Characterization of the crosstalk between immunity and metabolism in *Drosophila melanogaster* model

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Abstract

Immune and metabolic pathways collectively contribute to the containment of microbial invaders, and persistent activation of immune responses contribute to the development of severe metabolic disorders. To determine how interaction between innate immune system and metabolic responses affected host health as well as host response to microbial invaders, I used the *Drosophila* model. The fat body in *Drosophila* coordinates immune, growth and metabolic pathways which are evolutionary conserved between flies and mammals. The immune deficiency (IMD) pathway in *Drosophila* is very similar to the mammalian TNF-α pathway, a key regulator of vertebrate immunity and metabolism. To determine how a prolonged immune response impacts metabolism, I induced a constitutive inflammatory response in the *Drosophila* fat body, a key regulator of humoral immunity and metabolic homeostasis. Through whole-genome microarray analysis, I found that persistent immune activity in the larval fat body resulted in suppression of expression of genes involved in glycolysis, lipid synthesis and insulin signaling. In contrast, I found genes involved in glycogenolysis and apoptosis were highly expressed in larvae with consistent IMD activity in the fat body. I looked at macronutrients levels in whole larvae as well as in the hemolymph and found reduction in triglyceride contents as well as hyperglycemia in circulatory hemolymph. I also showed that elevated IMD activity in the larval fat body replicated key features of a suppressed insulin pathway such as delayed development, reduced body size, and hyperglycemia. Consistent with the role of IMD in metabolic homeostasis, I found that *imd* mutants weigh more, are hyperlipidemic, and have impaired glucose tolerance. Combined these results showed that persistent activation of IMD in the larval fat body has detrimental effects on metabolism, development and growth of the larvae and IMD is required for metabolic homeostasis. To test the importance of metabolic regulation for host responses to bacterial infection, I challenged

insulin pathway mutants with lethal doses of several *Drosophila* pathogens and I measured survival and bacterial load.

I found that loss-of-function mutations in the insulin pathway impacted host responses to infection in a manner that depends on the route of infection and the identity of the infectious microbe. Specifically, I found that enteric pathogen, *Vibrio cholerae*, disrupts glucose metabolism in adult flies and results in elevation of circulatory glucose. I showed oral infection of adult females with a holidic diet supplemented with glucose extends survival of flies upon enteric *V. cholerae* infection. Combined, my results support a role for coordinated regulation of immune and metabolic pathways in host containment of microbial invaders. Mechanistically, it is unclear how an immune–metabolic axis influences host responses to bacterial infection. Immunity encompasses resistance mechanisms that kill infectious microbes and tolerance mechanisms that mitigate disease severity without effects on microbial load. I studied how modulation of IMD or insulin signalling in the fat body affects host defence response towards oral infection with *V. cholerae*. I found that inhibition of insulin pathway in the fat body results in increased survival of flies upon oral infection with the *V. cholerae* while the bacterial number remained unchanged and *V. cholerae* persisted longer in the host in the absence of pathogen feeding as well. Additionally, suppression of IMD pathway in the fat body resulted in survival extension after oral infection with *V. cholerae* while the bacterial load was significantly higher in these flies. These observations suggest an improved tolerance towards *V. cholerae* is induced in the fly once IMD or insulin pathway are not functional in the fat body. Combined, my data reveal persistent immune activity in the fat body disrupts host growth and metabolism, uncovers the role of host insulin signaling in host-pathogen interaction, and indicate the protective role of immune and metabolic suppression in a metabolic tissue against an intestinal pathogen, *V. cholerae*.

Preface

This thesis contains original work by Saeideh Davoodi. Portions of the data presented here involved collaboration with members of Dr. Foley lab as well as Dr. Savraj Grewal at the University of Calgary and republished with their permission.

Parts of chapter 1 of this thesis were published as Saeideh Davoodi and Edan Foley. (2020) Host-Microbe-Pathogen interactions: A review of *Vibrio cholerae* pathogenesis in *Drosophila*, Frontiers in Immunology. https://doi.org/10.3389/fimmu.2019.03128

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All data presented in chapter 3 was produced by Saeideh Davoodi with the exception of the following figures: figure 3-13 was produced from data generated by Anthony Galenza. Dr. Edan Foley analyzed the microarray data presented in figures 3-2, 3-3 and 3-6. Meghan Ferguson analyzed data and generated graph for figures 3-2 (panel C &D), figure 3-5 (panel C). Data in figure 3-10 (panel B) and 3-11 was produced by Rujuta Deshpande through a collaboration with Dr. Savraj Grewal Lab at the University of Calgary. Parts of chapter 3 were published as Davoodi S, Galenza A, Panteluk A, Deshpande R, Ferguson M, Grewal S, and Foley E. (2019) The immune deficiency pathway regulates metabolic homeostasis in *Drosophila*. J Immunol. 202:2747-2759.

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All data presented in chapter 5 was produced by Saeideh Davoodi.

Dedication

Dedicated to the memory of University of Alberta Students, Alumni and Faculty members who were among 176 passengers perished in the downing of Flight PS752 near Tehran, Iran on January 8th, 2020.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Chapter 3. The Immune Deficiency pathway regulates metabolic homeostasis in *Drosophila*

Chapter 4. Modulation of host insulin signaling alters host response to pathogens

Chapter 5. Suppression of insulin and IMD signaling in the fat body has protective effects against enteric *V. cholerae* infection

Chapter 6. Discussion

List of Abbreviations

AKH	Adipokinetic hormone
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
BDSC	Bloomington *Drosophila* Stock Center
CAFE	Capillary feeding
CC	Corpora Cardiaca
CFU	Colony forming units
CT	Cholera Toxin
DAP-PGN	Diaminopimelic acid-type peptidoglycan
Dredd	Death related ced-3/Nedd2-like protein
Ecc15	*Erwinia carotovora carotovora, isolate 15*
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated protein with death domain
flyPAD	Fly proboscis and activity detector
GFP	Green fluorescent protein
GI	Gastro-intestinal
Glut	Glucose transporter
GO	Gene ontology
GS	GeneSwitch
Iap2	Death-associated inhibitor of apoptosis 2
IIS	Insulin/insulin-like growth factor signaling
ILP	Insulin-like peptide
ILP2-FH	Flag and HA epitope-tagged ILP2
IMD	Immune deficiency
ImpL2	Ecdysone-inducible gene L2
InR	Insulin receptor
IPC	Insulin-producing cell

IRS	Insulin receptor substrate
ISC	Intestinal stem cell
JNK	c-Jun N-Terminal Kinase
LB	Luria-Bertani
LD	Lipid droplet
MAPK	Mitogen-activated protein kinase
Mkk4	Mitogen activated protein Kinase kinase 4
Mkk7	Mitogen activated protein kinase kinase 7
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor of kappa light polypeptide gene enhancer in B-cells
PBS	Phosphate buffered saline
PGN	peptidoglycan
PGRP	Peptidoglycan receptor protein
PGRP-LC	Peptidoglycan recognition protein LC
PGRP-LE	Peptidoglycan recognition protein LE
PI3K	Phosphoinositide 3-kinases
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Tab2	Tak1-associated binding protein
Tak1	Tumour necrosis factor-B activated kinase
TCA	Tricarboxylic acid cycle
TG	Triglyceride
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TOR	Target of rapamycin
ts	Temperature sensitive
T6SS	Type VI secretion system
UAS	Upstream activated sequence
V. cholerae	*Vibrio cholerae*
w	White

Chapter 1:

Introduction

Portions of this chapter have been published as:

Saeideh Davoodi and Edan Foley. (2020) Host-Microbe-Pathogen interactions: A review of *Vibrio cholerae* pathogenesis in *Drosophila,* Frontiers in Immunology. https://doi.org/10.3389/fimmu.2019.03128

1. Introduction

1.1 Immunometabolism

Multicellular organisms rely on their defense systems to be protected against harmful microbes. The gastrointestinal tract processes ingested materials and activation of metabolic signalings initiate food storage and allocation of ingested material for host survival, growth, reproduction and overall fitness. Therefore a crosstalk between metabolic and immune responses is essential to ensure host survival. Immune systems are typically focused on defence and repair, but chronic activation of immune responses are frequently harmful for the host. To provide energy for an optimal immune response against pathogens, alterations in some physiological functions such as metabolic adaptation are required in order to facilitate allocation of nutrients and energy for host immune activation (1). In this case, hosts balance traditional metabolic needs against the immediate threat presented by the microbe and alter metabolic pathway activity accordingly. The earliest reported association of metabolic pathologies with infections dates back to 1884 in which patients with meningitis showed a transient diabetic syndrome (2). Later it was found that metabolic reprogramming such as increased glycolytic activity and decreased oxidative phosphorylation occurs in macrophages upon exposure to microbes (3–7). Another example of metabolic reprogramming induced by a pathogen is Tuberculosis in which the disease is characterized by a metabolic wasting syndrome that leads to weight loss and muscle atrophy (8).

In some infections, microbes benefit from the alteration in metabolite availability that results from host metabolic reprogramming while fighting the infection (9–12). For example, *Mycobacterium tuberculosis* reprograms macrophage metabolism to utilize fatty acid instead of glucose, providing nutrient for the pathogen (13–15). In the 1960s, increased infiltration of macrophages into adipose tissue was reported in animal models of obesity (16–19). Later it was found that macrophages secrete Tumor Necrosis Factor-α (TNF-α) which leads to insulin resistance in adipocytes, and glucose metabolism impairment (20–25). In diabetic animal models, administration of TNFα resulted in insulin resistance and disruption of glucose metabolism (26–29). The mechanism of TNFα-induced insulin resistance was shown to be through inhibition of insulin-stimulated tyrosine phosphorylation of insulin receptor β-subunit and insulin receptor substrate (30). Increased production of other cytokines such as IL-1β from pancreatic islets as well as increased islet-associated macrophages have been reported in type 2 diabetic patients in response to glucotoxicity (31,32). These data emphasize the relationship between immune cytokines and

metabolic disorders such as obesity and diabetes (33). Suppressing the inflammatory responses via anti-inflammatory antagonists such as Etanercept (34), Salsalate or Aspirin lowers systemic inflammation and improves hyperglycemia (35–37). However, metabolic syndromes associated with inflammation are multifactorial which makes the interpretation of clinical finding complicated. For example, although anti-TNF therapy have shown some improved insulin sensitivity (38), the anti-inflammatory treatments lacks consistency and significant efficiency (39,40). Therefore, understanding the impact of immune-metabolic crosstalk in the context of infectious disease broaden our perspective on how to treat the cause of pathology. Therefore, it is essential that we develop animal models to study molecular mechanisms involved in tissue-specific immune-metabolism.

1.2 *Drosophila melanogaster* as a model organism

Drosophila melanogaster has been used as a model organism for over a century (41) and has contributed to many discoveries on the fundamental mechanisms of biology. *Drosophila* has three autosomal and one pair of sex chromosomes which encode about 14,000 genes (42,43). *Drosophila* has four stages in its life cycle: embryo, larva, pupa and adult. The larvae undergoes three instar stages until pupariation and consequently metamorphosis into an adult fly. The duration of *Drosophila* life cycle is around 10 days at 25°C and 19-20 days at 18°C. As mammals have functional redundancies in their genome, studies of basic molecular mechanisms has been facilitated by *Drosophila* due to its reduced complexity (44,45). Other technical advantages of *Drosophila* are inexpensive and easy maintenance, availability of transgenic fly lines, and numerous molecular techniques for gene manipulation (46–52). There are many genes and signaling pathways in *Drosophila* that are highly conserved in mammals. For example, 75% of human disease genes have homologues in *Drosophila* (53,54). Also, internal organs involved in metabolism have functional analogues to vertebrates counterparts (51,55,56). Similar to the mammalian gastrointestinal tract, the *Drosophila* intestine takes food, absorbs, digests and processes nutrients. In addition to that, the *Drosophila* intestinal tract is in constant exposure to microbes similar to vertebrates gastrointestinal tract (57,58). *Drosophila* insulin producing cells (IPCs) are located in the brain and are responsible for the production and secretion of insulin like peptides (ILPs) as well as coordination of energy metabolism. Pancreatic β cells are the functional equivalent of IPCs in vertebrates (59). Corpora Cardiaca cells are endocrine cells in the *Drosophila* brain that secrete adipokinetic hormone (AKH), which is the equivalent of glucagon produced by

the pancreatic α cells of vertebrates (60). In flies, the integration of immune and metabolic pathways is particularly apparent in the fat body, which simultaneously regulates energy storage and humoral immunity. The fly fat body has similar functions to the human liver and white adipose tissue (61) (Figure 1-1). Although *Drosophila* lacks adaptive immunity, flies are able to combat infections via innate immune pathways such as Toll and Immune deficiency pathway (IMD) that are similar to the vertebrate Toll like receptor and Tumor Necrosis Factor (TNF) Receptor pathway, respectively (62,63). Overall, *Drosophila* has become a powerful tool to study immune-metabolic crosstalk, the role of innate immunity in metabolic diseases such as obesity and diabetes, and understanding of the infection-induced pathologies and immunometabolism (64–69).

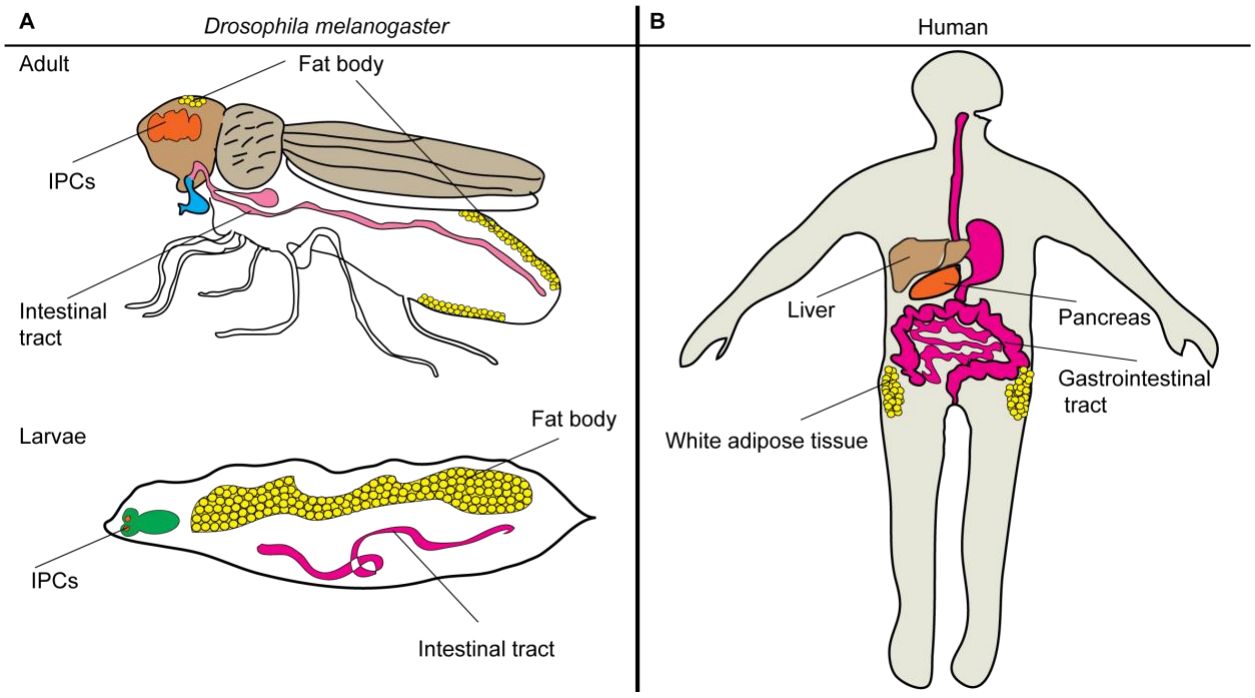


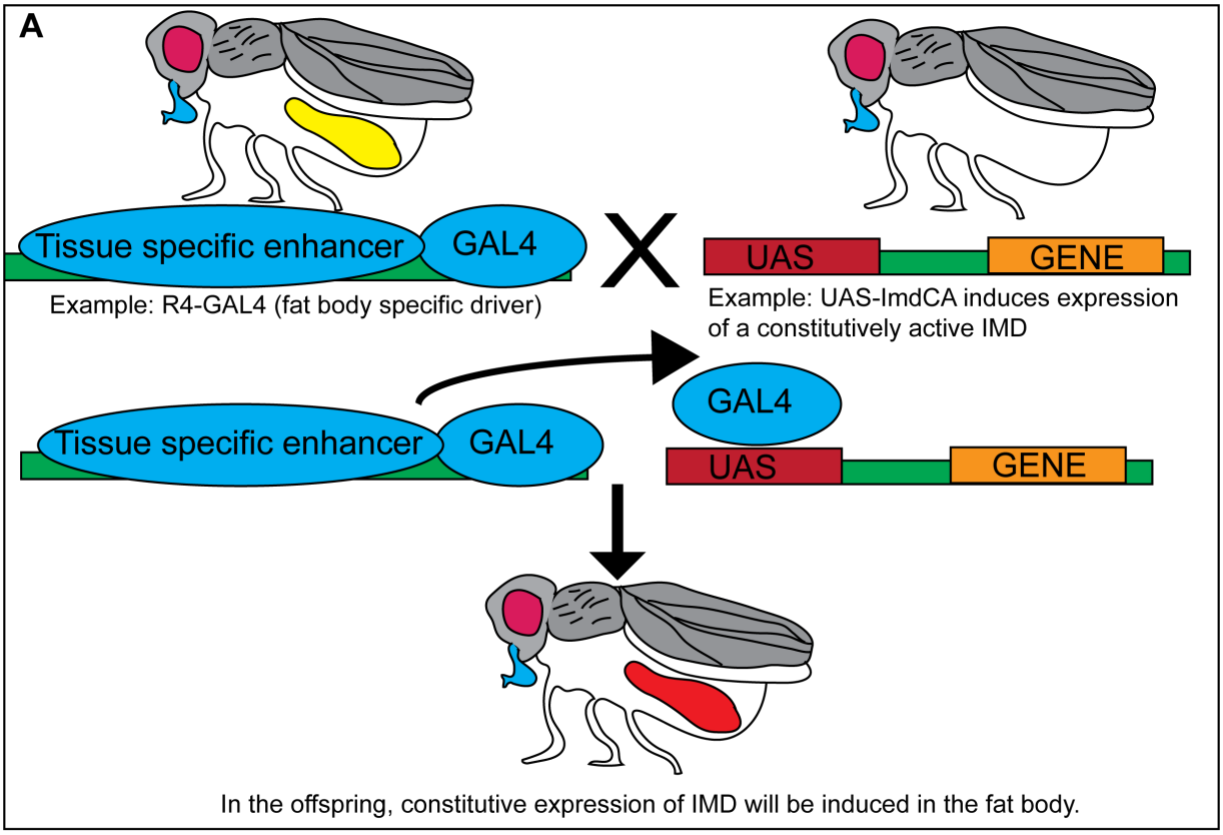
Figure 1-1 Comparison of organs between *Drosophila* and humans
Internal organ comparison between (A) adult *Drosophila*, larvae and (B) humans. Identical colors represents tissues with similar or analogous functions.

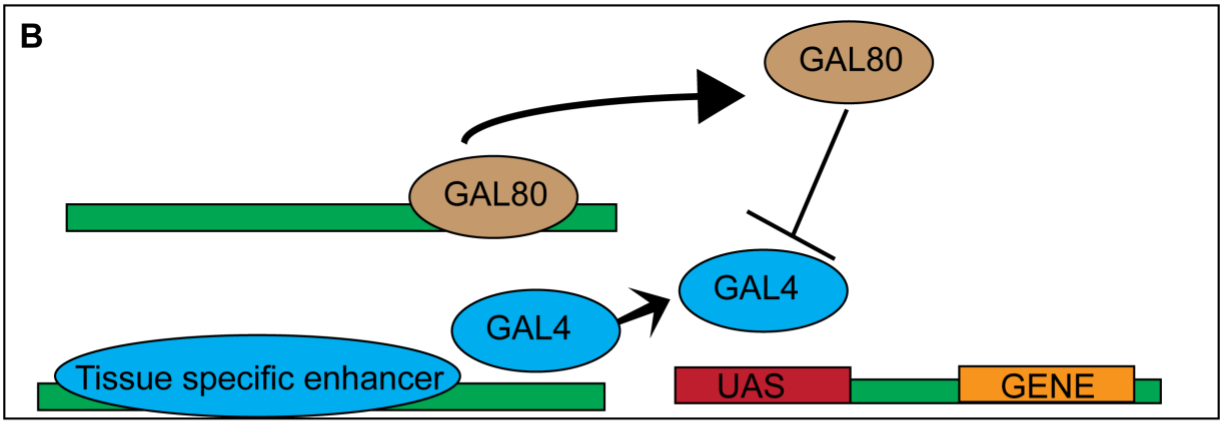
1.2.1 GAL4-UAS expression systems

The GAL4-upstream activating sequence (UAS) system was developed by Brand and Perrimon to activate the target genes in a cell or tissue-specific pattern (70). Many other techniques were later introduced in *Drosophila* to be able to control the gene expression level in both spatial and temporal manner (71). The GAL4-UAS expression system has two components: the yeast GAL4 transcription factor, which promotes the transcription of the target genes through its binding

to the UAS cis-regulatory sites (72). The other component is a transgene that is cloned under the control of a UAS promoter, however, it is not activated in the absence of GAL4 (70,73). By mating the GAL4 and UAS fly strains, we are able to observe the expression of transgene in the cell or tissue of interest in the offspring as they contain both UAS and GAL4-promoter constructs.

For spatial and temporal control over targeted gene expression in *Drosophila*, the GAL4-UAS system is combined with the temperature-sensitive Gal4 repressor ($Gal80^{ts}$). The temporal and regional gene expression targeting system or TARGET uses a temperature sensitive GAL80 protein, which is active at 18°C and inhibits expression of GAL4, however by shifting flies to a restricted temperature, 29°C, this inhibition will no longer exist and GAL4 drives the expression of our gene of interest (74,75). Another tissue specific GAL4 system is called GeneSwitch which consists of a GAL4-progesterone receptor fusion protein that induces expression of the gene of interest once the activator RU486 (mifepristone) is added to the food (76–78). The chimeric GAL4 protein is consist of the GAL4 DNA-binding domain, the ligand-binding domain of the human progesterone receptor and the p65 transcriptional activation domain (79). Upon feeding on the food containing the mifepristone, binding of RU486 to the chimeric GAL4 protein results in conformational change that enables the GeneSwitch to bind to a UAS sequence and activate transcription of target gene (78) (Figure 1-2).


A
Tissue specific enhancer
GAL4
Example: R4-GAL4 (fat body specific driver)
UAS
GENE
Example: UAS-ImdCA induces expression of a constitutively active IMD
Tissue specific enhancer
GAL4
GAL4
UAS
GENE
In the offspring, constitutive expression of IMD will be induced in the fat body.


B
GAL80
GAL80
GAL4
GAL4
Tissue specific enhancer
UAS
GENE

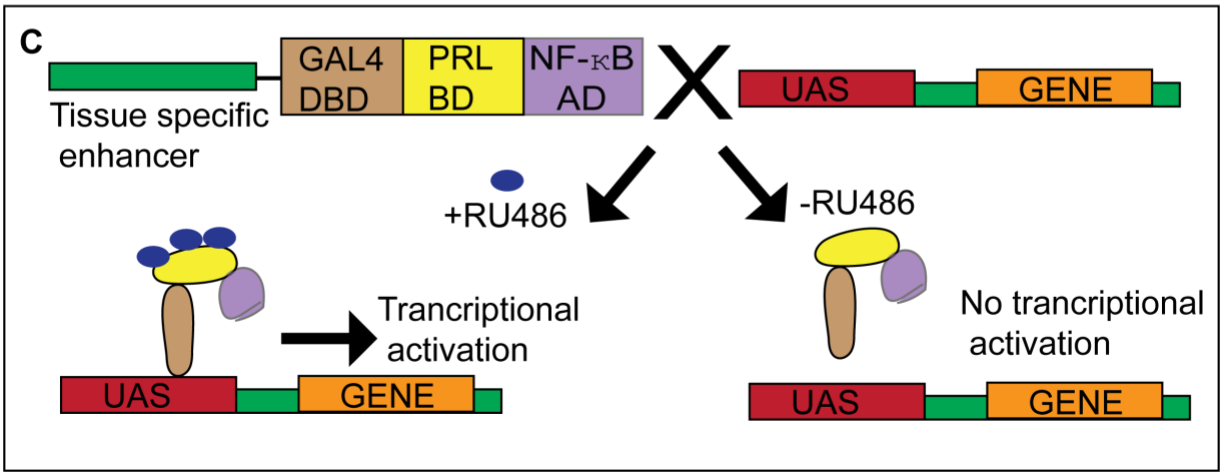

C
GAL4 DBD
PRL BD
NF-κB AD
Tissue specific enhancer
UAS
GENE
+RU486
-RU486
Trancriptional activation
No trancriptional activation
UAS
GENE
UAS
GENE

Figure 1-2 UAS-GAL4 gene expression system in *Drosophila*
(A) GAL4 is a transcriptional activator that has a tissue-specific promoter (eg, R4-GAL4). GAL4 binding to the upstream activating sequence (UAS) activates expression of our gene of interest (eg, ImdCA). (B) A temperature sensitive GAL80 protein which is expressed at permissive temperature inhibits expression of GAL4, however by shifting flies to a restricted temperature, this inhibition will no longer exist and GAL4 drives the expression of our gene of interest. (C) GeneSwitch (GS) system, which consists of a GAL4-progesterone receptor fusion protein that induces expression of the gene of interest once the activator RU486 (mifepristone) is added to the food.

1.2.2 The *Drosophila* fat body

The fat body in *Drosophila* has different roles, including regulating the humoral immune response, storing lipid and glycogen, and sensing nutrients (61). The fat body tissue in *Drosophila* is derived from mesoderm during embryogenesis (80) and early in the larval stage, the fat body acquires immune competency (62). Thin lobes of the fat body are composed of large lipid droplets that contain triglycerides. The larval fat body contains around 2200 cells (81–83) which are organized into sheets of attached fat cells (trophocytes). In contrast, the adult fat body is a loose tissue that is attached to the interior side of the abdomen wall, and a small population of fat cells reside in the fly head (61). The fat body tissue disassociates via apoptosis during metamorphosis in a process called fat-body remodelling (84). The remaining larval fat cells provide nutritional support during the non-feeding wandering third instar developmental stage and for young eclosed adults (85–87). Direct exposure of the fat body to hemolymph allows rapid detection of alteration in energy levels or circulating molecules in the hemolymph (61). The fat body stores nutrients as glycogen and triglyceride and upon request by other tissues, triglyceride or glycogen degradation products are released to provide energy and sustain growth (61,88–90). In addition to its role in energy storage, the fat body functions as a nutritional sensor which is accomplished by activation of conserved signaling pathways of the insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR) pathways. Furthermore, it has been found that fat body tissue serves as an endocrine organ, which interacts with other tissues such as the brain to coordinate growth and development (60,91). The fat body also regulates humoral immunity through activating the evolutionary conserved IMD or Toll pathways in response to bacterial infection (92,93). Given the *Drosophila* fat body has homologous function to liver and adipose tissue in mammals and its ability to respond to both metabolic and immune responses of the host through activation of signaling pathways that are conserved between *Drosophila* and vertebrates, therefore it provides an ideal system to extend our understanding of immuno-metabolic interactions in mammals.

1.2.3 Insulin like peptides production, secretion and regulation

ILPs mediate several physiological functions such as growth, behavior, metabolism, and lifespan in *Drosophila* (94–97). In flies, eight ILPs (ILPs 1-8) have been identified (94,98–100). ILPS 1-7 bind to the *Drosophila* insulin receptor (InR), which is a homologue of the mammalian insulin receptor, while ILP8 binds to a Leucine-rich repeat-containing G protein-coupled receptor 3, which is a *Drosophila* homolog of mammalian relaxin (100,101). Upon binding of ILPs to the specific receptors on the cell surface, an intracellular insulin signaling cascades initiates, which results in activation of different biological responses. *Drosophila* ILP 1-5 show similar homology to human insulin and mouse insulin/insulin-like growth factors (98,102) and are involved in carbohydrate and lipid metabolism (94,103). A group of 14 median neurosecretory cells in the brain, which are called Insulin Producing Cells (IPCs), produce and secrete ILP2, ILP3 and ILP5 (104). Removal of IPCs results in elevation of the circulatory carbohydrate levels which resembles a diabetes-like phenotype (104). Insulin deficient *Drosophila*, *ilp2-3,5* mutants, are viable, live longer and have a reduced body size compared to their wild-type counterparts (103). The fat body secretes ILP6, which has a similar structure to human insulin-like growth factor, and its secretion is important during larval transition to pupal stages as well as lipid and carbohydrate metabolism (105–107).

While specific role for each ILPs exist, ILPs can act redundantly to compensate for loss of individual ILPs. For example, expression of *ilp5* was up-regulated in single or double mutants of *ilp2* and *ilp2–3* mutants and *ilp3* expression was up-regulated in *ilp2* and *ilp5* mutants (94). In another study, *ilp2-3,5* mutants phenotypes were rescued by expression of *ilp2* (104). Spatial and temporal expression of insulin peptide genes reveals different physiological and developmental function for each ILP (91,108,109). For example, in larvae, *ilp2* is expressed in the IPCs, imaginal discs, salivary glands as well as glial cells, while in adults expression of *ilp2* has been only detected in IPCs. However, it has been shown recently that median neurosecretory cells which produce diuretic hormone 44 innervate the crop in adult flies and express *ilp2* as well (110). In adult *Drosophila, ilp3* is expressed in both IPCs and midgut muscle while in larvae, *ilp3* is restricted to the IPCs (111). For regulation of metabolism, specific ILPs are involved and their expression is altered in response to different diets. For example, in a low protein diet, *ilp2* expression increases, while *ilp5* mRNA levels increases in response to high protein diet (112,113). A recent study showed that *ilp2* mutation results in reduced glycogen levels independent of diet (114) which is

consistent with another study showing *ilp2* suppression increases whole body trehalose levels (115). ILP3 has been shown to be involved in regulation of trehalose metabolism during a low protein diet (96) as well as lipid metabolism regulation (116).

