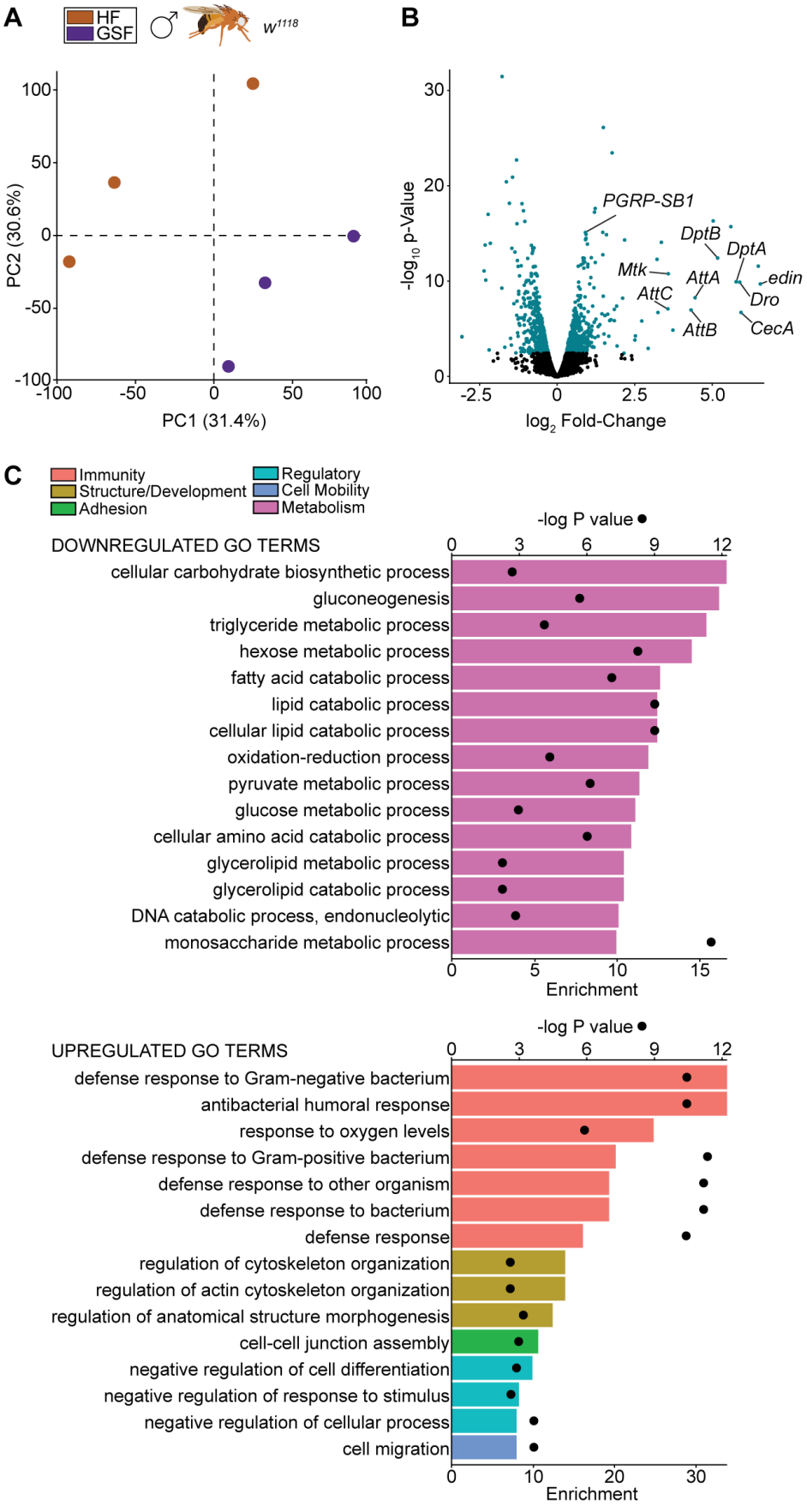

A
HF
GSF
♂
w1118
PC2 (30.6%)
PC1 (31.4%)
B
-log10 p-Value
log2 Fold-Change
PGRP-SB1
DptB
DptA
Mtk
AttA
edin
AttC
Dro
AttB
CecA
C
Immunity
Structure/Development
Adhesion
Regulatory
Cell Mobility
Metabolism
DOWNREGULATED GO TERMS
-log P value
cellular carbohydrate biosynthetic process
gluconeogenesis
triglyceride metabolic process
hexose metabolic process
fatty acid catabolic process
lipid catabolic process
cellular lipid catabolic process
oxidation-reduction process
pyruvate metabolic process
glucose metabolic process
cellular amino acid catabolic process
glycerolipid metabolic process
glycerolipid catabolic process
DNA catabolic process, endonucleolytic
monosaccharide metabolic process
Enrichment
UPREGULATED GO TERMS
-log P value
defense response to Gram-negative bacterium
antibacterial humoral response
response to oxygen levels
defense response to Gram-positive bacterium
defense response to other organism
defense response to bacterium
defense response
regulation of cytoskeleton organization
regulation of actin cytoskeleton organization
regulation of anatomical structure morphogenesis
cell-cell junction assembly
negative regulation of cell differentiation
negative regulation of response to stimulus
negative regulation of cellular process
cell migration
Enrichment

Figure 4-11 RNA-Seq comparison of flies raised on GSF or HF

(A) Principal component (PC) analysis of RNA-sequencing data comparing 20-day old male flies raised on GSF or HF. Each dot represents one replicate (B) Volcano plot of differentially expressed genes from comparison of flies raised on GSF versus HF. Each dot represents a single gene. Teal indicates $p < 0.01$, FDR < 0.05. (C) Gene Ontology (GO) analysis from down- or up-regulated differentially expressed genes from comparison of flies raised on GEF versus HF. Bars (bottom x axis) represent enrichment scores and black circles (top x axis) represent -logP values for each enriched GO term.

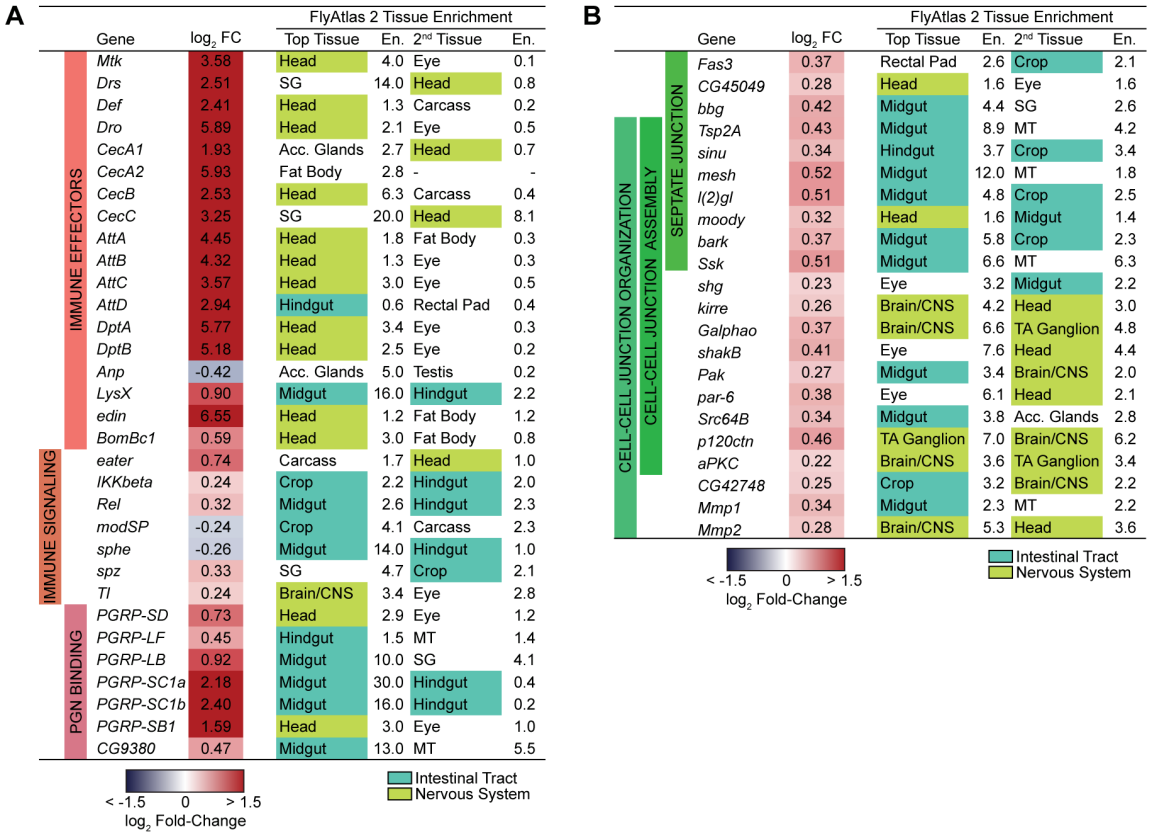


Figure 4-12 Differentially expressed genes of interest between flies raised on GSF or HF

(A-B) Differentially expressed ($p < 0.05$) (A) immune-related genes or (B) cell junction genes from RNA-Seq comparison of flies raised on GSF versus HF. Tissue enrichment is shown for tissues with the first and second highest enrichment scores based on FlyAtlas2 output of these genes.

To verify the expression pattern of these genes of interest, I compared transcription by RT-qPCR of representative septate junction and immune effector genes in whole flies, dissected heads, and dissected midguts from 20-day old male flies raised on HF. Immune effector genes were not expressed significantly different between tissues **(Figure 4-13A)**. Conversely, for cell junction genes I noted enriched expression in the intestinal tract relative to whole flies, or dissected heads **(Figure 4-13B)**, raising the possibility that GSF impacts organization of the gut epithelial barrier.

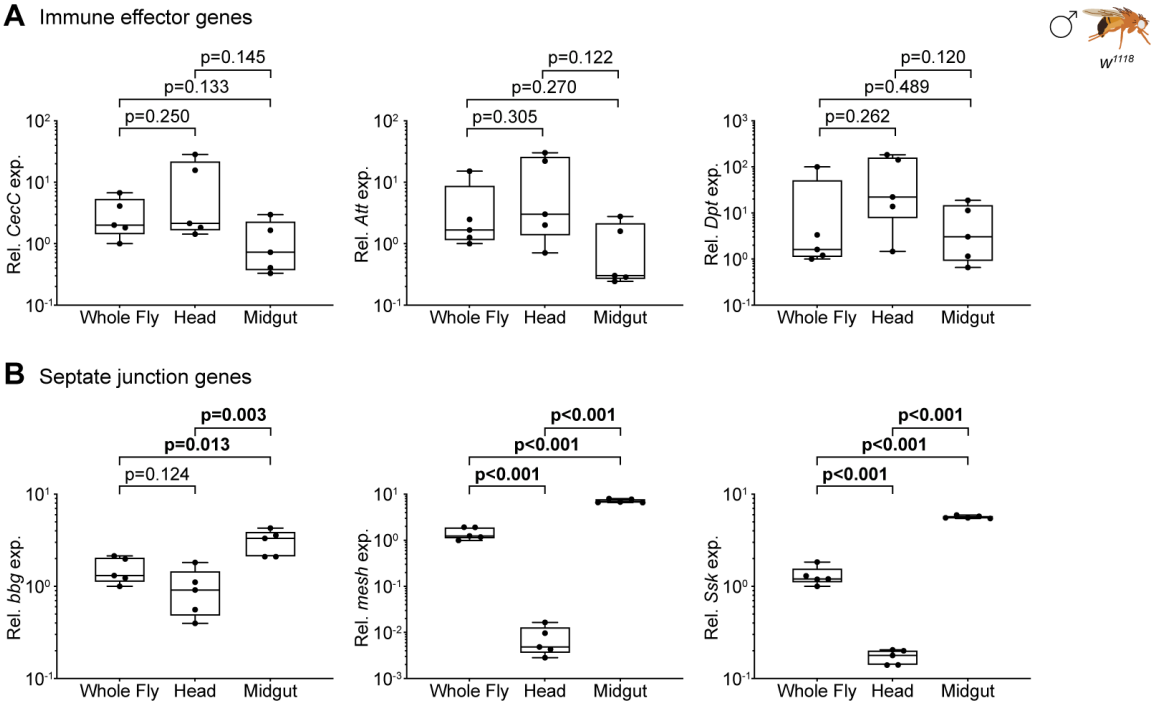


Figure 4-13 Tissue expression comparison of representative immune effector and septate junction genes.

(A-B) Quantification of the relative expression of representative (A) immune effector genes or (B) septate junction genes in whole flies, heads, or midguts from of 20-day old w^{1118} flies raised on HF. Statistical significance compared to whole fly samples determined by Student's T-test.

4.2.5. GSF enhances intestinal barrier integrity

4.2.5.1. Blood-brain barrier integrity is comparable between flies raised on GSF or HF

Barriers are maintained through occluding junctions, known as tight junctions in mammals or septate junctions (SJ) in invertebrates (Izumi and Furuse, 2014). Invertebrates have two morphologically distinct types of SJ: pleated SJ (pSJ) in ectodermally derived tissues, such as the blood-brain barrier (BBB), and smooth SJ (sSJ) in endodermally derived tissues, such as the midgut (Izumi and Furuse, 2014). Both perform a similar function and have proteins in common, such as Coracle, though less is known about the structure and components of the sSJ. In *Drosophila*, the BBB is essential to protect the vulnerable neuronal tissue, as a compromised BBB is associated with mortality (Daneman

and Barres, 2005). Although many GSF-responsive cell junction genes are associated with sSJ, several are thought to be specific to pSJ. I initially investigated the hypothesis that GSF strengthens the BBB.

To measure the integrity of the BBB, I used a dye (10 kDa-Texas Red dextran) that is normally excluded from the brain tissue unless the barrier is weakened. Initially, I raised flies for 40 days on HF or GSF, injected dye into their hemolymph, waited overnight, then dissected their brains for visualization. I found that this procedure is fairly harmful, especially in older flies, resulting in a low sample number (n=2). However, I observed that flies raised on HF had a weakened BBB compared to those raised on GSF, as dye penetrated the BBB of both flies raised on HF but not those raised on GSF **(Figure 4-14A)**. As I observed a potential difference in BBB integrity in older flies, I asked if there is a difference in younger 20-day old flies. I raised flies on either HF or GSF for 20 days and performed the dye injection assay with a higher sample number. I observed no penetration of dye into the brain in any samples, suggesting that 20 days is not long enough to detect any diet-dependent effect on the BBB (n=5-7) **(Figure 4-14B)**. As my initial sample number was very low, I repeated the dye injection assay with flies raised on either HF or GSF for 40 days. Unexpectedly, I observed no dye penetration in any samples (n=9-10) **(Figure 4-14C)**. I repeated the assay with even older 50-day flies and again did not observe dye penetration in any samples (n=14) **(Figure 4-14D)**. Combined, it appeared that the initial result was likely a false positive. As 50 days exceeds the age when I see phenotypic differences between flies raised on GSF and HF, I reasoned that GSF extends lifespan through a different mechanism than improved BBB integrity.

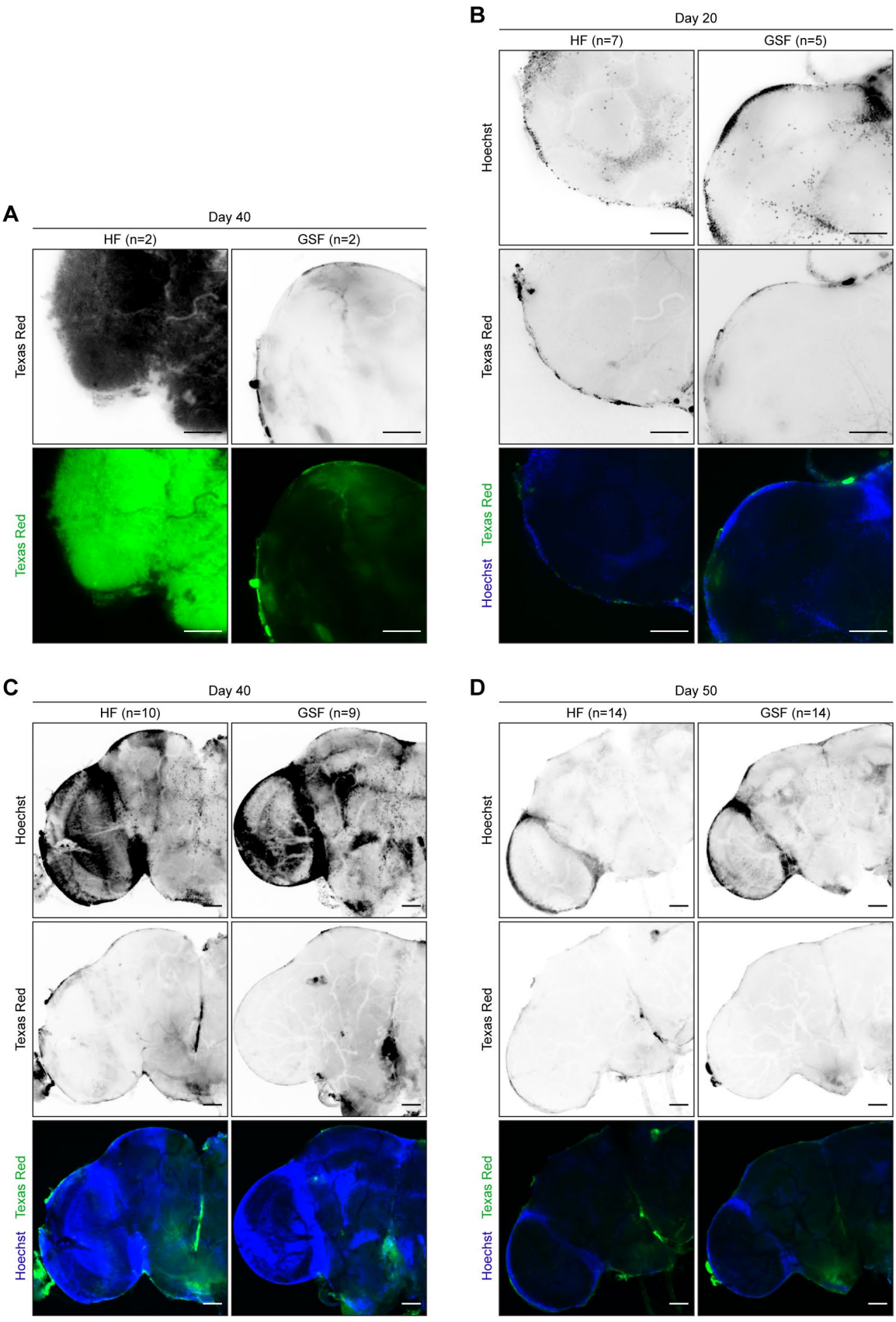

A
Day 40
HF (n=2)
GSF (n=2)
Texas Red
Texas Red
B
Day 20
HF (n=7)
GSF (n=5)
Hoechst
Texas Red
Hoechst Texas Red
C
Day 40
HF (n=10)
GSF (n=9)
Hoechst
Texas Red
Hoechst Texas Red
D
Day 50
HF (n=14)
GSF (n=14)
Hoechst
Texas Red
Hoechst Texas Red

Figure 4-14 Dye penetration of blood-brain barrier in flies raised on GSF or HF

(A-D) Immunofluorescent images of dissected brains from w^{1118} males raised on GSF or HF for (a) 40 days, (B) 20 days, (C) 40 days, or (D) 50 days labeling DNA (Hoechst, blue) and 10kDa-Texas Red dextran dye (green).

4.2.5.2. Intestinal Coracle maintains localization with age in flies fed GSF

Intestinal barrier integrity deteriorates with age and a weakened barrier is associated with reduced lifespan (Rera et al., 2012). As I observed increased expression of cell junction genes in GSF-treated flies, particularly midgut-associated SJ genes, I asked if GSF extends lifespan by improving intestinal barrier integrity.