In mammalian pancreatic islets, depolarization of the β-cell membrane potential by glucose results in suppression of the ATP-sensitive potassium channels (K^{ATP}) and consequently opening of voltage-dependent Ca^{2+} channels (117). In adult flies, IPCs directly sense circulating glucose in the hemolymph and similar to human β-islet cells, Glut1 transporter on IPCs mediates glucose uptake which leads to the stimulation of mitochondrial ATP-production, and closure of potassium channels (118,119). In contrast to the adults, larval IPCs are not capable of responding to circulating glucose directly as they do not express K^{ATP} channels (120). Instead, AKH hormone which is produced by corpora cardiaca (CC) is involved in indirect carbohydrates regulation by IPCs in larvae via axon projections which connects IPC and CC to each other and provides communication between them for nutrient sensing (104,120,121).

1.2.4 Regulation of insulin secretion via fat to brain communication

Adipose tissue in mammals contains a number of adipokines, which signal to the brain to maintain energy homeostasis. In *Drosophila*, signaling from the fat body to the brain is critical for controlling growth and metabolism in response to nutrient availability. For example, in low-nutrient conditions, the fat body releases signaling molecules to the brain which leads to the suppression of insulin signaling and body growth. In contrast, when nutrient levels are high, fat-brain communication stimulates expression and secretion of ILPs which results in increased insulin signaling in peripheral tissues and growth (60,122,123). For example, the fat body signals to the IPCs through multiple mechanisms including the secretion of Stunted (124), Eiger (125), Neural Lazarillo (126,127), the Unpaired 2 cytokine (128), the Activin-like ligand dawdle (129), or CCHamide-2 (130). Once Upd2 is released from the fat body, it activates the Jak/STAT pathway in GABAergic neurons and consequently reduces their suppressive effect on IPCs, triggering secretion of ILPs into the hemolymph (128). Dietary sugars activate *dawdle* expression, which encodes an Activin-like ligand of the transforming growth factor beta superfamily in the fat body in a Mondo-Mlx-dependent mechanism. Then, secreted Dawdle regulates the secretion of ILP2 and ILP5 by binding to the Activin-like receptor Baboon on IPCs (129). The fat body and enteroendocrine cells in the midgut expresses the peptide hormone CCHamide2 which binds to its receptor in the brain and functions as a nutrient-dependent regulator of ILPs secretion (130). In

nutritional stress condition such as starvation, the fat body secrets Imaginal morphogenesis protein-Late 2 (ImpL2), which is an ortholog of the mammalian IGF-binding proteins (131) and is able to form protein complexes with ILP2 and ILP5 and inhibit insulin signaling in adult *Drosophila* (131,132). In addition to that, FOXO regulates transcript levels of *ilp6* in the fat body which has an inhibitory effect on the production of *ilp2*, *ilp3* and *ilp5* by the brain (105,133).

1.2.5 Intracellular Insulin/TOR signaling in *Drosophila*

The *Drosophila* insulin pathway is highly conserved (134) and has different roles in the host such as regulation of growth, metabolism, lifespan, resistance to stress, and fecundity (102,135–140). Mutations in *Drosophila* insulin signaling induce similar phenotypes to those observed in mice with suppression of genes that encodes insulin-like growth factors (IGFs) or the IGF-I receptor (103,141,142). Tissue specific suppression of insulin signaling in the *Drosophila* fat body impairs larval development and results in reduced body size in adults (143). The *Drosophila* insulin receptor (InR) has two α and two β subunits as well as a cytoplasmic tyrosine kinase domain that becomes activated upon ILP binding (144). Due to structural conservation of the insulin-binding sites, *Drosophila* InR shows high affinity to mammalian insulin (145,146). ILPs binding to the InR results in autophosphorylation of InR and subsequent recruitment of the insulin receptor substrate, Chico (145,147). Then, Chico activates phosphatidylinositol-3-kinase (PI3K), which catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) in the cell membrane into phosphatidylinositol 3,4,5-trisphosphate (PIP3). PI3K function is countered by a phosphatase and tensin homolog (PTEN) which acts as a negative regulator of insulin signaling (148). Accumulation of PIP3 recruits phosphoinositide-dependent kinase 1 (PDK1) and serine/threonine protein kinase PKB (Akt). PDK1 then phosphorylates Akt which results in activation of the biological processes that mediate metabolic functions of the insulin signaling, growth, and longevity (149–153). Akt-dependent phosphorylation of FOXO suppresses nuclear accumulation and transcriptional activity of FOXO (154). FOXO target genes are involved in protein synthesis, mitochondrial function, and carbohydrate, lipid and protein homeostasis (152,155,156). In the low insulin signaling conditions such as starvation, increased FOXO activity increases *InR* expression, which provides a regulatory feedback mechanism (157)(Figure 1-3).

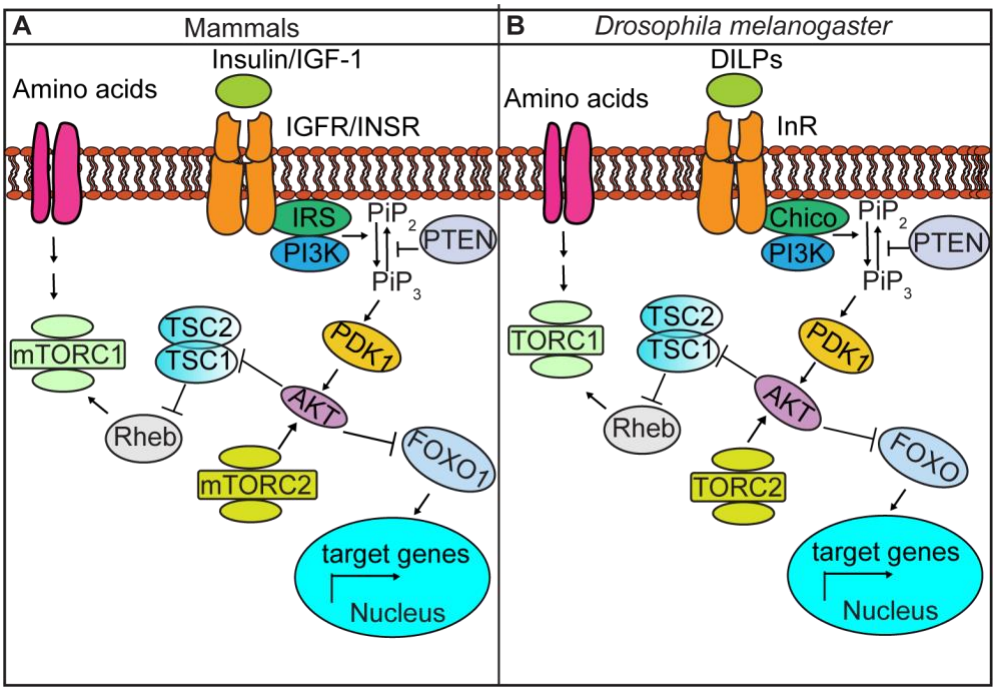


Figure 1-3 Comparison of the *Drosophila* and mammalian IIS/TOR pathways
Schematic representation of the Insulin/insulin-like growth factor signaling in (A) Mammals and (B) *Drosophila*. Similar colors represents orthologous proteins between fly and mammalian pathways.

The insulin pathway intersects with TOR signaling, which is an evolutionarily conserved pathway and homologous to the mechanistic target of rapamycin (mTOR) (158). TOR pathway regulates growth, protein synthesis, proliferation and metabolism (159–162).

1.2.6 Innate immunity in *Drosophila*

As *D. melanogaster* feeds on decaying substrates such as rotting fruits, flies are continuously exposed to a variety of microbes including bacteria, and fungi. To combat these pathogens, *Drosophila* has evolved cellular and humoral immune responses (62,163). However, *Drosophila* lacks the somatic rearrangement or hypermutation of immune receptors which are linked with adaptive immunity in vertebrates (164). The innate immune defenses in *Drosophila* include: the chitinous peritrophic matrix that lines the midgut and acts as a physical barrier, circulating cellular immune cells called hemocytes with phagocytosis ability, antimicrobial peptides (AMPs) which are produced either locally in the intestine or systemically in the fat body, and RNA interference (RNAi) mediated anti-viral defenses (62,163,165). In *Drosophila*, pathogen detection and production of AMPs occurs through activation of two main pathways: the Toll pathway and IMD pathway which share homologues components to the Toll-like Receptor (TLR)

and Tumor Necrosis Factor Receptor (TNFR) cellular pathways in mammals, respectively (2,8,9). The TNF and IMD pathways are activated by different receptors and ligands. For example, the TNF receptor reacts to soluble TNF homo-trimers ligand, while the *Drosophila* IMD pathway detects bacterial peptidoglycan (PGN) that contains diaminopimelic acid (DAP) (166,167). The Toll pathway responds to Gram-positive bacteria and fungi, while the IMD pathway is activated by Gram-negative bacteria as well as a subset of Gram-positive bacteria with DAP-type peptidoglycan such as *Bacillus spp.* (168). Mutations in different components of IMD pathway results in a high level of vulnerability to Gram-negative bacteria (169,170). During systemic infections, the fat body produces AMPs via activation of Toll and IMD pathway while intestinal epithelial immunity occurs via activation of the IMD pathway (163,171–173).

1.2.6.1 Antimicrobial peptides

One of the common feature of immune response in all living organisms is producing AMPs which indicates their significant role as host antibiotics in the fight against infections (174). AMPs are small cationic peptides that induce their antimicrobial effects through disruption of microbial cell wall integrity (175–178). In *Drosophila*, seven AMP families with 21 AMP and AMP-like genes have been identified (179). Although other tissues such as the intestinal epithelium (180–182) or circulating hemocytes produce AMPs (183), the fat body is the primary source of AMP production during systemic infection (62). Different sets of AMPs are transcribed by activation of the Toll and IMD pathways. For example, activation of the Toll pathway drives the expression of *metchnikowin*, *drosomycin* and *defensin* against Gram positive bacteria and fungi (184,185), while IMD pathway activity promotes *attacin* and *diptericin* expression against Gram negative bacteria (63,186–190). Both *cecropins* and *defensin* have antibacterial and antifungal properties (191–194). A recent study showed a high level of specificity in AMP and pathogen interactions and demonstrated that *Drosophila* AMPs could act additively or synergistically against pathogens (195).

1.2.6.2 The IMD pathway

The IMD pathway was first discovered in *Drosophila* when mutations in the *imd* showed disruption in only AMPs but not antifungal peptides (196). Flies that lack *imd* show a normal immune response towards Gram positive bacteria and fungi but died after Gram negative bacterial challenges. Overexpression of IMD in flies results in production of AMPs in the absence of infection (197). Members of a broad family of PGN recognition proteins (PGRPs) help *Drosophila*

to identify bacterial PGN (198). Synergistic activity of two PGRPs, PGRP-LC and PCRP-LE, identifies PGN and relays this information to the IMD pathway (167,199–203). Once the bacterial DAP-type PGN binds to PGRP-LC, receptor dimerization occurs which results in recruitment of the IMD protein, which is homologous to the mammalian receptor interacting protein 1 (RIP1) and contains a death domain (197,200,201). Interaction of the IMD death domain with the adaptor protein Fas-associated death domain (Fadd) (204,205) results in recruitment of the mammalian caspase-8 homolog, death-related Ced-3/Nedd2-like protein (Dredd) to the signaling complex, which is now consist of IMD, Fadd, and Dredd (205–210). Then, a ubiquitin E3 ligase death associated inhibitor of apoptosis 2 (Diap2) activates Dredd via ubiquitination (209,211), which enables Dredd to cleave IMD and subsequently exposes a binding site for association of IMD with the Diap2, followed by K63-polyubiquitination of IMD (210). The next step in the IMD signaling cascade is the recruitment of the *Drosophila* homolog of the mammalian mitogen-activated protein kinase kinase kinase (MAPKKK), TGF-β-activated kinase (Tak1), and its adaptor protein Tak-binding protein 2 (Tab2) (212–215). The Tak1/Tab2 phosphorylates the *Drosophila* IKK (IκB kinase) complex, which is consist of *kenny* and *ird5*. In *Drosophila*, Relish is orthologous to the mammalian p100/105 NF-kB family member, and is activated by Dredd via endoproteolytic removal of an autoinhibitory C-terminal domain from Relish (216–218). Following Relish phosphorylation by the IKK complex, the C-terminal domain of Relish remains in the cytoplasm while the N-terminal domain translocates to the nucleus. Once inside the nucleus, Relish induces expression of immune effectors such as AMPs (219)(Figure 1-4).

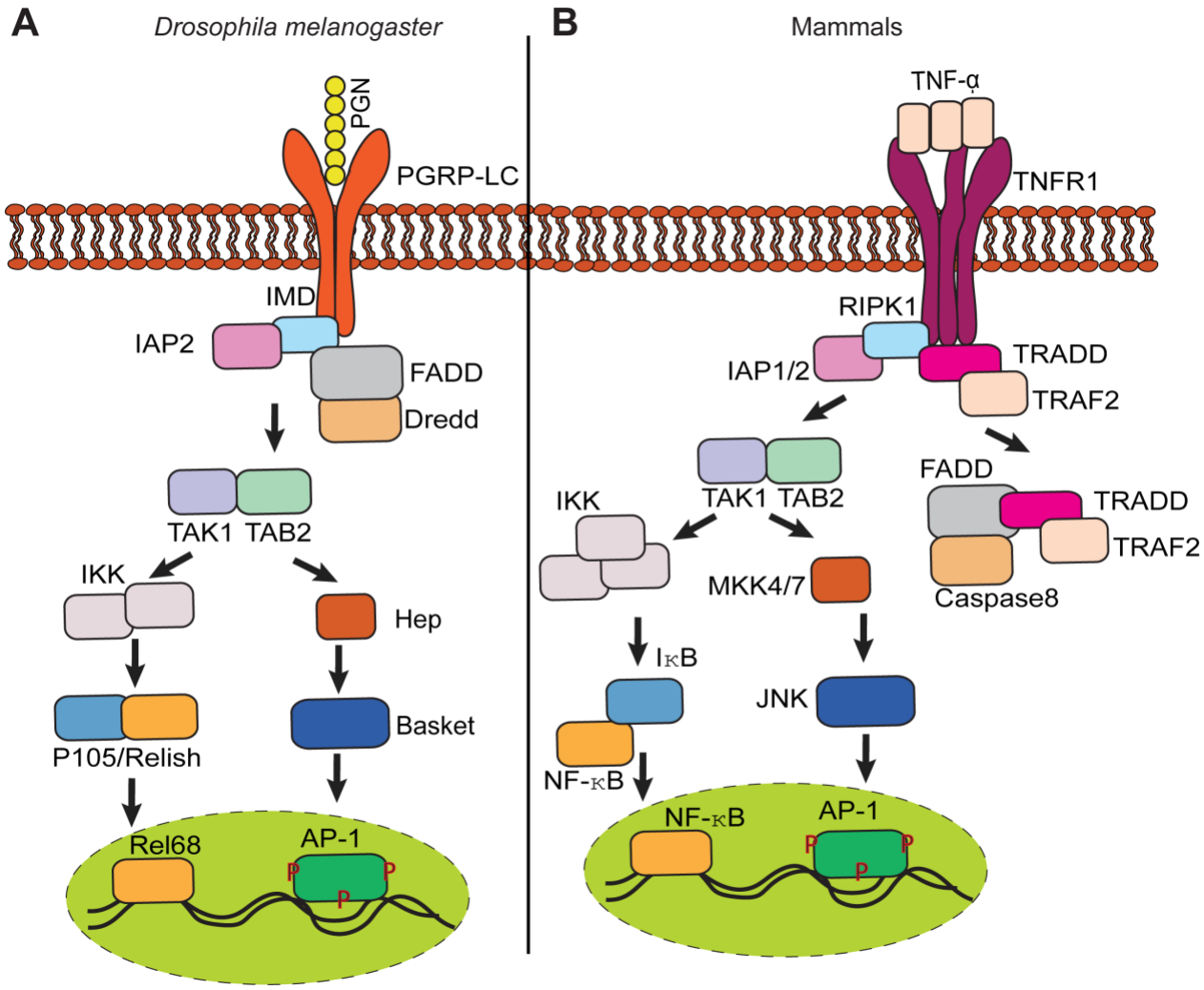


Figure 1-4 Comparison of the *Drosophila* IMD pathway and mammalian TNFR-1 pathway
Schematic representation of the (A) *Drosophila* Immune Deficiency Pathway (IMD) and the (B) human Tumor Necrosis Factor-alpha (TNF-α). Similar colors represents orthologous proteins between fly and mammalian pathways.

1.2.6.3 The *Drosophila* JNK pathway

The IMD pathway, like the mammalian TNFR pathway, branches into two arms at the Tak1 level: Relish and caspase c-Jun N-terminal Kinase (JNK) pathway (220,221). The JNK pathway, belong to the MAPK family and is activated by inflammatory cytokines and environmental stress (222). JNK pathway activation involves the sequential phosphorylation of a number of kinases. Activation of MAPKKK complex which contains a number of kinases including Tak1 results in phosphorylation of the JNK kinase *hemipterous*, which in turn activate *Drosophila* JNK, *basket*. Ultimately, basket phosphorylation activates JNK transcription factors such as AP-1 and Fos (223–

226). The JNK pathway functions as a regulator of insulin pathway as well and is able to restrict insulin signaling activity (227). In the fat body, JNK promotes nuclear translocation of FOXO and results in FOXO-induced transcription of stress response genes, which leads to systemic suppression of insulin signaling via suppression of ILPs expression (228). In addition to that, FOXO activation downstream of JNK in *Drosophila* IPCs suppresses *ilp2* transcription (229).

1.2.7 Carbohydrate metabolism in *Drosophila*

The regulation of glucose homeostasis is very strict and requires a number of metabolic tissues as well as signaling pathways (69). *Drosophila* has demonstrated to be an effective model for studying regulatory mechanisms in carbohydrate metabolism (230), as many metabolic pathways involved in carbohydrate metabolism are highly conserved between flies and mammals. The response of *Drosophila* to glucose ingestion is very close to that of mammals, which starts with glucose identification by taste-sensing neurons, followed by intestinal absorption, and distribution of circulating sugar to target tissues and storage of excess glucose (69,231,232). Trehalose, which is a non-reducing disaccharide made up of two glucose molecules, is the most prevalent circulating sugar in *Drosophila*. However, adult hemolymph has a higher glucose content compared to larvae, which implies differences between circulatory carbohydrate proportions at different stages of development (233). Trehalose is produced in the fat body, secreted into the hemolymph, and then is absorbed and utilized by different tissues (234). In adult flies, trehalose is essential for providing the energy needed for flight (235). Glucose and trehalose levels in the hemolymph seems to be regulated independently. As trehalose is a very stable disaccharide, circulating trehalose levels are not significantly impacted by caloric challenges or dietary sugars (236).

Excess dietary glucose can be stored as glycogen in the fat body and muscle (69). Glycogen provides a stored source of glucose for trehalose synthesis and the mobilization of trehalose to glucose has been found to be critical for metabolic homeostasis (237). Glucose homeostasis is regulated through a hormonal insulin/glucagon axis in mammals and a similar hormonal regulation exists in *Drosophila* via insulin/AKH signaling (238,239) in which intracellular signaling of both insulin and AKH are closely related to mammals. In vertebrates, increased circulatory glucose leads to secretion of insulin from pancreatic β-islet cells, while low glucose concentration triggers glucagon release from pancreatic α-cells (240). Insulin promotes anabolic activities such as glycogen synthesis from glucose and increases cell membrane permeability for glucose while

inhibiting glycogen-digesting enzymes (241). In *Drosophila*, mutations in different components of the insulin signalling pathway such as InR, Chico, Akt, PTEN and FOXO induces defects in the carbohydrate and lipid metabolism (64,147,242–244). Another regulator of glycogen metabolism is glycogen synthase kinase-3 beta (GSK3β), which stimulates glycogenesis in mammals. The *Drosophila* homologue of GSK3β is called *shaggy*, which is phosphorylated in a PI3K-dependent manner (245). During low insulin signaling, FOXO translocates to the nucleus and initiates transcription of genes involved in catabolic processes to provide energy (152,246). In contrast, AKH, which is the homologue of glucagon in vertebrates (247) activates glycogen phosphorylase to break down glycogen stores in the fat body and increases circulating glucose and trehalose levels (69,120,121,248). Removal of neurons that produce AKH by overexpression of the proapoptotic gene *reaper* results in a significant reduced trehalose level in the larval hemolymph (120,121,249).

Consumption of large amounts of dietary sugars stimulates activation of insulin signaling that consequently initiates conversion of glucose or trehalose into glycogen via glycogenesis. Conversely, during starvation or when nutrient are at scarcity, generation of glucose either through glycogen breakdown, or glycogenolysis, or glucose biosynthesis from non-carbohydrates carbon substrates which is called gluconeogenesis facilitates glucose generation. Gluconeogenesis and glycolysis which are reciprocal pathways are highly conserved. In gluconeogenesis, phosphoenolpyruvate carboxykinase (Pepck), fructose-1,6-bisphosphatase (fbp), and glucose-6-phosphatase (G6P) are important for glucose generation (250). For example, G6P converts glucose-6-phospate into glucose. Insulin signaling activation initiates gluconeogenesis through Akt-mediated regulation of glucogenic enzymes. Akt phosphorylation inhibits FOXO nuclear translocation and therefore expression of FOXO target genes. For example, FOXO regulates *pepck* expression (156,250). Insulin also positively regulates the gene expression of glucokinase and pyruvate kinase that are essential enzyme in glycolysis pathway for conversion of glucose to pyruvate. Glycogen synthase kinase 3 (GSK3), which regulates glycogen metabolism in mammals is important for glycogenesis and is activated by insulin signaling initiation. Insulin signaling leads to the phosphorylation and inactivation of GSK3, and therefore promotes the formation of active glycogen synthase required for glycogen synthesis. In *Drosophila*, release of ILPs into the hemolymph negatively regulates expression of Tobi which is an α-glucosidase and catalyzes glycogen breakdown or glycogenolysis (251). *Drosophila* GSK3β homologue or *shaggy* has been

shown to be phosphorylated in a PI3K-dependent manner in vivo (245). Under fasting conditions, glycogenolysis metabolises stored glycogen into glucose, which is then released into the bloodstream.

1.3 Tolerance and Resistance strategies during infection in *Drosophila*

Resistance mechanisms aim to recognize, neutralize, and remove pathogens while tolerance mechanisms reduce the damage induced either by the pathogen or increased activation of the host immune system without affecting pathogen burden. The concept of disease tolerance is a developing area that originally was discovered in plant biology research (252–254). One of the earliest studies compared effects of infection with the fungus *Puccinia triticina* on varieties of different winter wheat and assessed changes in fitness such as yield. They found that a specific variant, Fulhard wheat, had higher pathogen load but still had the highest yield grain (255). In a rodent malaria model, genetic variation for tolerance against infection with *Plasmodium chabaudi* was observed and despite of having similar pathogen burden the effects of infection with *P. chabaudi* varied between different mouse strains (256).

Production and secretion of the AMPs against pathogens is an evolutionary conserved resistance mechanism that exists across different animals including *Drosophila* (257–259). As AMPs are produced through activation of innate immune pathways, such as IMD and Toll, mutations in these pathways disrupt the production of AMPs, diminishing the ability of the host to restrict pathogen proliferation, which results in reducing host resistance (63,196,205,260–263). Another defense pathway is the Dual oxidase pathway that produces reactive oxygen species (ROS) to fight against bacterial pathogens (172,264). However, increased production of ROS also damages host cells, which results in reduced host tolerance (65,265). Antibacterial ability of autophagy processes also act as resistance mechanism in *Drosophila*. For example, increased autophagy in hemocytes of flies infected with *L. monocytogenes* results in reduced pathogen growth and increased survival of the flies (33). Different tolerance mechanisms have been identified in *Drosophila* although many others remain to be investigated. Genetic variation is an important factor that contributes to tolerance of the host against pathogens. For example, infection of eleven different wild-derived fly lines with *Pseudomonas aeruginosa* resulted in variation across genotypes in respect to infection outcomes which was measured by survival and bacterial burden and showed that increased host fitness did not equal reduced bacterial load (266). Flies that lack a

p38 mitogen-activated protein kinase show increased mortality against *Salmonella typhimurium* infection. This kinase is important for regulation of phagocytic encapsulation so that the bacteria is contained and further damages to the host is prevented (267). Therefore, p38 mitogen-activated protein kinase confers tolerance to the host against Salmonella-induced infection by inhibiting damages induced from pathogen without reducing the pathogen burden. In the *Drosophila* intestine, tolerance mechanisms improve host fitness upon microbial challenges that include tissue repair through increased proliferation of stem cells to replace damaged intestinal epithelial cells (268), epithelium thinning followed by enterocyte recovery (269) and inhibition of oxidative stress by production of antioxidants to inhibit the damage induced by ROS production (270,271).

Both resistance and tolerance strategies occur at a cost for the host. In resistance responses, the cost includes providing energy for production of AMPS, and for tolerance mechanisms the costs include processes involved in repair and fitness improvement. For example, *Mycobacterium marinum* infection induce a wasting phenotype in *Drosophila* through suppression of insulin signaling and a subsequent increase in transcriptional activity of FOXO. It was shown that reducing FOXO activity improves survival of host to *M. marinum* infection with no alteration in pathogen burden which is an indicator of increased tolerance (64). Loss of appetite or anorexia is another strategy that improves host fitness against infection and seems to be pathogen specific (272–274). For example, in flies that lack gene encoding the gustatory receptor *gr28b*, taste perception is impaired and flies are constitutively anorexic. These *Gr28b* mutants show increased survival towards *S. typhimurium* but are more sensitive to *L. monocytogenes* via septic infection (260). These observations suggest that diet restriction has a pathogen specific impact on host survival, and was later showed that diet restriction improves host diseases tolerance to *S. typhimurium* while reduces host resistance to *L. monocytogenes* (275). Effects of diet or different metabolites during different microbial infections has been shown in vertebrates as well. For example, feeding glucose to the mice during *L. monocytogenes* infection worsen survival while improves survival against a viral infection such as influenza (276).

1.3.1 Infection models in *Drosophila*, oral and septic

Drosophila has shown to be an excellent model for studying the host-pathogen interactions of a wide range of human-relevant microbes (277). Many bacteria use similar infection mechanisms

in both mammalian and fly hosts (278–280). For example, early stage of *M. marinum* infection in *Drosophila* shows similar pathologies to human tuberculosis infection (281,282). *Drosophila* is typically infected through two main routes: oral (fly feeding) and systemic (fly pricking). Measuring survival following oral or septic infection is useful for determining variations in the host reaction to infection. In addition to that, counting the number of colony forming units (CFU), which is representative of live bacteria at the time of sample collection serves as a quantitative method for measuring bacterial load (283). In systemic infection, a sterile needle is dipped into the desired bacterial suspension concentration and is used to prick *Drosophila* in the dorsal thorax or abdomen. For inoculation of exact doses of microbes into the fly, a microinjector could be used which has the disadvantage of slower speed and requirement of heavy injector equipment for infection. Using pricking technique, the pathogen is directly deposited into the body cavity and bypasses the intestinal epithelium barriers (280,283–286).

The route of infection has an important role in evaluating host-pathogen interactions and can affect disease outcome (287,288), evolutionary response of host to the pathogens (289), protective effects of *Drosophila* endosymbionts, *Wolbachia* (290), and activation of different immune pathways (268,291). As flies forage on decomposing fruits or organic matter, they are naturally exposed to a variety of microbes therefore feeding represents the most common route of pathogen entry. For oral infection, flies are fed with pathogens suspended in a sucrose solution, or added to the surface of cotton plugs (292–295). Different bacterial species have been used to study intestinal epithelium immune responses during oral infections such as *Erwinia carotovora* (*Ecc15*, non-lethal, Gram-negative), *Serratia marcescens* (lethal, Gram-negative), and *Pseudomonas entomophila* (lethal, Gram-negative) (268,296–298) which all have the ability to cross the gut barrier and induce a systemic immune response as well (293,294,299). Other bacterial pathogens that are commonly used in *Drosophila* host-pathogen models include: *Providencia rettgeri* which is an opportunistic Gram-negative pathogen, and causes traveler's diarrhea and is responsible for opportunistic infections in hospitalized patients (300–302). *P. rettgeri* has been extracted from *Drosophila* hemolymph (266,303,304) and induces a moderately lethal infection in the fly upon systemic infection (304–306). In contrast, *Providencia sneebia* induces higher mortality rate and does not provoke a strong immune response (304). *Enterococcus faecalis* is an opportunistic nosocomial human pathogen that is able to naturally infect flies (307,308). This Gram-positive

bacterium induces an intermediate mortality during systemic infection but is non-lethal upon oral infection (306,309).

1.3.2 *Drosophila* Intestinal physiology

The adult *Drosophila* gastrointestinal tract (GI) is composed of three sections: foregut, midgut, and hindgut, each of which has a specific function and cell composition (310). The foregut is composed of the esophagus, crop, and cardia (proventriculus). Upon food entry to the GI tract, the proventriculus initiates mechanical breakdown of the food (311) and is also responsible for the synthesis of the peritrophic matrix. The peritrophic matrix which is located above the apical surface of the gut epithelium lines the midgut epithelium, protects the intestinal epithelium from microbial insults, and prevents entry of bacterial pathogens to the hemolymph (312,313). The midgut epithelium is composed of six different anatomical regions in which each region is characterized by specific gene expression profiles, division rate of stem cells and different cellular composition and function (314–317). The midgut in *Drosophila* serve as primary region for digestion, nutrient absorption and hormone production (232,318). The posterior midgut is equivalent to the mammalian small intestine structurally and functionally while *Drosophila* hindgut is more similar to human colon. The midgut epithelium consists of large absorptive enterocytes, hormone-secreting enteroendocrine cells and intestinal stem cells (319–321). Enteroendocrine cells are marked by expression of the *prospero* transcription factor and subpopulations of enteroendocrine cells secretes peptide hormones that so far more than 24 of these peptides have been discovered, which are involved in the lipid metabolism, gut motility, energy homeostasis and feeding behaviour (111,319,322–327).