In the fly midgut, the epithelial barrier is maintained by SJs, which are analogous to mammalian tight junctions (Izumi and Furuse, 2014). Coracle (Cora), a *Drosophila* protein 4.1 homolog, is an essential component of SJs (Lamb et al., 1998). As flies age, Cora and other SJ proteins partially lose their cell junction localization and accumulate in the cytosol, leading to breaches in the barrier, paracellular leak of lumenal material into interstitial tissue, and ultimately, death (Rera et al., 2012; Resnik-Docampo et al., 2017). To determine the effects of GSF on the intestinal barrier, I used immunofluorescence to examine the cellular distribution of Cora in the intestines of 40-day old flies raised on HF or GSF compared to 5-day old flies raised on HF. The intestines of 5-day old flies raised on HF contained orderly arrangements of large, polyploid nuclei of differentiated absorptive enterocytes, and smaller, evenly spaced nuclei of progenitor cells or secretory enteroendocrine cells **(Figure 4-15A**, **Hoechst)**. At this young age, SJs are easily identified as fine margins of Cora staining at cell junctions **(Figure 4-15A**, **Coracle)**. In 40-day old flies raised on HF, I noted classic hallmarks of age-dependent epithelial degeneration. Specifically, I detected unevenly distributed, large enterocyte nuclei, interspersed by irregular populations of smaller nuclei from progenitor/enteroendocrine cells **(Figure 4-15A, Hoechst)**. In addition, I detected cytosolic accumulations of Cora **(Figure 4-15A, asterisk)**, including enrichments in punctae **(Figure 4-15A, arrowhead)**. In contrast, age-matched intestines of flies raised on GSF looked more similar to younger flies raised on HF, with regularly spaced nuclei **(Figure 4-15A, Hoechst)**, while the distribution of Cora appeared more localized to junctions than in HF-fed samples **(Figure 4-15A, Coracle)**. 3D

reconstruction of 40-day old intestines highlights the difference in Cora localization between flies raised on HF or GSF **(Figure 4-15B)**. In flies raised on GSF, Cora retained a reticulated pattern associated with points of cell-cell contact at SJs. In contrast, I detected uneven, diffuse Cora distribution in intestines of age-matched flies raised on HF.

To quantify food-dependent impacts on the subcellular distribution of Cora, I determined the bicellular junction to cytoplasm ratio of Cora in the midguts of flies raised on HF or GSF for 40 days. Here, I detected significantly higher junction to cytosol ratios of Cora in 40-day old GSF-treated flies than in age-matched HF-treated flies **(Figure 4-15C)**, supporting the hypothesis that GSF improves maintenance of Cora association with SJs as flies age.

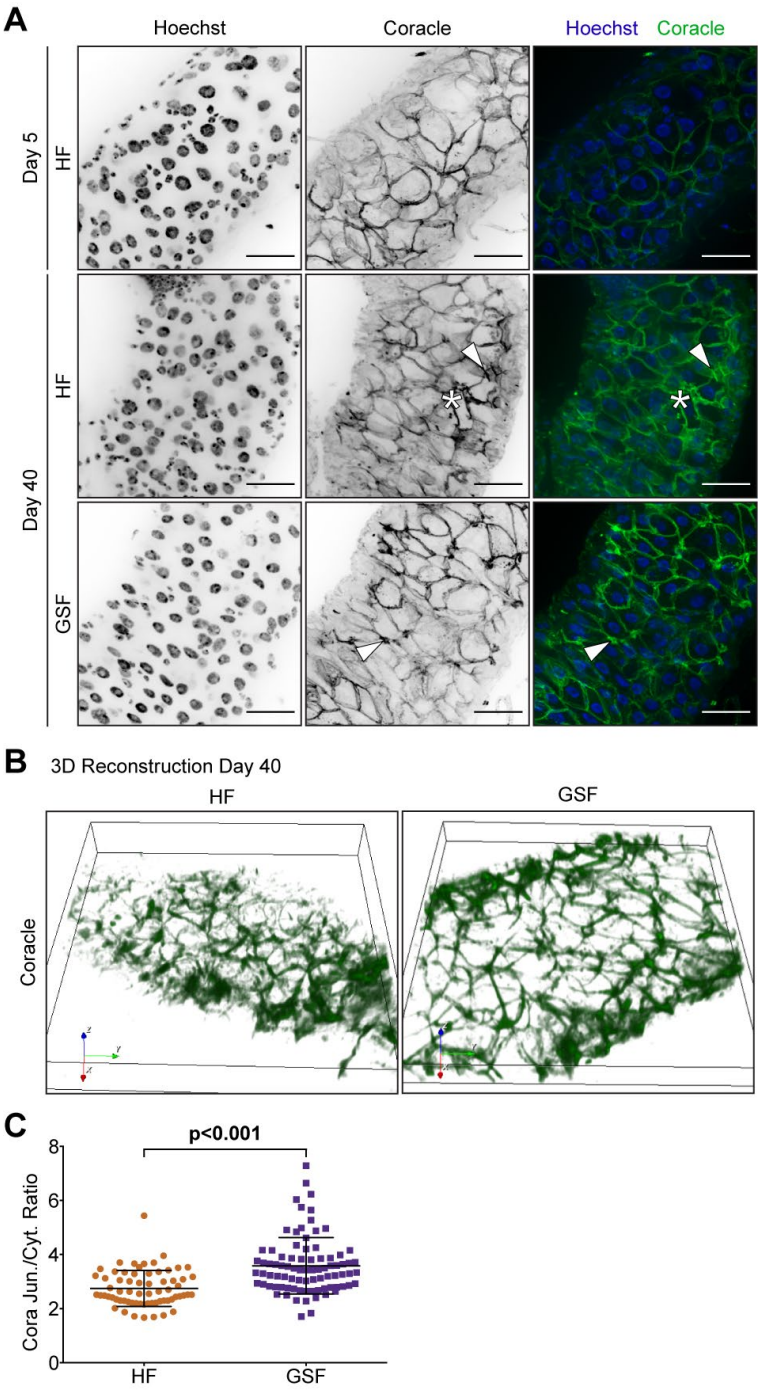


Figure 4-15 Coracle localization in flies raised on GSF or HF

(A) Immunofluorescent images of the posterior midgut of 5- or 40-day old w^{1118} male flies raised on HF or GSF labeling DNA (Hoechst, blue) and Coracle (green). Scale bars, 25 µm. (B) 3D reconstruction images of Coracle in the posterior midgut of 40-day old w^{1118} flies raised on HF or GSF. (C) Quantification of Coracle as a ratio of bicellular junction to cytoplasm localization in the posterior midgut of 40-day old w^{1118} flies raised on HF (n = 7 guts, 66 cells) or GSF (n = 8 guts, 84 cells). Statistical significance determined by Student's T-test.

4.2.5.3. Flies fed GSF maintain barrier function with age

As flies fed GSF had higher transcription of cell junction genes and maintained intestinal localization of the SJ protein Cora with age, I hypothesized that GSF-treatment improves intestinal barrier integrity with age. To determine if GSF functionally improves barrier integrity in aged flies, I performed a smurf assay, in which a non-permeable dye, that only crosses the epithelium upon loss of barrier integrity, is added to the food. I raised w^{1118} males on either HF or GSF with the addition of blue dye and monitored flies each day for leakage of dye outside the intestinal lumen, described as 'smurfing'. By counting smurfed flies over time, I found that flies raised on GSF smurfed significantly later than those on HF **(Figure 4-16)**, suggesting that GSF enhanced barrier integrity in flies.

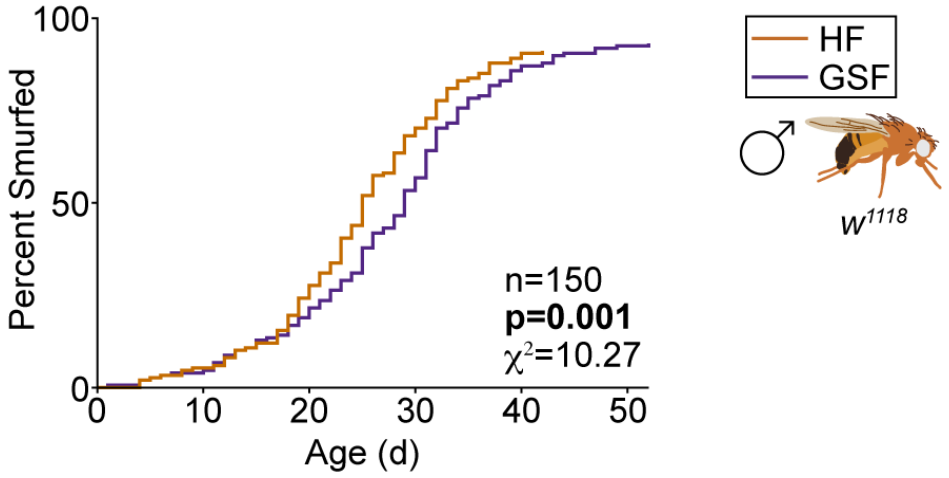


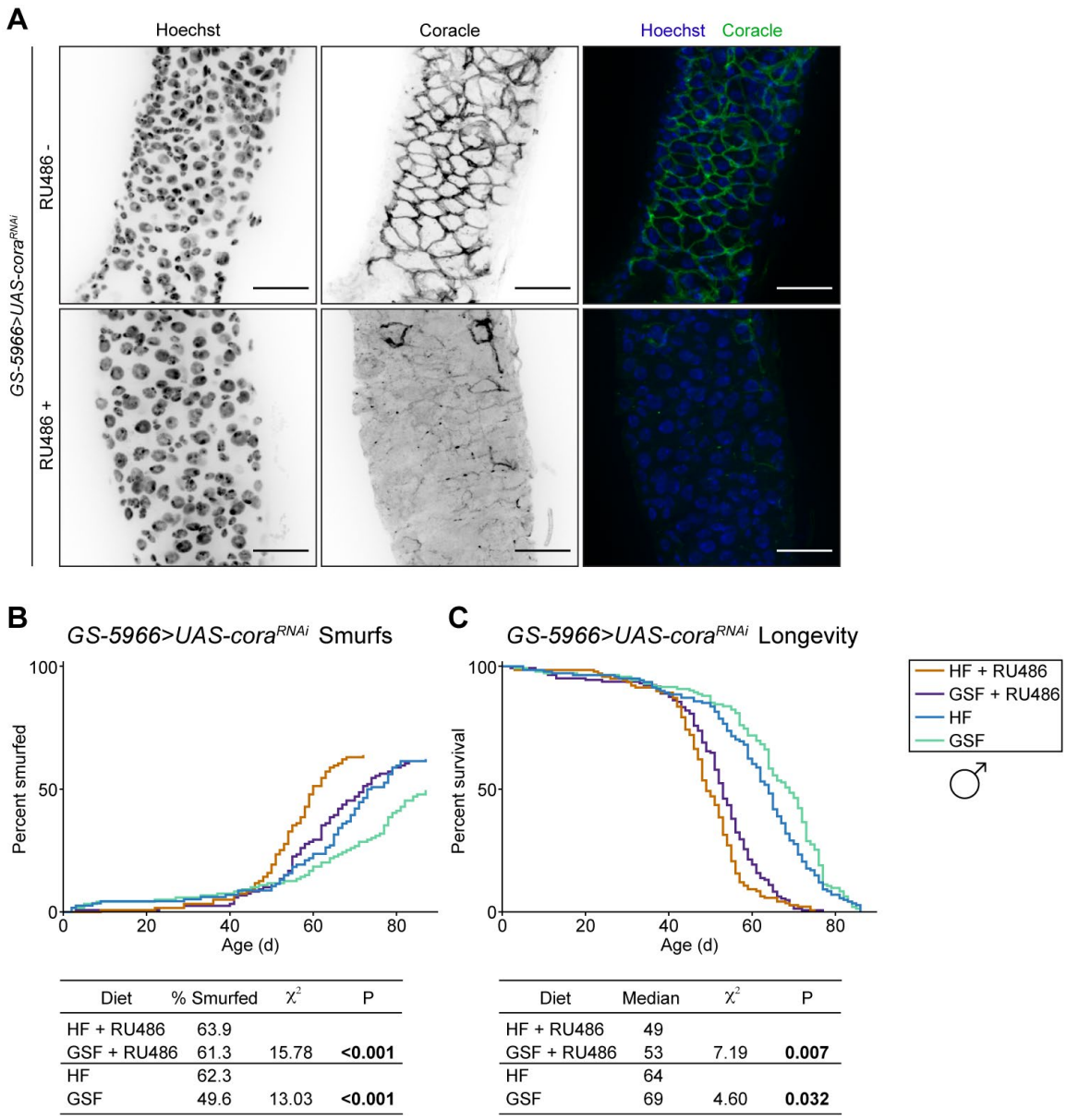
Figure 4-16 Smurf assay of flies raised on GSF or HF

Cumulative number of smurfs over time in w^{1118} flies raised on GSF or HF. Statistical significance compared to HF determined by log-rank (Mantel-Cox) test.

4.2.5.4. Knockdown of intestinal Coracle is not sufficient to remove GSF-dependent lifespan extension

Knockdown of intestinal Cora weakens the function of sSJs (Bonnay et al., 2013). As GSF maintained Cora localization with age, I asked if intestinal Cora is required for GSF-dependent lifespan extension. To test this, I expressed tissue-specific RNAi knockdown of *cora* in intestinal enterocytes using the GeneSwitch (GS) expression system. GS flies allowed me to chemically induce expression by adding the chemical RU486 to food (Osterwalder et al., 2001).

I expressed *cora* RNAi knockdown in intestinal enterocytes with the *GS-5966* driver. Initially, I used immunofluorescence to verify that Cora was depleted in the midguts of *GS-5966>UAS-cora^{RNAi}* flies and found that the RNAi knockdown was effective **(Figure 4-17A)**. To test if the knockdown of Cora impacted barrier integrity, I performed a smurf assay of *GS-5966>UAS-cora^{RNAi}* flies raised on either HF or GSF. I found that Cora knockdown decreased the age of smurfing, indicating that Cora expression in enterocytes is important for barrier maintenance **(Figure 4-17B)**. Similar to what I observed previously with *w^{1118}* flies, GSF-treatment delayed smurfing in *GS-5966>UAS-cora^{RNAi}* flies, with or without RU486-induced activation of *cora^{RNAi}*. Interestingly, a lower proportion of flies smurfed compared to the earlier experiment with *w^{1118}* flies. As the knockdown of Cora was both successful and reduced barrier integrity, I asked if Cora is required for GSF-dependent lifespan extension. I raised *GS-5966>UAS-cora^{RNAi}* flies on either HF or GSF and with or without the addition of RU486 to induce expression of *cora^{RNAi}*, then measured lifespan. I found that knockdown of *cora* with the *GS-5966* driver did reduce lifespan, however, it did not remove the GSF-dependent lifespan extension **(Figure 4-17C)**. While Cora expression is altered by GSF-treatment, it is not required for GSF-dependent lifespan extension. The lower proportion of smurfs than in previous experiments with *w^{1118}* flies suggests that knockdown of Cora alone does not sufficiently weaken the intestinal barrier to overcome the diet-dependent advantages.



Diet	% Smurfed	χ^2	P
HF + RU486	63.9		
GSF + RU486	61.3	15.78	**<0.001**
HF	62.3		
GSF	49.6	13.03	**<0.001**

Diet	Median	χ^2	P
HF + RU486	49		
GSF + RU486	53	7.19	**0.007**
HF	64		
GSF	69	4.60	**0.032**

Figure 4-17 Gene-Switch RNAi knockdown of *cora* in intestinal enterocytes of flies raised on GSF or HF

(A) Immunofluorescent images of *GS-5966>cora*^{RNAi} flies raised on HF with or without RU498 for 20 days labeling DNA (Hoechst, blue) and Coracle (green). Scale bars, 25 µm. (B) Cumulative number of smurfs over time in *GS-5966>cora*^{RNAi} flies raised on GSF or HF with or without RU486. Statistical significance determined by log-rank (Mantel-Cox) test. (C) Survival curve of *GS-5966>cora*^{RNAi} flies raised on GSF or HF with or without RU486. Statistical significance determined by log-rank (Mantel-Cox) test.

4.2.5.5. Chemical disruption of intestinal barrier removes GSF-dependent survival advantage

As genetic knockdown of Cora was not sufficient to induce smurfing in a large proportion of flies, I asked if chemical disruption of the epithelial barrier would revert the lifespan benefits associated with GSF. For this experiment, I raised flies on GSF or HF for 20 days, at which point I transferred them to HF or GSF that I supplemented with 5% dextran sodium sulfate (DSS), a detergent that disrupts the gut barrier, for the remainder of their lives **(Figure 4-18A)**. By increasing intestinal permeability with DSS, I found that flies raised on GSF completely lost their survival advantage **(Figure 4-18B)**, perishing at the same time as flies raised on HF. Combined, these data indicate that the lifespan extension I observe in flies raised on GSF is through a mechanism that involves maintenance of the intestinal epithelial barrier with age.

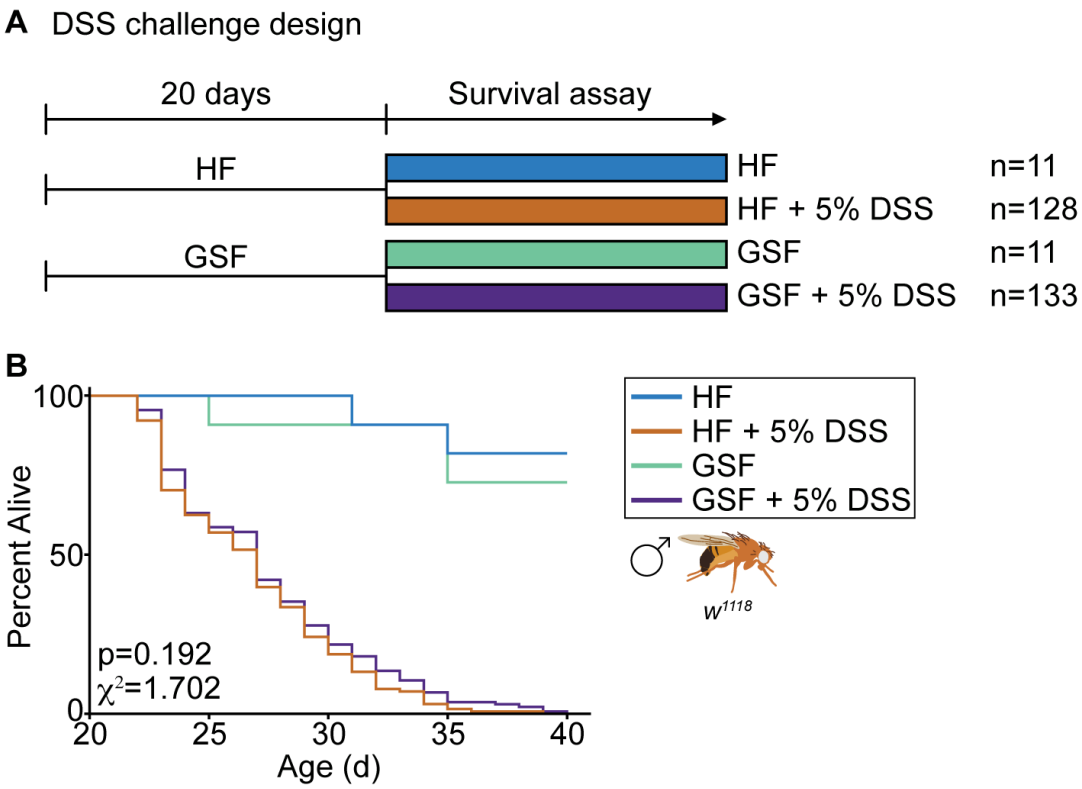


Figure 4-18 Survival with chemical disruption of intestinal barrier integrity

(A) Experimental design and (B) survival curve of w^{1118} flies raised on HF or GSF for 20 days, then transferred to food supplemented with 5% dextran sodium sulfate (DSS) or control food. Statistical significance for survival curves determined by log-rank (Mantel-Cox) test.