The apical surface of the fly intestinal epithelium contains microvilli brush borders that facilitate nutrient uptake from the intestinal lumen. The intestinal stem cells are located at the basal surface of the epithelium along with the basement membrane and visceral muscle (328). The multipotent intestinal stem cells are essential for regulation of epithelium homeostasis by controlling the balance between self-renewing and differentiating into enterocyte and enteroendocrine cells that occurs every one to two weeks (319,320). The intestinal stem cells are also important in immune response of the intestinal epithelium, as they increase their proliferative activity in response to infection (173,268,296,297,328,329), oxidative stress (330,331), and tissue damage (332). In enterocytes, digested glucose is absorbed through glucose transporters and de

novo synthesized and dietary fatty acids are converted into triglycerides and diacylglycerols to be transported to the fat body or other organs via lipoprotein particles (333). In addition to their role in carbohydrate and lipid absorptions, enterocytes produce AMPs and ROS against bacterial infections (268,334,335). Upon infection or stress-induced damage to the intestinal epithelium, damaged enterocytes produce a leptin-like (Interleukin-6 family) cytokine called unpaired 3, which is a ligand for the Domeless receptor, an Interleukin-6 type receptor, and activates the JAK-STAT pathway in intestinal stem cells resulting in intestinal stem cells mitotic division and differentiation into enterocytes (173,268,298). Similarly, in mammalian gut, activation of interleukin 6 and STAT3 during inflammation, inflammatory bowel disease and colitis results in promotion of a proliferative and regenerative response in the intestinal epithelium (336–338)(Figure 1-5).

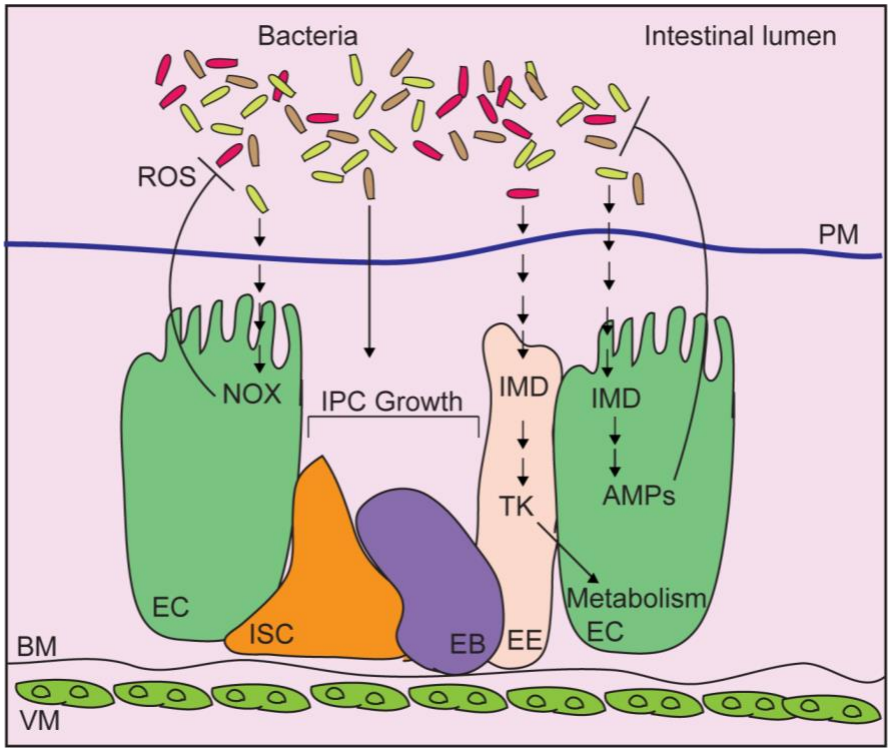


Figure 1-5 Schematic representation of the adult *Drosophila* midgut
Intestinal bacteria are contained within the lumen by a chitinous peritrophic matrix (PM). Bacteria diaminopimelic acid-type peptidoglycan activates the immune deficiency (IMD) pathway in enterocytes (EC), leading to production of antimicrobial peptides (AMP). In enteroendocrine cells (EE), IMD controls expression of the metabolism-regulatory hormone Tachykinin (Tk). Epithelial reactive oxygen species (ROS) generated by NADPH oxidases (NOX) also contribute to bacterial killing while cues from the bacterial microbiome promote the growth of intestinal progenitor cells (IPC), composes of intestinal stem cells (ISC), and enteroblasts (EB).

1.3.3 Vibrio disease, pandemic and pathogenesis

Vibrio cholerae is a curved, Gram-negative member of the *Vibrionaceae* family of Proteobacteria (339). It inhabits aquatic environments, and copepods and chironomids are reported as natural reservoirs in marine ecosystems (340,341). Intestinal colonization by *V. cholerae* causes the diarrheal disease, cholera, and is considered a substantial public health threat, especially in countries with poor sanitation and contaminated water (342). The first cholera pandemic emerged in 1817, with an expansion of cholera beyond the Indian subcontinent (343). Since then, the world has witnessed an additional six pandemics, with the seventh pandemic ongoing (344). Models that estimate cholera burden predict ~3 million cases of disease per year, resulting in roughly 100,000 deaths (345). *V. cholerae* strains are divided into classical and non-classical serotypes, with classical ones expressing the O1 antigen on their surface (346,347). Classical serotypes are further subdivided into two biotypes—classical and El Tor—that differ in the expression of a number of markers, such as hemolysins (348–351). The outbreak of epidemic cholera that spread through southeast Asia in 1992 is caused by the non-classical strain of *V. cholerae* O139 (352), whereas the ongoing pandemic that originated in Indonesia in 1961 is caused by the El Tor biotype (353). El Tor causes a milder cholera disease (354), with infected individuals frequently remaining asymptomatic early in infection (355).

V. cholerae encodes several virulence factors that regulate survival, colonization, and pathogenicity (356–359). Cholera toxin (CT) is a hexameric adenosine diphosphate-ribosyl transferase that contains one A subunit surrounded by five B subunits (360,361). Upon release into the intestinal lumen via a type two secretion system (362), the B pentamer of CT interacts with host GM1 ganglosides (363), permitting toxin endocytosis, and a subsequent cytosolic release of the A1 subunit (364). A1 ADP-ribosylates the Gs alpha subunit, locking $G\alpha_s$ in an active state (365). Active $G\alpha_s$ elevates adenylate cyclase activity, greatly increasing levels of 3′,5′-cyclic AMP, resulting in excess protein kinase A (PKA) activity (366). PKA stimulates an efflux of chloride ions through the cystic fibrosis transmembrane conductance regulator channel (367), leading to an uncontrolled flow of water, sodium and potassium ions into the intestinal lumen. This extreme, and rapid, dehydration results in the voluminous rice-water diarrhea that hallmarks cholera disease (368). In addition to CT, *V. cholerae* require the toxin co-regulated pilus virulence factor for pathogenesis (369). Toxin co-regulated pilus is a type IV pilus system that mediates colonization of the small intestine by a self-associate mechanism that supports the formation of bacterial microcolonies (370). Toxin co-regulated pilus also serves as the receptor for the CTXφ

bacteriophage. CTXφ encodes *ctxAB*, and converts benign *V. cholerae* to pathogenic strains. The ability to synthesize toxin co-regulated pilus is advantageous for *V. cholerae* in aquatic environments, as it improves *V. cholerae* fitness by facilitating inter-bacterial interactions during colonization of host chitinaceous surfaces (371). Although fluid replacement through oral rehydration solutions, antibiotic therapy, and vaccines are effective treatment options for patients with cholera, increased rates of antibiotic resistance among classical (372) and non-classical (373) strain of *V. cholerae* complicate treatment of the disease. Therefore, new antibacterial strategies that effectively target *V. cholerae* virulence factors are critical to contain this deadly disease. Over the last century, a variety of animal models that include rabbits, mice, fish, and flies, have been used to study *Vibrio*-host interactions and each of these models have added to our understanding of virulence, host responses to infection, interactions between *Vibrio* and host microbes, and cholera vaccine development.

1.3.4 Vibrio fly model

Insects such as chironmids (340) and houseflies (374) are candidate reservoirs of *V. cholerae*, and some studies suggest a correlation between disease transmission and increases in fly population, during cholera outbreaks, or in areas where the disease is endemic (375). Given the association of *V. cholerae* with arthropod vectors, researchers tested the utility of *Drosophila* as a model to characterize *V. cholerae* pathogenesis. *Drosophila* infections typically involve oral delivery of the pathogen, or introduction of the pathogen into the body cavity of the fly through a septic injury (286). In contrast to non-pathogenic *Vibrio* strains, injection of *V. cholerae* into the body cavity resulted in a rapid death of infected flies, raising the possibility of using flies as a model to study *V. cholerae* pathogenesis (376). In a foundational study from 2005, researchers showed that continuous feeding of adult flies with *V. cholerae* caused a cholera-like disease characterized by loss of weight, and rapid death that required a functional $G\alpha_s$ in the host (377), establishing flies as a valuable model to characterize *V. cholerae* pathogenesis. However, in contrast to vertebrates, *ctx* mutants remain lethal to flies, suggesting CT-independent pathogenic mechanisms in adult flies. Furthermore, *Vibrio* polysaccharide-dependent biofilm formation is important for persistent colonization of the fly rectum and for *V. cholerae*-mediated lethality (378), whereas *Vibrio* polysaccharides interfere with colonization of the host intestine (379). Thus, the fly is a useful tool to identify uncharacterized virulence factors that affect interactions between *V.*

cholerae and an arthropod host. As studies with this model progress, it will be interesting to determine how such virulence factors impact pathogenesis in vertebrate models.

1.4 Thesis objectives

Molecular links between immune and metabolic pathways are conserved across vast evolutionary distances, and abnormal immune–metabolic signals are linked to several pathological states. Host defense strategies encompasses resistance mechanisms that kill infectious microbes and tolerance mechanisms that mitigate disease severity without effects on microbial load. Adaptation of host metabolism is a common theme in the host response to infection although the impact of an immune–metabolic axis on host responses to bacterial infection remains unclear. In my project, I used the *Drosophila* model to understand the effects of increased innate immune signaling on metabolic homeostasis, to characterize the relationship between insulin and IMD-dependent containment of infectious microbes, and to identify the role of immune-metabolic signaling in the fat body in the host response to enteric infection with *V. cholerae.*

1.4.1 Study aims for Chapter 3

The primary aim for chapter three was to investigate how increased innate immune signaling in the *Drosophila* fat body affects metabolism and development of larvae. A previous observation in our lab showed that increased IMD signaling in intestinal progenitor cells has a profound effect on the transcription of genes involved in the regulation of host metabolism (380). This specific finding prompted me to determine the consequence of persistent immune signaling in a metabolic tissue such as the fat body. Therefore, I hypothesized that increased activation of IMD signaling in the fat body of third instar larvae would have a significant impact on fly metabolic homeostasis, growth and development.

1.4.2 Study aims for Chapter 4

My observations from chapter three showed that activation of IMD in the fat body has the molecular, genetic, and phenotypic signatures of alterations to host metabolism. Transcriptionally, activation of IMD resulted in a gene expression signature consistent with diminished insulin activity. Physiological defects induced by elevated IMD activity in the fat body such as hyperglycemia, delayed development, and a reduction in adult size phenocopied loss of function mutation in the insulin pathway. Given the apparent links between IMD and metabolism, I

hypothesized that loss of key of metabolic regulators, such as insulin pathway components, will have a measurable impact on the ability of *Drosophila* to survive microbial infection. I asked how suppression of metabolic regulation in insulin mutants flies affects host response to infection and if interactions between host insulin signaling and infectious microbes is specific to each pathogen and how the route of introducing the microbe into the host alters vulnerability of *Drosophila* to each pathogen.

1.4.3 Study aims for Chapter 5

A study in our lab showed that cell specific inhibition of IMD signaling in enterocytes and progenitor cells results in improved or reduced survival against oral infection with *V. cholerae*, respectively (381). Improved survival against *V. cholerae* has been reported in *imd* mutants flies, suggesting that inactivation of the intestinal IMD pathway confers resistance against *V. cholerae* (382). Infection of the *Drosophila* intestine triggers a systemic antimicrobial response in the fat body that emphasizes immunological crosstalk between distant organs (383). As both immune and metabolic pathways are integrated in the *Drosophila* fat body, and considering that *V. cholerae* infection engages both host immune and metabolic signaling pathways in *Drosophila*, I explored the effect of immune and metabolic signaling suppression in the fat body on the host response to the intestinal infection with *V. cholerae*. I hypothesized that suppression of immune and metabolic signaling in the fat body improves host tolerance to oral infection with *V. cholerae*.

Chapter 2:

Material and Methods

Portions of this chapter have been published as:

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2.1 *Drosophila* method

2.1.1 *Drosophila* husbandry

Adult flies and larvae were raised on standard corn meal medium (NutriFly Bloomington formulation, https://bdsc.indiana.edu/information/recipes/ bloomfood.html; Genesse Scientific). All adult experiments were performed using virgin male and female flies. Flies were maintained at 30 flies per vial and raised at 25˚C in a humidified incubator with a 12-hour light/12-hour dark cycle. Flies that were used in this study are as follows:

Table 2-1 *Drosophila* melanogaster stocks and strains

Name	Genotype	Source
w^{1118} (Wild-type)	*w[1118]*	BDSC (Stock#5905)
R4-GAL4	*y[1] w[*]; P{w[+mC]=r4-GAL4}3*	BDSC (Stock #33832)
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
ΔAMPs	*w[1118]; Def SK3, Dro SK4, Dro-AttABSK2, Dpt SK1, Drs R1, Mtk R1; AttD SK1*	Bruno Lemaitre
Iso wildtype	*w[1118]*	Bruno Lemaitre
$ImpL2^{def2}$	$y,w;;ImpL2^{def20}$	Young Kwon
UAS-ImdCA	*w;;UAS-ImdCA*	Foley Lab
GeneSwitch-108	*P{w[+mW.hs]=Switch1}106*	BSC(Stock#8151)
$UAS\text{-}InR^{DN}$	*y[1] w[1118]; P{w[+mC]=UASInR.K1409A}3*	BSC(Stock#8253)

The *imd* mutants and $UAS\text{-}InR^{DN}$ used in this study were back-crossed to the w^{1118} flies for eight generations prior to use. For experiments with gene-switch (GS) flies, mifepristone (RU486) (M8046; Sigma), was dissolved in 80% ethanol to achieve 4 mg/mL working solution. Standard Bloomington food was poured into each vial, 3-4 ml, and after 3 hours, 100 µL from 4 mg/mL RU486, or 100 µL from 80% ethanol was added to the top of the food in each vial and kept overnight to evaporate the ethanol. For experiments using flies maintained on the holidic diet, the holidic medium was prepared following the published protocol and recipe using the original amino acid solution (Oaa) at 100 mM biologically available nitrogen (384) (Table 2-2). For modified version of holidic medium in this study, which is mentioned as holidic diet supplemented with glucose, 50g per litre has been used.

Table 2-2 Holidic medium recipe

Ingredient	Amount per liter (g or ml)
Sucrose	17.12
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
cholesterol	0.3
choline chloride	0.05
myo-inositol	0.005
insoine	0.065
uridine	0.06
KH2PO4	3
NaHCO3	1
CaCl2	0.25
CuSO4	0.0025
FeSO4	0.025
MgSO4	0.25
MnCl2	0.001
ZnSO4	0.025
thiamine	0.0014
riboflavin	0.0007
nicotinic acid	0.0084
Ca pantothenate	0.0108
pyridoxine	0.0017
biotin	0.0001
folic acid	0.0005
acetic acid	3
abs. ethanol	15
propionic acid	6
nipagin	15
agar	20

2.1.2 Lifespan and starvation analysis

For starvation analysis, flies were kept at vials with 1% agar in water and maintained in a 25˚C humidified incubator. Death was recorded every 2-3 hours and flies were flipped into fresh vials every two days. For lifespan analysis, 30 flies per vial were kept in a humidified incubator and flies were flipped to fresh food every 2 days. Deaths were recorded daily.

2.1.3 Pupariation timing and developmental analysis

For pupal measurement, 24 hours after egg laying, larvae were collected and put into food vials in groups of 50 larvae. Using a paintbrush, 1 day old pupae were picked off the side of the vial. Pupae were imaged using a Zeiss Stereo Discovery V8 microscope using a 314 magnification. AxioVision software was used to measure the length and width of each pupae. Pupal volume was calculated with the assumption that the pupae are cylindrical using the formula $(4/3\pi)$ X (length/2) X $(\text{diameter}/2)^2$ (124). To measure developmental rates, 25 age-matched feeding third instar larvae were cultured at 25˚C and monitored for the formation of wandering third instar larvae, pupae, and eclosed adults. For pupariation timing, 25 age-matched third instar larvae were cultured at 25˚C and monitored for the length of time required for development to the P13 pupal stage. Developmental and pupariation assays were performed in quadruplicate.

2.2 *Drosophila* nutritional assays

2.2.1 Nutritional assays

For total triglyceride (TG) measurement, 10 third instar larvae or 5 adult flies were weighed and homogenized in TE buffer with 0.1% Triton X-100. TG content was measured in larval homogenate using the serum TG determination kit (TR0100; Sigma) according to the manufacturer's instructions. Total glucose was measured by homogenizing 10 third instar larvae or 5 adult flies in TE buffer and measuring glucose using the GAGO glucose assay kit (GAGO20; Sigma) according to the manufacturer's instructions. For trehalose hemolymph measurements, groups of 15 third instar larvae were dipped in halocarbon oil 700 (Sigma), and the epidermis was punctured to start hemolymph bleeding. Accumulated hemolymph on the oil drop was aspirated using a glass pipette and immediately frozen on dry ice. One μL of hemolymph was mixed with 99 ml of trehalase buffer (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl) and heated at 70˚C for 5 min to inactivate endogenous trehalase. To measure circulating sugar in adult flies, hemolymph

was extracted from samples of 20-25 female flies. Flies were pricked in the thorax with a 26G needle and place into a filtered collection tube, centrifuged at 9000g for 5 min at 4˚C and the hemolymph, around 1.5 µL was collected and diluted 1:100 in trehalase buffer. Trehalase solution was prepared by diluting 3 µL Porcine Kidney Trehalase (T8778-1UN; Sigma) (1 UN) in 1 mL trehalase buffer.

Each sample was divided into two 50 µL aliquots, one to measure glucose and one to measure trehalose. Then one aliquot of each sample was treated with 50 µL of trehalase solution and the other half of each sample was treated with 50 µL trehalase buffer. Then samples, standards and blanks were incubated at 37˚C for 16 hours and then the reaction was started by adding glucose assay reagent (GAGO20; Sigma), incubated at 37˚C for 30 min, and stopped by adding 12 N sulfuric acid. Absorbance was measured at 540 nm. To calculate trehalose levels, glucose levels in untreated samples was subtracted from glucose levels of samples that were treated with trehalase. For protein measurements, 10 third instar larvae per replicate were homogenized, and measurements were performed in 96-well plates using DC Protein Assay kit (Bio-Rad, 500-0116) commercial kit and absorbance was read at 750 nm. For all macronutrient measurements, standard curves were generated using reagents with defined concentrations provided in the commercial kits and the optical valued was obtained for each standard concentration using a microplate spectrophotometer (Molecular Devices, SpectraMax M5).

2.2.2 CAFÉ

For CAFÉ assay, capillaries delivered liquid food (5% sucrose and 5% yeast in deionized water) to the fly. Each chamber contained 3 capillaries with 10 adult flies and were kept in a 25˚C humidified incubator (385). Total consumption was calculated after 24 hours. The initial liquid food volume in each capillary was marked at the start of the experiment and control vials with no flies with the same food in the capillary tubes were used to monitor for food evaporation during the experiment. The amount of food consumed by flies were calculated based on the amount of food reduced in each capillary subtracted from the initial food volume and corrected by the food evaporation using a ruler.

2.2.3 Fly Proboscis and Activity Detector (FlyPAD)

Flies used for this experiment were starved for 16 hours prior to flyPAD assay. The flyPAD instrument was acquired from Dr. Pavel M. Itskov and Dr. Carlos Ribeiro (386). On the day of experiment, 50% carbohydrate and 50% protein solution was made using agarose and melted at 95˚C and then maintained at 60˚C to facilitate pouring the food on each arena. Individual flies were placed in each FlyPAD arena with a mouth aspirator at n = 32 for each genotype. Eating behavior was recorded for 1 hour.

2.2.4 Oral glucose tolerance test (OGTT)

w^{1118} and *imd* males 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 five flies were obtained after initial starvation, after 2 hours on 10% glucose, and then at both 2 and 4 hours following re-starvation. Samples of five flies were weighed and then mashed in 125 ml TE of buffer (10 mM Tris, 1 mM EDTA, 0.1% Triton X-100, pH 7.4). Glucose was measured using the Glucose Oxidase (GO) Assay Kit (GAGO20; Sigma).

2.3 Bacterial assays

2.3.1 Bacterial culture

For infection experiments, the following bacteria were used: *Providencia sneebia*, *Providencia rettgeri*, *Enterococcus faecalis*, *Serratia marcescens DB 11*, *E.coli DH5α* and *Vibrio cholerae* (*C6706* strain). For oral infections, all bacteria except *E. faecalis* were streaked from glycerol stocks onto lysogeny broth (LB)- agar plates and grown overnight at 37˚C. *E. faecalis* was streaked from glycerol stocks onto brain heart infusion (BHI) plates and grown overnight at 37˚C. For Vibrio infections, *V. cholerae* was grown on LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) at 37°C in the presence of 100 μg/ml streptomycin (Sigma, SLBK5521V).

2.3.2 Oral and septic infections

For septic infection, 0.15-mm minutin pins (Fine Science Tools) were dipped into the OD600 = 1 dilution of bacterial, which were grown overnight in media at 37˚C and then pricked into the thorax of Six- to seven-day old virgin female flies. A sterile 0.15-mm minutin pin was used to prick flies in the thorax and served as control. Flies were then transferred to normal food and kept at 29˚C for the rest of the experiments. For oral infections, the day before the infection, for

each pathogen, steaked agar plates were made from glycerol stocks and kept overnight in the incubator at 37˚C. On the next day, single colonies were grown in medium to an OD600 of 0.245 and soaked a sterile cotton plug with 3 ml of the bacterial culture in LB or BHI medium (for *E. faecalis*). Six- to seven-day-old virgin female flies were fed on the cotton plug, and death was recorded at the indicated time points. A cotton plug soaked with LB medium or BHI medium was used (for *E. faecalis*) for our control in oral infection experiments.

2.3.3 Colony forming unit (CFU) measurement

For bacterial load quantification, at indicated time points 25 live flies from five biological replicates (five flies from each biological replicate) were collected, anesthetized for 20 minutes at -20˚C and then surface-sterilized by rinsing in 20% bleach, 70% EtOH, and distilled water. Then, flies were randomly distributed into five groups (five flies in each 1.5-ml tube) and then homogenized in respective media. Serial dilutions of fly homogenates were made in 96-well plates, and 10 µL of spots were plated on LB agar supplemented with 100 mg/ml streptomycin (to select for *V. cholerae*), BHI agar (to select for *E. faecalis*), and LB agar for the rest of the bacteria. Plates were incubated overnight at 37˚C. For calculating CFUs per fly, CFU/ ml calculated for each bacterial culture was divided by five. To normalize the CFUs for weight, the CFUs for *ilp2,3,5* flies were divided by the ratio of the average weights of w^{1118} and *ilp2,3,5* mutants.

2.4 Molecular biology and microscopy

2.4.1 Reverse Transcription and quantitative real time polymerase chain reaction (RT-PCR)

Quantitative PCR (qPCR) measurements were performed with RNA purified from whole larvae using TRIzol, and the ΔΔCT cycle threshold method was used to calculate relative expression values. For adult fly qPCR measurements, 10 heads or 12 midguts or 5 whole flies were homogenized in 250 µL TRIzol. Then samples were incubated at room temperature for 5 minutes followed by centrifugation at 12000 g for 10 minutes at 4˚C. Clear homogenate with no fly residues was transferred to a 1.5 ml Eppendorf tube, then 50 µL of chloroform was added and the tubes were shaken vigorously for 15 seconds, and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12000 g for 15 minutes at 4˚C. The clear aqueous layer was transferred to

a new 1.5 ml Eppendorf tube. To precipitate the RNA, 125 µL isopropanol was added to each tube and the sample were kept at -20˚C overnight for gut samples (to increase RNA yield) or 30 minutes at room temperature for non-intestinal samples. Samples were centrifuged at 12000 g for 10 minutes at 4˚C followed a washing step for the RNA pellet with 500 µL 75% ethanol, then the tubes were centrifuged at 7500 g for 5 minutes at 4˚C, and allowed to air dry. To dissolve RNA pellet, RNAse free water was used followed by for 30 minutes incubation with 1 µL DNAse at 37˚C. cDNA was generated from 1µg of RNA using BIORAD iScript cDNA Synthesis Kit as described in the manufacture's guidelines. cDNA was synthesized with an Eppendorf Mastercycler thermocycler with the following program 25°C for 5min, 42°C for 30min, 85°C for 5min. For qPCR analysis, cDNA was diluted in 1:16 and qRT-PCR mixtures consisted of 2.5µl cDNA, 7.5µl master mix (2.5µl of a 1.6µM primer mix and 5.0µl of SYBR Green SuperMix (Quantabio, 023917) was used. Then the 96well PCR plates were sealed with Eppendorf heat sealing film, vortexed, and briefly centrifuged. Transcripts were amplified with an Eppendorf realplex2 PCR machine with the following program: 95°C for 10min followed by a 40x repeat of 95°C for 15seconds, 60°C for 1min. Gene expressions were normalized to actin. The following primers were used in this study: *wisp* (forward (F): 5`-CAACAACAGTCACTCGTGGG-3`, reverse (R): 5`-TGGAAGAACGAAGATGGTTGC-3`), *pathetic* (F: 5`-TACTACAGAACTCGCCGCAC-3`, R: 5`-CAGACCAAACAGGATGGAGAAC-3`), *odc1* (F: 5`-ATCTGCGACCTGTCTAGCGT-39, R: 5`-CATTGGATCGTCATTGCACTTG-3`), *tep1* (F: 5`-AGTCCCATAAAGGCCGACTGA-3`, R: 5`-CACCTGCATCAAAGCCATATTG-3`), *tsf1* (F: 5`-CGATTGTGTGGTGGCTCTGACCAAG-3`, R: 5`-AAGGACATCATCCTGAGCCCTCTGC-3`), *diptericin* (F: 5`-ACCGCAGTACCCACTCAATC-3`, R: 5`-ACTTTCCAGCTCGGTTCTGA-3`), *ilp2* (F: 5`-TCCACAGTGAAGTTGGCCC-3`, R: 5`-AGA TAATCGCGTCGACCAGG-3`), *ilp3* (F: 5`-AGAGAACTTTGGACCCCGTGAA-3`, R: 5`-TGAACC GAACTATCACTCAACAGTCT-3`), *ilp5* (F: 5`-GAGGCACCTTGGGCCTATTC-3`, R: 5`-CATGTG GTGAGATTCGGAGCTA-3`), and *actin* (F: 5`-TGCCTCATCGCCGACATAA-3`, R: 5`-CACGTCACCAGGGCGTAAT-3`), *gstd1* (F:5`-TCCTGAACACCTTCCTGGAG-3`, R:5`-CTTGCTGATCTCGAATTTGG-3`), *irc* (F:5`-TAGCAAGCCGGTGTCGCAATCAAT-3`, R:5`-ACGGCCAGAGCACTTGCACATAG-3`), *sod1* (F:5`-CCAAGGGCACGGTTTTCTTC-3`, R:5`-CCTCACCGGAGACCTTCAC-3`), *jafrac1* (F: 5`-CCCGAAAACTTTTAGACTCA-3`, R:5`-TTTTCAAACATTTCCATCGT-3`), *MtnB* (F:5`-AAGGGTTGTGGAACAACTGC-3`,

R:5`-GTCCTTGGGCCCATTCTT-3`), *Attacin* F: 5'-AGTCACAACTGGCGGAC-3', R: 5'-TGTTGAATAAATTGGCATGG-3'.

2.4.2 Lipid droplet staining

For Nile red staining, the fat body of 10 third instar larvae were dissected in PBS and fixed in 4% formaldehyde for 30 minutes. After twice washing with 1X PBS, fat tissues were stained with 1:1000 of a Nile red stock (0.5 mg/ml in acetone) and 1:500 of Hoechst 33258 for 30 minutes. Stained tissue was mounted on slides and visualized using a spinning disc confocal microscope (Quorum WaveFX). Lipid area was quantified with Columbus software (PerkinElmer).

2.4.3 Immunofluorescence and microscopy of midgut samples

For dissection of midguts, first flies were anesthetized with CO2, washed briefly in 95% ethanol to reduce water surface tension and then transferred to 1X PBS to isolate midguts. For measuring macronutrients from midgut samples, 12-13 guts per replicate was dissected. The dissected guts were kept on ice cold 1X PBS until fixation. Guts were fixed for 30 minutes at room temperature in 500 µL of 8% formaldehyde in PBS followed by a washing step with 1X PBS + 0.2% Triton-X (PBT) for 30 minutes at room temperature. Then, guts were transferred into blocking buffer, which contains PBT + 3% bovine serum albumin (BSA) (Sigma-Aldrich A3059-10G) for 1 hour at room temperature. Guts were then stained with primary antibody Phospho-Histone H3 (PH3) rabbit anti-PH3 (EMD milipore, #3256620) in 1:2000 dilution and for Prospero staining with mouse Prospero antibody (DSHB MR1A) in 1:100 dilution in blocking buffer overnight at 4°C in 96 well plates while rotating gently. The next day, guts were washed with PBT for 30 minutes and stained with anti-mouse 488 in 1:500 dilution secondary antibody (Alexa Fluor® 488) (ab150113) for Prospero and anti-rabbit 568 in 1:1300 dilution secondary antibody (Alexa Fluor® 568) (ab175471) for PH3 as well as 1:500 dilution of DNA staining Hoechst (ThermoFisher H3569) were made and rotated at room temperature for 1 hour.