4.3. Discussion

In this chapter, I asked how glucose-supplemented food (GSF) extends lifespan in male w^{1118} flies. I found that GSF extends lifespan independent of caloric restriction, or effects on insulin pathway activity. Instead, I showed that GSF extends the lifespan of adult flies by improving the maintenance of intestinal barrier integrity in aging flies. GSF-treated flies have increased expression of cell junction proteins and higher levels of the SJ protein Coracle localized to bicellular junctions. Flies raised on GSF maintain barrier function to a later age than their control counterparts. Furthermore, treatment with a barrier disrupting detergent removes lifespan benefits of glucose supplementation. Combined, these data presented here identify a relatively uncharacterized diet-dependent mechanism of lifespan extension.

In summary, this chapter shows that moderate levels of glucose can extend *Drosophila* lifespan through improved intestinal barrier integrity. In humans, the intestinal barrier deteriorates with age, as well as in chronic diseases such as inflammatory bowel disease. With population aging becoming a growing global concern, further investigation of how dietary components can help maintain intestinal barrier integrity will be essential. I believe that these findings contribute to our understanding of intestinal health and may help efforts to develop preventative measures to limit the effects of aging and disease.

Chapter 5

The immune deficiency pathway regulates lipogenesis

Portions of this chapter have been published as:

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5. The immune deficiency pathway regulates lipogenesis

5.1 Introduction

In chapter 3, I found that 100 g/L glucose-supplemented food improved the survival response of female w^{1118} flies when challenged with oral *V. cholerae* infection. Additionally, microarray analysis of female w^{1118} flies raised on glucose-supplemented or unmodified food for 10 days revealed that supplemental glucose increases expression of immunity genes. Similarly, in chapter 4, RNA-Seq comparison of male w^{1118} flies raised on GSF or HF for 20 days showed that GSF increases expression of immunity genes, particularly immune effectors such as antimicrobial peptides. Together, these data suggest that supplemental dietary glucose impacts the immune response in flies.

Studies of immunometabolism, or interactions between immunity and metabolism, have increased in prominence with the discovery of inflammatory components to metabolic diseases such as Type 2 diabetes (Hotamisligil et al., 1993; Weisberg et al., 2003; Xu et al., 2003). As *Drosophila* is widely used to study both immune signaling (Buchon et al., 2014) and the regulation of metabolism (Nässel et al., 2015; Owusu-ansah and Perrimon, 2014) *in vivo*, the fly has considerable potential as a tool to build our understanding of the molecular and cellular bridges that connect immune and metabolic pathways.

In this chapter, I initially asked how glucose-supplemented food impacts survival to infection. I found that GSF-fed flies have improved survival response to both oral and systemic infection with *V. cholerae*. While testing if *imd* is required for GSF-dependent longevity, I observed that *imd* mutants have increased weight compared to wild-type flies. Following this observation, I asked how the lack of *imd* impacts metabolic homeostasis. I found that *imd* flies have disrupted energy stores, reduced insulin activity, and alter expression of lipid metabolism genes.

5.2 Results

5.2.1. GSF improves response to *V. cholerae* infection

5.2.1.1. GSF improves survival following systemic or oral challenge with *V. cholerae*

In chapter 3, I found that supplementing a holidic diet with 100 g/L glucose improved the response of w^{1118} female flies against oral infection with *Vibrio cholerae*, though it did not have an effect on male survival **(Figure 3-4)**. In chapter 4, I switched to 50 g/L glucose supplemented food (GSF), as it had a greater benefit on lifespan. As I observed increased expression of immune genes, particularly antimicrobial peptides, in male flies raised on GSF **(Figure 4-12A)**, I hypothesized that GSF has a functional impact on the immune response.

As I performed the RNA-Seq comparison on whole fly samples, I did not know where in the fly GSF-responsive immune effector genes were being upregulated. I initially speculated that GSF-treatment may alter the systemic immune response. To test this, I raised flies on GSF or HF for 20 days, then infected them systemically by puncture with needles dipped in *V. cholerae* culture, and measured survival over time. Control flies were mock-infected with sterile LB. All flies exposed to *V. cholerae* succumbed to infection within one day, but I found that GSF extended survival compared to HF **(Figure 5-1A)**. To assess if GSF improves the efficiency of the survival response, bacterial clearance can be measured by estimating bacterial persistence in the fly (Neyen et al., 2014). To estimate bacterial persistence, I measured the colony forming units (CFU) of *V. cholerae* in live infected flies at two time points during the infection. In this experiment, a lower pathogen CFU count would suggest a more efficient immune response. I repeated the systemic infection protocol and measured the *V. cholerae* CFU in flies raised on either HF or GSF at both 4h and 8h. After 4h, I found that flies raised on GSF have lower CFU of *V. cholerae*, though not significantly lower **(Figure 5-1B)**. After 8h, I observed higher variability in CFU, and found no difference between flies raised on either food. Combined, these data suggest that male w^{1118} flies raised on GSF have improved survival against systemic *V. cholerae* infection, but with no significant difference in CFU recorded, it remains unclear how GSF is improving the survival response. It will likely require a more detailed analysis

of infection progression with more time points to examine how GSF affects the infection survival response.

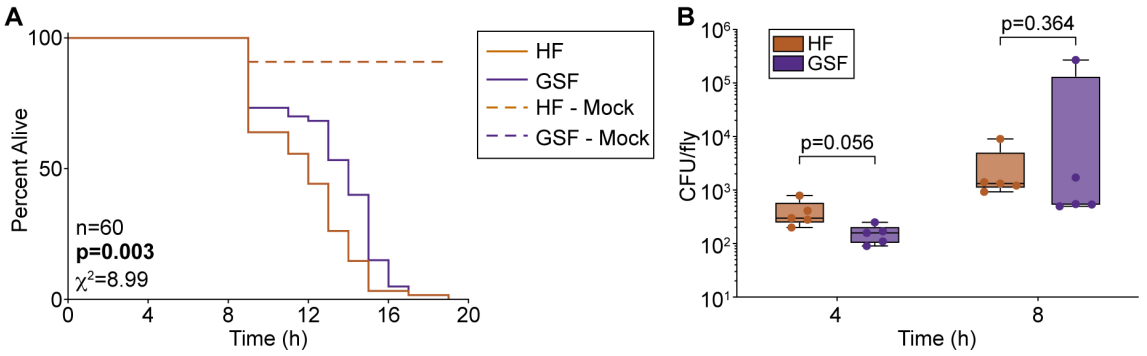


Figure 5-1 Systemic *V. cholerae* infection of flies raised on GSF or HF

(A) Survival curve of 20-day old w^{1118} male flies raised on glucose-supplemented food (GSF) or unmodified holidic food (HF) either infected with *V. cholerae* systemically or mock-infected with LB broth. Significance between infected samples determined by log-rank (Mantel-Cox) test. (B) Colony forming units (CFU) of *V. cholerae* per fly at 4- or 8-hours after initial infection. Significance compared to HF determined by Student's T-test.

I previously found that 100 g/L glucose-supplemented food did not improve the infection response of males against oral *V. cholerae* infection. However, as 50 g/L glucose-supplemented food (GSF) had a greater effect on lifespan **(Figure 4-1)** and improved survival against systemic *V. cholerae* infection, I wondered if GSF-treatment improves survival against oral *V. cholerae* infection. To test this, I raised male w^{1118} flies on HF or GSF for 20 days, infected them orally with *V. cholerae*, and measured survival. Similar to a systemic infection, I found that GSF-treatment improved survival **(Figure 5-2A)**. To investigate how GSF improved survival to oral infection, I measured the *V. cholerae* CFU at 24h or 48h in flies raised on either HF or GSF. I found significantly lower CFU at 24h in flies fed GSF, though there was no difference at 48h **(Figure 5-2B)**. The lower CFU at 24h suggests that GSF-treatment extends survival through a reduced pathogen burden on the host. However, more work is required to determine how GSF-treatment leads to a reduced *V. cholerae* CFU during oral infection. Potential mechanisms may involve an increase in the fly's ability to directly clear the pathogen, or an indirect effect on the pathogen's fitness. Combined with the increased immune effector gene expression, this

finding raises the possibility that flies fed GSF have greater resistance against *V. cholerae* through earlier or increased expression of antimicrobial peptides.

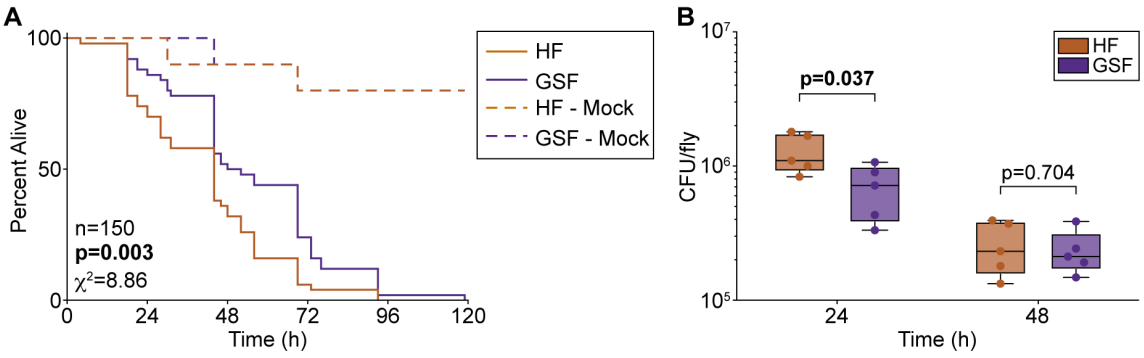


Figure 5-2 Oral *V. cholerae* infection of flies raised on GSF or HF

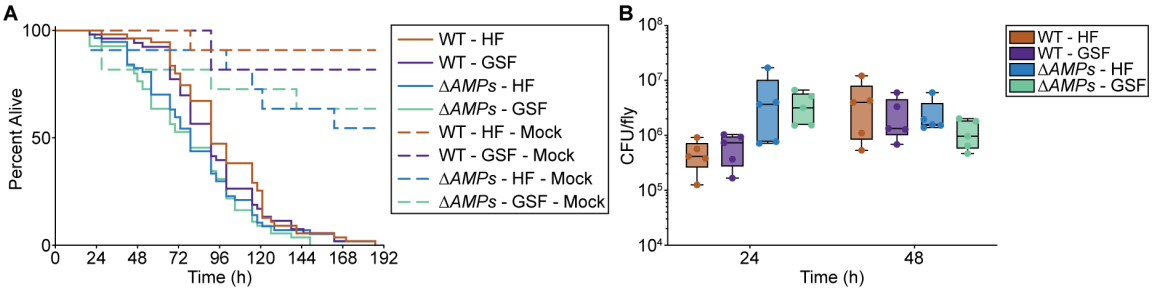
(A) Survival curve of 20-day old w^{1118} male flies raised on glucose-supplemented food (GSF) or unmodified holidic food (HF) either infected with *V. cholerae* orally or mock-infected with LB broth. Significance between infected samples determined by log-rank (Mantel-Cox) test. (B) Colony forming units (CFU) of *V. cholerae* per fly at 24- or 48-hours after initial infection. Significance compared to HF determined by Student's T-test.

5.2.1.2. Δ*AMPs* flies orally infected with *V. cholerae*

Antimicrobial peptides (AMPs) are small cationic peptides that disrupt the negatively charged membranes of microbes, with 14 known immune-inducible AMPs in *Drosophila* (Hanson and Lemaitre, 2020). To determine if AMPs are required for GSF-dependent survival response against oral *V. cholerae* infection, I used flies lacking 10 of the 14 known immune-inducible AMP genes (Δ*AMPs*) (Hanson et al., 2019). If the higher expression of *AMP* genes in GSF-treated flies contributes to the better survival response, then I predict that Δ*AMPs* flies raised on either HF or GSF will have a comparable survival curve following infection. I raised male Δ*AMPs* and isogenic wild-type (WT) control flies on either HF or GSF for 20 days, then performed an oral infection with *V. cholerae* and measured survival. Mock infected flies were provided with sterile LB broth. I also measured CFU of *V. cholerae* in live flies at both 24 and 48 hours. For the survival curves, I found that mock-infected control flies survived better than their infected counterparts, as expected **(Figure 5-3A)**. In support of my hypothesis, GSF-fed Δ*AMPs* flies did not survive

significantly better than their HF-fed counterparts and had no diet-dependent difference in *V. cholerae* CFU at 24h or 48h **(Figure 5-3B)**. However, GSF-treatment did not improve survival in the WT flies, nor was there a difference in CFU levels between diet treatments. This was unexpected as I previously found that GSF-treatment improved survival against oral *V. cholerae* infection and decreased CFU count in w^{1118} flies. Notably, this WT fly line is not the same as the w^{1118} flies previously infected. As GSF-treatment did not improve survival in WT controls, I cannot make conclusions on whether AMPs are required for GSF-dependent improvement to survival against *V. cholerae* infection. These data suggest that the benefits of GSF to infection survival may be genotype dependent. Further studies will be required to test this hypothesis.

While it remains unclear if increased infection survival in GSF-fed flies requires AMPs, the result that Δ*AMPs* survived significantly worse than WT flies, suggesting that antimicrobial peptides contribute to the immune response against *V. cholerae*. Expression of the either *AttacinA* or *Metchnikowin*, both mutated in Δ*AMP* flies, were previously found to improve survival against *V. cholerae* infection (Park et al., 2005). This is intriguing as IMD pathway mutants have increased survival against *V. cholerae* (Berkey et al., 2009). Combined, these data suggest that the improved survival response in IMD pathway mutants is not dependent on AMP production. Rather than through the production of AMPs, IMD signaling may influence the response to *V. cholerae* by regulating the proliferative response of intestinal stem cells (Wang et al., 2013).



Comparison			
Sample 1	Sample 2	χ^2	P
WT - HF	WT - GSF	0.67	0.414
WT - HF	ΔAMPs - HF	4.47	**0.035**
ΔAMPs - HF	ΔAMPs - GSF	0.53	0.467
WT - GSF	ΔAMPs - GSF	3.73	0.053

Comparison		P value	
Sample 1	Sample 2	24h	48h
WT - HF	WT - GSF	0.456	0.432
WT - HF	ΔAMPs - HF	0.155	0.415
ΔAMPs - HF	ΔAMPs - GSF	0.625	0.203
WT - GSF	ΔAMPs - GSF	**0.021**	0.227

Figure 5-3 Oral *V. cholerae* infection of Δ*AMPs* flies raised on GSF or HF

(A) Survival curve of 20-day old Δ*AMPs* and WT male flies raised on glucose-supplemented food (GSF) or unmodified holidic food (HF) either infected with *V. cholerae* orally or mock-infected with LB broth. Significance between infected samples determined by log-rank (Mantel-Cox) test. Color-coding in table corresponds with colors used in survival graph. (B) Colony forming units (CFU) of *V. cholerae* per fly at 24- or 48-hours after initial infection. Significance determined by Student's T-test.

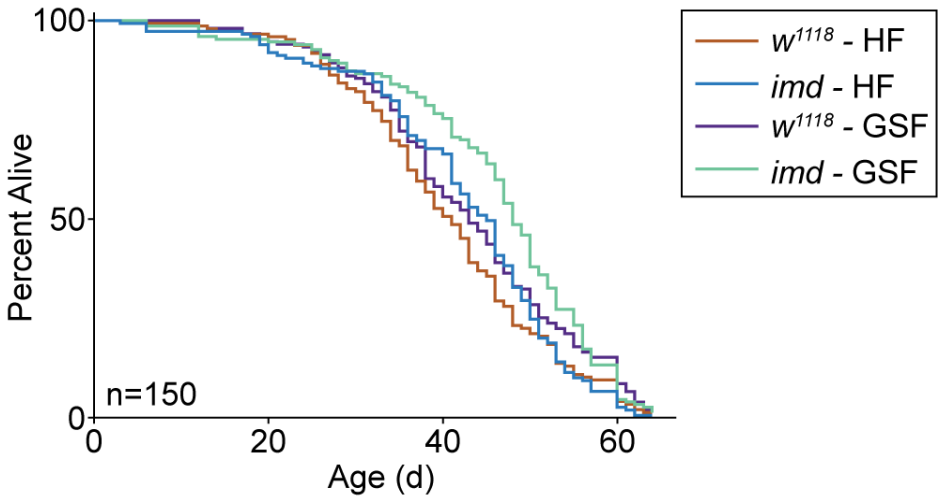
5.2.2. *imd* mutants have increased weight and increased energy stores

5.2.2.1. *imd* is not required for GSF-dependent lifespan extension

Immunometabolism is a growing field that has revealed extensive connections between immunity and metabolism (Galenza and Foley, 2019; Hotamisligil, 2017a, 2017b; Lee et al., 2018). Furthermore, diet influences immunity and survival to infection (Childs et al., 2019; Ponton et al., 2020; Wu et al., 2019). In chapter 4, I found through RNA-Seq comparison of flies fed GSF or HF that GSF-treatment caused widespread upregulation of immunity-associated genes **(Figure 4-12A)**. In particular, I noticed that many of these upregulated genes are associated with the immune deficiency (IMD) signaling pathway. While IMD signaling regulates expression of metabolic genes in response to the microbiota (Broderick et al., 2014; Dobson et al., 2016; Erkosar et al., 2014; Guo et al., 2014), the role of IMD signaling in metabolic homeostasis was unknown. As my lab has a long-standing interest in the IMD pathway, I wondered what role IMD signaling may have

in regulating metabolism, and the response to diet. Specifically, I was curious if *imd* is involved in the health and lifespan responses to GSF.

I initially asked if *imd* is required for GSF-dependent lifespan extension. To test this, I used *imd* null mutants, which have been backcrossed with my wild type w^{1118} flies to avoid genetic background effects. I raised *imd* null mutants on HF or GSF alongside w^{1118} controls and measured survival. As expected, w^{1118} males fed GSF lived longer than those fed HF, though not statistically significant in this particular experiment **(Figure 5-4)**. As others have observed, I found that *imd* mutants live longer than their wild-type counterparts (Lin et al., 2018). Contrary to my hypothesis, GSF-treatment significantly extended the lifespan of *imd* flies compared to those raised on HF. These data suggest that *imd* is not required for GSF-dependent lifespan extension.



Group	Median	χ^2	P
w^{1118} - HF	41		
w^{1118} - GSF	43	3.29	0.070
imd - HF	45		
imd - GSF	48	9.94	**0.002**

Figure 5-4 GSF extends lifespan of *imd* mutants

Survival curve of *imd* and w^{1118} male flies raised on glucose-supplemented food (GSF) or unmodified holidic food (HF). Significance determined by log-rank (Mantel-Cox) test.

5.2.2.2. *imd* mutants have increased weight and disrupted macronutrient stores

While working with *imd* mutants, we were struck by their visibly larger size than their wild-type counterparts. To my knowledge, this phenotype had not been reported in previous studies of the IMD pathway. This seemingly novel finding suggested that *imd* may potentially have a role in metabolic homeostasis or growth. With little known about how IMD signaling affected metabolism, I wondered why *imd* mutants are larger than w^{1118} flies. As I had previously found GSF-dependent increases in both glucose and triglyceride levels without a change in weight **(Figure 4-2)**, I was also curious how *imd* mutation would impact these diet-dependent effects on weight and macronutrients.

To test if *imd* flies have increased weight compared to wild-type flies, I raised *imd* and w^{1118} male flies on either HF or GSF, then measured weight at day 20 and day 40. As this experiment had three different independent variables; age, diet, and genotype; I performed a 3-way ANOVA to analyse the effect on weight of each independent variable separately and interacting.