Then guts were washed with PBT at room temperature for 2x 30 min. Guts were mounted on slides in Fluoromount (Sigma-Aldrich F4680), and R4/R5 region of the posterior midgut was visualized. using a spinning disk confocal microscope. Once top and bottom of guts were identified, Z-stacks images were collected that were spanned through the entire tissue depth. The Velocity software was used to capture the images. The collected z-slices were compressed into single images

using Fiji software (387). Positive PH3 cells for each midgut (10 for each replicate) were counted manually via scanning through the entire midgut via eyepiece of the Olympus IX-81 microscope and for Prospero positive cells, first the number of nuclei was counted manually and the number of Propsero positive cells was divided to the number of nuclei for each single image and reported as the percentage of Prospero positive cells per nuclei.

2.4.4 Enzyme-linked immunosorbent assay (ELISA)

To measure circulating and total ILP2 levels, the *ilp2 gd2HF* fly stock was used and protocols acquired from Dr. S.K. Kim (119). For sample preparation, the black posterior end of the abdomen was dissected away and 10 dissected male bodies was transferred to 60 ml of PBS, followed by a 10 minutes vortex at maximum speed. Then, the tubes were centrifuged at 1000 g for 1 minute and 50 µL of the supernatant was transferred to a PCR tube as circulating ILP2HF sample. Then, 500 µL of PBS with 1% Triton X-100 was added to the tubes with the remaining flies and mashed the samples using a pestle and cordless motor (VWR 47747-370), followed by a 5-min vortex at maximum speed. The tubes were centrifuged at maximum speed for 5 minutes and then transferred 50 µL of the supernatant to a PCR tube as total ILP2HF sample. For standards, FLAG(GS)HA peptide standards (DYKDDDDKGGGGSYPYDVPDYA amide, 2412 d; LifeTein) was used. One 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 (44-2404-21; Thermo Fisher Scientific) was coated with 100 µL of anti-FLAG Ab diluted in 0.2 M sodium carbonate/bicarbonate buffer (pH 9.4) to 2.5 µg/ml and then the plate was incubated at 4˚C overnight. The plate was washed twice with PBS with 0.2% Tween 20 and then blocked with 350 µL of 2% BSA in PBS at 4˚C overnight. Anti-HA–Peroxidase, High Affinity (clone 3F10, 25 mg/ml, no. 12013819001; Roche) 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 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, and the plate was sealed with adhesive sealer (MSB-1001; Bio-Rad) 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. Then, 100 µL of 1-Step Ultra TMB-ELISA Substrate (no. 34028; Thermo Fisher Scientific) was

added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by adding 100 μL of 2 M sulfuric acid, and absorbance was measured at 450 nm on a SpectraMax M5 (Molecular Devices).

2.4.5 Western blot

For western blots, larvae were lysed in lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 1 mM EDTA, 25% glycerol, 1% NP-40, 50 mM NaF, 1 mM PMSF, 1 mM DTT, 5 mM Na3VO4, protease inhibitor mixture [cat. no. 04693124001; Roche], and phosphatase inhibitor [cat. no. 04906845001; Roche]), and protein concentrations were measured using the Bio-Rad DC Protein Assay Kit II. For each experiment, equal amounts of protein lysates (usually 15–40 μg) were subjected to western blot analysis. Primary Abs used were anti–atubulin (a-tubulin E7; *Drosophila* Studies Hybridoma Bank), anti–phospho-*Drosophila* Akt Ser505 (no. 4054; Cell Signaling Technology), and anti–phospho-S6K Thr398 (no. 9209; Cell Signaling Technology). For immunoblot quantifications, the area under each peak, subtracting the background, was quantified. The p-Akt was normalized to total Akt, and the p-S6K was normalized to tubulin.

2.5 Bioinformatics

For microarray studies, the GeneChip *Drosophila* Genome 2.0 Array (Affymetrix) was used to measure gene expression in triplicate assays. Total RNA was extracted from third instar larvae using TRIzol. Then, 100 ng of purified RNA was used to make labeled cRNA using the GeneChip 39 IVT Plus Reagent Kit (Affymetrix). Transcriptome Analysis Console software (Affymetrix) was used for preliminary analysis of gene expression data. Array data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (accession number GSE109470, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109470). Transcriptome data from *R4/ImdCA* relative to *R4GAL4/+ (R4/+)* larvae were analyzed using Gene Set Enrichment Analysis (388) to identify KEGG pathways that were differentially regulated upon activation of IMD. The data from the GSEA analysis were then visualized using the Enrichment Map plugin in Cytoscape (version 3.6.1) to generate the gene interaction network (389). The resulting network map was curated to remove uninformative nodes, resulting in the simplified network shown in Figure 2 of chapter 3. Panther database (390) was used to identify biological process that were affected by

IMD activation as well as FlyMine (391) to determine tissue enrichment of the respective genes in third instar larvae. GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool) was used to identify biological processes influenced by R4/ImdCA (392). From the transcriptome data, two gene lists were created that contained significantly upregulated or downregulated genes in response to ImdCA. Each of these lists was run in GOrilla against the background gene set (all microarray genes) with a p-value cutoff of 10^{-4} . The top 15 Gene Ontology (GO) terms sorted by p value were selected for both upregulated and downregulated analyses, ranked by enrichment score, and visualized using the easyggplot2 package in R (version 1.1.442).

2.6 Statistical analysis

All statistical analyses were performed with GraphPad Prism. qPCR data were analyzed with unpaired Student t tests (p , 0.05). Survival data were analyzed with log-rank (Mantel–Cox) tests. For pupariation timing and pupae counting, Kolmogorov–Smirnov tests and unpaired Student t tests were used (p , 0.05), respectively. Pupal volumes were compared with unpaired Student t tests (p , 0.05). For analyzing the bacterial load difference, we used two-way ANOVA with Sidak correction. All analysis in Figure 5-5 was performed using two way ANOVA with Sidak correction.

Chapter 3:

The Immune Deficiency pathway regulates metabolic homeostasis in *Drosophila*

Portion of this chapter have been published as:

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3.1 Introduction

Molecular links between immune and metabolic pathways are conserved across vast evolutionary distances, and abnormal immune–metabolic signals are linked to several pathological states. For example, persistent inflammation is involved in the development of chronic metabolic disorders, such as insulin resistance and type two diabetes (393,394). During an infection, immuno-metabolic interactions between the host and pathogens cost energy for the host, however, host has evolved physiological adaptation such as alteration to energy allocation to improve fitness while fighting an infection (395,396). Recent studies have shown that mammalian adipose tissue serves more than just a fat reservoir but it functions as an endocrine organ that maintain metabolic hemostasis (397,398). Although the prevalence of metabolic diseases induced by immune system dysfunction have been reported, we do not fully understand the mechanisms of crosstalk between metabolic and immune function. *D. melanogaster* is widely used as a whole-animal model system to study metabolism, and immunity. *Drosophila* shares many conserved pathways with humans. The fat body in *Drosophila* is an invaluable tissue to study immune-metabolic interactions as both immune and metabolic pathways are integrated in this tissue. It synthesizes most hemolymph proteins and circulating metabolites, and responds to nutritional challenge. In addition, to its metabolic function, the fat body responds to infection via the evolutionary conserved IMD pathway which is homologous to the mammalian TNF-α pathway.

To synchronize metabolic homeostasis, the fat body produces growth signals and communicate with other tissues (399). The proper immune and metabolic function in the fat body is very important for *Drosophila* health. Recent study in our lab showed that constitutive IMD signaling in intestinal progenitors alters the expression of genes involved in metabolism and stress responses (380). This observation raised the question what would be the consequence of elevated IMD activity in metabolic tissues such as the fat body. Given the existing research showing links between increased inflammation and metabolic disorders, I asked what would be the consequence of consistent IMD activity in the fat body. To answer this question, I used the R4-GAL4 driver line to express a constitutively active IMD (ImdCA) construct exclusively in the fat body (380). This approach allowed me to ask how persistent immune activity influences host physiology without collateral damage through the introduction of pathogenic microbes. I used a GAL4-UAS system, a powerful tool for targeted gene expression, to induce a tissue-specific activation of IMD activity in the fat body. Then, I first characterized the effects of elevated IMD activity in the larval fat body

on the transcriptome profile of whole larvae as well as disruption at the physiological levels such as development, metabolism and growth. I also investigated the effect of increased IMD activation in the fat body of adult *Drosophila* on the lifespan, sensitivity to starvation as well as food behaviour. Furthermore, I asked if IMD contributes to metabolic homeostasis in the absence of an activating signal. To test this question, I raised adult flies on a defined holidic diet, which provides all nutrients needed to sustain adult life and allows investigators to monitor host physiology on a chemically defined food, and measured different macronutrients levels involved in regulating metabolism, energy storage and insulin activity.

3.2 Result

3.2.1 Activation of IMD signaling via a constitutively active IMD construct in the fat body

To investigate the effect of increased IMD activity in the *Drosophila* fat body, I used the UAS-GAL4 system and a fat body specific driver called R4-GAL4 that is exclusively expressed in the larval and adult adipose tissue. The UAS-GAL4 system is the most widely used method for spatially restricted transgene expression in *Drosophila.* This system which consist of a driver, with a tissue specific promoter upstream of the gene encoding yeast GAL4 transcription factor, and a responder transgenic fly line that carries the gene of interest placed downstream of a GAL4 upstream activating sequence that controls transcription of the gene of interest only when GAL4 is present. To confirm the specificity of the R4-GAL4 driver, I crossed R4-GAL4 fly line with a UAS-GFP reporter line and visualized the control larvae (*R4>+*) and larvae with GFP expression in the fat body (*R4>GFP*) with a fluorescence microscope. I observed that adipose tissue of larvae with GFP expression had an increased GFP intensity compared to control larvae (Figure 3-1A). Next, I used the R4-GAL4 driver to express a constitutively active IMD (ImdCA) in the fat body of larvae. This transgenic fly line has been previously generated in the Foley lab (380). The inhibitory N-terminal is truncated in ImdCA flies but IMD is still able to interact with *Drosophila* Fas-associated protein with death domains (Fadd). Then recruitment of the caspase-8 homolog Death-related ced-3/Nedd2-like protein (Dredd) to the IMD-Fadd complex results in cleavage of IMD by Dredd. As a result, the IMD signaling pathway and downstream target genes are activated in the absence of microbes.

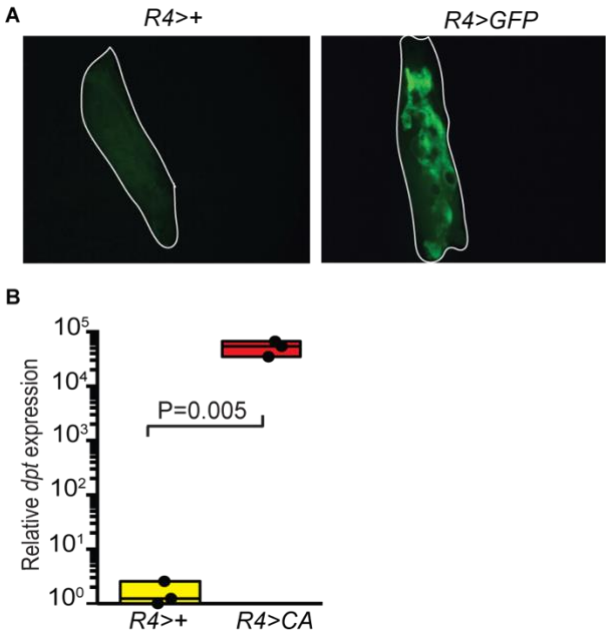


Figure 3-1 Verification of *UAS-ImdCA* and *R4-GAL4* transgenic fly lines
(A) Verification of fat-body derived line, *R4-GAL4,* using a green fluorescence protein (GFP) that expresses GFP in the fat body of larvae. *R4>+* Larvae represent progeny of R4-GAL4 flies crossed with the wildtype flies and *R4>GFP* represents progeny of R4-GAL4 flies crossed with the UAS-GFP flies which resulted in the expression of GFP in the fat tissues of larvae. White line outlines the larvae (B) Verification of *UAS-ImdCA* fly line that constitutively expresses IMD. mRNA expression of *diptericin* as a readout for IMD pathway activation from control (*R4>+*) and larva with consistent IMD expression in the fat body (*R4>CA*). Whole larvae from feeding third instar stage of the development was used for the RNA extraction. Fold change of *diptericin* was calculated relative to the *R4>+ diptericin* expression and cycle threshold of each replicate was normalized to the actin as housekeeping gene. Statistical significance was determined with an unpaired Student t test.

The *Drosophila* fat body is a central organ for induction of humoral immunity by activation of Toll and Immune Deficiency (IMD) pathways upon invasion of bacterial pathogens. Activation of IMD and Toll signaling results in production and release of AMPs into the hemolymph to combat the microbes. To validate the ImdCA fly line, I measured expression levels of *diptericin*, an antimicrobial peptide that is expressed in the fat body in response to IMD activation by Gram negative bacteria. I found a significant increase in expression of *diptericin* in the larvae with IMD expression in the fat body (*R4>CA*) compared with control larvae (*R4>+*), which confirms expression of ImdCA in the larval fat body results in the increased activation of IMD signaling in adipose tissue (Figure 3-1B).

3.2.2 Whole-genome microarrays shows elevated IMD activity in the larval fat body modifies host transcription profile

The *Drosophila* fat body is an invaluable tissue to investigate the crosstalk between immune and metabolic pathways as the fat body is capable of responding to metabolic needs as well as inducing humoral immunity once a pathogen threatens the host. The evolutionary conserved IMD and Toll pathways are activated in the fat body in response to microbial infection while IIS and TOR pathways are essential regulators of metabolism in the fat body in respond to metabolic stimulus. Different studies have addressed the effect of Toll activation in the fat body on the metabolic homeostasis of *Drosophila* (400) or how intestinal IMD activity affects metabolic and developmental process (401,402). A recent study by Petkau et al. showed that constitutive IMD signaling in intestinal progenitors alters expression of genes involved in metabolism and stress responses (380). Although the impact of increased immune responses on metabolic homeostasis has been extensively studied, we do not fully understand the mechanism of immune-metabolic crosstalk.

Given the existing research showing links between increased inflammation and metabolic disorders, I asked what would be the consequence of chronic IMD activity in an endocrine tissue such as the fat body. To answer this question, I devised a microarray experiment to investigate how elevated IMD expression in the fat body affects biological systems and cellular pathways in the host. I dissected total RNA from third instar feeding larvae to look at the gene expression profile of the larvae with persistent IMD activity in the fat body along with control larvae (Figure 3-2A). Following statistical analyses, I found that 1218 genes were differentially expressed between *R4>CA* and control group, *R4>+*. As expected, *R4>CA* larvae showed upregulation of genes related to AMP expression;10.12 $\log_2$ fold increase for *attacinD*, 8.2 $\log_2$ fold increase for *diptericinB* (Figure 3-2B) and genes responsible for production of peptidoglycan recognition proteins; 7.27 $\log_2$ fold increase for *PGRP-SB1*, 1.69 $\log_2$ fold increase for *PGRP-LB* (Figure 3-2B). KEGG pathway analysis showed that most of the downregulated genes were involved in different aspects of carbohydrate metabolism. For example, I found a significant reduction in expression of enzymes involved in glycolysis; -1.74 $\log_2$ fold reduction for *hexokinase-C* and -0.69 $\log_2$ fold reduction for *aldolase* (Figure 3-2B and Figure 3-3). I also found that further steps in cellular respiration such as gluconeogenesis; -0.68 $\log_2$ fold reduction for *fbp*, -1.65 fold reduction

for *MDH-2* (Figure 3-3) tricarboxylic acid cycle (TCA) cycle, mitochondrial respiratory activity, and ATP production were significantly reduced (Figure 3-2C). In addition, fatty acid beta oxidation and lipid metabolism process showed a significant downregulation as well (Figure 3-2C&D). Trehalose is the main circulating sugar in insects that is synthesized from glucose by trehalose-6-phosphate synthase 1 and is stored in the fat body (403). Larvae with increased IMD expression in the fat body showed downregulation of *Tps1* gene compared to control; -1.21 $\log_2$ fold reduction. Consistent with reduced lipid metabolism in *R4>CA* larvae, two lipid droplet storage genes, *Lsd1* and *Lsd2*; -0.97 $\log_2$ fold reduction for *Lsd-1*, and -0.88 $\log_2$ fold reduction for *Lsd-2* showed significant downregulation (Figure 3-2B).

Insulin signaling is one of the main regulators of carbohydrate metabolism and lipid synthesis (404). I found that transcription profile of genes involved in regulation of insulin activity was significantly affected by chronic IMD expression in the fat body. For example, *ilp3* gene was downregulated; -0.86 $\log_2$ fold reduction and in contrast gene expression of *ilp6* and *ImpL2* that are antagonists of insulin pathway were upregulated; 0.98 $\log_2$ fold increase and 0.91 $\log_2$ fold increase, respectively (Figure 3-2B). The IIS pathway activation results in phosphorylation of Akt and subsequent phosphorylation of FOXO by Akt inhibits FOXO shuttling to the nucleus which inhibits transcription of FOXO target genes. I found that FOXO target gene, *Thor,* which is the human 4E-BP orthologue was upregulated in the *R4>CA* larvae; 0.88 $\log_2$ fold increase (Figure 3-2B). TOR is an evolutionary conserved signaling pathway that respond to amino acid stimulus and regulates growth and development. Zhang et al showed that mutation of TOR results in delay in larval development (160). In my microarray, comparing transcriptional profile of *R4>CA* and control larvae, I found suppression of TOR pathway (Figure 3-2C) and cellular amino acid metabolism (Figure 3-2B) in larvae with increased IMD activity in the fat body. These results suggest that persistent IMD activity in the fat body reduces insulin signaling activity, which results in disrupted carbohydrate metabolism in larvae.

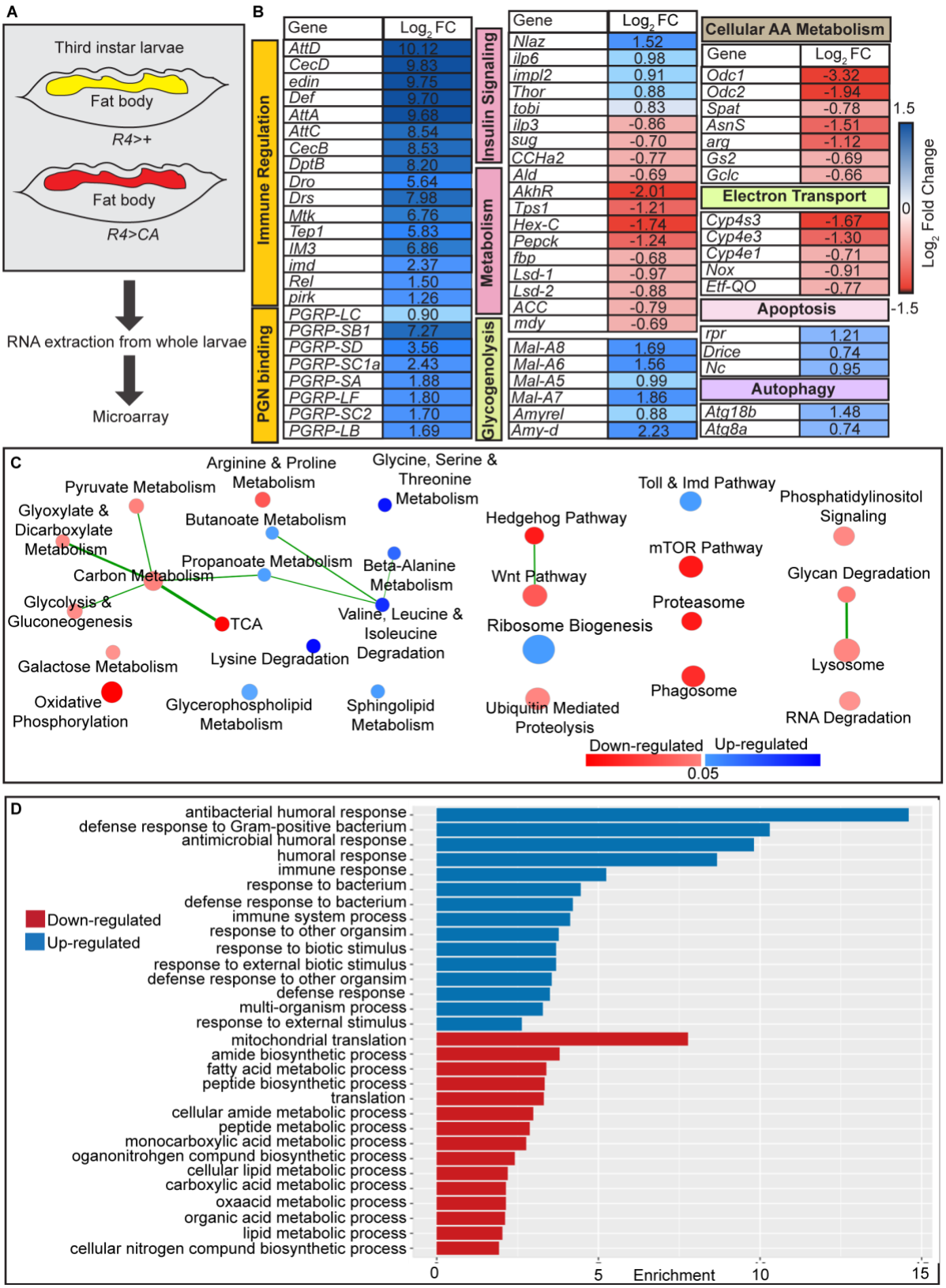


Figure 3-2 Elevated IMD expression in the larval fat body alters host biological processes

(A) Experimental design for the collection and preparation of *R4>+* and *R4>CA* third instar larvae for transcriptional studies via microarray (B) List of differentially expressed genes in *R4/CA* larvae compared to control (*R4>+*) based on their Log_2 fold change and are organized according to the similarities of their biological functions (C) Gene interaction network of upregulated and downregulated KEGG terms altered in *R4>CA* larvae relative to *R4>+* larvae. Red and blue nodes indicate downregulated and upregulated KEGG terms, respectively. Lines indicate genes shared between nodes, and node size indicates the number of genes represented by that KEGG term. Graphs are the output from Enrichment Map plugin in Cytoscape (D) Biological processes altered in *R4>CA* larvae compared with *R4>+* larvae. Red and blue bars indicate downregulated and upregulated GO terms, respectively. The height of the bar indicates the enrichment score of the GO term. The graph is the output from GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool). For all terms shown, the p value is $<10^{-4}$.

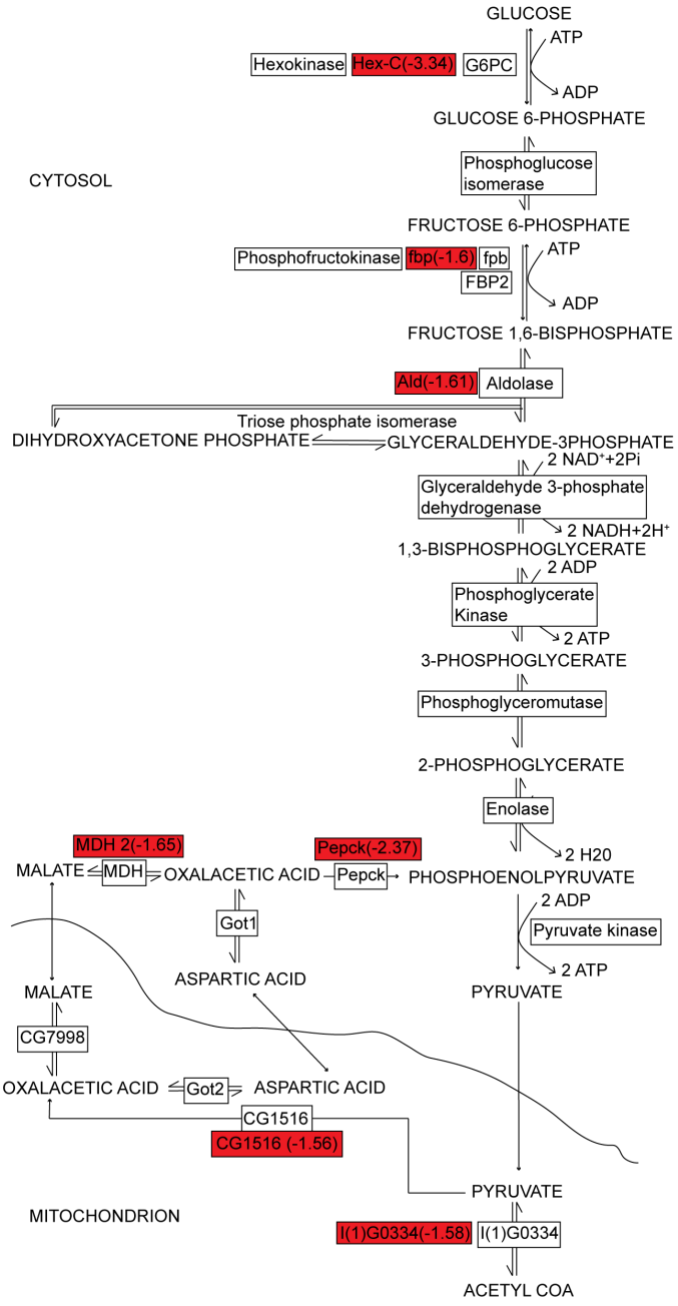


Figure 3-3 Constitutive IMD activation in the fat body disrupts glycolysis
KEGG illustration of glycolysis pathway, the red boxes show downregulated enzymes in *R4>CA* larvae and their fold change reduction for each gene relative to *R4>+*.

Upregulated biological process in *R4>CA* larvae suggest that larvae with elevated IMD activity in the fat body switch to glycogenolysis to produce glucose from stored glycogen as we found upregulation of amylases and maltase; 0.88 $\log_2$ fold increase in *amylase distal (Amy-d)* and 1.86 $\log_2$ fold increase in *maltase A7* (Figure 3-2B). These enzymes are involved in glucose production from different sources such as glycogen, starch and maltose in the gut. This observation suggests that effects of elevated IMD expression is not limited to the fat body and affects gene expression in distant tissues. Apoptotic processes were significantly upregulated;1.21 $\log_2$ fold increase for *reaper,* an important gene involved in activation of apoptosis in *Drosophila* (405), which suggest that a dysfunctional mitochondria and low level of energy production contribute to upregulation of cell death signaling. I observed upregulation of multiple genes involved in autophagy and proteolysis, which suggest that larvae with elevated IMD activity use amino acids from autophagic degradation to maintain cellular ATP production (Figure 3-2B). Overall, these results suggest that constitutive activation of IMD signaling in the larval fat body results in diversion of metabolism from anabolic to catabolic metabolism and from building energy reservoirs to degrading the stored nutrients to respond to the high innate immune activation demands of the host which is indicated by suppression of glycolysis and gluconeogenesis and increased glycogenolysis. Further investigation of the mechanisms involved in the alterations in the glycolysis, and glycogenolysis during elevated IMD activation in the fat body will be required to fully understand the mechanisms involved in IMD-induced alterations in these metabolic pathways.

To validate gene expression results from the microarray experiment, I measured the expression of three genes as representative of downregulated genes and two genes as representative of upregulated genes via quantitative real time PCR (qPCR). I used the same RNA used in the microarray experiment to make the cDNA for *R4>CA* and *R4>+* samples. For downregulated genes, I looked at the expression levels of *pathetic*, *odc1* and *wisp* and I confirmed that expression level of all three genes was significantly suppressed in *R4>CA* larvae compared with control (Figure 3-4A-C). For upregulated genes, I measured mRNA level of *tep1* and *tsf1* and confirmed higher expression of both genes in *R4>CA* larvae (Figure 3-4D&E). These results confirm that qPCR measurement of gene expression is consistent with the microarray expression results.

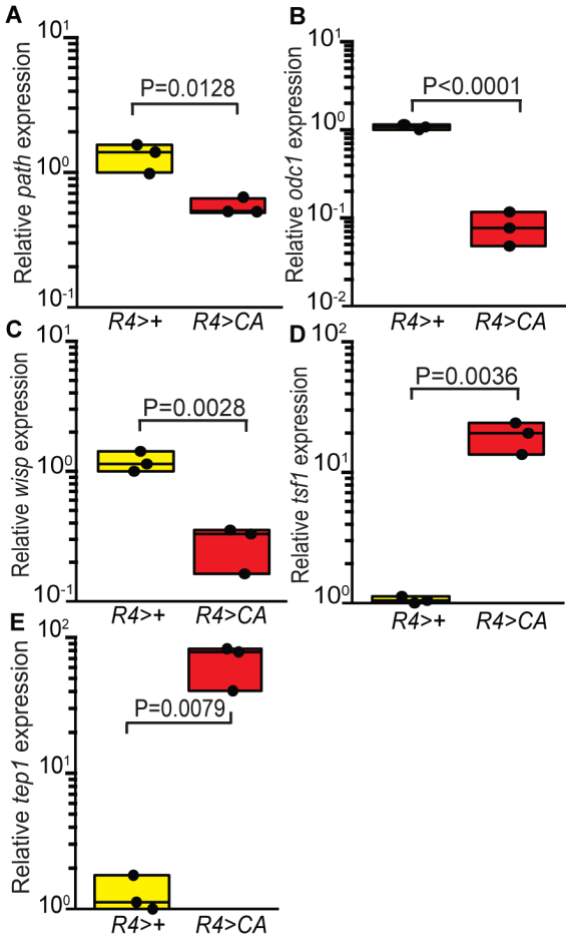


Figure 3-4 Confirmation of microarray experiment via qPCR
Quantification of relative gene expression from whole third instar *R4>CA* and *R4>+* larvae by qPCR (A) *pathetic* (B) *odc1* (C) *wisp* (D) *tsf1* and (E) *tep1*. In each case, gene expression is reported for *R4>CA* flies relative to the corresponding gene in *R4>+* flies. All statistical significance was determined using an unpaired Student t test.

A study by Troha et al. 2018 investigated the effect of systemic infection with different bacteria on the host transcriptional profile. They found that each bacterium exerts a unique effect on the host gene expression profile but they found core genes that were similarly affected by the majority of bacterial pathogens. The core downregulated genes were annotated as genes responsible for sugar metabolism and cellular respiration, which are suppressed by activation of *crebA* in the fat body after infection (306). As I observed similarities between elevated IMD expression in the fat body and core downregulated genes in this study, I compared the expression profiles of *R4>CA* and gene expression profile of wild type flies challenged with a panel of different pathogens and found overlapping genes between downregulated or upregulated gene lists

(Figure 3-5A&B). The pathogen that had the highest overlap between downregulated (28.62% overlap with *R4>CA*) and up regulated genes (34.48 % overlap with *R4>CA*) with larvae with consistent IMD expression in the fat body was *P. entomophila*. This is a highly pathogenic bacteria for *Drosophila* and septic infection with *P. entomophila* results in 100% mortality within 96 hours of stabbing the flies in the thorax (306). Oral infection with *P. entomophila* induces more than 70% morality in larvae and the remaining larvae will not survive pass the pupal stage (293). This observation is interesting as suggest ImdCA expression in the fat body affects expression of genes that are similarly affected by a highly virulent Gram-negative bacterial pathogen. Among Gram-negative bacteria, *Ecc15* showed the lowest overlap withing downregulated genes to *R4>CA* condition. This could be explained that most of the host infected by *Ecc15* are able to clear this bacteria by 132 hours post infection (306). I also compared the core biological processes regulated by *R4>CA* and septic bacterial infections and found that similar to ImdCA expression, systemic infection of *Drosophila* with a set of different bacterial infection modified the expression of host transcriptional response in which the main suppressed biological processes are involved in the regulation of metabolism (Figure 3-5C). Combined, these data indicates a crosstalk between activation of immune and metabolic responses in the fat body upon environmental challenges, such as infection.

A

Condition	#genes	% Overlap	P value
R4>CA	594		
E. coli	795	14.48	$4.19*10^{-20}$
E. faecalis	755	13.47	$3.23*10^{-18}$
Ecc15	484	3.70	0.1354
M. luetus	832	13.30	$2.98*10^{-15}$
P. entomophila	1325	28.62	$6.37*10^{-51}$
P. rettgeri	974	22.22	$1.89*10^{-41}$
P. sneebia	910	10.27	$2.57*10^{-6}$
S. aureus	1100	25.93	$1.01*10^{-50}$
S. marcescens Db11	944	16.67	$4.77*10^{-22}$
S. marcescens	717	12.12	$2.93*10^{-15}$

B

Condition	#genes	% Overlap	P value
R4>CA	525		
E. coli	768	11.81	$1.55*10^{-11}$
E. faecalis	932	17.52	$1.56*10^{-22}$
Ecc15	946	17.14	$6.04*10^{-21}$
M. luetus	834	18.48	$2.61*10^{-29}$
P. entomophila	1000	30.48	$1.1*10^{-69}$
P. rettgeri	988	24.38	$3.53*10^{-44}$
P. sneebia	714	16.38	$2.58*10^{-28}$
S. aureus	1285	26.48	$5.7*10^{-39}$
S. marcescens Db11	995	22.86	$4.63*10^{-38}$
S. marcescens	925	17.14	$1.29*10^{-21}$

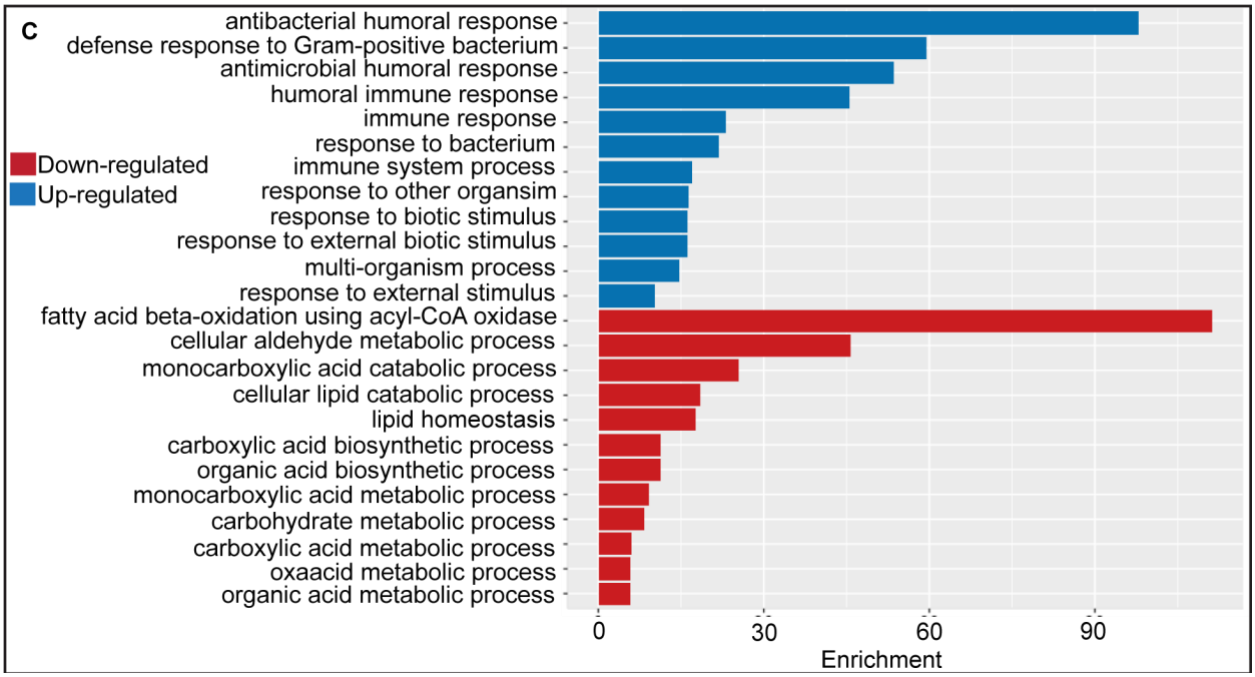


Figure 3-5 Core biological processes regulated by chronic IMD expression in the fat body and septic bacterial infection

(A) Upregulated and downregulated biological process GO terms from a core list of genes similarly regulated by R4>CA and or more bacterial infection from Troha et al., 2018. Red and blue bars indicate downregulated and upregulated GO terms, respectively. The height of the bar indicates the enrichment score of the GO term. The graph is the output from GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool). For all terms shown, the p value is <10-4.

To investigate if the effect of IMD activation in the fat body is specific to this tissue or has a general consequence for the host, I compared the transcriptional profile of larvae with chronic activation of IMD in the fat body with the gene profile of *Drosophila* adults with elevated activation of IMD in intestinal progenitor cells. Humoral immunity in *Drosophila* is regulated in the fat body while the intestine is where local immunity against oral pathogens is initiated by activation of IMD signaling. I found that among all genes altered in *R4>CA* larvae, only 9.8% showed an overlap with genes differentially expressed in the progenitor cells with elevated IMD expression (Figure 3-6A). Minimal overlap was also observed between IMD activation in the fat body and IMD activation in the intestinal progenitor cells for upregulated and downregulated genes with only 8% and 5.2% overlap respectively (Figure 3-6B&C). These observations suggest broad tissue autonomy in IMD responses and suggests elevated IMD activity induces tissue specific alterations in the host.

A study from Mussleman et al., 2018 showed that suppression of insulin signaling via inhibition of the insulin receptor in the larval fat body results in the increased expression of genes involved in defense responses (406). This allowed me to compare the gene expression profile of larvae with chronic IMD activation in the fat body to larvae with suppression of insulin signaling in the fat body. I found that 29.3% of genes differentially expressed by IMD expression in the fat body were also altered by insulin inhibition in the fat body (Figure 3-6D&E). These result suggest an overlap between IMD and insulin-induced transcriptional regulation in the *Drosophila* fat body.

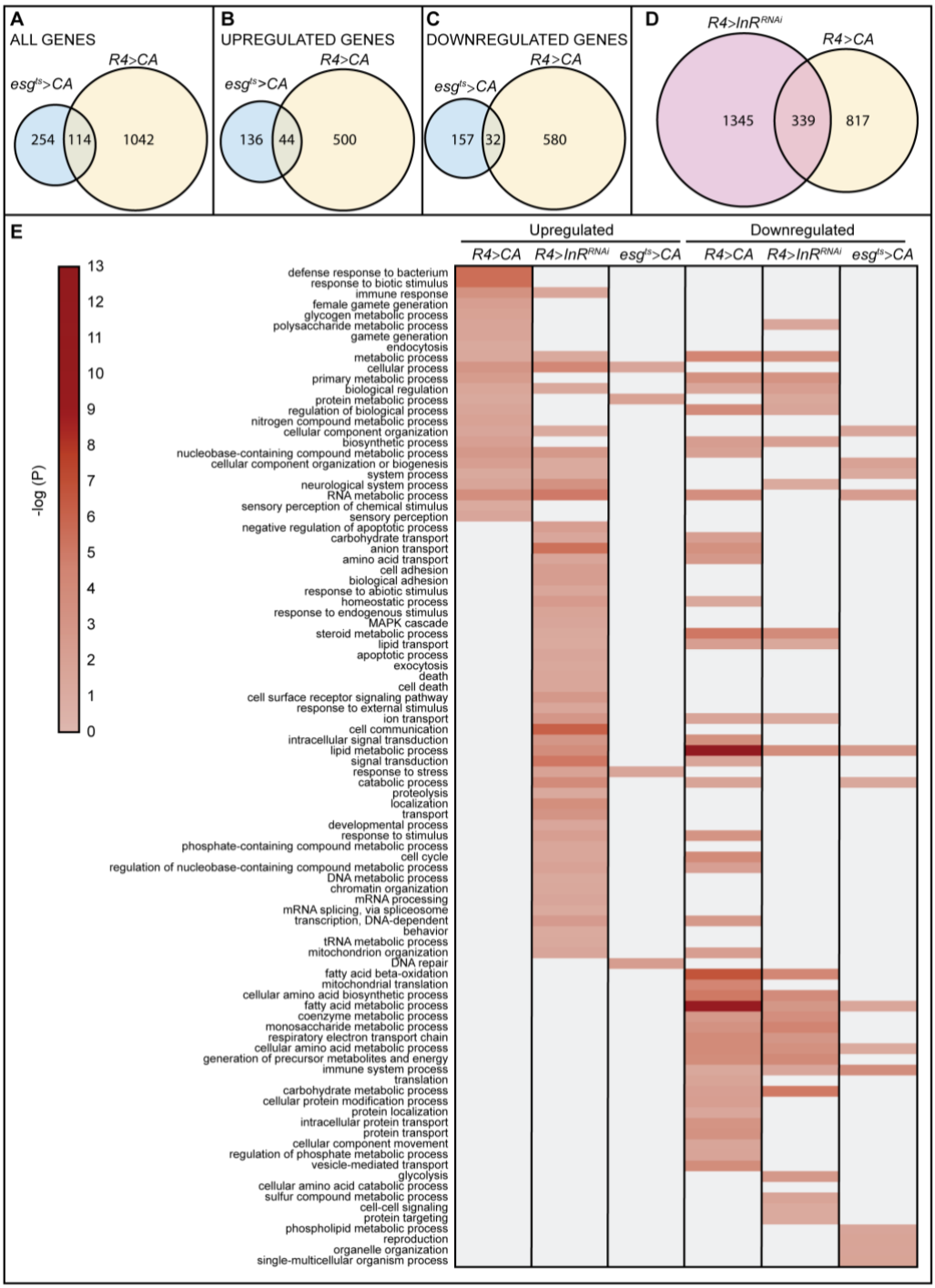


Figure 3-6 Comparison between constitutive IMD activation in the fat body, intestinal progenitor cells, and larvae with insulin signaling inhibition in the fat body

(A) Overlap between all dysregulated genes in *R4>CA* larvae and *esg^{ts}>CA* intestines. The esg^{ts} transgenic line allows inducible transgene expression in intestinal stem cells (B) Upregulated genes in *R4>CA* larvae and *esg^{ts}>CA* intestines (C) Downregulated genes in *R4>CA* larvae and *esg^{ts}>CA* intestines (D) Overlap between dysregulated genes in *R4>CA* larvae and *R4>InR^{RNAi}* larvae (E) Heat-map of dysregulated GO terms in *R4>CA* larvae, *esg^{ts}>CA* intestines, and *R4>InR^{RNAi}* larvae.

3.2.3 Activation of IMD in the fat body disrupts carbohydrate and lipid metabolism

Most of the biological processes that were altered in the gene expression profiling of the larvae with chronic IMD expression in the fat body were related to metabolism. Therefore, I hypothesized that suppression of metabolic processes in the larvae with increased IMD activation in the fat body will be reflected in the macronutrient levels and metabolic reservoirs. As I observed a significant depletion of genes involved in lipid droplet storage and lipid metabolic processes, I hypothesized a reduced triglyceride level in the larvae with increased IMD activity in the fat body. To test these hypotheses, I looked at the total level of triglyceride, glycerol, glucose and protein as well as the circulating trehalose in the hemolymph of *R4>CA* and *R4>+* larvae. As triglyceride is composed of glycerol and free fatty acid, therefore measuring the amount of free glycerol is used as a readout for lipolysis in which increased levels of free glycerol in larvae with elevated IMD activity in the fat body will indicate increased lipolysis but reduced levels of glycerol along with total triglyceride levels will be an indicative of reduced triglyceride synthesis.

I found that there was no significant difference between the control and *R4>CA* larval weight and total glucose levels (Figure 3-7A&B). In contrast, consistent expression of IMD in the larval fat body resulted in increased circulating trehalose levels twice as high as controls (Figure 3-7C) and significant reduction in total triglyceride and glycerol levels (Figure 3-7D&E). Rising level of circulating trehalose in larvae with elevated IMD activation in the fat body is in agreement with the increased expression of genes involved in glycogen degradation which suggests increased level of glycogen degradation leads to increased trehalose levels in hemolymph. In addition to that, reduced expression of genes involved in in glycolysis suggests combined disruption of glycogen breakdown and diminished glycolytic flux during increased immune responses results in elevated hemolymph sugar in the larvae. I also measured the total protein level and found no change in *R4>CA* larvae compared with control (Figure 3-7F). These observations demonstrate that increased IMD activity in the fat body disrupts lipid metabolism and negatively affects sugar homeostasis in the *Drosophila* larvae.

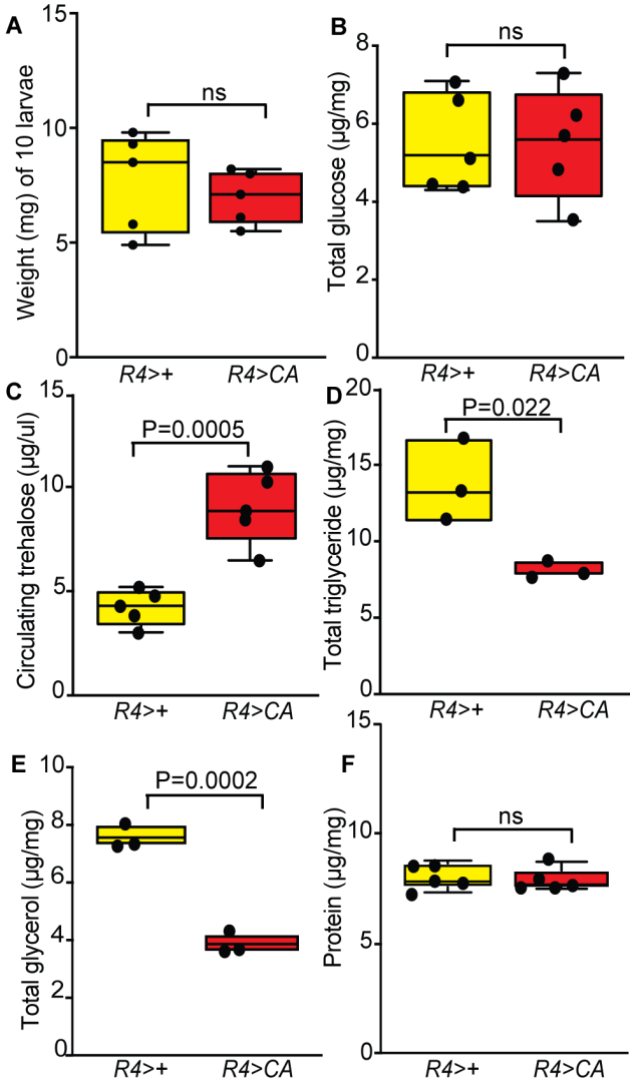


Figure 3-7 Effects of IMD activation in the fat body on the macronutrients of the larvae
(A) Weight of 10 third instar larvae (B) Total level of glucose from whole third instar larvae (C) Total level of hemolymph trehalose from third instar larvae (D) Total level of triglyceride from whole third instar larvae (E)Total level of glycerol from whole third instar larvae (F) Total level of protein from whole third instar larvae collected from *R4>+* or *R4>CA* larvae. For hemolymph trehalose graph, each dot represent pool of 15 larvae to extract 1ul of hemolymph. All statistical significance was determined using an unpaired Student t test.

3.2.4 IMD activation in the fat body depletes lipid stores in the larvae

Comparing the transcriptional profile of *R4>+* and *R4>CA* third instar larvae, I found reduced expression of genes involved in fatty acid metabolic process, cellular lipid metabolic process and phosphatidylinositol signaling (Figure 3-2C&D). Specifically, I found that activation

of IMD in the larval fat body reduced expression of *Lsd1* and *Lsd2* genes (Figure 3-2B), which are *Drosophila* homologues of mammalian perilipins (407). *Lsd1* regulates lipid homeostasis by recruiting lipase for lipid mobilization once the animal is starved while *Lsd2* protects lipid stores from lipolysis (408). Lipid droplets are storage compartments in the fat body for triacylglycerol deposition and mobilization. As I showed reduced triglyceride level in *R4>CA* larvae, I asked if lipid droplets in larvae with consistent IMD activity in the fat body have diminished amount of neutral lipids compared to control. To test this question, I first starved both control and *R4>CA* larvae for 6 hours, and then dissected the larval fat body and stained with Nile red, which is used for qualitative assessment of size and shape of lipid droplets. I found that larvae with elevated IMD expression in the fat body had a reduced Nile red intensity and smaller lipid droplets compared to control (Figure 3-8A). Depletion of neutral lipids in the lipid droplets of *R4>CA* larvae correlates with the reduction of total triglyceride in *R4>CA* larvae (Figure 3-8D). I quantified the Nile red staining area of lipid droplets from *R4>+* and *R4>CA* larvae and showed a significant reduction in total Nile red staining area in *R4>CA* larvae compared with control (Figure 3-8B). These results indicate that elevated IMD expression in the larval fat body depletes lipid storages in the fat body.

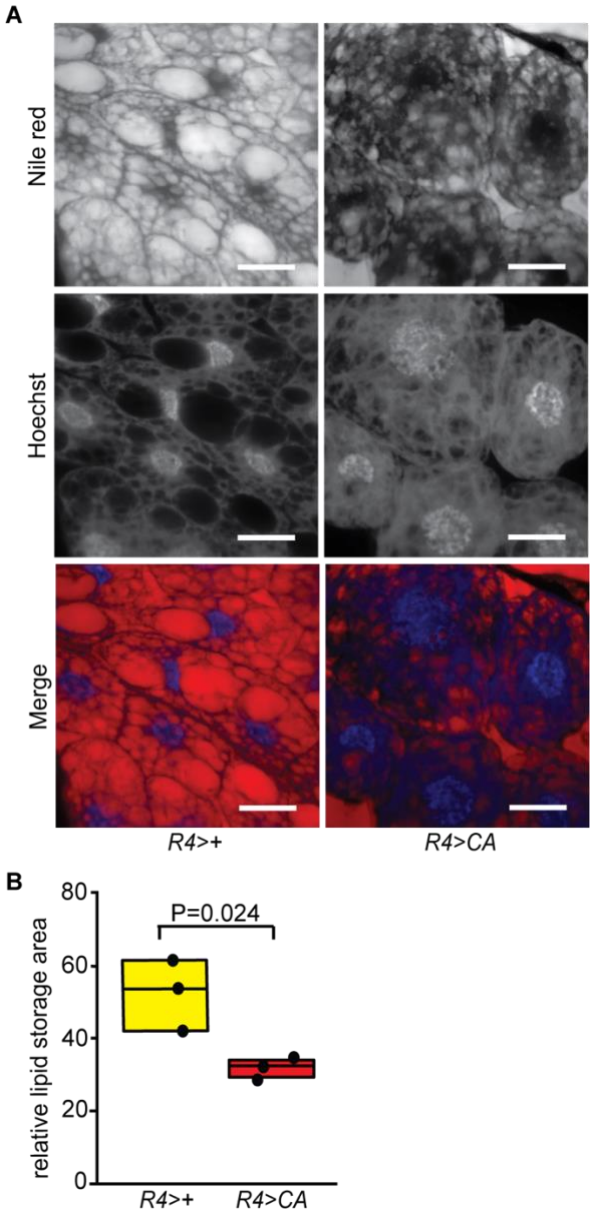


Figure 3-8 Increased IMD activity in the larval fat body depletes lipid reservoir
Staining of the lipid droplets from the dissected fat bodies of *R4>+* or *R4>CA* third instar larvae after 6 hours of starvation (A) Fat tissue was stained with Nile red (lipid droplets) and Hoechst (nuclei) and imaged using confocal microscopy. Scale bar, 25 mm (B) Quantification of total Nile red staining area of lipid droplets from third instar larvae. Statistical significance was determined using an unpaired Student t test.

3.2.5 IMD activation in the fat body delays development and reduces pupal size

Drosophila larvae undergo a rapid growth after hatching in which the body mass increases 200 fold within three days. Coordination between metabolism and development is crucial to provide energy requirement for the growing larvae. As I observed depletion of lipid resources in

the fat body of larvae with chronic IMD activity, as well as disruption in lipid metabolism and circulating sugar, I asked if growth and development in the larvae with increased IMD activity in the fat tissue is compromised as well. To answer this question, I measured the timing of pupariation for third instar *R4>+* and *R4>CA* larvae until they reach the P13 stage of pupal development. By this stage, wings turn black and are visible from pupal case (409). I showed that *R4>CA* larvae had approximately 18 hours delay to reach P13 stage of pupal development compared with *R4>+* larvae (Figure 3-9A). As delays in development frequently affect body size and growth, I measured pupal size and found a 10% reduction in pupal volume in *R4>CA* larvae compared with control (Figure 3-9B). Delay in development and reduced pupal size suggested that the rate of eclosed adults may also be affected, therefore, I counted the number of adults that eclose from control and *R4>CA* larvae, and found a significant reduction in the rate of pupal eclosion in larvae with increased IMD expression in the fat body (Figure 3-9C). These results indicate that constitutive IMD activity in the fat body negatively affects larval development, pupal size and has a semi-lethal effect on larval development by reducing the pupal eclosion rate.

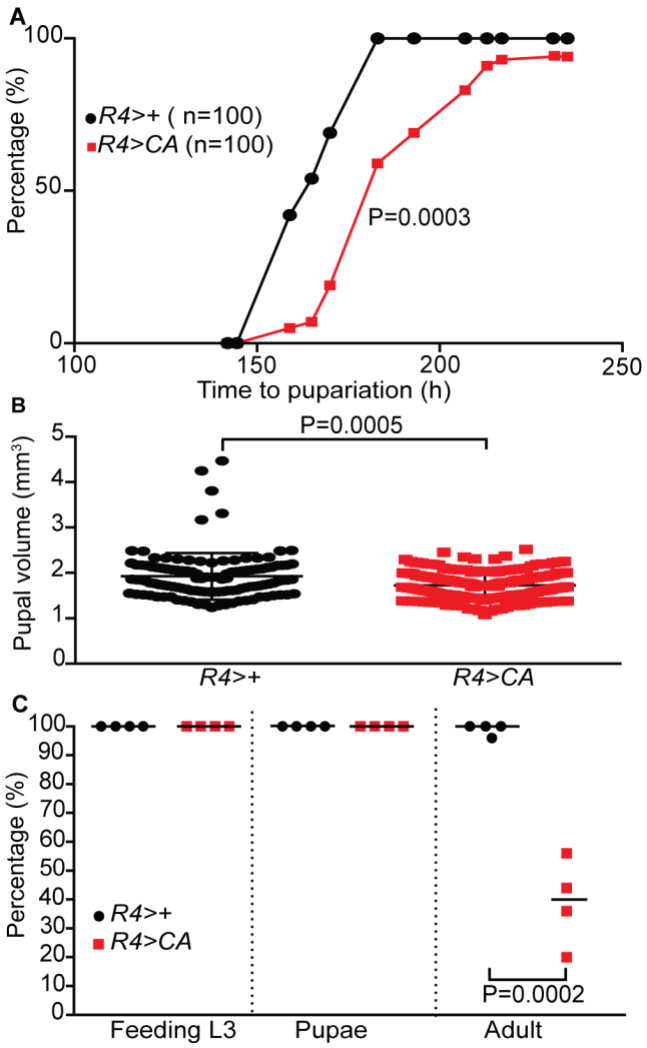


Figure 3-9 IMD activation impacts larval development
(A) Pupariation timing of third instar larvae to P13 stage of pupal development. 100 larvae per genotype were used; and four biological replicates, each containing 25 larvae, were used in this graph. Statistical significance was determined using a Kolmogorov–Smirnov test (B) Quantification of pupal volume in *R4>CA* and *R4>+ Drosophila*. Statistical significance was determined using an unpaired Student t test (C) Twenty-five feeding third instar larvae of the indicated genotypes were monitored for their development as third instar larvae, pupae, and adults. Results are shown for four independent measurements. Statistical significance was determined using an unpaired Student t test.

3.2.6 Increased IMD expression in the larval fat body suppresses insulin/TOR signaling

Previous studies have shown that flies with mutation in different component of insulin signaling pathway, for example flies with ablated IPCs, *insulin like peptide 1-5* mutants, mutation in the insulin receptor substrate *chico*, and *insulin receptor* trans-heterozygotes, showed disruption

in glucose homeostasis and growth and development impairment (98,103,104,410,411). Similarly, mice lacking Insulin receptor substrate-1 (IRS-1) had growth defects and high blood sugar levels (412,413). I showed that consistent IMD activity in the fat body results in impaired metabolism and growth defects as indicated by increased level of circulating sugar, reduced total triglyceride, developmental delay and small pupal size. As these observations are similar to symptoms reported for insulin deficient flies, I asked if elevated IMD activity in the larval fat body suppresses insulin signaling activity. To answer this question, through a collaboration with Dr. Savraj Grewal at the University of Calgary, we measured Akt phosphorylation at serine 505, which is homologous to mammalian phosphorylation site at serine 473, to total Akt protein via western blot. Akt phosphorylation is used as a readout for insulin signaling activity. We found that *R4>CA* larvae had reduced Akt phosphorylation compared to control larvae (Figure 3-10B&C), while total Akt levels remained the same in both control and *R4>CA* larvae (Figure 3-10A). This observation is in line with study from Michelle Bland lab in 2019 in which they showed constitutive expression of Toll signaling, another innate immune signaling pathway, in the *Drosophila* fat body results in insulin resistance in the fat body, although in my experiment I did not test if increased IMD activity in the fat body induces insulin resistance and future experiment testing this possibility will be useful to fully understand the mechanisms by which IMD affects insulin dysfunction in the fat body (414).

As the TOR pathway is another key regulator of metabolism and energy homeostasis, and given the suppression of genes involved in TOR regulation in the *R4>CA* larvae, along a collaboration with Dr. Savraj Grewal, we looked at the activity of the TOR pathway by measuring phosphorylation of p70 S6 kinase (p-S6K) which is downstream of TOR pathway at threonine 398. I found a significant reduced phosphorylation of S6K in whole lysate of *R4>CA* larvae compared with controls (Figure 3-10B&D). These observation indicate that increased IMD activation in the fat body suppresses IIS/TOR activity systemically in *Drosophila* larvae.

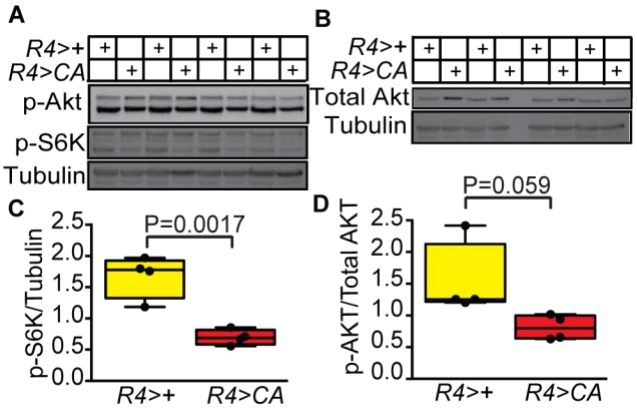


Figure 3-10 Effect of IMD activation in the fat body on systemic activity of IIS/TOR pathway in the larvae

Western blots of whole lysate from *R4>+* and *R4>CA* third instar larvae probed for (A) p-S6K, p-Akt (B) total Akt. Tubulin and total AKT levels were visualized as loading controls (C) Quantification of immunoblots of whole lysate from third instar larvae for p-S6K (D) Quantification of immunoblots of whole lysate from third instar larvae for p-Akt. Statistical tests were performed using an unpaired Student t test.