Since the 3-way ANOVA is infrequently used, I will briefly explain this statistical method. As a 2-way ANOVA is named for having two independent variables, likewise, a 3-way ANOVA has three independent variables. A 3-way ANOVA involves one dependent variable, **y**, and three independent variables, **a, b, c**. This statistical test can analyze whether **a, b, or c** affect variance in **y**. The different variables tested can affect **y** individually, through interactions between two of the variables, as in **a x b**, or interactions between all three variables, as in **a x b x c**. Interestingly, **a or b** may not statistically affect **y**, but the interaction between **a x b** could still affect variance in **y**.

I compared the weight of *imd* and w^{1118} male flies raised on either HF or GSF for 20 or 40 days. Similar to my previous results, GSF-treatment did not alter weight significantly, while older flies weighed more than younger flies **(Figure 5-5A)**. As I hypothesized, genotype had a significant impact on weight, as *imd* mutants weigh more than w^{1118} flies.

While increased weight suggests that *imd* mutants have a metabolic defect, an alternative explanation is that the IMD pathway affects growth, and *imd* mutants are simply larger than wild-type flies. To differentiate between these hypotheses, I compared

macronutrient content of *imd* and w^{1118} flies. By measuring macronutrient levels and normalizing to weight, I can determine how *imd* affects relative levels of macronutrients. No difference in normalized macronutrient levels between *imd* and w^{1118} flies would indicate that *imd* impacts growth, whereas a difference would suggest that *imd* mutation affects metabolism, although there may also be interactions between growth and metabolism.

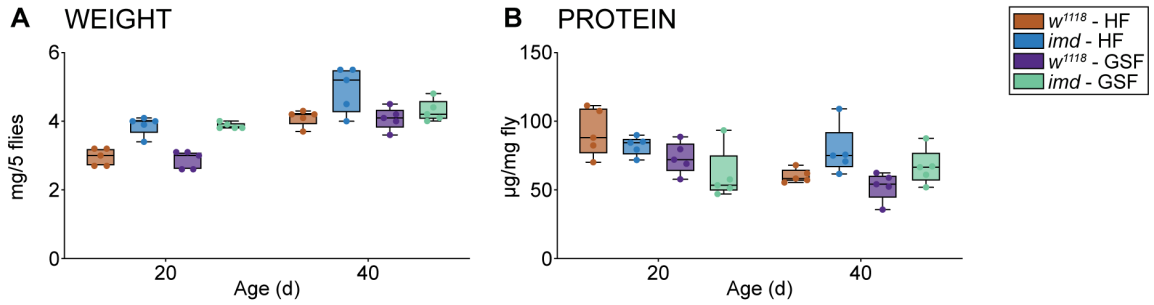
To address this question, I raised *imd* and w^{1118} male flies on either HF or GSF, then measured protein, glucose, and triglyceride content at day 20 and day 40. Again, I performed a 3-way ANOVA to determine significant impact on macronutrient level by either age, diet, or genotype. I found that both age and diet had a significant effect on protein levels, with older flies and GSF-treatment both leading to lower levels of protein **(Figure 5-5B)**. However, ANOVA suggests that these variables account for a relatively low percent of the variation in protein levels, 13.1% and 18.4% respectively. Interestingly, the interaction between age and diet was also found to impact variation in protein levels. Specifically, *imd* affected protein levels dependent on age, as 20-day old *imd* flies had lower protein than w^{1118} flies, while 40-day old *imd* flies had higher protein than w^{1118} flies. It appears that protein levels decrease in age in w^{1118} flies but remain steady with age in *imd* flies.

Measurement of glucose levels revealed significant differences between *imd* and w^{1118} flies. I found that age was a source of variation in glucose levels (26.88%), while diet (6.36%) and genotype (8.04%) also had a smaller influence on glucose levels. The largest source of variation in glucose was from the interaction between diet and genotype (30.13%) **(Figure 5-5C)**. While *imd* mutants raised on HF have increased glucose levels compared to w^{1118} flies, GSF-treatment does not increase glucose levels further as it does for w^{1118} flies. As I previously found, GSF increased the glucose levels of w^{1118} flies, but unexpectedly, *imd* flies raised on GSF had lower glucose than those raised on HF. Combined, the lack of *imd* appears to increase glucose levels on HF, while GSF-treatment leads to a decrease in glucose levels. These data suggest that *imd* is required for regulation of glucose homeostasis.

Similar to glucose, the interaction between diet and genotype was the greatest source of variation in triglyceride levels (34.37%) **(Figure 5-5D)**. As with glucose, I found that *imd*

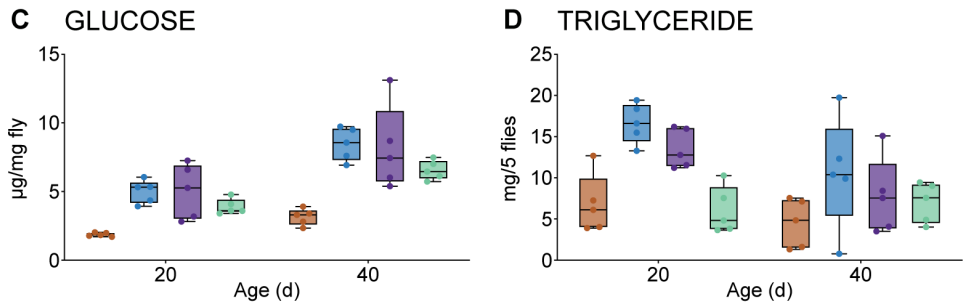
mutants raised on HF have increased triglyceride levels compared to w^{1118} flies. However, GSF-treatment, which increases triglycerides in w^{1118} flies, decreases triglycerides in *imd* flies. Age also had an effect on triglyceride levels (10.17%), with 40-day old flies generally have decreased triglyceride levels than 20-day old flies. The interaction between age, genotype, and diet also had a small influence on triglyceride levels (6.45%). This suggests that *imd* affects triglyceride levels differently dependent on the diet, but this interaction is more pronounced in 20-day old flies than 40-day old flies.

In summary, these data confirm that *imd* mutants weigh more than w^{1118} flies and suggest that IMD signaling is required for metabolic homeostasis rather than growth. Interestingly, while GSF-treatment increases glucose and triglycerides of w^{1118} flies, GSF-treatment has no effect on glucose and decreases triglyceride levels in *imd* mutants. The mutation of *imd* has a clear effect on macronutrient levels, as I observed increased glucose and triglyceride levels compared to w^{1118} flies raised on HF. Furthermore, GSF-treatment increases glucose and triglyceride stores in w^{1118} flies has the opposite effect in *imd* mutants. These data show that *imd* mutation affects macronutrient stores, but also the response to GSF-treatment. Combined, these data further support the hypothesis that IMD signaling is involved in metabolic regulation.



Three-way ANOVA		
Source of variation	% of variation	P
age	46.42	**<0.001**
diet	1.82	0.086
genotype	27.58	**<0.001**
age x diet	0.98	0.201
age x genotype	2.22	0.059
diet x genotype	0.99	0.201
age x diet x genotype	1.46	0.123

Three-way ANOVA		
Source of variation	% of variation	P
age	13.10	**0.007**
diet	18.42	**0.002**
genotype	0.50	0.572
age x diet	2.33	0.228
age x genotype	15.99	**0.003**
diet x genotype	0.28	0.673
age x diet x genotype	0.01	0.952



Three-way ANOVA		
Source of variation	% of variation	P
age	26.88	**<0.001**
diet	6.36	**0.009**
genotype	8.04	**0.004**
age x diet	0.25	0.581
age x genotype	0.74	0.348
diet x genotype	30.13	**<0.001**
age x diet x genotype	1.67	0.161

Three-way ANOVA		
Source of variation	% of variation	P
age	10.17	**0.010**
diet	1.06	0.381
genotype	3.50	0.117
age x diet	0.71	0.472
age x genotype	0.56	0.524
diet x genotype	34.37	**<0.001**
age x diet x genotype	6.45	**0.036**

Figure 5-5 Macronutrient comparison between *imd* and w^{1118} flies

(A-D) Quantification of (A) weight, (B) protein, (C) glucose, and (D) triglycerides in *imd* and w^{1118} flies raised on glucose-supplemented food (GSF) or unmodified holidic food (HF) for 20 or 40 days (n = 5). Each dot represents 5 flies. Significance determined by 3-way ANOVA.

5.2.2.3. *imd* mutants do not consume more food

Although the comparison of GSF-fed *imd* and w^{1118} flies was intriguing, I decided to focus on comparing *imd* and w^{1118} flies raised on HF to reduce complexity of analysis. I wondered how *imd* mutation leads to increased weight alongside both increased glucose and triglyceride levels. One possibility is that IMD signaling is involved in feeding behavior and *imd* mutants consume more food. Alternatively, the IMD pathway may be directly involved in regulation of metabolism. To test the former hypothesis, I compared the feeding behavior of *imd* mutants with w^{1118} flies using both the flyPAD and the CAFE assays.

Initially, I measured feeding frequency with the flyPAD. The flyPAD records an individual fly's interactions with solid food and measures individual sips; bursts, which are clusters of sips; and bouts, which are clusters of bursts. I raised male *imd* and w^{1118} flies on HF for 20 days, then starved them for 2 hours prior to feeding in a flyPAD arena for 1 hour. I found that *imd* flies had significantly more feeding bouts than w^{1118} flies, a 1.5-fold increase **(Figure 5-6A)**. Bouts represent how often a fly approaches the food, suggesting that *imd* flies are more attracted to the food. However, the number of sips and bursts, which have a greater correlation to ingested volume (Itskov et al., 2014), were not significantly different between genotypes. These data suggest that *imd* mutants consume a similar amount of food as w^{1118} controls, though increased bouts raise the possibility that the lack of *imd* affects olfaction.

To measure feeding behavior over a longer period of time and quantify consumption, I used the CAFE assay. In the CAFE assay, flies are fed liquid food through capillary tubes and consumption can be determined by measuring the displaced volume of food. I raised male *imd* and w^{1118} flies on HF for 20 days, then transferred them to the CAFE setup at 10 vials of 10 flies/vial, where they were fed a liquid version of HF over a 3-day period. I found that *imd* mutants consumed a similar volume of food as w^{1118} flies on each day **(Figure 5-6B)**. Combined with the flyPAD results, these data argue that *imd* flies are not consuming more food. As *imd* flies have increased weight without higher food consumption, I wondered if IMD signaling is involved in the regulation of metabolic homeostasis.

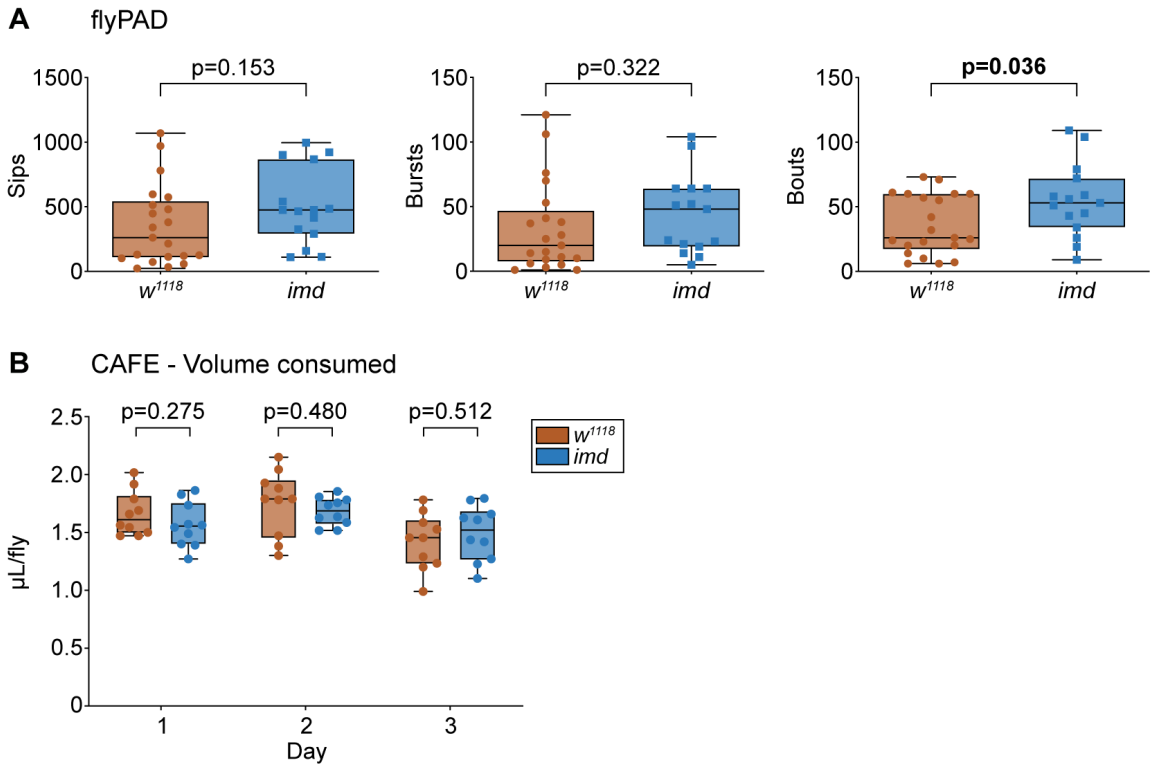


Figure 5-6 Comparison of feeding behavior of *imd* and w^{1118} flies

(A) Quantification of number of sips, duration of feeding bursts, and total feeding bouts in 20-day old *imd* and w^{1118} flies raised on unmodified holidic food (HF) using a flyPAD. Statistical significance determined by Student's T-test. (B) Quantification of volume of liquid food consumed in 20-day old *imd* and w^{1118} flies raised on HF using a CAFE assay. Statistical significance determined by Student's T-test.

5.2.3. *imd* mutants have altered insulin signaling

5.2.3.1. *imd* is required for normal insulin production

As I found that *imd* flies have increased weight, glucose, and triglyceride levels, without a change in food consumption, I hypothesized that IMD signaling is involved in metabolic signaling. One of the most important pathways for systemic coordination of metabolic homeostasis is the insulin/ insulin-like growth factor signaling (IIS) pathway, where insulin is directly involved in nutrient-sensing, maintenance of macronutrient stores, and body weight (Nässel et al., 2015; Owusu-ansah and Perrimon, 2014). With established

connections to metabolic homeostasis and weight, I hypothesized that IMD signaling is required to maintain functional IIS activity in flies.

To test this hypothesis, I asked if the lack of *imd* affects insulin activity. Initially, I quantified transcription of the insulin-like peptides (Ilp) *ilp2*, *ilp3*, and *ilp5*, in male *imd* and w^{1118} flies raised on HF for 20 days. In adults, these three Ilps are thought to coordinate the systemic insulin response and are primarily produced in insulin-producing cells in the brain (Broughton et al., 2005; Nässel and Broeck, 2015). I isolated RNA from dissected heads and quantified the expression of these three peptides with RT-qPCR. I found that the expression of *ilp3* was decreased in *imd* mutants, but both *ilp2* and *ilp5* transcription were unaffected **(Figure 5-7A-C)**. Interestingly, Ilp3 is also expressed in intestinal visceral muscle in adults (Veenstra et al., 2008), though the intestinal expression of *ilp3* is thought to have a paracrine function rather than a systemic role (O'Brien et al., 2011). Similar to my findings, IMD signaling was recently reported to affect intestinal expression of *ilp3* specifically, concomitant with increased glucose and triglyceride levels (Kamareddine et al., 2018). However, my findings extend this to suggest that IMD affects the systemic regulation of Ilp3.

As previously mentioned, IIS signaling is complex, and transcription does not necessarily correspond to circulating peptides (Nässel and Broeck, 2015). As *imd* flies have decreased *ilp3* transcription specifically, it would be of interest to measure Ilp3 levels. However, as I did not have reagents to measure Ilp3 readily available, I measured Ilp2 levels using an ELISA to quantify total and circulating amounts of FLAG and HA epitope-tagged Ilp2 (Ilp2-FH) in male *imd* flies raised on HF for 20 days. I generated a fly line that combined the *imd* null mutation and the epitope-tagged Ilp2 under control of the endogenous Ilp2 promoter. I found that total levels of Ilp2-FH are lower in *imd* flies compared to controls **(Figure 5-7D)**, however circulating levels are higher **(Figure 5-7E)**. Despite no difference in *ilp2* transcription, *imd* mutation appears to affect Ilp2 peptide levels. As higher levels of circulating insulin is a hallmark of insulin resistance (Graham and Pick, 2017), I hypothesized that the lack of *imd* leads to insulin resistance.

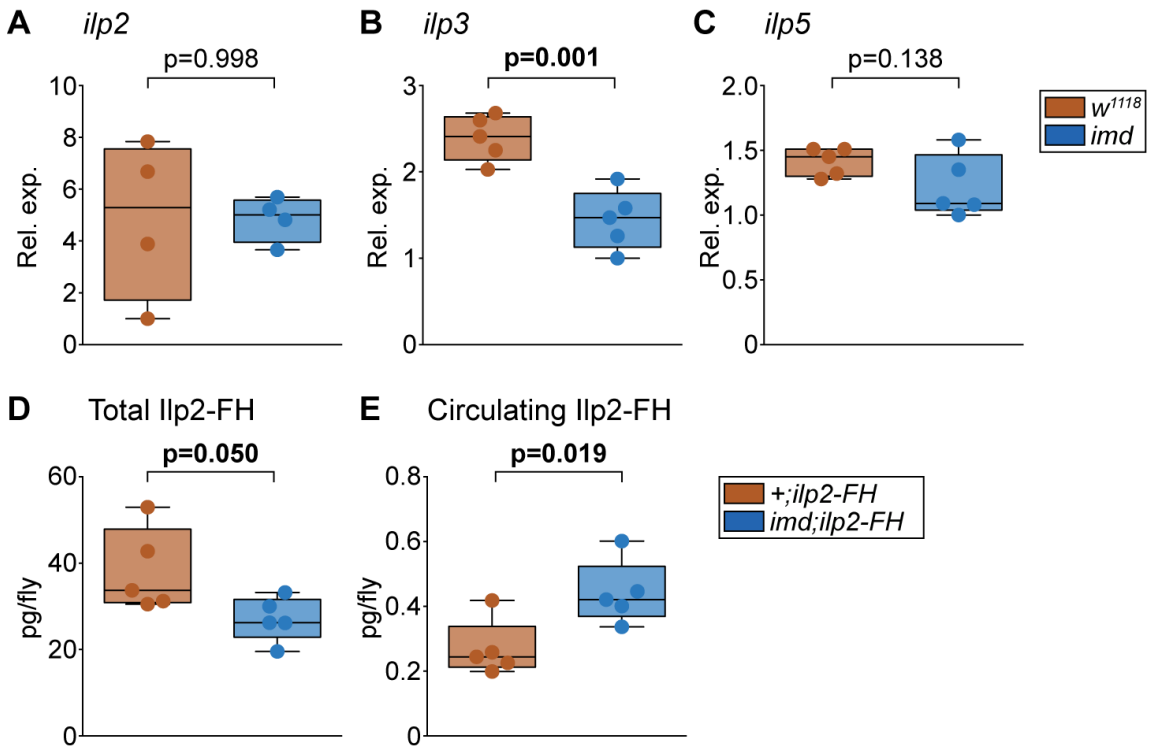


Figure 5-7 *imd* alters insulin production

(A-C) Quantification of the relative expression of (A) *ilp2*, (B) *ilp3*, and (C) *ilp5* in *imd* or w^{1118} flies raised on unmodified holidic food (HF) for 20 days. (D-E) Quantification of (D) total and (E) circulating Ilp2-FH in *imd;ilp2-FH* or *+;ilp2-FH* flies raised on HF for 20 days. Statistical significance determined by Student's T-test.

5.2.3.2. *imd* is required for an efficient insulin response

To test the insulin response in *imd* mutants I performed starvation and oral glucose tolerance test (OGTT) assays. Reduced insulin activity improves survival during starvation (Oldham et al., 2002; Post et al., 2018) and impairs OGTT response (Haselton et al., 2010). If the lack of *imd* disrupts insulin activity, possibly leading to insulin resistance, then *imd* mutants should survive longer than w^{1118} flies in a starvation assay and perform worse in an OGTT. To address this, I initially raised both *imd* and w^{1118} flies on HF for 10 and 20 days., prior to transfer to a nutrient-deprived medium (1% agar in water). Contrary to my hypothesis, *imd* flies had no difference in survival upon starvation at either time point **(Figure 5-8)**.

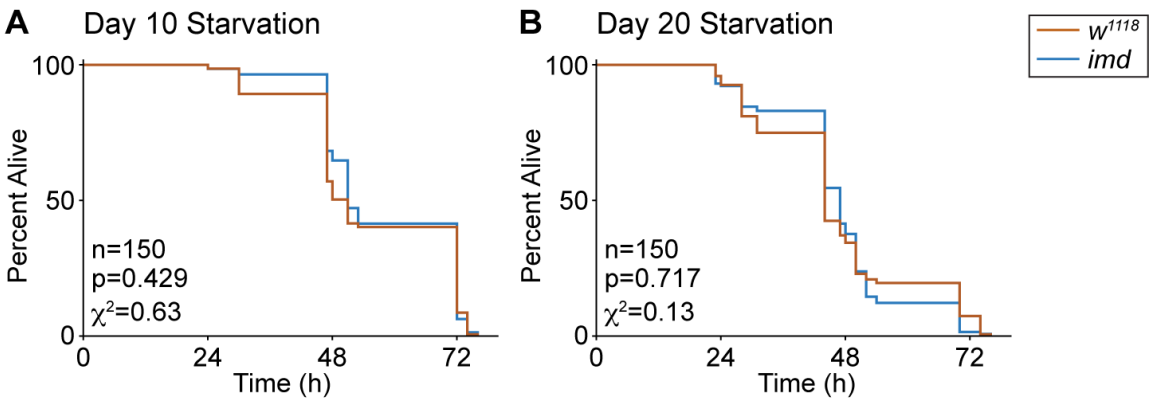


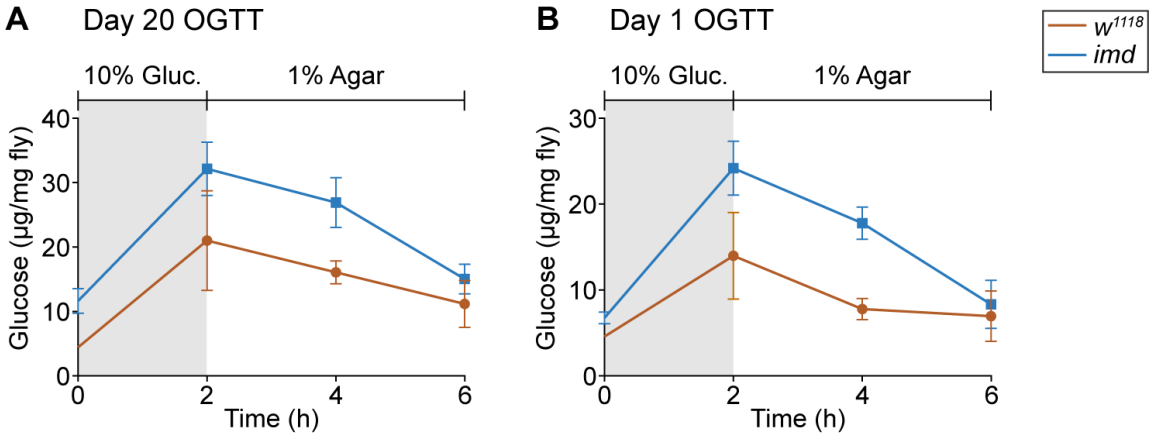
Figure 5-8 Starvation comparison of *imd* and w^{1118} flies

(A-B) Survival curve upon starvation of *imd* and w^{1118} flies raised on unmodified holidic food (HF) for (A) 10 or (B) 20 days. Statistical significance determined by log-rank (Mantel-Cox) test.

For the OGTT, I raised *imd* and w^{1118} flies on HF for 20 days. Prior to the assay, flies were starved for 16h, then fed 10% glucose medium for 2 hrs, followed by a re-fast. I quantified total glucose in flies following the initial fast (0h), after feeding on 10% glucose (2h), and twice during the re-fast period (4h, 6h). If flies are insulin-sensitive, I expect that their glucose levels will increase during feeding, then decrease quickly during the re-fast period through insulin-mediated uptake of glucose. I saw a significant difference in the OGTT response in 20-day old *imd* and w^{1118} flies **(Figure 5-9A)**. *imd* flies had a greater increase in glucose following feeding and a slower clearance of glucose, suggesting that *imd* is required for a functional insulin response.

The increased weight and higher levels of circulating Ilp2 in *imd* flies indicate development of insulin resistance (Graham and Pick, 2017). I was curious to see if this phenotype developed with age or if *imd* mutants had a reduced insulin response from the start of adulthood at eclosion. To test this, I compared freshly emerged 1-day old *imd* and w^{1118} flies in an OGTT. Strikingly, I observed a similar result as with 20-day old flies, where *imd* mutants took longer to control glucose levels following feeding **(Figure 5-9B)**. These results suggest that *imd* is required for an efficient functional insulin response, rather than *imd* mutants develop insulin resistance over time. Together, these results show that *imd* influences systemic insulin signaling. Previous research found that activating the Toll

pathway, but not the IMD pathway, affected insulin activity (DiAngelo et al., 2009). However, the expression of a constitutively active Imd in the fat body was found to affect expression of several insulin pathway genes (Davoodi et al., 2019). Together with my findings, these data suggest that IMD signaling is involved in the regulation of insulin activity.



Time Point	P
0h (Fasted)	**<0.001**
2h (Fed)	**0.022**
4h (Re-fasted)	**<0.001**
6h (Re-fasted)	0.080

Time Point	P
0h (Fasted)	**<0.001**
2h (Fed)	**0.005**
4h (Re-fasted)	**<0.001**
6h (Re-fasted)	0.467

Figure 5-9 Oral glucose tolerance of *imd* and w^{1118} flies

(A-B) Oral glucose tolerance test (OGTT) performed on *imd* and w^{1118} flies raised on unmodified holidic food (HF) for (A) 20 or (B) 1 days. Statistical significance determined by Student's T-test.

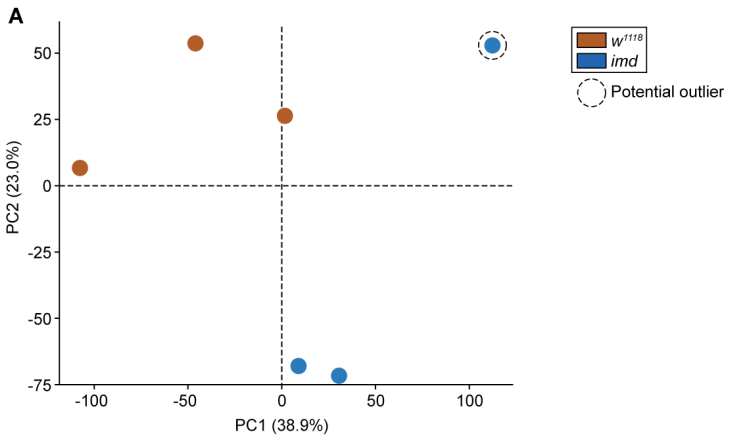
5.2.4. RNA-Seq comparison of w^{1118} and *imd* males

5.2.4.1. *imd* mutation alters expression of lipid metabolism genes

imd mutants have increased weight, altered macronutrient content, and a reduced insulin response, suggesting that *imd* is required to regulate metabolic homeostasis. However, little is known about how IMD signaling affects metabolism. To address this, I performed RNA

sequencing (RNA-Seq) to compare the transcriptome of *imd* mutants with w^{1118} control flies. I raised either *imd* or w^{1118} flies on HF for 20 days, then isolated total RNA from whole fly samples for RNA-Seq. Initially, I performed principal component (PC) analysis to verify that replicates grouped together and detect potential outliers. Looking at PC analysis, one of the *imd* replicates did not appear to group with the other *imd* samples, indicating a potential outlier **(Figure 5-10A)**. However, the low sample number makes it difficult to distinguish between biological variability or variability due to technical factors and identify a true outlier. While there is no clear established standard to define an RNA-Seq outlier, clustering of replicates in PC analysis is the most recommended method to detect outliers (Conesa et al., 2016). To address this, I decided to analyze the data both with and without the potential outlier.

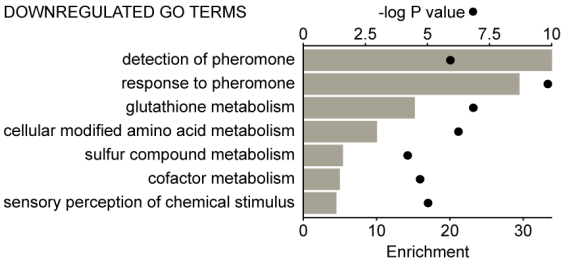
For analysis that includes all *imd* samples, I found that the *imd* mutation led to the downregulation of 195 genes and upregulation of 231 genes ($p < 0.01$, FDR < 0.05). Of these downregulated genes, many encode poorly characterized CG or long non-coding RNA (lncRNA) genes **(Figure 5-10B)**. lncRNAs are a class of RNA sequences that do not encode proteins, and are greater than 200 nucleotides long (Kapranov et al., 2007). Understanding the function of lncRNAs is a relatively new field, but lncRNAs appear to be important regulators of gene expression involved in diverse processes such as development, behavior, and immunity (Li et al., 2019; Mongelli et al., 2019). The human lncRNA, *Dnm3os*, can bind to and increase activity of NFκB (Das et al., 2018). One *Drosophila* lncRNA, *lncRNA-IBIN*, has also been associated with the immune response (Valanne et al., 2019), though I did not find it was differentially regulated in *imd* mutants. As the expression of many lncRNAs appears to be affected by *imd* mutation, it will be of interest to further examine their role in the immune response. Gene ontology (GO) term analysis to examine functional enrichment in this gene list found few enriched terms. However, the two GO terms with the highest enrichment score were associated with olfaction and the response to pheromone. As I previously observed increased feeding bouts in *imd* flies, these data support my hypothesis that the lack of *imd* may affect olfaction. Similar to downregulated genes, many of the genes upregulated in *imd* mutants are CG or lncRNA genes. GO term analysis of upregulated genes found no functional enrichment **(Figure 5-10C)**.



B

TOP DOWNREGULATED GENES

Gene	log_2 FC	Function
lncRNA:CR44641	-4.74	long non-coding RNA
lncRNA:CR44640	-4.71	long non-coding RNA
lncRNA:CR44344	-3.43	long non-coding RNA
CG42876	-3.10	unknown
lncRNA:CR45317	-3.00	long non-coding RNA
NimC1	-2.37	bacterial phagocytosis
lncRNA:CR44602	-2.34	long non-coding RNA
lncRNA:CR44645	-2.32	long non-coding RNA
CG44623	-2.25	unknown
Obp56h	-2.23	odorant binding protein
CG6421	-2.17	lysozyme
CG42821	-2.14	unknown
CG33958	-2.11	guanylate cyclase
CG14419	-2.04	unknown
GstE1	-2.02	glutathione S-transferase



C

TOP UPREGULATED GENES

Gene	log_2 FC	Function
Drsl2	6.94	antifungal peptide
lncRNA:CR44631	5.34	long non-coding RNA
CG43920	5.26	unknown
lncRNA:CR43808	4.81	long non-coding RNA
lncRNA:CR45045	4.36	long non-coding RNA
y	3.25	yellow color
ppk6	2.92	sodium ion channel
CG10764	2.68	unknown
lncRNA:CR45425	2.63	long non-coding RNA
CG31091	2.45	unknown
BomT1	2.27	Toll-induced peptide
lncRNA:CR45229	2.23	long non-coding RNA
CG14499	2.20	unknown
Su(Ste):CR42407	2.11	unknown
Jon66Ci	2.11	elastase-like

Figure 5-10 RNA-Seq comparison of *imd* and w^{1118} flies with all samples

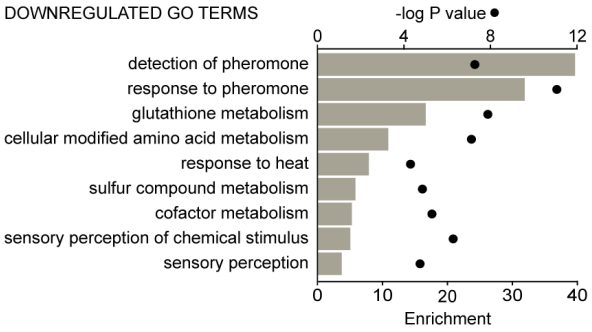
(A) Principal component (PC) analysis of RNA-sequencing data comparing 20-day old *imd* or w^{1118} male flies raised on unmodified holidic food (HF). Each dot represents one replicate (B) Top downregulated differentially expressed genes in *imd* flies compared to w^{1118} flies ($p < 0.01$, FDR < 0.05) and Gene Ontology (GO) analysis from downregulated differentially expressed genes. Bars (bottom x axis) represent enrichment scores and black circles (top x axis) represent -logP values for each enriched GO term. (C) Top upregulated differentially expressed genes in *imd* flies compared to w^{1118} flies ($p < 0.01$, FDR < 0.05).

Next, I removed the potential outlier from the *imd* samples and repeated analysis of the data. I found that *imd* mutation led to the downregulation of 199 genes and upregulation of 280 genes ($p < 0.01$, FDR < 0.05). Again, I found that many of these downregulated genes encoded CG or lncRNA genes, the function of which is still poorly understood **(Figure 5-11A)**. GO term analysis of downregulated genes was similar to the previous analysis with the outlier included. Removal of the potential outlier had a greater impact on the number of upregulated genes in *imd* mutants **(Figure 5-11B)**. GO term analysis of upregulated genes found several terms associated with immunity as well as synthesis of lipid molecules. As the *imd* mutation effectively blocks one of the main bacterial response pathways, perhaps the upregulation of other immune genes is a form of compensation. It will be of interest to explore how *imd* mutation impacts regulation of other immune pathways, such as Toll signaling or the DUOX pathway. Interestingly, the enrichment of GO terms associated lipid synthesis support my hypothesis that *imd* mutants have disrupted lipid metabolism. Looking at lipid metabolism genes that were upregulated in *imd* mutants, I found genes associated with *de novo* lipogenesis, synthesis of triglycerides, and lipid transport. Combined with previous evidence that *imd* mutants have increased weight, increased triglycerides, and disrupted insulin activity, it will be of interest in future studies to investigate how IMD signaling interacts with these specific aspects of lipid metabolism.

A TOP DOWNREGULATED GENES

Gene	log_2FC	Function
lncRNA:CR44505	-7.83	long non-coding RNA
lncRNA:CR43399	-4.68	long non-coding RNA
lncRNA:CR44640	-4.58	long non-coding RNA
lncRNA:CR44641	-4.51	long non-coding RNA
lncRNA:CR44344	-4.23	long non-coding RNA
lncRNA:CR45317	-3.40	long non-coding RNA
CG42876	-3.08	unknown
Cpr49Ah	-3.03	cuticle protein
lncRNA:CR43412	-2.74	long non-coding RNA
BomBc1	-2.51	Toll-induced peptide
CG44622	-2.44	unknown
CG6421	-2.40	unknown
CG44623	-2.37	unknown
GstE1	-2.35	glutathione S-transferase
NimC1	-2.31	bacterial phagocytosis

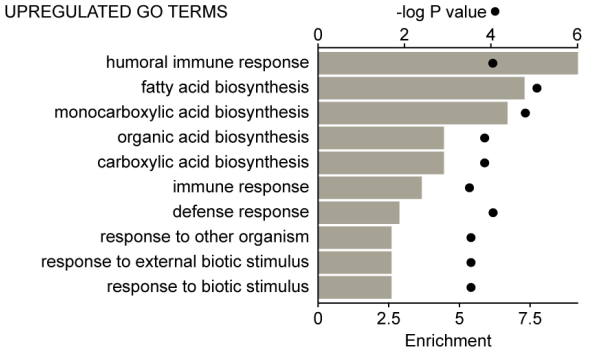
DOWNREGULATED GO TERMS



B TOP UPREGULATED GENES

Gene	log_2FC	Function
Drsl3	3.51	antifungal peptide
ppk6	2.91	sodium ion channel
CG31091	2.83	unknown
CG10764	2.62	trypsin-like
lncRNA:CR45045	2.47	long non-coding RNA
Jon66Ci	2.31	elastase-like
APC7	2.19	cell cycle
Su(Ste):CR42418	2.04	unknown
w	2.03	eye color
Su(Ste):CR42407	2.03	unknown
CG9377	1.98	unknown
Lsp1alpha	1.93	unknown
CG14499	1.91	unknown
vanin-like	1.90	unknown
CG30091	1.90	unknown

UPREGULATED GO TERMS



C UPREGULATED LIPID METABOLISM GENES

Gene	log_2FC	Function
CG1946	1.69	enzymatic synthesis of TAG
FASN3	0.97	*de novo* lipogenesis and dietary lipid digestion
CG6283	0.77	*de novo* lipogenesis and dietary lipid digestion
Apolpp	0.67	lipid transport
FASN2	0.55	*de novo* lipogenesis and dietary lipid digestion
Mdy	0.51	enzymatic synthesis of TAG
bgm	0.49	regulation of lipolysis
LpR2	0.39	lipid transport
LpR1	0.29	lipid transport

Figure 5-11 RNA-Seq comparison of *imd* and w^{1118} flies with potential outlier removed

(A-C) RNA-sequencing analysis comparing 20-day old *imd* or w^{1118} male flies raised on unmodified holidic food (HF). (A) Top downregulated differentially expressed genes in *imd* flies compared to w^{1118} flies ($p < 0.01$, FDR < 0.05) and Gene Ontology (GO) analysis from downregulated differentially expressed genes. Bars (bottom x axis) represent enrichment scores and black circles (top x axis) represent -logP values for each enriched GO term. (C) Top upregulated differentially expressed genes in *imd* flies compared to w^{1118} flies ($p < 0.01$, FDR < 0.05) and Gene Ontology (GO) analysis from downregulated differentially expressed genes. Bars (bottom x axis) represent enrichment scores and black circles (top x axis) represent -logP values for each enriched GO term. (C) Upregulated lipid metabolism genes of interest in *imd* flies compared to w^{1118} flies.

5.3. Discussion

In this chapter, I found that GSF-treatment improves survival in male flies against infection with *V. cholerae*, though the mechanism remains to be determined. The observation that *imd* mutants are larger than wild type w^{1118} flies led me to ask if *imd* is required for metabolic homeostasis. I found that *imd* flies have increased weight, glucose, and triglyceride levels, despite no difference in consumption. The lack of *imd* appears to disrupt insulin activity as *imd* mutants have slower clearance of glucose than w^{1118} flies. Through RNA-Seq comparison of *imd* and w^{1118} flies, I found that IMD signaling affects lipid homeostasis. Combined, these data demonstrate a clear impact of *imd* mutation on metabolic homeostasis. I hypothesize that IMD signaling is involved in regulation of lipid metabolism, perhaps through IIS signaling, but future studies will be required to explore this mechanism.