3.2.7 Loss of IMD disrupts metabolic homeostasis in *Drosophila*

I showed that consistent expression of IMD in the larval fat body disrupts metabolic homeostasis, diminishes energy stores, delays development, reduces pupal size and suppresses systemic insulin signaling activity. Given the impact of increased IMD expression in the fat body on larval metabolism, I hypothesized that IMD has a systemic role in regulation of metabolic homeostasis in *Drosophila.* To test this hypothesis, I used a null mutant *imd* that is unable to activate IMD pathway to measure insulin activity and insulin function as well as macronutrient levels in adult flies. I raised both *imd* mutant and w^{1118} flies on a chemically defined, holidic food, that contains all nutrients required for fly survival and fecundity and allows for investigation of the nutrient manipulation on the host as well as tracking host physiological responses to a controlled nutritional diet (384). The three main ILPs, ILP2, ILP3 and ILP5 are produced in the IPCs in the *Drosophila* brain and released into hemolymph after each meal to facilitate glucose uptake in peripheral tissues. As *ilp2*, *ilp3* and *ilp5* are expressed in the brain IPCs, I first measured the transcriptional level of *ilp2*, *ilp3* and *ilp5* from dissected heads of adult males and compared their expression level to the wildtype (w^{1118}) adult male flies.

I found that expression of *ilp2* and *ilp5* was not different in *imd* mutants compared to control (Figure 3-11A&C), however, mutation of *imd* reduced expression of *ilp3* (Figure 3-11B). As

expression of genes encoding for insulin peptides is complex and inulin peptides transcription does not necessarily correlate with the amount of total or circulatory peptide (415), therefore, I asked if mutation of *imd* affected the production of insulin peptides in the IPC or had any effect on the release of insulin peptides into the hemolymph. The total ILP2 measurement is an indicator of insulin production and circulatory ILP2 represents the secreted insulin peptide. To measure ILP2 peptide levels, I used a fly line that express hemagglutinin (HA) and flag-epitope-tagged ILP2. This fly line enables us to quantify the circulating and total ILP2HF by using an enzyme-linked immunosorbent assay (ELISA) (119). I observed a significant reduction in total ILP2 levels and significant increase in circulating ILP2 in *imd* mutants compared to controls (Figure 3-11D&E). This observation suggests that IMD affects insulin production and secretion in *Drosophila*.

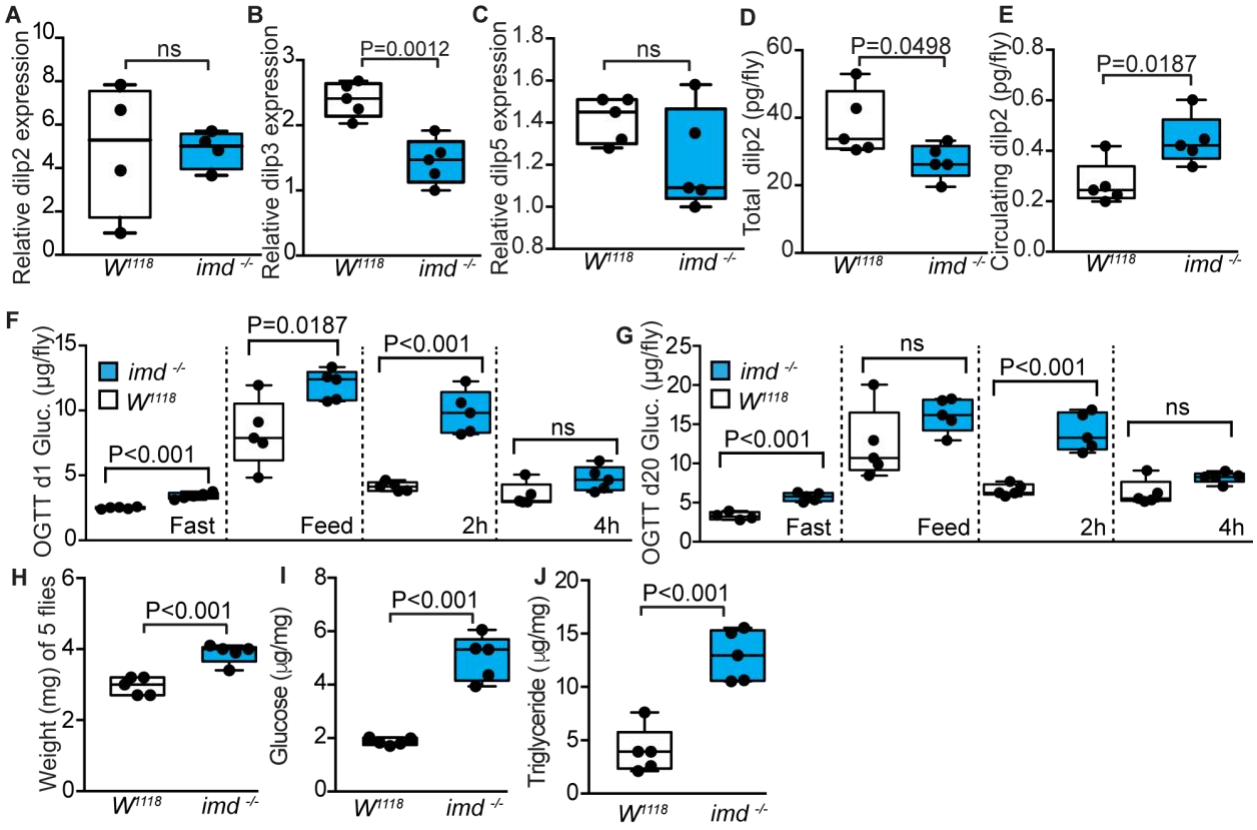


Figure 3-11 Metabolic homeostasis in the *imd* deficient flies
(A) Quantification of the relative expression of (A) *ilp2* (B) *ilp3* (C) *ilp5* from dissected heads of adult male w^{1118} vs *imd* mutants flies raised on holidic diet for 20 days (D) Total ILP2 protein, (E) circulating ILP2 protein in male w^{1118} and *imd* mutant flies raised on holidic diet (F) Oral glucose tolerance test performed on 1 day old (G) or 20 days old male w^{1118} and *imd* mutant flies raised on a holidic diet (H) Measurements of weight for w^{1118} and *imd* and each dot represent 5 adult male

(I) Total glucose (J) Total triglyceride level of male w^{1118} and *imd* mutant flies raised on the holidic diet for 20 days. Comparisons were performed using unpaired Student t tests.

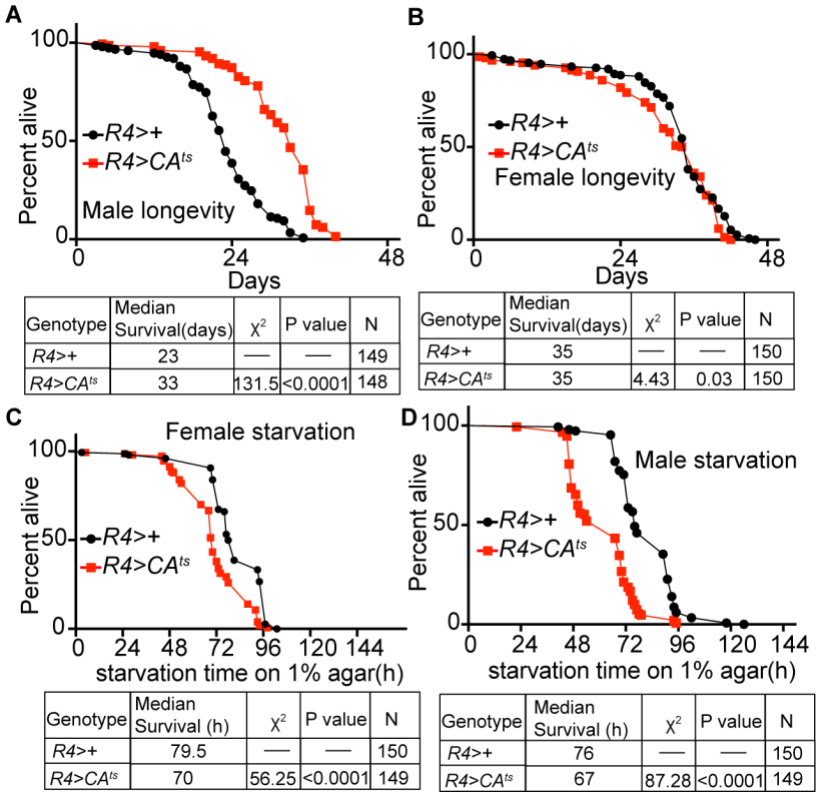
As insulin is one of the key hormones that regulates glucose metabolism, clearance of blood sugar after a meal is an indication of a functional insulin hormone. As lack of IMD had a significant effect on insulin synthesis and release into the hemolymph, I asked if mutation in *imd* will negatively affect the ability of flies to clear glucose. To answer this question, I tested the ability of 1 or 20 days old *imd* mutants along with controls to clear a glucose meal after a period of fasting. For the oral glucose tolerance test (OGTT), *imd* mutant and w^{1118} flies were raised for 1 or 20 days on holidic diet. I starved *imd* mutant and w^{1118} for 16 hours and then fed 10% glucose medium for 2 hours followed by fasting for 2 and 4 hours. Then, glucose levels from whole flies was measured in each timepoint of sample collection. In contrast to w^{1118} flies, *imd* mutants showed a higher glucose level upon glucose feeding and slower glucose clearance, which is indicative of an impaired insulin response (Figure 3-11F&G). This observation suggests that IMD is required for proper function of insulin. As I showed a disruption in glucose tolerance and increased insulin release in circulation, I asked how mutation of *imd* affects macronutrient levels. Therefore, I measured weight, glucose and triglyceride levels in *imd* mutants and wild type flies. I found that *imd* mutants weighed more (Figure 3-11H) and had elevated glucose (Figure 3-11I) and triglyceride levels (Figure 3-11J) compared with wildtype flies. These observations suggest IMD has a systemic effect on regulation of insulin, glucose and lipid metabolism in adult flies and its impact on metabolic homeostasis is essential.

3.2.8 Increased IMD expression in the fat body of adult *Drosophila* alters host response to starvation and affects lifespan

Previous studies in *Drosophila* showed over expression of PGRP-LE, a peptidoglycans (PGN) sensor in the fat body that activates IMD pathway, during adult stage negatively affected lifespan, however, acute expression of PGRP-LE for only 50 hours did not affect *Drosophila* lifespan compared with control (416). So far, I characterized the effects of elevated IMD expression in the fat body in the juvenile stage of *Drosophila* development, however, if increased expression of IMD in the fat body during adult stage affects adult lifespan was still unclear for me. I asked if expression of ImdCA in the fat body of *Drosophila* exclusively during adulthood affects longevity. To answer this question, I expressed ImdCA in the fat body of adult flies using the GAL4

temperature shift UAS system. This method enabled me to activate the specific gene only by shifting the temperature to 29°C and avoid possible negative effects of IMD activation on development. I expressed ImdCA in the fat body of male and female flies by changing temperature to 29°C (*R4>CA^{ts}*) as soon as flies emerged and monitored their survival over time until the last fly died.

I found that consistent activation of IMD in the fat body of adult male results in extension of lifespan by 43.5% compared with control (Figure 3-12A). In contrast, adult female flies with elevated IMD expression in the fat body did not show significant extension or reduction in their median survival compared with controls (Figure 3-12B). My results contradicts previous studies showing reduced lifespan in flies with activation of IMD signaling through overexpression of PGRP-LC and PGRP-LE in the adult fat body (416,417), however, the difference in the experimental design and transgenic fly line used could explain the different observations. Another study showed that elevated IMD expression in the intestinal progenitor cells also did not affect lifespan in adult flies (380). These observations suggest a sex-dependent and tissue specific effect on lifespan with consistent IMD expression in the fat body. It will be important to repeat this experiment by using R4-GAL4 flies which are backcrossed to the wild type *w^{1118}* flies to confirm there is no genetic background effect that masks the difference in lifespan.



Genotype	Median Survival(days)	χ^2	P value	N
R4>+	23	—	—	149
R4>CA^ts	33	131.5	<0.0001	148

Genotype	Median Survival(days)	χ^2	P value	N
R4>+	35	—	—	150
R4>CA^ts	35	4.43	0.03	150

Genotype	Median Survival (h)	χ^2	P value	N
R4>+	79.5	—	—	150
R4>CA^ts	70	56.25	<0.0001	149

Genotype	Median Survival (h)	χ^2	P value	N
R4>+	76	—	—	150
R4>CA^ts	67	87.28	<0.0001	149

Figure 3-12 Effect of chronic IMD activity in the fat body on lifespan and survival sensitivity
(A) Lifespan of adult male flies with increased IMD activity in the fat body kept at 29°C for consistent activation of IMD (B) Survival of adult male flies with increased IMD activity in the fat body starved on 1% agar as they emerged (C) Lifespan of adult female flies with increased IMD activity in the fat body kept at 29°C for consistent activation of IMD (D) Survival of adult female flies with increased IMD activity in the fat body starved on 1% agar as they emerged. Significance was compared to *R4>+* lifespan determined by log-rank (Mantel–Cox) test.

I showed previously that shortage of energy stores in the larvae with increased IMD activity had a semi-lethal effect on larval development. One of the mechanisms that is important for lifespan extension is the ability to deal with scarcity of energy or food resources. Animals that have an improved starvation resistance outlive their counterparts. I asked if adult flies with consistent IMD activation in the fat body are more sensitive to starvation compared to the control flies. To test this question, I expressed ImdCA in the adult male and female flies upon eclosion by shifting the *R4>CA^ts* flies to 29°C incubator while flies were kept in vials with 1% agar during the experiment and measured their lifespan by monitoring the survival. I found that both male and females *R4>CA^ts* flies showed 12 % reduction in survival compared with *R4>+* counterparts (Figure 3-12C&D). These results suggest that increased expression of IMD in the fat body increases sensitivity of adult *Drosophila* to starvation.

3.2.9 Increased IMD activity in the fat body of adult *Drosophila* alters feeding behaviour

I showed that IMD activation in the fat body results in disruption of metabolic homeostasis, lipid depletion and increased expression of genes involved in glycogen degradation. Therefore, I asked if expression of ImdCA in the fat body alters food consumption and food behavior of these flies in order to compensate for the diminished resources. To answer this question, I used an automated monitoring device to precisely measure feeding behavior. The fly Proboscis and Activity Detector (FlyPAD) is based on a capacitive sensing and records changes every time the proboscis is in contact with the food on the surface arena of the device and allows for an automated, high-throughput measurements of feeding behavior and consumption (386). The FlyPAD uses solid food which is placed as a droplet on the capacitor and allows precise recording of a *Drosophila* interaction with the food. Using this device, we can monitor and record the number of proboscis contacts with food or sips, number of bursts that are defined as sips clusters and number of bouts that means each time fly interacts with food or intervals between going for a meal. However, this assay has its own limitations as the interaction of individual flies with the food can only be measured over a short period of time, up to 1 hour and requires starving flies prior to the assay (Figure 3-13A).

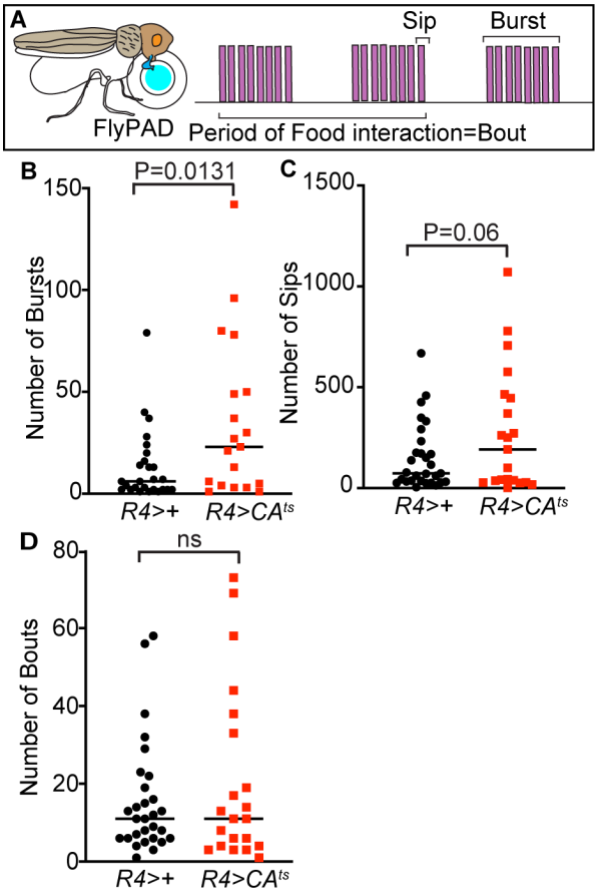


Figure 3-13 Acute IMD expression in the fat body alters feeding behavior in *Drosophila*
(A) Quantification of food consumption over two days in c*g*>+ and *cg*>*CA*^{ts} adult female flies after induction of IMD expression for two days. Food consumption was measured by CAFÉ assay over 5 hours in each day. Each dot represent 10 adult female flies (B) Quantification of total number of bursts (C) Total feeding sips and (D) Total feeding bouts using FlyPAD. Experiments was performed on *R4*>+ and *R4*>*CA*^{ts} adult female flies after induction of IMD expression for 48 hours in 29°C. Comparisons were performed using unpaired Student t tests.

To understand the feeding behaviors of adult flies with increased IMD activity in the fat body, I tested how ImdCA expression in the fat body could affect *Drosophila* interaction with the food. To answer this question, I first expressed ImdCA in the fat body of female adults for 48 hours and then starved them for 16 hours before starting the FlyPAD assay. Then I monitored fly behavior for 1 hour in the food area that were containing drops of 50% carbohydrate and 50% protein. I chose this recipe as I was only interested to look at the feeding behavior not food preference by *Drosophila,* therefore a similar levels of protein and carbohydrate was used for this experiment. I found that *R4*>*CA*^{ts} flies had a significant higher level of feeding bursts compared to control (Figure 3-13B) and slightly higher number of sips as well (Figure 3-13C). However, there was not

a significant difference between *R4>CA^{ts}* and control flies for bouts frequency (Figure 3-13D). These results suggest that flies with elevated IMD activity in the fat body have an altered food behavior compared with control flies as it was shown by increased feeding bursts or increased consecutives sips for food intake.

3.3 Summary

A balanced function between immune activity and metabolism is very crucial for the animal health, therefore, in this chapter I investigated the interaction of immunity and metabolism in the fat body in maintaining *Drosophila* health. I showed through microarray studies that larvae with elevated IMD activity in the fat body switched from glycolysis to glycogenolysis, reduced their carbohydrate metabolism, lowered the insulin gene expression and up-regulated apoptotic pathways. As I observed a significant disruption in the genes regulating insulin/TOR signaling, I further measured the activity of these two metabolic pathways at the protein level in whole larvae and found a systemic reduction in the insulin and TOR signaling activity in larvae with increased IMD activity in the fat body. As disruption in insulin signaling activity is an indicator of interruption of lipid and carbohydrate metabolism, I measured levels of circulatory trehalose and stored triglyceride and observed a diminished lipid reservoirs in the larval fat body as well as elevated levels of circulatory trehalose in the hemolymph of larvae with increased IMD activity in the fat body. As metabolism affects development and growth, I showed that consistent IMD activity in the fat body delayed the development of larvae and increased pupariation timing. In addition to that, elevated IMD in the larval fat body had a semi-lethal effect on larval development as I observed reduced number of larvae emerged as adults. As delay in development results in growth defects, therefore, I asked if consistent IMD activity in the fat body affects pupal size and found reduced pupal size in the larvae with increased IMD activity.

In the adult stage, activation of IMD in the fat body reduced starvation resistance of both sexes, induced a sex specific effect on adult lifespan and altered feeding behaviour of flies as well. Combined, my findings showed increased innate immune system activation in the metabolic tissue of *Drosophila* larvae causes hyperglycemia, depletion of lipid stores, delay in pupariation, reduced pupal size reduced systemic insulin signaling activity. My data suggests that IMD activation in the fat body affects the cross-talk between fat body and brain through deregulation of systemic insulin signaling. This hypothesis is consistent with the phenotypic overlaps between larvae with elevated

IMD activity in the fat body and insulin deficient flies. However, the exact molecular basis by which the IMD pathway contributes to the regulation of the fat–brain axis needs to be further investigated. To follow the effects of IMD on metabolism more closely, I monitored metabolic activity of adult flies with IMD pathway mutation and found wide range of effects of IMD on insulin, metabolism, and energy storage in adult flies raised on holidic diet which support a role for IMD in the maintenance of metabolic homeostasis. In summary, this chapter contributes to the existing literature showing the interaction between immune and metabolic signaling and the physiological impact of consistent immune activation on the host metabolism. Given that IMD and insulin signaling in *Drosophila* are evolutionary conserved, the result from this chapter emphasizes the advantage of using *Drosophila* in immunometabolism studies.

Chapter 4:

Modulation of host insulin signaling alters host response to pathogens

Portion of this chapter have been published as:

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4.1 Introduction

In chapter 3, I found that a constitutive immune response in the *Drosophila* fat body results in similar phenotypes observed in flies with suppressed insulin signaling that includes hyperglycemia, reduced pupal size and delay in development. These observations raised a question that if suppression of insulin signalling benefits the host against pathogens. Insulin signaling pathway is one of the main metabolic signaling that is evolutionarily conserved and regulate host metabolism and growth (418). There is an established relationship between different components of insulin pathway and pathogens, however, the impact of insulin signaling in response to each individual pathogen on the host is complex. In this chapter I asked how metabolic deregulation affects the ability of *Drosophila* to combat microbial pathogens. As insulin is one of the principal regulators of metabolic homeostasis, I examined the immune responses of *ilp2-3,5* mutant flies to oral and septic challenges with a panel of bacteria that range from low to high pathogenicity in *Drosophila* infection models.

I showed that, loss of insulin has microbe-dependent effects on bacterial pathogenicity in *Drosophila*. Previous studies have shown that *V. cholerae* which is an enteric pathogen and the cause of cholera diseases, suppresses insulin signaling in *Drosophila* (419) while inactivation of IMD signaling extends the survival of adult flies infected with *V. cholerae* (420). Given these studies suggest that *V. cholerae* modulates the host immune-metabolic responses, I characterized the effect of El Tor strain, *C6706 V. cholerae* infection on host macronutrients and energy stores. As I observed *V. cholerae* disrupts glucose homeostasis and increases circulating glucose, I asked if a define holidic diet supplemented with glucose improves survival of flies infected orally with *C6706*. I also examined the role of AMPs which are downstream of IMD pathway in fly survival upon infection with *V. cholerae* through either oral or septic route of entry. Overall, in this chapter I investigated the immune-regulatory role of insulin signaling in host-pathogen interactions.

4.2 Results

4.2.1 *V. cholerae* intestinal infection disrupts carbohydrates levels

One of the intestinal pathogens that has become a public health threat in developing countries is *V. cholerae* (342). *V. cholerae* has a cell surface lipopolysaccharide O antigen that is used to classify its strains into more than 200 serogroups from which classical and non-classical serotypes caused epidemic or pandemic cholera, with classical ones expressing the O1 antigen on

their surface (346,347). Classical serotypes composed of two biotypes—classical and El Tor—that each express a distinct number of markers, such as hemolysins (348–351). The *V. cholerae* attacks intestinal epithelial cells, inverts the host metabolism for its own advantages, and competes with the gut-resident symbionts for space and nutrients. Arthropods serve as natural reservoirs of *V. cholerae,* as it has been shown that chironmid egg masses contain the non-O1, and non-O139 *V. cholerae* (340). Colonization of *V. cholerae* in the *Drosophila* intestinal tract produces symptoms similar to the mammalian cholera illness (377). Therefore, flies are an excellent inexpensive model to study host-pathogen interaction in the context of cholera pathogenesis. Investigations on the effect of oral infection with *V. cholerae* MO10 strain, an O139 serogroup, in the lab of Dr. Paula Watnick showed that after 72 hours of oral infection with *V. cholerae*, glucose, glycogen and lipid levels in the host reduced compared to flies fed on Lysogeny Broth (LB) medium (419). These results suggest that *V. cholerae* negatively affects metabolic homeostasis in *Drosophila*.

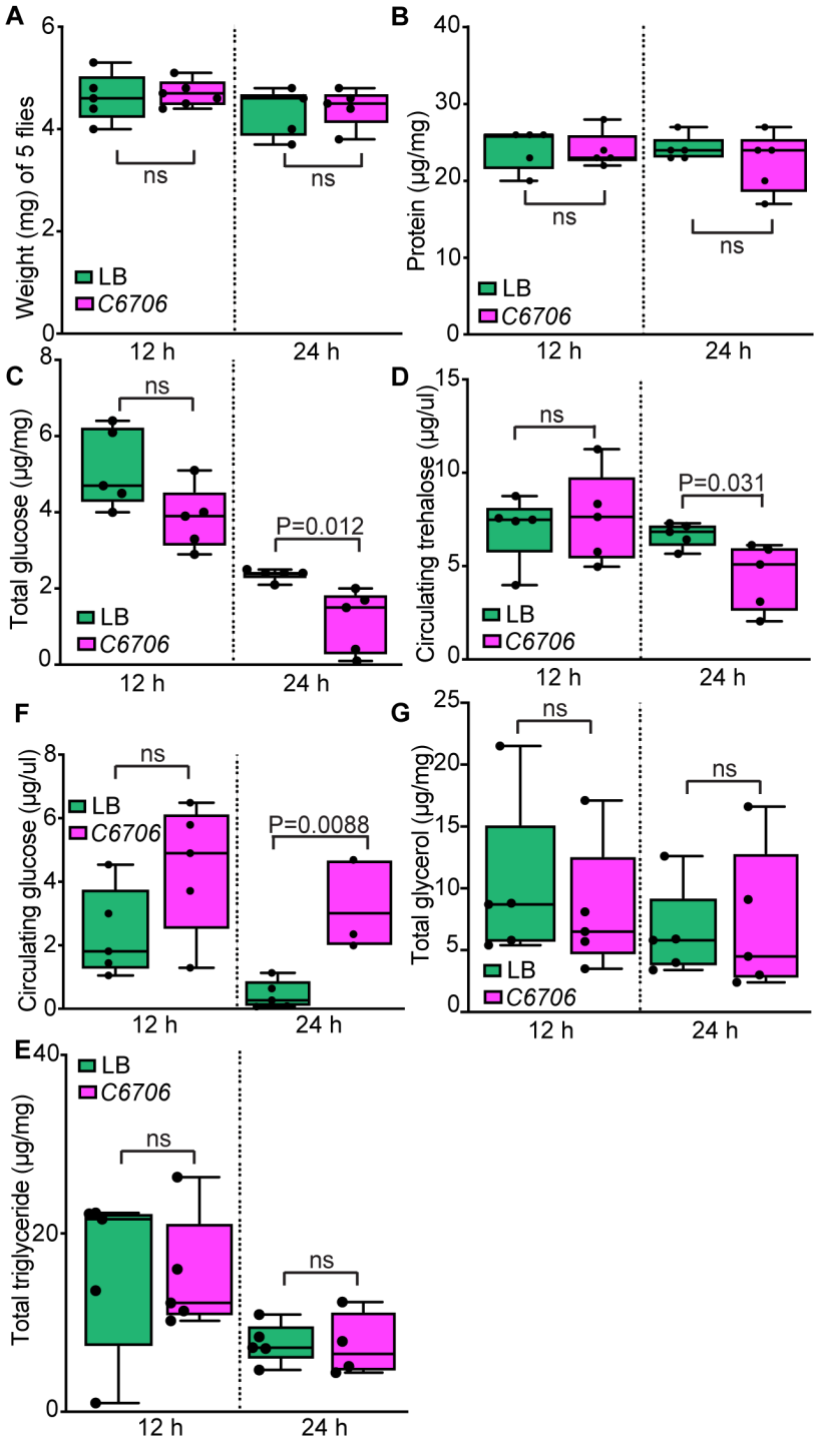
Although *V. cholerae* El Tor and O139 serogroups are closely related and patients infected with both pathogens show severe clinical symptoms associated with cholera disease (421,422) the *V. cholerae* O139 strain and El Tor serogroup have major differences. For example, the *V. cholerae* O139 has a different monosaccharide composition for O antigen (423), contains a polysaccharide capsule, is resistant to antibiotics (424–427), makes biofilm faster on abiotic surfaces (379) and is more resistant to antibodies produced by immune system (428). The O1 El Tor *C6706* strain is associated with the current 7th pandemic and was originally obtained from a Peruvian clinical isolate in 1991. The Watnick lab have shown oral infection of *Drosophila* with *C6706* was not lethal for the host as quorum sensing attenuates pathogen virulence (429), however, the *C6706* isolate I used in this study is lethal for the flies due to reduced expression of *hapR*, the master regulator of quorum sensing (430).

Given previous observations that *V. cholerae* intestinal infection affects host metabolism, I asked how oral infection of w^{1118} flies with *V. cholerae* El Tor strain *C6706* will impact the metabolic homeostasis of adult flies. To test this question, I measured macronutrients of 7-8 day-old female w^{1118} flies that were fed either with *C6706*, or a mock group that were only fed with LB. One day before infection, *C6706* was streaked from glycerol stocks onto lysogeny broth (LB)-agar plates supplemented with Streptomycin and grown overnight at 37˚C. On the day of infection, single colonies grew from overnight culture were suspended in the LB agar plates supplemented with Streptomycin in medium to an OD600 of 0.245 and soaked a sterile cotton plug with 3 ml of

the bacterial culture in LB. Vials with cotton plug soaked with LB alone was used for uninfected controls. Then, I kept vials at 29˚C for the duration of the experiment and death was recorded at indicated timepoint. Then, after 12 or 24 hours of oral infection, flies were harvested, weighed and processed for measurements of individual macronutrients.

I found no significant difference between body mass or protein levels of wildtype flies fed on LB or *C6706* (Figure 4-1A&B). The classical biotype of *V. cholerae* metabolizes glucose into an acidic product, which results in lower PH and a non-favorable environment for bacterial growth. In contrast, the *V. cholerae* El Tor biotype metabolizes glucoses to a neutral fermentation by-product called 2,3-butanediol or acetoin that does not inhibit bacterial proliferation (431). Given the ability of El Tor biotype to metabolize glucose, I predicted that flies infected with *C6706* show reduced glucose levels compared to mock group. I measured the total glucose level of w^{1118} after 12 and 24 hours post oral infection. I found that, after 24 hours of infection, *C6706*-infected flies showed a significant reduction in total glucose levels (Figure 4-1C). This observation suggests that feeding female flies with *V. cholerae* disrupts glucose metabolism in the host and this observation is in line with a previous report showing oral infection of adult male Oregon-R flies with *V. cholerae* MO10 reduced total glucose levels (419). As glucose levels were dysregulated by *C6706* oral infection, I then asked if oral infection with *V. cholerae* altered the levels of circulating trehalose and glucose. Trehalose is a non-reducing sugar and is the main circulating sugar in *Drosophila* hemolymph. To answer this question, I collected hemolymph from w^{1118} mock or *C6706*-infected female flies after 12 and 24 hours of oral infection and then measured glucose and trehalose levels.

I found that circulating trehalose was significantly reduced after 24 hours of infection in *C6706* flies (Figure 4-1D), while hemolymph glucose was twice as high as in flies orally infected with *V. cholerae C6706* (Figure 4-1E). These observations suggest that oral infection of wild type flies with *C6706* reduces glucose uptake as indicated by higher levels of circulating glucose. In *Drosophila*, increased blood sugar or hyperglycemia has been reported previously as a host response to bacterial infections such as *M. marinum* and *Streptococcus pneumoniae* (64,432). For lipid measurements, I found no significant difference in the triglyceride or glycerol levels between infected and non-infected w^{1118} flies (Figure 4-1G&E). Overall, these results suggest that *V. cholerae* El Tor *C6706* strain depletes glucose levels and increases circulatory glucose in the *Drosophila* host within 24 hours of oral infection.


A
Weight (mg) of 5 flies
6
4
2
0
ns
ns
LB
C6706
12 h
24 h
B
Protein (µg/mg)
40
30
20
10
0
ns
ns
LB
C6706
12 h
24 h
C
Total glucose (µg/mg)
8
6
4
2
0
ns
P=0.012
LB
C6706
12 h
24 h
D
Circulating trehalose (µg/ul)
15
10
5
0
ns
P=0.031
LB
C6706
12 h
24 h
F
Circulating glucose (µg/ul)
8
6
4
2
0
ns
P=0.0088
LB
C6706
12 h
24 h
G
Total glycerol (µg/mg)
25
20
15
10
5
0
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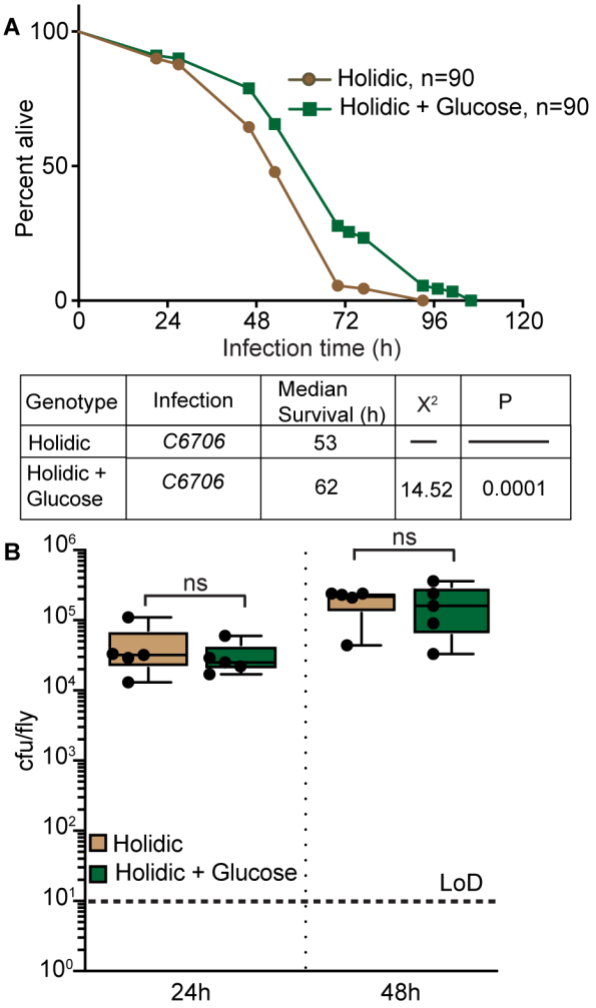
LB
C6706
12 h
24 h
E
Total triglyceride (µg/mg)
40
20
0
LB
C6706
ns
ns
12 h
24 h

Figure 4-1 *V. cholerae* intestinal infection affects host sugar homeostasis
(A) Weight measurements (B) Total protein level (C) Total glucose level (D) Circulating trehalose from hemolymph (E) Circulating glucose from hemolymph (F) Total glycerol level (G) and total triglyceride levels of eight days old w^{1118} female flies after 12 and 24 hours of oral infection with *C6706* or fed with LB. For hemolymph data, each dot represents a combined hemolymph of twenty five flies and for the rest of the data, each dot represents five flies that were homogenized for macronutrient measurements. Student unpaired t test was used to compare statistical significance between *C6706* fed and LB fed flies, $P<0.05$.

4.2.2. Glucose supplementation extends host survival upon enteric infection with *V. cholerae*

Previous studies have shown the impact of macronutrient balance and dietary modification on the host immune response, as well as tolerance and resistance to infection. Resistance is defined as mechanisms that either kill or inhibit proliferation of the pathogens, while tolerance is referred to strategies that reduce the negative impacts of a pathogen on the host health with a neutral or positive impact on the pathogen load (267,275,433,434). Dietary modification alters host defence towards pathogens and its effect is pathogen-specific, as restricting food intake in flies increased tolerance towards *S. typhimurium* while dietary restriction reduced fly resistance to *L. monocytogenes* (275). Restricting protein uptake benefits the host while fighting a pathogen as it was shown for increased tolerance of *Drosophila* toward *E. coli* infection (435). Another study showed flies raised on a low protein to carbohydrate diet survived longer in response to infection with *Micrococcus luteus* compared to when flies were raised on high protein to low carbohydrate ratio (436). My previous observation showed that *V. cholerae* disrupts glucose homeostasis and increases circulating glucose (Figure 4-1). Given the ability of *V. cholerae* El Tor biotype in metabolizing glucose which facilitates its growth, I asked how a glucose diet will impact the host response to *V. cholerae*.

Monitoring host survival to infection is a comprehensive method to analyse the host response to different bacterial infections or different genotype response to a similar infection (280,292,437). To measure infection survival, I raised wild type flies on a holidic diet or a holidic diet supplemented with glucose concentration (50 g/liter) for 7 days and then started oral infection with *C6706* and monitored the survival of flies at 29°C. A holidic diet allows precise manipulation of individual nutrients and enables investigating the impact of each ingredient on the organism (384).



Genotype	Infection	Median Survival (h)	X^2	P
Holidic	*C6706*	53	—	———
Holidic + Glucose	*C6706*	62	14.52	0.0001

Figure 4-2 Dietary glucose affects host response against *V. cholerae*
(A) Survival curves of freshly emerged female w^{1118} flies raised on holidic and holidic food supplemented with high glucose for eight days followed by oral infection with *C6706* (B) Bacterial load of w^{1118} flies raised on holidic and holidic food supplemented with high glucose for eight days followed by oral infection with *C6706* and homogenized at 24 and 48 hours of infection for CFU measurements. Each dot represents five flies homogenized and plated for bacterial load measurement and statistical significance was determined using one-way ANOVA with Sidak correction for multiple comparisons. Statistical significance for survival curves was determined using a Log-rank (Mantel-Cox) test that represents the survival significance between holidic and holidic food supplemented with high glucose flies. For survival experiments, 90 flies per condition (30 flies in three vials) were used for oral infection with *C6706*.

I found that flies raised on a holidic diet supplemented with glucose had a 17% survival extension compared to w^{1118} flies raised on holidic diet after an oral infection with *C6706* (Figure 4-2A). This result suggests that a glucose diet extends host survival upon infection with *V. cholerae C6706* strain. Measurement of bacterial burden in the host during an infection is a quantitative

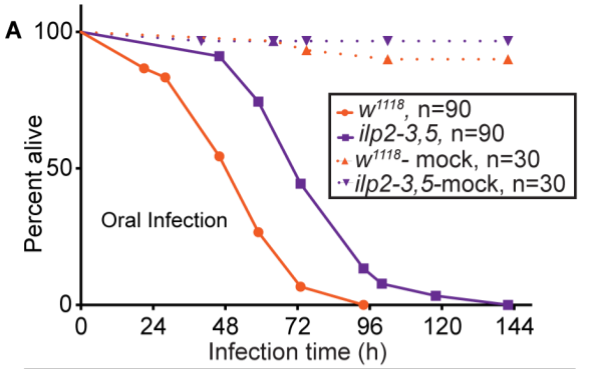
assessment of the ability of pathogens to grow in the host and also capacity of the host to clear the pathogen, and it also represents the live bacteria that are present at the time point in which samples were collected. This can be performed by counting the number of colony forming unit (CFU) per fly at different time point post infection. To test how the holidic diet or the holidic diet with glucose supplementation affects internal bacterial loads in flies infected with *C6706*, I collected flies at 24 and 48 hours during oral infection, and counted the CFUs for each timepoint and condition. If the glucose supplementation enables the host to eliminate the pathogen, I expect to see a lower CFU count in flies raised on glucose-supplemented holidic food. However, I found that at both timepoints there was no significant difference between bacterial loads in flies on either diets (Figure 4-2B). These results suggest that flies raised on a holidic diet supplemented with glucose have increased ability to survive *C6706* oral infection, although the bacterial load in unmodified holidic diet or flies raised on a holidic diet supplemented with concentration of glucose is similar. The increased host survival against infection with no effect on bacterial load suggests an improved tolerance induced by glucose-supplemented food in *Drosophila*.

4.2.3 Suppression of insulin signaling alters host response to *V. cholerae* infection

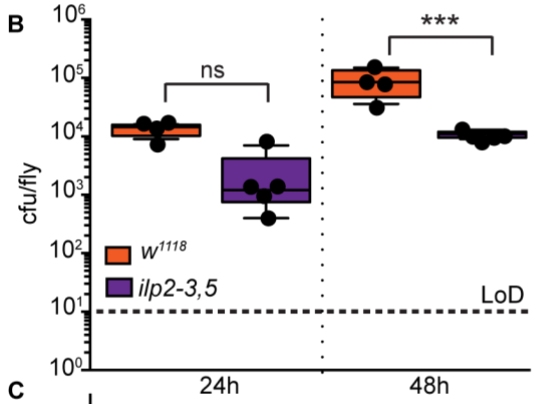
Insulin signaling pathway is one of the main metabolic signaling that is evolutionarily conserved and regulates host metabolism and growth (418). In response to elevated glucose after a meal, insulin secretion increases, which results in increased glucose uptake, activation of glycolytic enzymes and storage of glucose as glycogen (117,143,240). In addition to the regulatory role of IIS in metabolic homeostasis, crosstalk between different components of the insulin pathway in response to pathogens have been reported. For example, mutation in the upstream component of insulin signaling, insulin receptor substrate orthologue, *chico*, results in improved survival against *P. aueroginosa* and *E. faecalis* (438). As I found *V. cholerae* oral infection of wild type flies negatively affects glucose homeostasis and previous report on inhibitory effect of *V. cholerae* MO10 on systemic insulin signaling in *Drosophila* (419), I hypothesized that insulin alters host response to *V. cholerae C6706* strain infection.

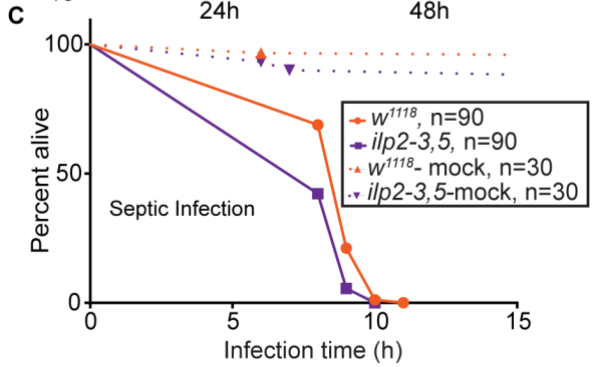
To test this hypothesis, I used a specific *Drosophila* null mutant for insulin like peptides 2, 3, and 5 (*ilp2-3,5* mutants). These peptides are secreted by neurosecretory cells located in the pars intercerebralis and are essential for regulation of development, growth and sugar homeostasis

(415,439). I infected *ilp2-3,5* mutant and wildtype flies with *C6706* via oral infection and monitored the survival of these flies. I also fed *ilp2-3,5* mutants and wildtype flies with LB media to serve as mock group for the experiment. I found that *ilp2-3,5* mutant flies showed a 45.8% survival extension compared with wild type flies (Figure 4-3A). This observation suggests that insulin peptides contribute to the lethality of *V. cholerae*. To understand if bacterial growth in *ilp2-3,5* mutant flies is altered compared with w^{1118} flies, I collected flies from both genotypes at 24 and 48 hours of starting the oral infection and measured the internal bacterial load at these timepoints. I found that bacterial load at 24 hours was not significantly different between *ilp2-3,5* mutant and wild type flies, however at 48 hours of infection, *ilp2-3,5* mutants showed a significant reduced CFU compared to their wildtype counterparts (Figure 4-3B). Increased survival of *ilp2-3,5* mutant flies upon oral infection with *V. cholerae* is in line with previous observation in flies in which suppression of insulin signaling protect flies against infection (64,440,441) and suggest an improved resistance towards *C6706* infection.



Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*C6706*	48	—	———
ilp2-3,5	*C6706*	70	57.66	<0.0001





Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*C6706*	8.5	—	———
ilp2-3,5	*C6706*	7	17	<0.0001

Figure 4-3 Insulin deficiency alters *Drosophila* response to oral and septic infection with *V. cholerae*

(A) Survival curves for eight days old female w^{1118} and *ilp2-3,5* mutant flies after oral infection with *C6706* as well as w^{1118} and *ilp2-3,5* mutant flies fed on LB only (B) Bacterial load of female w^{1118} and *ilp2-3,5* mutant flies after oral infection with *C6706* and homogenized at 24 and 48 hours of infection for CFU measurements (C) Survival curves for eight days old female w^{1118} and *ilp2-3,5* mutant flies after septic infection with *C6706* as well as w^{1118} and *ilp2-3,5* mutant flies pricked with sterile needle served as mock groups. For bacterial load measurements, each dot represents five flies homogenized and plated to count CFUs and statistical significance was determined using

one-way ANOVA with Sidak correction for multiple comparisons. Statistical significance for survival curves was determined using a Log-rank (Mantel-Cox) test that represents the survival significance between w^{1118} and *ilp2-3,5* mutant flies. For survival experiments, 90 flies per genotype (30 flies in three vials) were used for septic infection with *C6706* and 30 flies per genotype for mock groups.

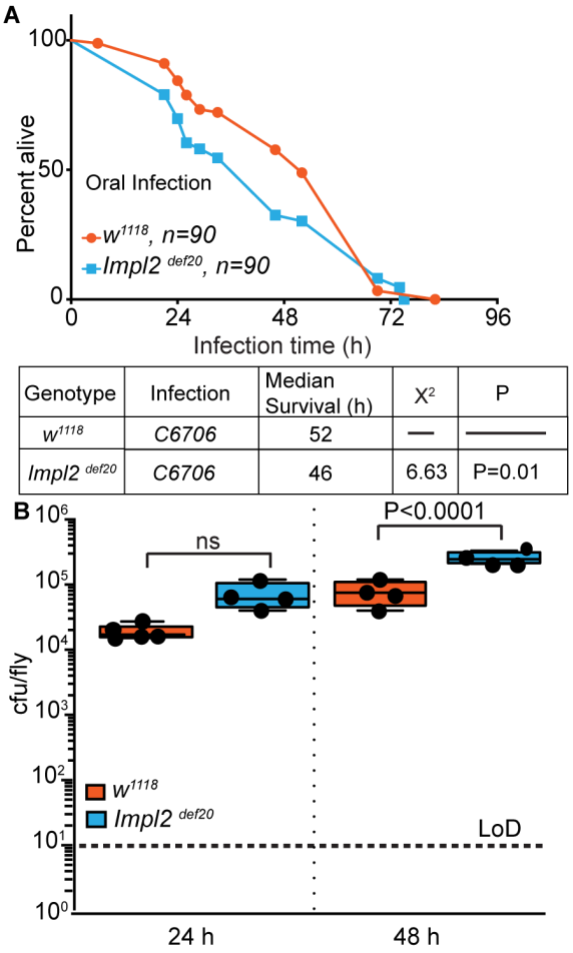
Route of pathogen entry into the host is important in infection models as *S. marcescens Db11* pathogenicity showed attenuated lethality in the oral infection model compared with septic route (294). Feeding pathogens to fly mimics the natural route of infection as *Drosophila* exposes to bacteria upon feeding on decaying fruits. In contrast, pricking the flies in the thorax or abdomen with a fine needle allows investigating how hosts respond to the systemic bacterial infection (287), although differences exist in host response to either of septic models. To understand how route of infection affects *ilp2-3,5* mutants response to *V. cholerae* infection, I pricked *ilp2-3,5* mutant flies along with wild type controls in the thorax with a needle dipped into *V. cholerae* bacterial suspension with OD600=1. I also pricked both w^{1118} and *ilp2-3,5* mutants with sterile needle to serve as a mock group. Deposition of *V. cholerae* into the body cavity of flies killed both genotypes within 10 hours of inoculating flies, however, *ilp2-3,5* mutant flies succumbed to death more rapidly than wildtypes as they had 17% survival reduction compared with w^{1118} flies (Figure 4-3C). Overall, these observations indicate lack of insulin peptide in *Drosophila* positively affects survival of host to *V. cholerae* oral infection while does not protect flies against septic infection with this pathogen.

4.2.4 Systemic increase in insulin signaling reduces survival to enteric *V. cholerae* infection

As I observed systemic reduction of insulin peptides increases survival of flies to oral infection of *C6706*, I asked if increased insulin signaling has a negative effect on host survival against *V. cholerae* infection. To answer this question, I used flies that lack a functional ImpL2 called $ImpL2^{def20}$. As ImpL2 regulates cell size and number, a loss of function mutation in this secretory protein results in elevated insulin signaling in the adult *Drosophila*. ImpL2 binds to ILP2 in *Drosophila* and human insulin and acts as antagonists of insulin signaling (442,443). Binding of ImpL2 to insulin results in suppression of insulin signaling at the ligand level. Flies that lack Imp-L2 have a larger body size but develop at a normal timing compared to wildtype flies. Suppression of insulin signaling by expression of Imp-L2 in starvation condition avoids lethal consequence of overexpression of insulin signaling once food is at scarcity (131). Using the $ImpL2^{def20}$ fly line, I

tested how host responds to *V. cholerae* infection once insulin signaling is systemically elevated. I aged *ImpL2*^{def20} and w^{1118} female flies for 7-8 days at 25°C followed by oral infection with *C6706* and monitored their survival and bacterial loads over the course of infection. I found flies that lack Imp-L2, which means higher insulin signaling activity for these flies, had a 12% reduced survival compared to wildtype flies (Figure 4-4A). This result suggests that increased insulin signaling activity in the host provides a favorable environment for *C6706* pathogenicity which increases the vulnerability of flies towards *V. cholerae* infection.

To understand how bacterial quantity of *C6706* is affected in the host with increased insulin signaling compared to wildtype flies, I measured the CFUs of *ImpL2*^{def20} and w^{1118} flies at 24 and 48 hours from starting oral infection with *C6706* and found in contrast to *ilp2,3,5* mutants flies, the bacterial load was significantly higher in *ImpL2*^{def20} mutant flies after 48 hours of infection with *C6706* (Figure 4-4B). Although for both *ImpL2*^{def20} and w^{1118} flies internal bacterial loads increased over time but *ImpL2*^{def20} mutant flies showed a 3.76 and 3.32 fold increase in the number of *V. cholerae* associated with *ImpL2*^{def20} mutants relative to wild type flies. As increased bacterial load adversely correlates with reduced survival of *ImpL2*^{def20}, these observation suggest an impaired resistance in the flies with increased insulin signaling activity to the intestinal infection of *C6706*.



Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	C6706	52	—	———
$Impl2^{def20}$	C6706	46	6.63	P=0.01

Figure 4-4 Increased host insulin signaling adversely affects host survival to *V. cholerae*
(A) Survival curves for eight days old female w^{1118} and $ImpL2^{def20}$ mutant flies after oral infection with *C6706* (B) Bacterial load of female w^{1118} and $ImpL2^{def20}$ mutant flies after oral infection with *C6706* and homogenized at 24 and 48 hours of oral infection for CFU measurements. For survival experiments, 90 flies per genotype (30 flies in three vials) were used. Statistical significance for survival curves was determined using a Log-rank (Mantel-Cox) test that represents the survival significance between w^{1118} and $ImpL2^{def20}$ mutant flies, n=90 flies for each genotype. For bacterial load comparisons, each dot represents five flies that were homogenized at respective timepoints and statistical significance was determined using one-way ANOVA with Sidak correction for multiple comparisons.

4.2.5 *ilp2-3,5* mutants weight does not affect bacterial quantity upon *V. cholerae* infection

There are different factors that affect the dynamics of pathogen load in the host such as rate of pathogen shedding, appetite, body size or consumption of contaminated food with the specific pathogen. In addition, bacterial growth rate or expression of virulence genes on early or later stage of growth can affects the pathogenicity of the bacteria and livelihood of the host. Flies lacking insulin peptides 1-5 have a smaller body size due to the systemic reduction of insulin signaling and

delay in their development compared with wildtype ones (103). *ilp2-3,5* mutants show a visible smaller body size compared to w^{1118} flies and as I observed reduced bacterial load in *ilp2-3,5* mutant flies compared with w^{1118} flies after oral infection with *V. cholerae*, I asked if the reduced bacterial load was due to direct effect of the small body size of the *ilp2-3,5* mutant flies and in contrast the increased bacterial load in flies with loss of function mutation of *ImpL2* flies is the result of the larger size of $ImpL2^{def20}$. To answer this question, I first weighed *ilp2-3,5* mutant flies and compared their body mass with wildtype flies and confirmed *ilp2-3,5* mutant weigh significantly less compared to control flies (Figure 4-5A).

In contrast, $ImpL2^{def20}$ mutants with increased insulin signaling activity showed slightly higher but not statically significant difference in their weight in comparison with wildtype counterparts (Figure 4-5B). Then, I normalized CFUs for weight by dividing the CFUs for *ilp2-3,5* flies to the ratio of the average weights of w^{1118} and *ilp2-3,5* mutants and expected if smaller body size affects the bacterial burden in the host, by correcting for weight for the CFUs of w^{1118} and *ilp2-3,5,* the difference for microbe loads between wildtype and insulin deficient flies will no longer be significant. However, once I corrected the *ilp2-3,5* mutant bacterial load for weight, the difference between insulin mutant and w^{1118} flies CFUs remained unchanged (Figure 4-5C, Figure 4-3B). Similarly, I still saw higher levels of bacterial load in $ImpL2^{def20}$ mutants compared with wildtype flies when correcting for differences in weight (Figure 4-5D, Figure 4-4B). These data suggest that size of the host does not affect the bacterial load in the *ilp2-3,5* mutant flies after oral infection with *V. cholerae.*

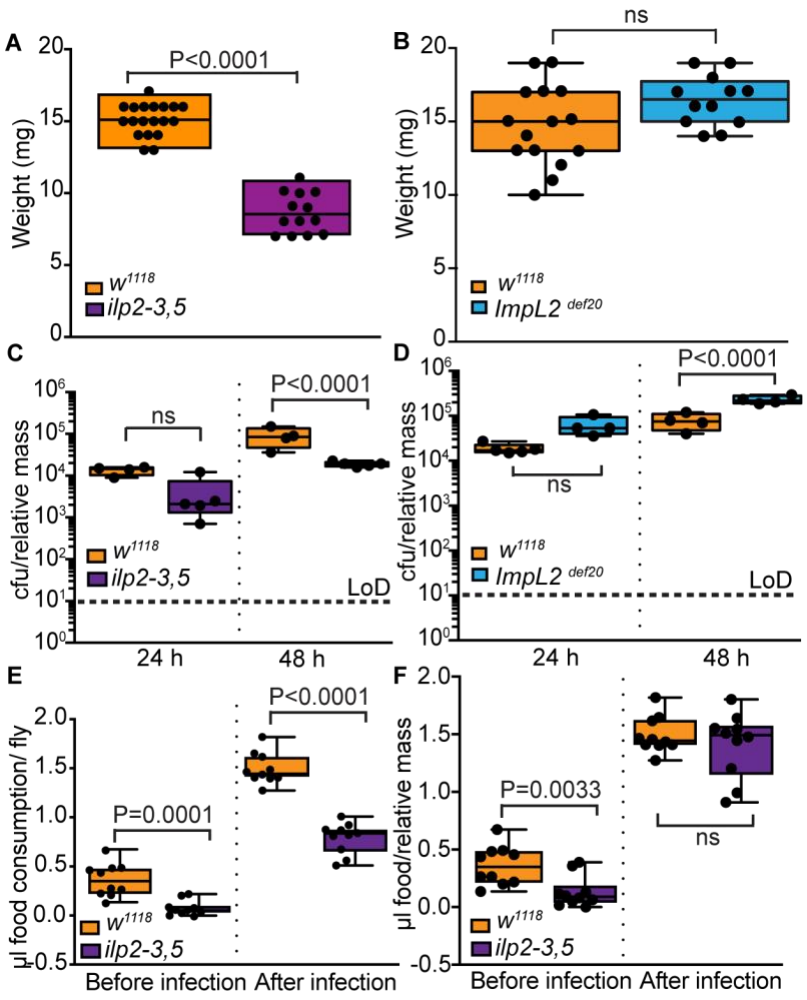


Figure 4-5 Effect of host weight on bacterial load and food consumption in *ilp2-3,5* mutant flies

(A) Weight measurements of female w^{1118} and *ilp2-3,5* mutant flies (B) Weight measurements of female w^{1118} and $ImpL2^{def20}$ mutant flies. Student unpaired t test was used to compare statistical significance between each genotype (C) CFUs of *ilp2,3,5* mutant flies normalized to weight after 24 and 48 hours of oral infection with *C6706* (D) CFUs of $ImpL2^{def20}$ mutant flies normalized to weight after 24 and 48 hours of oral infection with *C6706*. For CFU normalization to weight, statistical significance was determined using one-way ANOVA with Sidak correction for multiple comparisons (E) Food consumption rates per fly of w^{1118} and *ilp2-3,5* mutant flies before, and 48 hours after oral infection with *C6706* using CAFÉ assay, capillaries were filled with 5% sucrose and 5% yeast, each chamber contained 3 capillaries with 10 adult flies. Each dot represents average volume of food consumed per fly per replicate and total of 10 vials was used for each genotype, food consumption was monitored for 24 hours (F) Food consumption rates per fly normalized to weight for w^{1118} and *ilp2-3,5* mutant flies before, and 48 hours after infection with *C6706*. Student unpaired t test was used to compare statistical significance between each genotype, $P < 0.05$.

In *Caenorhabditis elegans,* as microbes are a food source therefore overtime the host has developed an aversive learning strategy in the neurons to distinguish between pathogenic and non-pathogenic microbes (444). An insulin neuropeptide that is encoded by *ins-11* and expressed in the intestine increases avoidance behaviour once *C. elegans* is exposed to pathogenic bacteria *P. aeruginosa* (445). In another study insulin receptor *daf-2* mutants were resistant to Gram positive bacteria *Bacillus thuringiensis* and showed reduced food intake compared to control group (446). In *Drosophila,* a direct correlation between higher glucose levels in the hemolymph and aversion to feeding was observed as well (447). As *ilp2-3,5* mutants have been shown to have higher hemolymph sugars and previous studies in *C. elegans* showed mutation in the insulin signaling affects food intake and considering insulin signaling is an evolutionary conserved metabolic pathway, I asked if reduced food intake in *ilp2-3,5* deficient flies is directly affecting the consumption of pathogen after oral infection with *V. cholerae* and consequently protecting the flies against *V. cholerae* infection.

To answer this question, I measured food consumption of *ilp2-3,5* mutant and wildtype flies before and 48 hours after oral infection with *V. cholerae* using Capillary Feeder (CAFE) assay. CAFÉ assay allows measurement of ingestion in real-time and provides quantitative analysis of food behavior in flies via capillary tubes (448). For this experiment, I aged *ilp2-3,5* mutants and wildtype flies for 7-8 days and measured food consumption for these flies over 24 hours while capillary tubes were filled with 5% yeast and 5% sucrose. I then infected both genotypes with *V. cholerae* via oral infection for 48 hours and measured their food consumption with CAFÉ assay for 24 hours. I found that *ilp2-3,5* mutants consume significantly less food compared to the controls in both before and after infection with *C6706* (Figure 4-5E). Although both wildtype and *ilp2-3,5* mutant flies showed higher volume of food consumption after being infected with *C6706* for 48 hours but flies that lack insulin peptides showed reduced food consumption in compared to w^{1118} flies. This result is in line with my previous observation that *ilp2-3,5* mutants have a reduced bacterial load and suggests that inactivation of insulin may reduce ingestion of pathogenic microbes. Then, I asked if small body size of insulin deficient flies results in reduced consumption of food by these flies. To answer this question, I normalized the food consumption per fly per weight for *ilp2-3,5* mutants and found there was still significant reduction in food consumption in *ilp2-3,5* mutant flies compared with control (Figure 4-5F), however, once volume of food consumed by *ilp2-3,5* mutants were normalized for weight after being infected with *V. cholerae*

for 48 hours, the significant difference between two genotypes disappeared (Figure 5-5F). This result suggests that reduced food consumption of *ilp2-3,5* mutant flies after being infected with *C6706* for 48 hours of is mainly due to the small body size of these flies in contrast to when *ilp2-3,5* mutants are not infected with *V. cholerae*. As I did not tested food consumption rate of *ilp2-3,5* mutant flies while the food is contaminated with *V. cholerae*, I cannot conclude that *ilp2-3,5* deficient flies showed an aversion behavior towards *V. cholerae* and therefore further experiments are required to test this hypothesis.