In summary, these findings contribute to the growing literature linking immunity and metabolism. With the high energy demand of an immune response, it seems reasonable that a diet with increased calories, such as GSF compared to HF, can improve the survival response to infection. Nutritional intervention is a promising approach to limit the damage of infection, and further study will be required to determine optimal nutritional regimes. My findings that IMD may have an important role in metabolic homeostasis, however, are more unexpected, as IMD signaling is traditionally studied for its role in pathogen sensing. However, as they both interact with the external environment, crosstalk between signaling pathways governing nutrient acquisition or the immune response may have long-standing evolutionary interactions that are receiving greater appreciation.

Chapter 6
Discussion

6. Discussion

Drosophila melanogaster is an excellent model to uncover fundamental aspects of immunity and metabolism. Interactions between diet, health, immunity, and lifespan involve numerous factors and are difficult to study in humans or mammalian models. Many important immune and metabolic pathways are highly conserved between flies and mammals, and metabolic organs in flies perform similar functions to those in mammals. In this project, I used the *Drosophila melanogaster* model to explore how diet impacts health, longevity, and immunity.

6.1 Diet and microbiota

Diet has a substantial role in shaping the composition of the intestinal microbiota, encouraging the growth or colonization of a particular bacterial strain over another (Bibbò et al., 2016; Brown et al., 2012). Switching between diets with different proportions of macronutrients causes rapid and dramatic changes in the microbiota composition (Turnbaugh et al., 2009). The interactions between diet and the microbiota are very complex, where diet-acquired nutrients affect microbes through direct and indirect mechanisms (Zmora et al., 2019). Numerous aspects of diet influence the microbiota, including the specific macronutrient content, the ratios of nutrients, quantity of food consumed, and time of food consumption for example. As diet-microbiota interactions are potentially beneficial or harmful to human health, it is important that researchers try to understand these complex interactions.

6.1.1 Diet and *Drosophila* intestinal microbiota

I found that different supplementations to the holidic diet had a dramatic impact on the intestinal microbial composition. The changes in microbiota composition after only 10 days on each given modified diet is quite striking, and demonstrates the sizeable impact that diet has on the microbiota. As the microbiota is known to have wide ranging effects on host health (Zmora et al., 2019), it seems reasonable that the large diet-dependent shifts in composition and diversity that I observed may contribute to differences in fly host health. Most fly labs generally report similar microbiota composition, with a dominance of

Acetobacter and *Lactobacillus* genera. While the different food consumed may alter the host intestinal environment, an alternative explanation is that the food itself supports a different microbial population. Differentiating between these hypotheses may be difficult, but rapidly transferring flies to fresh sterile food at least every day or even twice a day to limit bacterial growth may be one method. However, the stability of the microbiota in the fly gut would affect the possibility of this experiment.

Flies raised on glucose-supplemented (100 g/L) food in particular had the most diverse microbiota composition of all the experimental diets I tested. However, I found that flies raised on glucose-supplemented food still lived longer than flies raised on unmodified holidic food independent of the elimination of their intestinal microbiota through antibiotic treatment. Despite the phenotypic similarities between flies raised under germ-free conditions and flies raised on a glucose-supplemented diet, I established that glucose-supplemented food does not require a microbiota to improve immunity or longevity. Instead, my data suggest that host-intrinsic responses are key to the benefits of supplementation with glucose. An alternative interpretation is that having an intact microbiota decreases both lifespan and survival against *V. cholerae* infection in flies raised on an unmodified diet. In this case, glucose supplementation would be countering the negative effects of the microbiota. In our lab, we showed that mono-association with *L. plantarum* specifically, and not *A. pasteurianus*, was detrimental to host lifespan compared to the lifespan of germ-free counterparts (Fast et al., 2018a). Though I found that *Lactobacillus* occupied a larger percent of the microbial composition in flies raised on glucose-supplemented food, I did not specifically measure the abundance of *L. plantarum* in these flies. It would be interesting to measure how glucose-supplemented food affects the commensal *L. plantarum* population and to determine if glucose-supplemented food could counteract the decreased lifespan of flies that are mono-associated with *L. plantarum*.

However, while I found that glucose supplementation influenced lifespan and immunity in the absence of the microbiota, I cannot exclude that some dietary modifications act at least partially through effects on the microbiota in *Drosophila*. While I focused on glucose supplementation, I also found that flies fed a diet supplemented with moderate amounts (1%) of ethanol lived longer and survived infection better than control flies raised on an unmodified diet. Since wild flies develop in and consume decomposed fruit, ethanol is

likely a common constituent of their environment. As ethanol is a calorie source for *Acetobacter*, a prominent fly commensal that modifies insulin and TOR signals in the midgut (Shin et al., 2011; Storelli et al., 2011), it remains possible that the lifespan extension I see from ethanol depends on the microbiota.

6.1.2 Microbiota elimination in *Drosophila*

Consistent with other reports (Clark et al., 2015; Lee et al., 2019; Petkau et al., 2014), I found that elimination of the adult microbiota extended the lifespan of flies raised on holidic food. Increasing bacterial load with age may contribute to this observation. This phenomenon has also been observed in a vertebrate model, as eliminating the microbiota through antibiotic treatment extends lifespan in the African turquoise killifish (Smith et al., 2017).

Notably, for flies raised on glucose-supplemented food, antibiotic treatment did not significantly extend lifespan. One explanation may be that there is a limit to how far lifespan can be extended in this model. Perhaps flies raised on the glucose-supplemented food are near this maximum lifespan, and the removal of the microbiota has no further effect. Alternatively, the elimination of the microbiota and being raised on glucose-supplemented food may have overlapping mechanisms of lifespan extension. As I previously described, an alternative explanation may be that the microbiota has a negative effect on lifespan, and glucose supplementation might counter this. As bacterial load is a significant determinant of fly lifespan (Lee et al., 2019), it is possible that glucose-supplemented food encourages lower bacterial growth. While this might contribute to the glucose-supplemented lifespan extension, my finding that germ-free flies still live longer on a glucose-supplemented diet suggests that this is not the entire explanation.

While I used antibiotic treatment to remove the microbiota of adult flies, many labs generate axenic flies through bleach-mediated dechorionation at the embryo stage (Koyle et al., 2016). As the embryo itself is sterile, this technique helps avoid residual bacterial exposure and allows control over vial egg density. This axenic method also avoids potential off-target effects of antibiotic treatment (Brodersen et al., 2000). However, elimination of the microbiota at the embryo stage may have deleterious effects on development, as axenic flies generated with this method take significantly longer to develop and weigh less than

untreated flies (Heys et al., 2018). Interestingly, a recent study found that streptomycin treatment was the most effective at eliminating the microbiota and the least harmful to host health (Heys et al., 2018). While which method is most suitable for elimination of the microbiota remains under debate, it may be useful to determine if glucose-supplemented food enhances lifespan and immunity independent of the microbiota through the alternative axenic embryo method.

6.2 Glucose and lifespan

A prominent finding in my project was that 100 g/L glucose-supplemented food extends *Drosophila* lifespan, and that this lifespan extension is optimal with 50 g/L glucose supplementation. This lifespan extension was particularly effective in males, although females raised on 100 g/L glucose-supplemented food also lived longer than those raised on unmodified holidic food. I consider the differences observed between sexes in greater detail later in this discussion. While I mainly focused on wild-type w^{1118} flies, I found that glucose-supplementation also extended lifespan in flies from different genetic backgrounds including wild-caught flies, $esg^{ts}>InR$ flies, *ilp2-3,5* flies, and $GS\text{-}5966>UAS\text{-}cora^{RNAi}$ flies, suggesting that the lifespan extension from glucose supplementation is not genotype specific.

While I explored how glucose-supplemented food extends lifespan in this project, it is important to note that my findings may not be specific to glucose. It is possible that other types of sugar could lead to a similar result, such as supplementation with fructose or sucrose. Alternatively, as glucose-supplemented food has a lower protein to carbohydrate ratio than unmodified holidic food, the shift in macronutrient ratio may explain the observed lifespan extension as diets with low a protein to carbohydrate ratio have been found to extend lifespan (Lee et al., 2008; Simpson and Raubenheimer, 2009; Solon-Biet et al., 2015b, 2014). However, I found that starch supplementation, which is also an effective reduction in protein to carbohydrate ratio, reduced lifespan slightly in males and females, indicating that all carbohydrates do not have the same effect on lifespan. I also cannot rule out that glucose supplementation extends lifespan through an indirect effect, such as reducing the bacterial load on the food or within the fly.

In my perspective, diet-lifespan studies are separated into having one of two distinct, but overlapping, goals: to determine (1) what aspects or components of diet influence lifespan, and (2) what mechanisms diet acts through to affect lifespan. In this view, my project was largely focused on the latter. I will speculate why the glucose supplementation in particular extended *Drosophila* lifespan, but future studies will be required to explore what characteristics of glucose-supplemented food benefit lifespan when compared to unmodified holidic diet.

6.2.1. Lifespan studies with the holidic diet

Decreased caloric intake is often associated with increased lifespan (Dilova et al., 2007; Fontana et al., 2010). Glucose supplementation to the holidic diet is an effective increase of calories, but also extends *Drosophila* lifespan. Supplementation with either 50 g/L glucose (416.3 kcal/L) or 100 g/L glucose (616.3 kcal/L) raises the caloric density compared to the unmodified holidic diet (216.3 kcal/L). This may suggest that the unmodified holidic diet simply does not provide sufficient calories for optimal lifespan. Conversely, I found that flies raised on 50 g/L casein-supplemented food (416.3 kcal/L) or 22.2 g/L lard-supplemented food (416.3 kcal/L) either had no change or reduced lifespan, respectively, compared to an unmodified holidic diet. However, the source of calorie provided is important (Mair et al., 2005). As all the dietary modifications I tested increased the caloric density compared to the unmodified holidic diet, it would be of interest to test the effect of calorie reduction on lifespan in flies raised on the holidic diet. The holidic diet has not yet been used to study caloric restriction. As previous studies typically rely on dilution to reduce calorie, the holidic diet would be useful to manipulate the amounts of specific nutrients provided.

The authors of the holidic medium recipe methodically constructed the holidic diet to support longevity comparable to a previously described oligidic diet that was designed to promote longevity (Bass et al., 2007; Piper et al., 2014). However, they also aimed to optimize egg-laying, or fecundity, in flies raised on the holidic diet. As lifespan and reproduction have known trade-offs (Travers et al., 2015), this suggests that the holidic diet limits the potential maximum lifespan to allow sufficient fecundity. Conversely, I speculate that glucose-supplemented food may extend lifespan at the expense of reduced fecundity

compared to unmodified holidic food. It would be intriguing to compare the egg-laying capacity of flies raised on either a glucose-supplemented or unmodified holidic diet.

Interestingly, flies raised from the embryo stage on the holidic diet take longer to develop than those on Bloomington cornmeal food, and a smaller percentage of flies survived from embryo to adult (Piper et al., 2014). This developmental delay is rescued through provision of yeast extract, suggesting that the holidic diet does not provide all nutritional requirements necessary to replicate the Bloomington cornmeal food. However, for my experiments, I transferred flies to the holidic diet at the adult stage and did not investigate dietary effects on development. A high-sucrose diet (1.0M compared to 0.15M) delayed larval development and reduce the size of larvae (Musselman et al., 2011). However, the glucose-supplemented food used in my study was not intended to model a high-sugar diet. The unmodified holidic diet provides approximately 0.05M of sucrose, while 50 g/L glucose supplementation provides an additional 0.28M of glucose. Future studies will be required to investigate how glucose-supplemented holidic food impacts *Drosophila* development.

6.2.2. Nutritional geometry and lifespan

Several studies demonstrated that the ratio of protein to carbohydrate (P:C) in a diet exerts a substantial influence on the health of flies and mice, with extended lifespan observed in animals raised on diets with low P:C ratios (Bruce et al., 2013; Grandison et al., 2009; Lee et al., 2008; Mirzaei et al., 2014; Piper et al., 2011; Solon-Biet et al., 2015a, 2015c, 2014). Consistent with this hypothesis, I showed that supplementation of 100 g/L glucose (P:C of 1:10.9), an effective drop in P:C ratio compared to the unmodified holidic diet (P:C of 1:1.6), extended lifespan in males and females. Conversely, the supplementation of 70 g/L casein (P:C of 1:0.2), an effective increase in P:C ratio, reduced the lifespan of female flies, though it did not have a significant effect on male lifespan. I later found that supplementation with 50 g/L glucose (P:C of 1:6.3) optimized male lifespan on the holidic diet. At the same time, 50 g/L casein-supplemented food (P:C of 1:0.3) had no effect on male lifespan compared to unmodified food. These results suggest that simply changing the P:C ratio is not sufficient to regulate lifespan, and that the specific source of protein or carbohydrate is am important factor. Perhaps if I increased the amino

acids provided in the diet rather than supplemented with casein, I would have observed a different outcome in lifespan. While my results largely support what is seen in nutritional geometry studies, I mainly focussed on comparing the effects of two diets. A thorough, comprehensive study with several diets ranging in their P:C ratios would be required to determine if glucose-supplemented food extends lifespan because of its lower P:C ratio relative to the unmodified holidic diet.

6.2.3. Glucose and growth signaling

It has long been hypothesized that there is a trade-off between growth rate and lifespan (Blagosklonny and Hall, 2009; Lee et al., 2013). Reducing the activity of nutrient-sensing pathways that promote growth, such as IIS and TOR signaling, has often been shown to extend lifespan (Fontana and Partridge, 2015). For example, *Drosophila* insulin mutants (*ilp2-3,5*) are smaller than wild-type flies but live longer (Clancy et al., 2001; Tatar et al., 2001). I found through microarray analysis that 100 g/L glucose-supplemented food decreased the expression of wide-ranging growth and differentiation pathways in w^{1118} females compared to unmodified holidic food. I hypothesized that glucose-supplemented food extends lifespan through downregulation of MAPK signaling.

While genes associated with many different growth pathways were downregulated in flies raised on glucose-supplemented food, I initially focused on MAPK signaling through the Ras protein as Ras is a nexus for several pathways, including insulin signaling. In *Drosophila,* there are eight membrane-bound receptor tyrosine kinases (RTKs) that signal through the canonical MAPK signaling cascade, and seven of the eight have known mammalian homologs (Sopko and Perrimon, 2013). These include the insulin receptor (InR), Alk, Epidermal growth factor receptor (Egfr), Heartless (Htl), Breathless (Btl), PDGF- and VEGF-receptor related (Pvr), Sevenless (Sev), and Torso (tor). Diet-dependent regulation of lifespan through Ras has previously been observed in studies of caloric restriction in both *C. elegans* (Wei et al., 2008) and mice (Xie et al., 2007).

Unfortunately, due to experimental limitations, I was unable to test my hypothesis that glucose-supplemented food extends lifespan through downregulation of RTK signaling. Specifically, both *UAS-*Ras^{V12} and *Ilp3-GAL4* fly lines may have potential genetic background effects on lifespan. To address this, I would require a comprehensive screen

where all flies used have been backcrossed into the same genetic background. Initially, I would raise MAPK signaling mutants, such as *Lnk* mutants on either a glucose-supplemented or unmodified holidic diet. If I found that a MAPK mutant had a similar lifespan on either diet, then I could perform a follow-up screen where I knocked down the pathway of interest with a range of tissue-specific drivers.

Interestingly, RTK signaling has been implicated in regulation of lifespan in *Drosophila*. Insulin signaling has a well-studied association with lifespan but signaling through other RTKs may also influence lifespan. Lnk is an adaptor protein that is required to transduce signals from a number of RTKs, and *Lnk* mutants were found to have a longer lifespan than wild-type controls (Slack et al., 2010). Further down the MAPK signaling cascade, ubiquitous RNAi knockdown of Ras also extends lifespan, as does pharmacological treatment with the drug Trametinib, which inhibits the phosphorylation of ERK by Ras (Slack et al., 2015). Recently, the inhibition of the receptor tyrosine kinase, Alk, was found to extend longevity (Woodling et al., 2020). Combined, MAPK signaling appears to be important in the regulation of lifespan. Notably, all these studies performed interventions at a systemic level, and it is unknown if MAPK signaling in a specific tissue is required for regulation of lifespan. As I was unable to rule out that lifespan extension through glucose supplementation involves MAPK signaling, it remains possible that glucose-supplemented food extends lifespan through a mechanism that involves reduced growth signaling, possibly through downregulation of MAPK signaling.

6.3 Intestinal barrier and lifespan

Maintenance of the epithelial barrier is essential for health and longevity. Occluding junctions, known as tight junctions in vertebrates or the related septate junctions (SJs) of invertebrates, form this barrier, allowing for regulated movement across the epithelium (Zihni et al., 2016). Disruptions to the expression and localization of tight junction components are observed in both Crohn's disease (Zeissig et al., 2007) and sepsis (Yoseph et al., 2016), with the upregulation of pore-forming claudin-2 and downregulation of sealing claudin-5 in both cases. Intestinal permeability also increases with age, as occluding junction proteins are downregulated (Parrish, 2017). In flies, formation and maintenance of SJ protein complexes relies on several proteins including Mesh (Izumi et

al., 2012), Snakeskin (Ssk) (Yanagihashi et al., 2012), and Coracle (Cora) (Lamb et al., 1998). Similar to vertebrates, disruption of SJs affects both health and longevity. For example, loss of Ssk affects composition of the gut bacterial community, while upregulation of Ssk extends lifespan (Salazar et al., 2018).

I found that 20-day old flies raised on 50 g/L glucose supplemented food had increased expression of cell junction proteins, many associated specifically with smooth septate junctions that form in endoderm-derived epithelial tissue, such as the midgut. When I looked at localization of the septate junction protein Coracle in midgut enterocytes, I found that 40-day old flies raised on glucose-supplemented food had higher localization of Coracle to the bicellular junction. Flies raised on glucose-supplemented food also had increased barrier function with age, and chemical disruption of the intestinal barrier removed their survival advantage. Combined, these data suggest that glucose supplementation may extend lifespan through enhancing intestinal barrier integrity **(Figure 6-1)**.

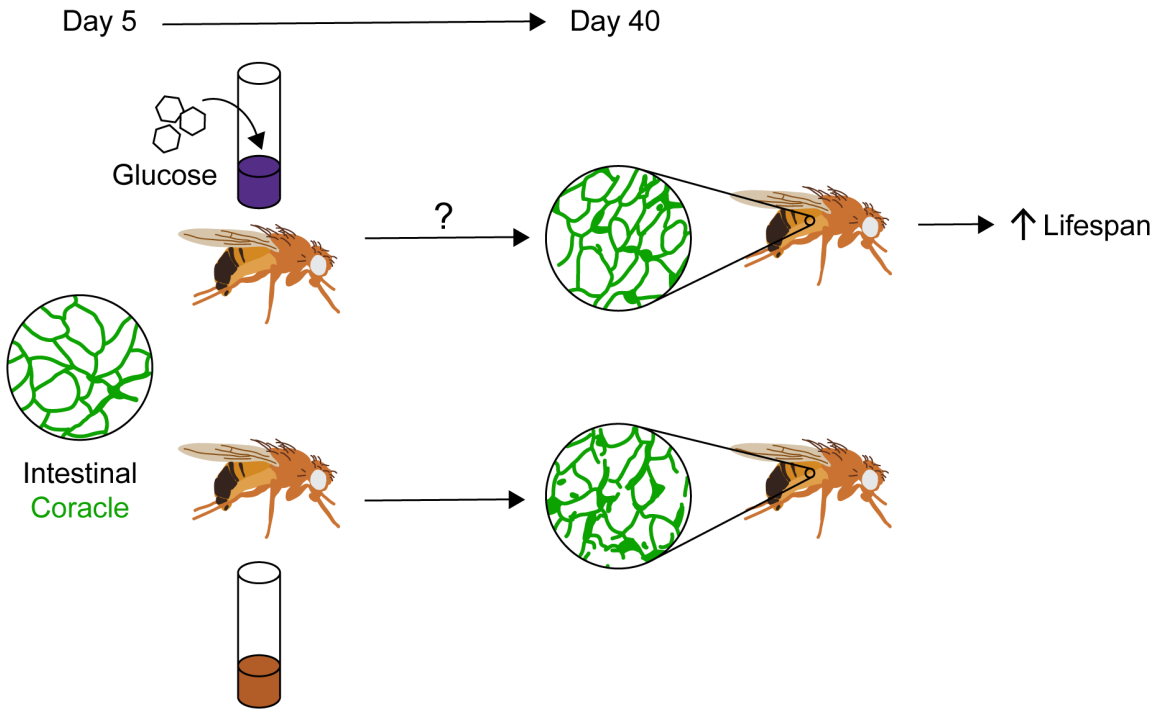


Figure 6-1 Glucose-supplementation enhances Coracle localization and extends lifespan

Flies raised on 50 g/L glucose-supplemented holidic food (GSF) have increased lifespan compared to those raised on unmodified holidic food (HF). GSF-fed flies also have enhanced intestinal barrier integrity (represented here by visualization of Coracle) with age compared to those raised on HF. However, the mechanism for how GSF improves intestinal barrier integrity remains unknown.

While I found that glucose supplementation was associated with enhanced intestinal barrier integrity, my results are largely correlative rather than causative. Disruption of the intestinal barrier through DSS-treatment removed the survival advantage of GSF-fed flies but was also detrimental to flies raised on either diet compared to untreated controls. An experiment that more convincingly establishes a causative mechanism would be important. Specifically, an intervention that reduced the survival of flies raised on glucose-supplemented food to the level of flies raised on unmodified food, without having a severe impact on the lifespan of flies raised on unmodified food, would be ideal. The knockdown of Coracle in enterocytes, for example, could potentially have served this purpose. Importantly, while I used Coracle as a marker to examine the effect of diet on septate junctions, other proteins, such as Ssk, Mesh, or Bbg, may have a greater physiological role

in how diet affects septate junctions. Knockdown of these other septate junction proteins, alone or in combination, may possibly address if glucose-supplementation extends lifespan through improved intestinal barrier integrity. Alternatively, I could raise *bbg* mutants on both diets and measure the effect on lifespan. Further experimentation is needed to establish a causative link that glucose-supplementation extends lifespan through enhanced barrier integrity.

6.3.1. Diet and barrier maintenance

Effects of glucose-supplemented food on the intestinal barrier are consistent with previous literature that linked food intake to intestinal permeability, frequently by targeting occluding junctions (De Santis et al., 2015). For example, the amino acid glutamine has received interest for its therapeutic potential in intestinal health, as glutamine appears to directly and indirectly upregulate levels of tight junction proteins (Kim and Kim, 2017). Conversely, gliadin, a component of wheat, increases intestinal permeability in celiac disease through disassembly of tight junctions (Schumann et al., 2017). Gliadin binds CXCR3, inducing a MyD88-dependent release of zonulin, a known modulator of tight junctions (Fasano, 2011). Loss of zonulin weakens tight junctions by altering the localization of junction proteins (Lammers et al., 2008). With their analogous role and many conserved proteins, studying the septate junctions of *Drosophila* will provide a useful *in vivo* model to explore relationships between food and the integrity of occluding junctions.

6.3.2. Mechanism of glucose enhancement of intestinal barrier integrity

Although I did not identify the molecular mechanism by which glucose supplemented food improves intestinal barrier integrity, others have explored the effect of glucose on epithelial barriers. Exposure of human retinal pigment epithelial cells to high glucose (25 mM compared to 5.5 mM) improved barrier function by increased expression of tight junction proteins (Villarroel et al., 2009). Conversely, hyperglycemia in mice, induced by streptozotocin treatment, drives intestinal barrier dysfunction by global transcriptional reprogramming of intestinal epithelial cells, specifically by downregulation of N-glycan

biosynthesis genes (Thaiss et al., 2018), a critical pathway for tight junction assembly (Nita-Lazar et al., 2010). While the effect of glucose on barrier integrity is unclear, evidence suggests that glucose transporters may colocalize or interact with tight junction proteins (Rajasekaran et al., 2008).

A recent study in flies suggests that dietary restriction through reduced yeast enhances barrier function via Myc activity in intestinal enterocytes (Akagi et al., 2018). Though my study was not designed to limit protein intake, my CAFE assay data indicate that flies raised on glucose-supplemented food received 14% of their calories from protein, whereas flies raised on unmodified holidic food received 38% of their calories from protein. My RNA-Seq data did not uncover differential expression of the *myc* gene. However, I cannot exclude the possibility that glucose supplementation may improve barrier integrity in a Myc-dependent manner. Future studies will be required to determine the role of Myc in glucose supplementation-dependent enhancement of the intestinal barrier.

A major question that emerges from my data is how does glucose supplementation mechanistically lead to enhanced barrier integrity? While a more direct mechanism may be involved, such as enterocyte Myc expression, I speculate that neuronal regulation is involved. Neuronal regulation has been found to be involved in both glucose homeostasis and intestinal physiology. A pair of glucose-sensing neurons was found to regulate systemic insulin and glucagon signaling (Oh et al., 2019). Neurons have also been found to regulate intestinal physiology. For example, neuronal hedgehog signaling controls intestinal stem cell differentiation in the midgut (Han et al., 2015). The permeability of the peritrophic matrix, which lies on the lumenal side of the intestinal epithelium, is controlled by a subset of neurons (Kenmoku et al., 2016). Intriguingly, a subset of neurons was found to sense nutritive sugars, including glucose, and signal to the gut to control gut motility (Dus et al., 2015). Combined, I hypothesize that the supplementation of glucose is recognized by neurons, directly or indirectly through the fat body or intestine. These neurons then signal to the midgut leading to increased cell junction gene expression in enterocytes and an enhanced intestinal barrier **(Figure 6-2)**. This model also raises the question of why would the brain control intestinal barrier integrity in response to glucose? I speculate that perhaps the glucose level is sensed by the brain as an indirect indicator of the intestinal environment. If the brain interprets increased glucose levels as a proxy of

increased feeding and a corresponding intake of intestinal microbes, then it may respond by increasing expression of intestinal cell junction genes.

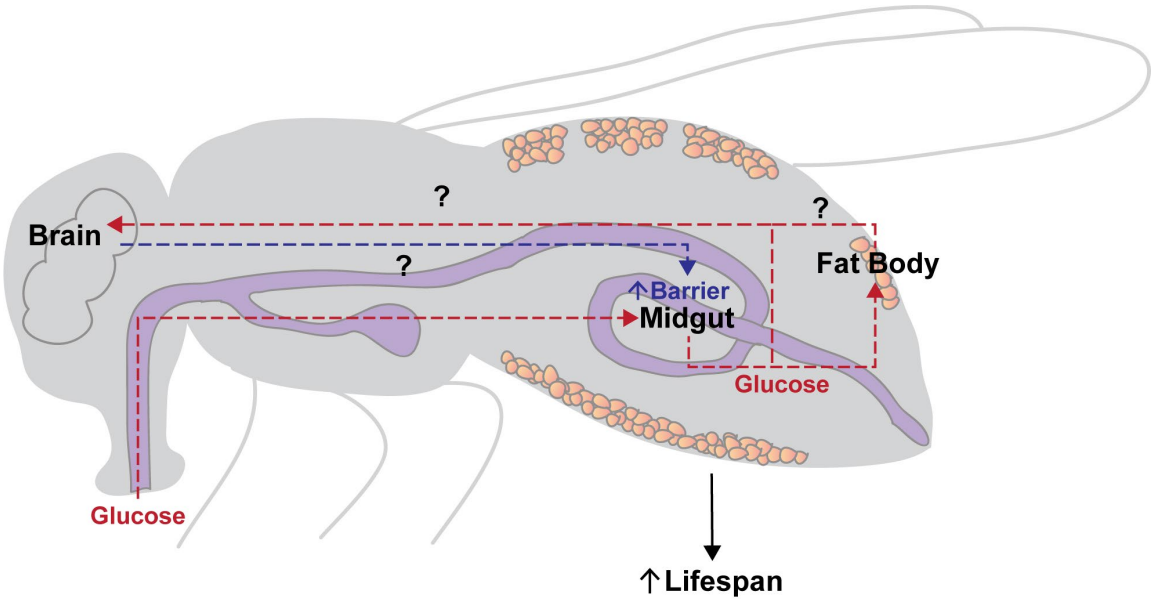


Figure 6-2 Proposed model for glucose-supplementation enhancement of intestinal barrier

The following is a hypothetical model of how glucose supplementation leads to improved intestinal barrier integrity. Dietary glucose crosses the intestinal epithelium via absorption by enterocytes. Increased circulating glucose is recognized by the fat body or brain directly. If by the fat body, an unknown cytokine signals to the brain. Following recognition in a change in glucose levels, the brain secretes an unknown neuropeptide to the midgut, signaling enterocytes to increase expression of genes involved in septate junction formation.

6.4. Differences between sexes

While I primarily performed experiments on male flies, 100 g/L glucose-supplemented food also improved lifespan in females, though not to the same extent as in males. Recent reports have revealed distinct sex differences in intestinal physiology, including a higher proliferative rate in intestinal stem cells of females, that could affect the response to dietary interventions (Hudry et al., 2016; Millington and Rideout, 2018). Dietary restriction, which extends lifespan partly by limiting proliferation, is much more effective in females (Regan et al., 2016), while high-sucrose diets are more detrimental to females (Chandegra et al.,

2017). In females, the high nutritional requirements of oogenesis may contribute to these distinct responses compared to males (Wu et al., 2020). The metabolic response to sucrose itself is distinct between sexes, as bi-directional communication between male gonads and the proximal intestine drives a male-biased increased expression of sugar metabolism genes in the midgut (Hudry et al., 2019). As I focused on males, it is worth considering that the lifespan extension associated with glucose supplementation and observed physiological changes may be different in females. In the future, it will be interesting to see if glucose-supplemented food improves intestinal barrier integrity of females.

6.4.1. Sex differences in nutritional responses

I found that dietary modifications that reduced lifespan, such as supplementation with palmitic acid or protein, were more detrimental to females than male flies. Conversely, dietary modifications that benefitted lifespan, such as supplementation with ethanol or glucose, had a greater effect on the lifespan of males than females. While both males and females appear to maximise lifespan on low protein to carbohydrate (P:C) diets, male lifespan appears to be more sensitive to differences in diet (Maklakov et al., 2008). Likewise, I observed similar trends between males and females. Interestingly, a study using nutritional geometry found that both male and females had a maximum lifespan raised on a diet with a P:C ratio of approximately 1:16 (Jensen et al., 2015). However, males maximised progeny production when raised on a diet with a P:C of 1:8, while females required a diet with P:C of 1:2 to maximise egg production. This difference illustrates the competing demands of lifespan and reproduction, and that this trade-off differs between the sexes.

As previously mentioned, glucose supplementation appeared to be particularly beneficial to male lifespan compared to female lifespan. This may be due to sex differences in carbohydrate metabolism. A high-sucrose diet (1.17M compare to 0.15M controls) was found to reduce the median lifespan of females by about 38% but only about a 12.5% reduction of median lifespan in males (Chandegra et al., 2017). The authors suggest that different reproductive requirements may explain this difference. However, this report contradicts with the previously described study that showed that male lifespan was more sensitive to diets with increasing P:C ratios (Maklakov et al., 2008). Despite, this

contradiction, both reports argue that females require higher protein levels to maximise reproductive potential, whereas this is not the case for males. Interestingly, the proximity of the male gonads to the midgut appears to partly account for sex differences in carbohydrate metabolism (Hudry et al., 2019). Communication between the midgut enterocytes and the nearby male testes leads to increased production of sugar metabolism genes in enterocytes that is not observed in females. Taking advantage of fly genetics, the authors showed that masculinization of female gonads could resulted in expression of sugar metabolism genes similar to males. This study demonstrates that the sexual identity at a cellular level orchestrates different metabolic responses. As glucose supplementation affected male and female lifespan to a different extent, it would be intriguing to take advantage of this technique and ask if masculinization of a particular organ allowed female flies to live as long as males on glucose-supplemented food.

6.4.2. Sex differences in immune responses

Males and females have dramatic differences in their immune responses (Belmonte et al., 2020). Survival against infection has a male- or female-bias depending on the pathogen. I found that female flies had an improved survival response to *V. cholerae* infection when raised on 100 g/L glucose-supplemented food, but males had no improvement in survival. It is interesting that this particular diet is more beneficial for male lifespan in the long-term, but benefits females during *V. cholerae* infection. Later I find that males raised on 50 g/L glucose-supplemented food do have improved infection survival compared to those raised on an unmodified diet. Combined, these data demonstrate that it is essential that diet and sex be taken into consideration when studying the immune response. As I only tested the male response, it would be useful to see how the 50 g/L glucose-supplemented food impacted infection survival in females against *V. cholerae*.

6.5. Diet and immunity

Diet has a profound influence on the response to infection. For example, dietary restriction has a complicated impact on host defenses. In some cases, it improves survival, such as after infection with *Salmonella typhimurium* or *Pseudomonas aeruginosa* (Lee et

al., 2017). Here, dietary restriction improves survival by lowering TOR activity which leads to higher abundance of Myc, a transcription factor that induces antimicrobial genes. In other cases, such as upon infection with *Listeria monocytogenes*, dietary restriction reduces survival (Ayres and Schneider, 2009). *L. monocytogenes* exerts its pathogenesis in part through its impact on host metabolism. Specifically, infection with *L. monocytogenes* leads to a decrease in both stored fats and glycogen (Chambers et al., 2012). These data have parallels in rodent models of infection, where starvation has differential effects on host survival after infection with a bacterial or viral pathogen (Wang et al., 2016). Feeding mice accelerates death after infection with *L. monocytogenes*, whereas feeding improves survival after infection with influenza virus.

6.5.1. Glucose and antimicrobial peptide expression

While I focused on cell junction genes in RNA-Seq analysis comparing flies raised on 50 g/L glucose-supplemented food with those raised on unmodified holidic food, I also observed a striking increase in expression of immune-related genes, particularly antimicrobial peptides, in flies raised on glucose-supplemented food. This was unexpected, as antimicrobial peptide expression increases with age (Pletcher et al., 2002) and promotes intestinal barrier dysfunction (Rera et al., 2012). Selective breeding for long-lived flies reduces age-dependent increase in antimicrobial peptide expression (Fabian et al., 2018). Furthermore, knockdown of individual antimicrobial peptides extends lifespan (Lin et al., 2018). The effect of overexpression of antimicrobial peptides on lifespan may be context-dependent as evidence suggests either detrimental (Badinloo et al., 2018) or beneficial outcomes (Loch et al., 2017). Higher baseline antimicrobial peptide expression in the long-lived GSF-treated flies suggests that the relationship between antimicrobial peptides and lifespan may be complex. As I performed RNA-Seq on 20-day old flies, it would be important to measure antimicrobial peptide expression in GSF-treated flies across their lifespan to determine changes with age.

6.5.2. *V. cholerae* infection model

My findings demonstrate that diet influences the survival response to *V. cholerae* infection, as female flies raised on 100 g/L glucose-supplemented food and males raised on 50 g/L glucose-supplemented food had an improved survival response compared to flies raised on an unmodified holidic diet. Oral infection with the enteric pathogen *Vibrio cholerae* has uncovered parallel involvements of host metabolism and immunity in the progression of cholera in *Drosophila* (Hang et al., 2014b). *imd* mutants display improved survival after infection with *V. cholerae*, suggesting that host immune signaling is a component of disease in the fly model of cholera (Wang et al., 2013), although I found that Δ*AMPs* flies succumbed to infection faster than controls. Interestingly, the improved survival of *imd* mutants appears to rely on interactions between *V. cholerae* and commensal *Acetobacter* (Fast et al., 2018b). *V. cholerae* uses a type six secretion system to kill *Acetobacter pasteurianus* and elimination of *Acetobacter* from the fly microbiome delays the onset of *V. cholerae*-dependent death. At the same time, the CrbRS two-component system of *V.cholerae* leads to consumption of intestinal acetate (Hang et al., 2014b). Depletion of intestinal acetate suppresses the insulin pathway and promotes intestinal steatosis, accelerating host death. Microbial-derived acetate activates IMD in enteroendocrine cells, and increases the relative numbers of enteroendocrine cells that express the Tachykinin endocrine peptide (Kamareddine et al., 2018). Tachykinin controls lipid metabolism, insulin signaling, and the differentiation of enteroendocrine cells (Amcheslavsky et al., 2014; Kamareddine et al., 2018; Song et al., 2014). Combined, these data implicate bacterial acetate in the parallel regulation of host IIS and IMD responses. Consumption of acetate by *V. cholerae* disrupts the activity of both pathways and accelerates the demise of infected flies.

6.6. IMD and metabolism

Several roles of IMD signaling have been found beyond conventional pathogen-sensing and production of immune effectors (Zhai et al., 2017). These diverse roles include regulation of apoptosis in development, neurodegeneration, or even regulation of sleep. As with mammalian TNFR signaling, the IMD pathway is implicated in cell death, as

overexpression of *imd* in the fat body leads to apoptosis (Georgel et al., 2001). Conversely, activation of PGRP-LF, a negative regulator the IMD pathway, is required for apoptosis during development (Tavignot et al., 2017). Inflammation is often associated with human neurodegenerative diseases (Lucin and Wyss-Coray, 2009). Similarly, the IMD signaling through Relish has been found to lead to neurodegenerative phenotypes in flies (Cao et al., 2013; Petersen et al., 2013), possibly linked with its role in apoptosis (Chinchore et al., 2012). Perhaps one of the most fascinating processes that IMD signaling may be involved with is the regulation of sleep. Infection appears to increase the length of time flies spend sleeping, and this increase in sleep is dependent on Relish (Kuo et al., 2010).

With increasing interest in immune-metabolic interactions, IMD signaling has also been explored for its role in metabolism. In this project, I found links between the IMD pathway and metabolism. Specifically, *imd* mutants have increased weight, increased energy stores, altered insulin activity, and increased lipid metabolism gene expression. Combined, these data suggest that *imd* is required for metabolic homeostasis.

6.6.1. IMD and lifespan

In my initial investigation of the IMD pathway, I asked if *imd* was required for lifespan extension in flies raised on glucose-supplemented food. I tested this by raising *imd* mutants alongside w^{1118} controls on either 50 g/L glucose-supplemented food or unmodified holidic food. Unexpectedly, I found that *imd* mutants survived longer than w^{1118} flies. At first it seems unusual that an immune-compromised fly would live longer than wild-type controls, however, a similar finding was previously reported as both male and female hypomorphic *imd* mutants were found to live longer than control flies (Lin et al., 2018). Conversely, *Relish* mutants have a shorter lifespan than wild-type controls (Valtonen et al., 2010). However, this latter study transferred flies to fresh food every 2 weeks, rather than every 2-3 days, which would likely lead to increased bacterial growth on the food, which could confound these findings.

In mammals, chronic inflammation is associated with decreased lifespan (Jurk et al., 2014; Rea et al., 2018). Similarly, the activation of the IMD pathway is thought to be detrimental to lifespan (DeVeale et al., 2004; Libert et al., 2006). Overexpression of PGRP-LE in the fat body reduces lifespan through a Relish-dependent mechanism (Libert et al.,

2006). Inhibition of negative regulators of IMD signaling has also been found to be detrimental to lifespan. Blocking *pirk*, a negative regulator of IMD signaling, reduces lifespan (Paredes et al., 2011). Similarly, mutation of *diedel*, another negative regulator of the IMD pathway, also reduces lifespan (Lamiable et al., 2016). Combined, these data suggest that, in the absence of infection, IMD signaling is detrimental to lifespan. However, expression of a constitutively active form of Imd in intestinal progenitor cells had no effect on lifespan (Petkau et al., 2017), suggesting that where IMD signaling is active is an important determinant on lifespan.

While it appears that overactive IMD signaling decreases lifespan, the impact of reduced IMD signaling on lifespan is less clear. In our lab, we found that inhibition of IMD signaling in either intestinal enterocytes or intestinal progenitor cells reduces lifespan (Shin et al., 2019). As I found that *imd* mutants live longer than controls, this suggests a difference between the loss of IMD signaling systemically compared to intestinal cells. One potential explanation may be that the holidic diet that I use limits bacterial growth compared to other lab diets, such as Bloomington cornmeal food. If this is case, then flies raised on holidic food would be exposed to a lower concentration of bacteria and be less vulnerable to the negative aspects of high bacterial load (Lee et al., 2019). An interesting experiment to test this would be comparing the bacterial CFU between the holidic diet and Bloomington cornmeal food, both from the food surface and in flies raised on either food. Further work is required to determine how inhibition of IMD signaling affects lifespan.

6.6.2. IMD and metabolism

As we look beyond the traditional immune effector response of IMD signaling, it is becoming more apparent that IMD signaling has a wider role in fly physiology. My findings here suggest that the IMD pathway may be required to regulate insulin signaling and lipid metabolism in flies, which may be important aspects of a comprehensive immune system.

Several studies suggest that IMD signaling in response to the microbiota is involved in metabolic regulation in the intestine. For example, the fly commensal *L. plantarum* promotes growth, at least in part, through induction of intestinal peptidases by the IMD pathway (Erkosar et al., 2015). Elevated levels of intestinal peptidases boost the digestion

of dietary protein and promote larval growth via the TOR pathway. The IMD response to infection may also involve metabolic regulation as infection with a panel of ten different bacteria found many effects on the expression of metabolism genes (Troha et al., 2018). These findings suggest a link between gut bacteria, IMD activity, and metabolism in the host. Consistent with this hypothesis, transcriptional studies of axenic flies, *imd* mutant flies, and flies with constitutive activation of IMD in the intestine (Broderick et al., 2014; Dobson et al., 2016; Erkosar et al., 2014; Guo et al., 2014; Petkau et al., 2017) demonstrated a regulatory impact of IMD on metabolic processes in the gut, including the expression of digestive peptidases.

6.6.3. IMD and insulin signaling

In an oral glucose tolerant test, I found that *imd* mutants have higher levels of glucose and slower clearance of glucose, suggesting that *imd* is required for a fully functional insulin response. The IMD-responsive NF-kB transcription factor Relish does not block insulin activity (DiAngelo et al., 2009). However, expression of a constitutively active IMD protein in the fat body suppresses systemic insulin signaling and mimics many phenotypes often associated with insulin loss of function (Davoodi et al., 2018). These data suggest that IMD acts upstream of Relish to attenuate insulin activity. The stress-responsive, JNK, is a likely candidate for IMD-dependent control of insulin, as JNK influences insulin activity in several physiological contexts (Agrawal et al., 2016; Karpac et al., 2009; Pasco and Léopold, 2012; Wang et al., 2005). Transcriptional studies of insulin resistant fat bodies revealed additional links between IIS and *Drosophila* immune responses (Musselman et al., 2018). Specifically, knockdown of the insulin receptor in the fat body suppresses expression of the peptidoglycan receptor proteins PGRP-SB2 and promotes expression of PGRP-SC2.

In *Drosophila,* the TNF-α homolog, Eiger, is involved in the regulation of insulin activity. Although Eiger does not directly activate the IMD pathway in the same way that TNF-α stimulates TNFR signaling in humans, Eiger may be involved in the regulation of IMD activity. The house fly homolog of Eiger affects AMP expression from both IMD and Toll pathways (Tang et al., 2019). Fat body-derived expression of Eiger controls the release of insulin-like peptides from the brain (Agrawal et al., 2016). In low-nutrient conditions,

Eiger is released by the fat body, and directly binds to the TNF-α receptor homolog, Grindelwald (Andersen et al., 2015), on insulin-producing cells to inhibit expression of insulin-like peptides. Thus, TNF-α acts as a direct link between nutrient sensing and insulin release in the fly. As Eiger signals through the JNK pathway (Igaki et al., 2002), it may be possible that the IMD signaling pathway regulates insulin through the JNK signaling arm.

While evidence suggests that IMD signaling regulates insulin activity, it is worth speculating if the reverse is true. Does insulin influence the immune response and would the hyperactivation of insulin signaling decrease the infection survival response? Reduction of systemic insulin activity through the mutation of *chico*, the *Drosophila* insulin receptor substrate ortholog, improves survival against both gram-negative, *P. aeruginosa*, and gram-positive, *Enterococcus faecalis*, bacteria (Libert et al., 2008). In contrast, *chico* mutants had no improvement in survival against infection with either *P. luminescens* or the non-pathogenic K12 strain of *E. coli* (McCormack et al., 2016). However, in this case, *chico* mutants did have increased resistance to infection, as well as increased melanisation and phenoloxidase activity. Future studies are required to determine the role of insulin signaling in immunity.

6.6.4. IMD and lipid metabolism

The *Drosophila* IMD signaling pathway is homologous with human tumor necrosis factor receptor (TNFR) signaling. However, while the IMD pathway in flies directly recognizes bacterial peptidoglycan, the TNFR pathway is activated in response to the cytokine tumor necrosis factor alpha (TNF-α). TNF-α is a multifunctional cytokine, with a well-described role in lipid metabolism (Chen et al., 2009; Sethi and Hotamisligil, 1999). While overactivation of TNFR in adipocytes is associated with obesity and metabolic diseases (Hotamisligil, 2017b), acute expression was found to be required for proper adipose tissue expansion and function (Asterholm et al., 2014). I found that *imd* mutants have increased weight and triglyceride stores, suggesting dysregulation of lipid metabolism. Upregulation of lipid catabolism genes in *imd* mutants further supports the hypothesis that *imd* is required for regulation of lipid metabolism.

Other studies have also linked IMD signaling with lipid metabolism. Similar to my findings, a study with hypomorphic *imd* mutants observed increased weight and increased

fat stores (Lin et al., 2018). Blocking IMD signaling in intestinal enterocytes also appears to increase whole fly triglyceride levels and weight (Shin et al., 2019). Conversely, the expression of a constitutively active form of Imd in the fat body leads to decreased triglycerides and fat stores (Davoodi et al., 2019).

The IMD pathway has been associated with changes in lipid storage, but little is known about how IMD signaling impacts lipid metabolism. When I compared the transcriptome of *imd* and w^{1118} flies, I observed upregulation of several lipid metabolism genes in *imd* mutants. Notably, major genes in both the transport of lipids and the *de novo* formation of lipids were upregulated **(Figure 6-3)**. Lipid transport involves shuttling lipids, primarily in the form of diacylglycerides, between organs through the hemolymph (Palm et al., 2012). The gene for the precursor of the major lipid carrier, *apolpp*, was upregulated in *imd* mutants. The two major lipoprotein receptors, *LpR1* and *LpR2*, were also upregulated in *imd* mutants. Combined, these data suggest that interorgan lipid transport is increased in *imd* mutants. Lipogenesis of triglycerides requires fatty acids, which are acquired either from the diet or formed *de novo* from acetyl-CoA (Heier and Kühnlein, 2018). *Drosophila* have three genes that encode fatty acid synthases, an enzyme required for the *de novo* formation of long-chain fatty acids from acetyl-CoA (Parvy et al., 2012). Two of these, *FASN2* and *FASN3*, were both upregulated in *imd* mutants. This suggests that *imd* is required to regulate *de novo* synthesis of triglycerides.

I also observed that *imd* mutants have increased expression of the hormone, *tachykinin* (*Tk*), which has a regulatory role in lipid metabolism (Song et al., 2014). However, the increased expression of *tachykinin* (*Tk*) is interesting, but difficult to evaluate. Six different Tk peptides are expressed from a single *Tk* gene and have different functions dependent on where they are expressed (Wegener and Gorbashov, 2008). As I performed RNA-Seq on whole flies, its unknown where *Tk* is upregulated in *imd* flies. The main tissues that express *Tk* are the brain and the midgut, where brain derived Tk has systemic effects that do not affect lipid levels (Birse et al., 2011), enteroendocrine derive Tk has been shown to increase enterocyte lipid droplet formation in a paracrine fashion (Song et al., 2014), possibly dependent on IMD signaling. However conflicting reports suggest that IMD signaling in enteroendocrine cells may either decrease (Harsh et al., 2019) or increase (Kamareddine et al., 2018) expression of *Tk*. The difference may be due to the use of systemic versus oral

infection routes, respectively. As I performed RNA-Seq on whole fly samples, further work will be needed to determine the tissue-specific regulation of *Tk* in *imd* mutants.

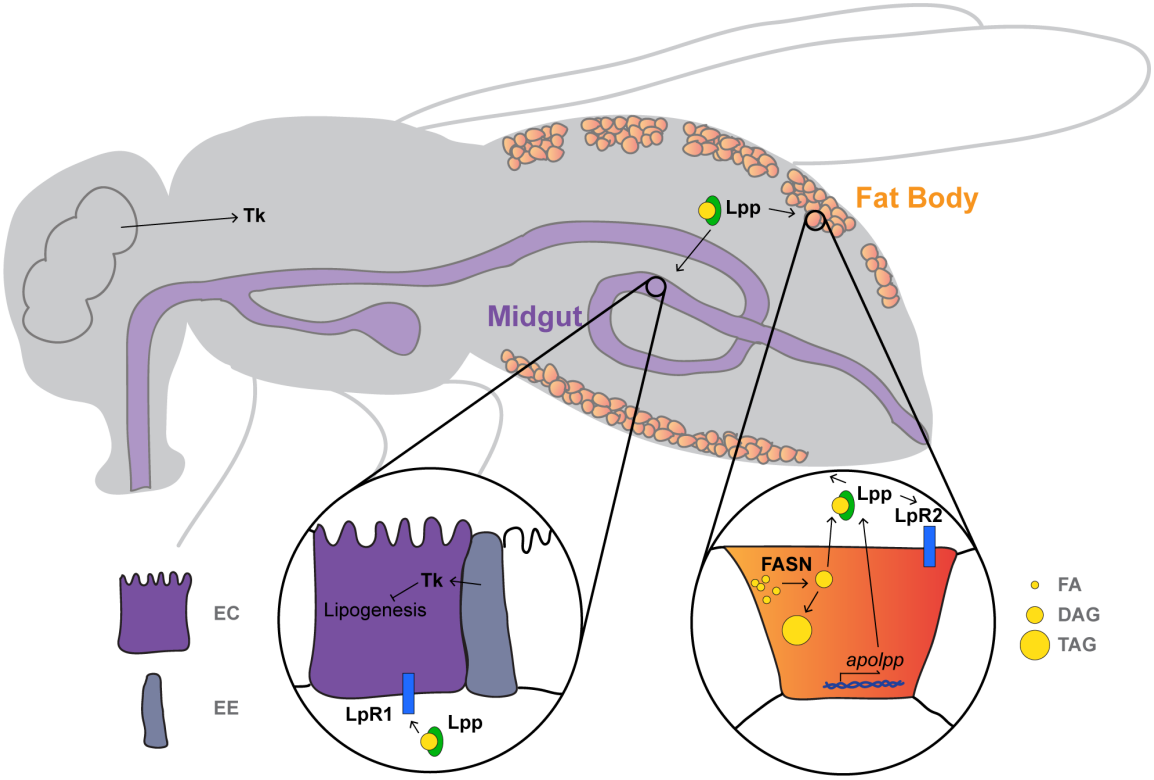


Figure 6-3 Function of lipid metabolism proteins with expression affected in *imd* mutants

RNA-Seq comparison of *imd* mutants and w^{1118} flies revealed increased expression of lipid metabolism genes. Shown here is the function of key proteins of interest whose gene expression was affected in *imd* mutants. In fat body cells, free fatty acids (FA) are formed into diacylglycerides (DAG) by Fatty acid synthase (FASN) enzymes. DAG can be further synthesized to triglyceride (TAG) for storage or released for transport to other tissues. *apolipophorin (apolpp)* encodes the lipid carrier, Lipophorin (Lpp). Lpp primarily transports DAG through the hemolymph. Lpp can bind two receptors, Lipoprotein receptor 1 (LpR1) and Lipoprotein receptor 2 (LpR2), which have different expression patterns in the adult fly (LpR1 is expressed throughout the body, while LpR2 is mainly expressed in the fat body). Tachykinin (Tk) can be expressed neuronally, where it does not appear to affect lipid metabolism. Tk is also expressed in intestinal enteroendocrine (EE) cells, where it inhibits lipid droplet formation in nearby enterocytes (EC).

6.6.5. Proposed model of IMD regulation of lipid metabolism

Why would IMD signaling suppress lipogenesis in an uninfected state? During an infection, IMD activity appears to mobilize resources through the depletion of lipid stores, but why does IMD signaling seem to inhibit the accumulation of those energy stores in the absence of an infection? I propose a model in which IMD signaling constitutively favors lipolysis over lipogenesis at the systemic level **(Figure 6-4)**. I believe that in the absence of an infection, negative regulators of IMD signaling would inhibit the IMD pathway, favoring lipogenesis. However, as *imd* mutation leads to increased triglyceride stores, basal levels of IMD signaling must limit this process. During an infection, the IMD pathway is activated and signals to express genes involved in lipolysis while repressing genes involved in lipogenesis.

While the IMD pathway may control lipid metabolism directly through signaling, I speculate that the most likely mechanism is that the IMD pathway reduces the expression of lipogenesis genes through the JNK signaling arm. The Relish arm of the IMD signaling cascade may have a role in the regulation of lipid metabolism, but it remains unclear. Mutants in the IMD pathway, including *kenny* (IKKγ), *Relish*, and *Dredd*, were found to have increased triglycerides compared to controls (Kamareddine et al., 2018). However, others have reported decreased triglycerides in *Relish* mutants (Molaei et al., 2019), and no effect on triglycerides when *Relish* was overexpressed in the fat body (DiAngelo et al., 2009). Contrary to my findings with *imd* mutants; *kenny*, *Relish*, and *Dredd* mutants appeared smaller in size and weighed less than controls (Kamareddine et al., 2018). Mutation of Cylindromatosis (CYLD), a negative regulator of Kenny, also leads to increased triglyceride levels (Tsichritzis et al., 2007). While Relish may be involved in lipid regulation, I speculate that JNK signaling is more important. Further studies are required to test this hypothesis, such as comparing the effects of the genetic knockdown the *Drosophila* JNK, *basket*, with the knockdown of *Relish*, on triglyceride stores.

IMD signaling may also have differential effects on lipid metabolism in different tissues. My model focuses on systemic IMD signaling, likely orchestrated from the fat body, but IMD has been found to have mixed effects on lipids in intestinal enterocytes, where lipid droplets may be involved in intestinal immunity (Harsh et al., 2019). Specifically, lipid droplet accumulation in intestinal enterocytes and increased whole fly

lipids are observed during infection with *Photorhabdus* bacteria, and this is replicated by activation of Relish in the fat body. Conversely, *Relish* and *PGRP-LC* mutants have increased midgut accumulation of lipid droplets (Kamareddine et al., 2018), and blocking the IMD pathway in intestinal cells leads to increased triglyceride stores (Shin et al., 2019). Future studies will be required to test my proposed model and dissect the separate role of IMD signaling in different tissues. Interestingly, the effect of IMD signaling on lipid metabolism may have a temporal factor, as a recent preprint suggests that IMD signaling regulates size and number of lipid droplets differentially during an acute or chronic infection (Wang et al., 2020).

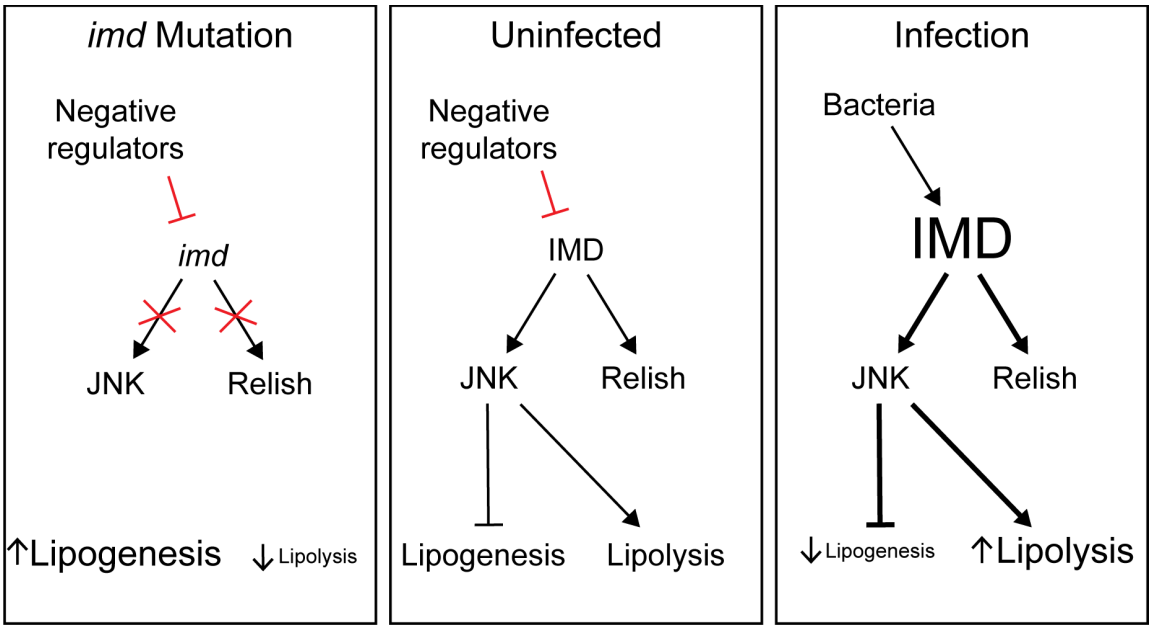


Figure 6-4 Proposed model of systemic IMD regulation of lipid metabolism

The following is a hypothetical model of how systemic IMD signaling regulates lipid metabolism. Uninfected flies have a low basal level of IMD activation that signals through JNK to favor lipolysis over lipogenesis. Negative regulators of the IMD pathway limit this response. During an infection, IMD is strongly activated, overcoming negative regulation, and leading to lipolysis, and mobilization of lipid energy stores to promote the infection response. In *imd* mutants, there is no IMD activation, leading to the favoring of lipogenesis over lipolysis, and an increase in triglyceride stores.

6.6.6. IMD and pheromone production

One of the more unexpected findings in my RNA-Seq comparison of w^{1118} and *imd* flies was an upregulation in genes associated with pheromone production. Interestingly, this may be linked to the upregulation of lipid metabolism genes as key pheromones are derived from fatty acids (Wicker-Thomas et al., 2015). Flies appear to have olfactory pathways that allow them to avoid other flies that have been infected with certain pathogens. Infection with *Pseudomonas entomophila* leads to production of attractive pheromones in infected flies and the IMD pathway was found to be required for this pheromone production (Keesey et al., 2017). Further work is required to examine the role of IMD signaling in pheromone production.

6.7. Concluding Remarks

Aging is a growing problem around the world, and solutions are required to limit the negative aspects associated with aging. We are not solely searching for mechanisms to extend lifespan, but also how to improve health in our later age. Exploring lifespan extension in model organisms, such as *Drosophila*, provides fundamental insights into the process of aging. I anticipate that determining a mechanism that allows a fly to live longer may one day be applied to improve the health in aging humans. Uncovering the complex interactions of diet, health, lifespan, and immunity requires comprehensive investigation from a wide range of approaches. I believe that my findings here have contributed to our expanding knowledge and in the future may translate to the improvement of human health.

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