4.2.6 Expression of *drosomycin* is increased in the intestine of *ilp2-3,5* mutants

I previously observed increased survival and reduced bacterial loads in *ilp2-3,5* mutants compared to wildtype flies after oral infection with *V. cholerae*, and the opposite trend by increasing insulin signaling in the host as observed in $ImpL2^{def20}$ mutant flies by reduced survival and increased bacterial load after intestinal infection with *V. cholerae*. These observations suggest an improved resistance of insulin deficient flies towards enteric infection with *V. cholerae* and reduced resistance once insulin signaling activity in the host is increased. As I showed earlier that loss of insulin results in reduced consumption of food and is independent of small body size of *ilp2-3,5* mutant flies before infection with *V. cholerae*, I asked if there are other factors contributing to the resistance of *ilp2-3,5* mutants to *V. cholerae C6706* infection. Previous studies showed that starvation of S2 cells leads to increased AMPs expression and is confirmed in vivo in adult *Drosophila* that starvation of flies results in suppression of insulin signaling and increased expression of AMPs (449). Another study showed FOXO activation in the enterocytes is required for increased resistance to *S. marcescense* oral infection through production of AMPs (440). As reduced insulin signaling results in nuclear translocation of FOXO and increased transcription of FOXO-target genes, I asked if that lack of insulin peptides improves resistance to *V. cholerae* by increased expression of AMPs.

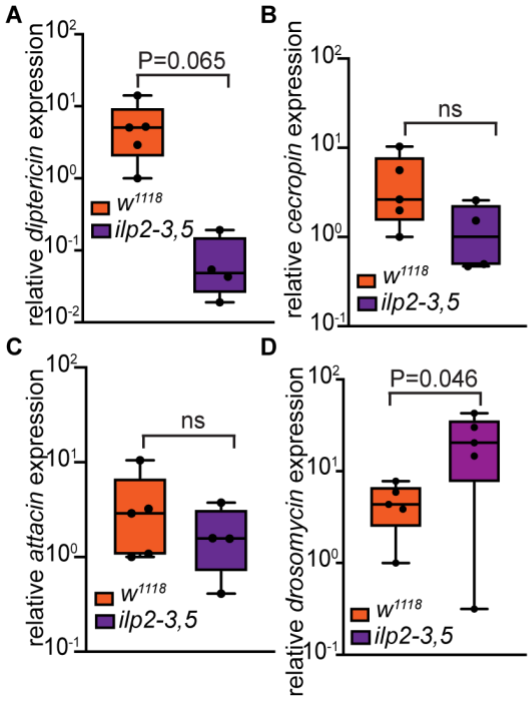


Figure 4-6 Comparison of antimicrobial peptide gene expression in the intestine of wildtype and *ilp2-3,5* mutant flies
(A) Quantification of relative gene expression of *diptericin* (B) *cecropin* (C) *attacin* and (D) *drosomycin* from intestine of 8 days old female w^{1118} and *ilp2-3,5* mutant flies. Each dot represents ten dissected guts per replicates. Student unpaired t test was used to compare statistical significance between w^{1118} and *ilp2-3,5* mutant flies, P <0.05.

To test this hypothesis, I aged female *ilp2-3,5* mutant and wildtype flies for 7-8 days and then dissected intestine from each genotypes and measured expression of *diptericin*, *cecropin*, *attacin* and *dosomycin* from intestine of *ilp2-3,5* mutants and w^{1118} flies before infection. Expression of *diptericin* was slightly reduced in *ilp2-3,5* mutant flies but not statistically significant (Figure 4-6A). I did not detect significant changes in *cecropin* or *attacin* expression in *ilp2-3,5* mutants compared with wildtype flies (Figure 4-6B&C). However, I found that *ilp2-3,5* mutants have a 10-fold higher level of *drosomycin* expression compared to controls (Figure 4-6D). This result suggests that lack of insulin peptides increases expression of *drosomycin* in the intestine of *ilp2-3,5* mutant flies through increased FOXO activation downstream of insulin signaling. These results is in line with an earlier report that FOXO controls the expression of *drosomycin* in the adult intestine and starvation of larvae or adult flies or chemical inhibition of insulin signalling results in increased expression of AMPs and is independent of presence of any infection (449). Among all AMPs, regulatory regions of *drosomycin* promoter showed more conserved FOXO binding sites

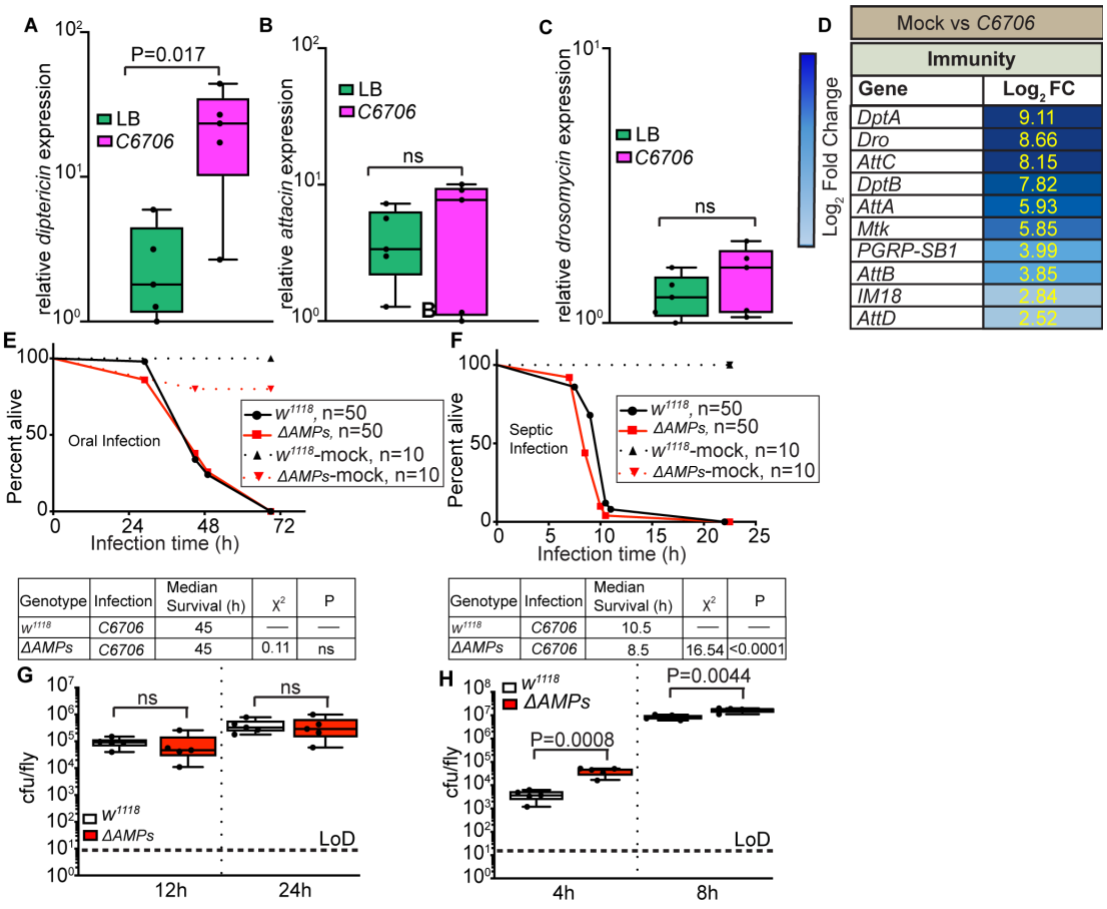
which might explain the increased expression of *drosomycin* in *ilp2-3,5* mutant flies compared with other AMPs. Although this result indicates *ilp2-3,5* mutants intestine has an increased expression of *drosomycin* but does not indicate a resistance mechanism for reduced bacterial load for *V. cholerae* as *drosomycin* is effective against Gram positive bacteria and fungi. Therefore, additional experiments are required to further understand the mechanisms involved in the *ilp2-3,5* mutants resistance to *V. cholerae* infection.

4.2.7 Antimicrobial peptides are not essential for protection of *Drosophila* against *V. cholerae*

The *Drosophila* Toll and IMD innate immune responses are essential for the survival of the host against Gram negative and positive pathogens, respectively (261,450,451). As both IMD and Toll are evolutionary conserved, investigation of host innate immune responses and host pathogen interaction using *Drosophila* model has been fundamental for expanding our knowledge for the role of immune responses in host-pathogen interaction. Mutation in IMD or Toll pathway components results in increased susceptibility of flies to infection. For example, IMD and Toll mutants were more susceptible to Gram negative *E. coli* or the fungus *Beuveria bassiana* infection compared with controls (452). However, the route of infection and level of pathogenicity of specific bacteria affects the degree of host vulnerability as *imd* mutant and wildtype flies showed no difference to septic infection with *S. marcescens*, while oral infection of *imd* mutants with the same pathogen results in reduced survival of flies (294). *P. sneebia*, a Gram negative bacteria does not induce immune responses and depositing this bacteria into the thorax via septic infection does not activate IMD signaling which suggests *P. sneebia* avoids recognition by host immune systems through mechanisms that are still unknown (304). IMD pathway activity contributes to the pathogenicity of *V. cholerae* as suppression of different components of IMD pathway including Relish, Dredd, Fadd and Kenney increased survival towards oral infection of *V. cholerae* MO10 biotype (420). As these studies suggest a crosstalk between IMD pathway and *V. cholerae* lethality, I asked how oral infection of flies with *V. cholerae* El Tor *C6706* biotype affects AMPs production which are downstream of IMD pathway.

To test this hypothesis, I first raised w^{1118} female flies for 7-8 days followed by oral infection with *C6706* or LB feeding for 24 hours and then measured systemic expression of *diptericin*, *attacin*, and *drosomycin* from whole flies. Although epithelial immunity is the first line of defense in an intestinal infection, but transition or escape of pathogen through peritrophic matrix

into the haemocoel of flies induces a systemic immune response. I found 10-fold increase in gene expression of *diptericin* after 24h of oral infection with *C6706* compared with mock group (Figure 4-7A) and not a significant change in either *attacin* or *drosomycin* gene expression level (Figure 4-7B&C). These results suggest that oral infection of *Drosophila* with *C6706* only affects systemic expression of *diptericin* and not a significant effect on expression of other AMPs. Then, I asked if expression of AMPs is affected in the intestine of flies infected with *V. cholerae*. To answer this question, I compared transcriptional profile data from Fast et al. who assessed the expression profile of intestine from flies orally infected with *C6706* and mock group via RNA-sequencing. Data from his study showed increased expression of intestinal AMPs after oral infection with *C6706* (453)(Figure 4-7D), which overall suggests feeding *V. cholerae* to flies induces activation of IMD pathway through epithelial immunity as well as systemic humoral innate immune responses.



Mock vs *C6706*	
Immunity	
Gene	**Log_2 FC**
DptA	9.11
Dro	8.66
AttC	8.15
DptB	7.82
AttA	5.93
Mtk	5.85
PGRP-SB1	3.99
AttB	3.85
IM18	2.84
AttD	2.52

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*C6706*	45	—	—
ΔAMPs	*C6706*	45	0.11	ns

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*C6706*	10.5	—	—
ΔAMPs	*C6706*	8.5	16.54	<0.0001

Figure 4-7 Contribution of antimicrobial peptide to *V. cholerae* infection is dependent on route of entry
(A) Quantification of relative gene expression of *diptericin* (B) *attacin* (C) and *drosomycin* from whole flies of 8 days old female w^{1118} fed on LB or *C6706* for 24 hours via qPCR. (D) Antimicrobial peptide gene expression from RNA-seq of *Drosophila* whole guts after oral infection with *C6706*. Fold change is based on comparisons between *C6706* and mock (E) Survival curves for iso w^{1118} and Δ*AMPs* infected via oral route of infection with *C6706* (F) Survival curves for iso w^{1118} and Δ*AMPs* infected through pricking flies with *V. cholerae* in the thorax (G) Bacterial load measurements from iso w^{1118} and Δ*AMPs* after 12 and 24 hours of oral infection with *C6706* (H) Bacterial load from iso w^{1118} and Δ*AMPs* after 4 and 8 hours of septic infection with *C6706*. Statistical significance for survival curves was determined using a log-rank (Mantel-Cox) test that represents the survival significance between iso w^{1118} and Δ*AMPs*, 50 flies per genotype were used (10 flies in five vials) and 10 flies per genotype for mock groups. Student unpaired t test was used to compare statistical significance between AMPs gene expression of w^{1118} fed on LB or *C6706*, $P < 0.05$ and each dot represents five flies that were homogenized. For bacterial load comparison, five flies were homogenized per replicate and statistical significance was determined using one-way ANOVA with Sidak correction for multiple comparisons.

Our lab and other group showed that *imd* mutants have an increased survival against oral infection with *V. cholerae* (382,454). We also showed that cell specific inhibition of IMD signaling in enterocyte and progenitor cells results in improved or reduced survival against oral infection with *V. cholerae*, respectively (381). Given the IMD pathway activation results in production of AMPs downstream of the pathway and mutation in the IMD pathway increases survival of flies to *V. cholerae*, I asked if removal of AMPs from the host will have the same beneficial effect as it is seen with upstream components of IMD signalling. To answer this question, I used a mutant fly line that lacks the AMPs (Δ*AMPs*) along with isogenic wildtype flies and measured the survival after oral and septic infection with *C6706*. This Δ*AMPs* mutants contain ten null mutations including mutations that affects *Defensin, Attacin C, Attacin D, Metchnikowin, Drosomycin, Diptericins A, Diptericins B*, *Drosocin, Attacin A* and *Attacin B*. In a previous study, removal of AMPs did not affect IMD and Toll pathway activity suggesting AMPs are only acting as immune effectors and are not required for a functional IMD or Toll pathway (195).

I found that survival of Δ*AMPs* flies was not significantly different from iso w^{1118} flies upon oral infection with *C6706* (Figure 4-7E), however, upon septic infection, Δ*AMPs* died significantly faster compared to control flies (Figure 4-7F). These results suggest that AMPs do not impact epithelial immunity of *Drosophila* to *C6706* infection while AMPs are required in providing protection against systemic infection of *V. cholerae*. Then, I asked how bacterial load of *V. cholerae* in the Δ*AMP* mutants is affected through either oral or septic route of entry. To answer

this question, I measured the internal bacterial load after 12 and 24 hours of starting oral infection and 4 and 8 hours of starting septic infection. I found no difference between bacterial load of the Δ*AMP* mutant and isotype control with oral infection (Figure 4-7G), however, there was significant increase in *V. cholerae* bacterial load at both 4 and 8 hours of septic infection (Figure 4-7H). These results suggest that lack of AMPs had neutral effect on resistance or tolerance of flies to *V. cholerae* oral infection but removal of AMPS in the host impaired resistance of flies to limit bacterial growth and increased mortality.

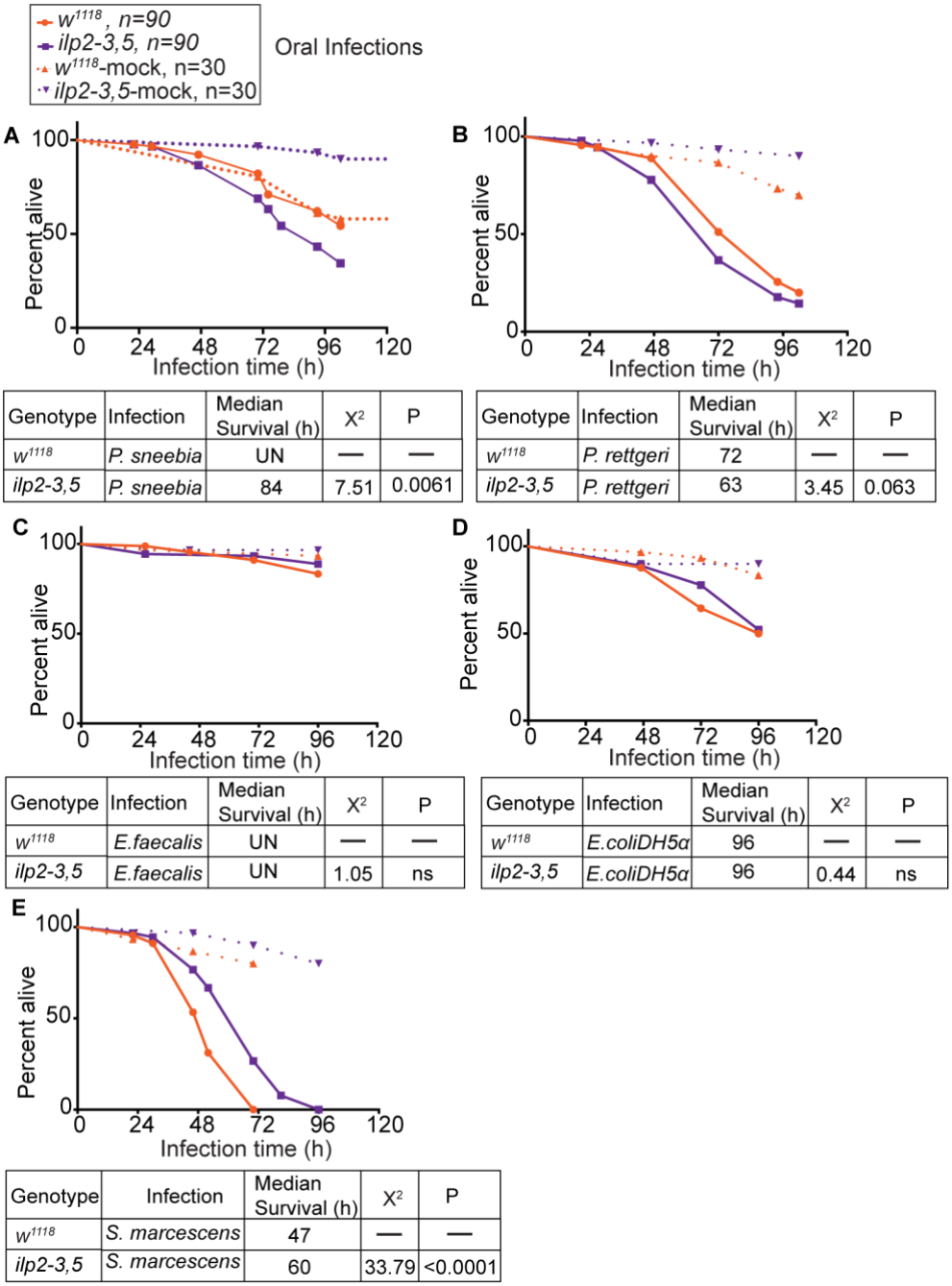
4.2.8 Systemic suppression of insulin signaling has pathogen specific effect on host response to oral infections

I observed that modulation of insulin signaling alters host survival and the dynamics of bacterial growth in the host after oral or septic infection with *V. cholerae* El Tor *C6706* biotype. The impact of insulin signaling in response to each individual pathogen on the host is complex as previous studies have shown that mutation of insulin signaling does not always result in protection of host against pathogens. For example, *chico* mutant flies showed an extended survival against *P. aueroginosa* and *E. faecalis* (438), however, another study showed that *chico* mutants did not show improved survival towards *P. luminescens* or *E. coli*, but an increased cellular immunity was observed for these flies (441). These studies underline the impact of host insulin signaling in response to bacterial infection and highlight a pathogen-specific interaction with host insulin signaling. As I showed that lack of insulin peptides positively contributes to the survival of the host to enteric infection of *C6706*, I asked if protective effect of insulin deficiency on host-pathogens interaction is specific to each infectious microbe introduced to the host.

To answer this question, and to achieve a comprehensive overview of host insulin and pathogens interaction, I challenged *ilp2-3,5* mutants flies with a panel of high to low virulent bacteria including *P. sneebia* (highly virulent), *P. rettgeri* (moderately virulent), *S. marcescens Db11* (highly virulent) , *E. faecalis* (moderately virulent) and *E. coli DH5α* (low virulent). Providencia species *P. rettgeri* and *P. sneebia* are Gram negative bacteria and have been isolated from hemolymph of wild caught *Drosophila* (304) and each induces a different level of mortality in the flies. *P. rettgeri* has been reported to be a cause of travellers' diarrhea (302), urinary tract infections and rare cases of nosocomial infections (455,456). *P. sneebia* septic infections results in 90% mortality in 48 hours of infection, however *P. rettgeri* systemic infection induces 40%

lethality in the same time period (304). *S. marcescens* is a Gram negative bacteria and a lethal enteric pathogen for *Drosophila*. This enterobacterium infects different hosts such as plants, nematodes and mammals (457). *S. marcescens* has been associated with the nosocomial infections identified in the neonatal and intensive care units (458) and many of them are resistant to antibiotics (458,459).

S. marcescens Db11 is a streptomycin-resistant bacteria that has been derived from a *S. marcescens Db10* strain (460) and is lethal in both septic and oral infection with the ability to cross the peritrophic matrix and reaching to the hemolymph, therefore accelerating the pathogenicity of the bacteria (294). *E. faecalis* is a Gram positive bacteria and a natural commensal for *Drosophila* (309). *E. faecalis* resides in the human intestinal tract as well (461) and enteric species causing infection are mostly detected in the hospitals and recently have become resistant to many antibiotics (462). The non-pathogenic *E.coli DH5α* is a Gram negative bacteria and wildtype flies survive systemic infection with this bacteria (463). To understand the *ilp2-3,5* mutants response to each bacterial infection, I fed 7-8 days female *ilp2,3,5* mutants or w^{1118} flies with each pathogen along with LB as mock control group. I monitored the survival of flies for 5 days post oral infection and terminated the survival experiment by day 5 to avoid counting death resulted from starvation.



Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*P. sneebia*	UN	—	—
ilp2-3,5	*P. sneebia*	84	7.51	0.0061

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*P. rettgeri*	72	—	—
ilp2-3,5	*P. rettgeri*	63	3.45	0.063

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*E.faecalis*	UN	—	—
ilp2-3,5	*E.faecalis*	UN	1.05	ns

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*E.coliDH5α*	96	—	—
ilp2-3,5	*E.coliDH5α*	96	0.44	ns

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*S. marcescens*	47	—	—
ilp2-3,5	*S. marcescens*	60	33.79	<0.0001

Figure 4-8 *ilp2-3,5* mutant flies induce a unique response after oral infection with bacterial pathogens

(A) Survival curves for oral infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. sneebia* as well as for w^{1118} and *ilp2-3,5* mutant flies fed with LB only. UN=Undefined (B) Survival curves for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *P. rettgeri* (C) Survival curves for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *E. faecalis* (D) Survival curves for oral infection of w^{1118} and *ilp2-3,5* mutant flies infected with *E.coli DH5α* (E) Survival curves for oral infection of w^{1118} and *ilp2-3,5* mutant flies infected with *S. marcescens Db11*. For survival experiments, 90 flies per genotype (30 flies in three vials) were used for oral infection with *C6706* and 30 flies per genotype for mock groups. Statistical significance for survival curves was determined using a Log-

rank (Mantel-Cox) test that represents the survival significance between w^{1118} and *ilp2-3,5* mutant flies.

I found that feeding *ilp2-3,5* mutants and w^{1118} flies with LB did not induce a lethal effect in all survival curves. *ilp2-3,5* mutant flies showed a reduced survival compared with wildtype after oral infection with *P. sneebia* (Figure 4-8A). Median survival identified for *ilp2-3,5* mutants was 93 hours, however, the median survival was undefined for w^{1118} flies as more than 50% of wildtype flies were still alive at the longest time reported for these flies post oral infection which was 102 hours. Although the median survival of *ilp2-3,5* mutants was smaller than w^{1118} after oral infection with *P. rettgeri* but comparison of survival curves between two genotypes did not result in a statistically significant difference between two genotypes (Figure 4-8B). Oral infection of *ilp2-3,5 mutants* with *E. faecalis* and *E. coli DH5*α showed no significant difference in survival between *ilp2-3,5 mutants* and w^{1118} flies (Figure 4-8C&D). These observation were not surprising as *E. coli* is a non-pathogenic bacteria and *E. faecalis* is also naturally observed as *Drosophila* commensals. I found *ilp2-3,5* mutants showed a significant survival extension compared to w^{1118} flies after oral infect with *S. marcescens Db11* (Figure 4-8E). Similar to *V. cholerae, S. marcescens Db11* is a lethal enteric pathogen and these observations suggest a possible crosstalk between insulin peptide and intestinal pathogens for the survival of host. Overall, I found that *ilp2-3,5 mutants* have a different respond to each pathogen and absence of insulin peptide in the host results in reduced, increased or neutral effect for the host survival.

4.2.9 Loss of insulin has microbe-dependent effects on bacterial burden

Tolerance and resistance are defence strategies that host use to combat infections. Tolerance is composed of processes that improves host fitness during infection with no distinct impact on bacterial load, however, resistance strategies protects host against infection by eliminating bacterial pathogens (1). Therefore, correlating survival outcome and pathogen load is a useful method to investigate the outcome of infection for different pathogens. I asked if insulin loss changes tolerance or resistance to pathogens with different pathogenicity levels. To answer this question, I first compared the survival of *ilp2-3,5* mutant flies with wildtype flies and correlated it with the internal bacterial load collected at 24 and 48 hours of oral infection. As *ilp2-3,5* mutants have a significantly reduced weight compared to wild-type flies, I also normalized the CFU per fly to weight to correct for the difference in body size of *ilp2-3,5* mutants and wildtype flies. If bacterial load in the *ilp2-3,5* mutant flies does not change after controlling for the weight, it suggests that

pathogen burden is not affected by *ilp2-3,5* mutation and is independent of smaller body size of *ilp2-3,5* mutant flies.

I fed *ilp2-3,5* mutants and wildtype flies with each pathogen and collected flies at 24 and 48 hours after starting the oral infection and then plated the homogenized flies on agar plates to count CFUs. To understand the difference between bacterial loads of wildtype and insulin deficient flies over 24 and 48 hours of oral infection, I used two-way ANOVA with both genotype and time as variables to test for a significant impact on variation in bacterial load. I found that bacterial load is similar in *ilp2-3,5* mutant and w^{1118} flies at both 24 and 48 hours of oral infection with *P. sneebia* (Figure 4-9A) and the CFUs remained similar after I corrected for host weight (Figure 4-9B). As I previously showed *ilp2-3,5* mutants had a reduced survival upon oral infection with *P. sneebia* (Figure 4-8A), combining the survival and bacterial load data, these results suggest an impaired tolerance in flies with insulin deficiency to *P. sneebia* oral infection. Oral infection of *ilp2-3,5* mutant flies with *P. rettgeri* resulted in no significant difference in bacterial load after 24 hours of infection but insulin deficient flies showed a slight reduced bacterial load compared to wildtype flies at 48 hours of oral infection (Figure 4-9C). However, once I normalized the CFUs per fly to the weight, the significant difference between bacterial load of *ilp2-3,5* mutant and w^{1118} flies disappeared (Figure 4-9D). Survival of *ilp2-3,5* mutant flies showed a slight reduction after infection with *P. rettgeri* (Figure 4-8B) and as bacterial load was lower after 48 hours of infection, these results suggest an impaired resistance towards *P. rettgeri* infection in insulin deficient flies.

S. marcescens DB11 bacterial load was not significantly different in *ilp2-3,5* mutants compared with control counterparts after 24 hours of oral infection but reduced significantly by 48 hours (Figure 4-9E), however, upon correction for weight there was no significant difference between *ilp2-3,5* mutants and wildtype flies bacterial load (Figure 4-9F). As insulin deficient flies showed a significant extension of survival upon intestinal infection with *S. marcescens DB11* (Figure 4-8E) and reduced bacterial load by 48 hours of infection, theses phenotypes suggest an improved resistance for *ilp2-3,5* mutant flies to this enteric pathogen. Oral infection with *E. coli DH5α* resulted in higher bacterial load in 48 hours of infection in *ilp2-3,5* mutants (Figure 4-9G). After I normalized the CFUs for the weight, the difference between bacterial load of *ilp2-3,5* mutant and w^{1118} flies remained significant (Figure 4-9H). Survival curves of *ilp2-3,5* mutant and wildtype flies was not significantly different upon oral infection with *E.coli DH5α* (Figure 4-8D),

and given the reduced bacterial load after 48 hours of infection, these results suggest insulin deficient flies have a neutral impact on resistance or tolerance of flies fed with *E.coli DH5α*. For *S. marcescens DB11* and *P. rettgeri* infections, normalizations of bacterial load to the weight altered the difference observed between CFUs of *ilp2-3,5* mutants and wildtype flies suggesting reduced bacterial load of pathogen in *ilp2-3,5* mutants is due to a direct effect of reduced body size. However, for *P. sneebia* and *E.coli DH5α* oral infections, correction for weight did not have an effect on bacterial load suggesting the difference observed between two genotypes`s bacterial burden is independent of smaller body size of *ilp2-3,5* mutant flies. Overall, these results indicate that host-pathogens interactions during an infection is complex and host insulin deficiency affects ability of each pathogens to proliferate in the host in a specific way.

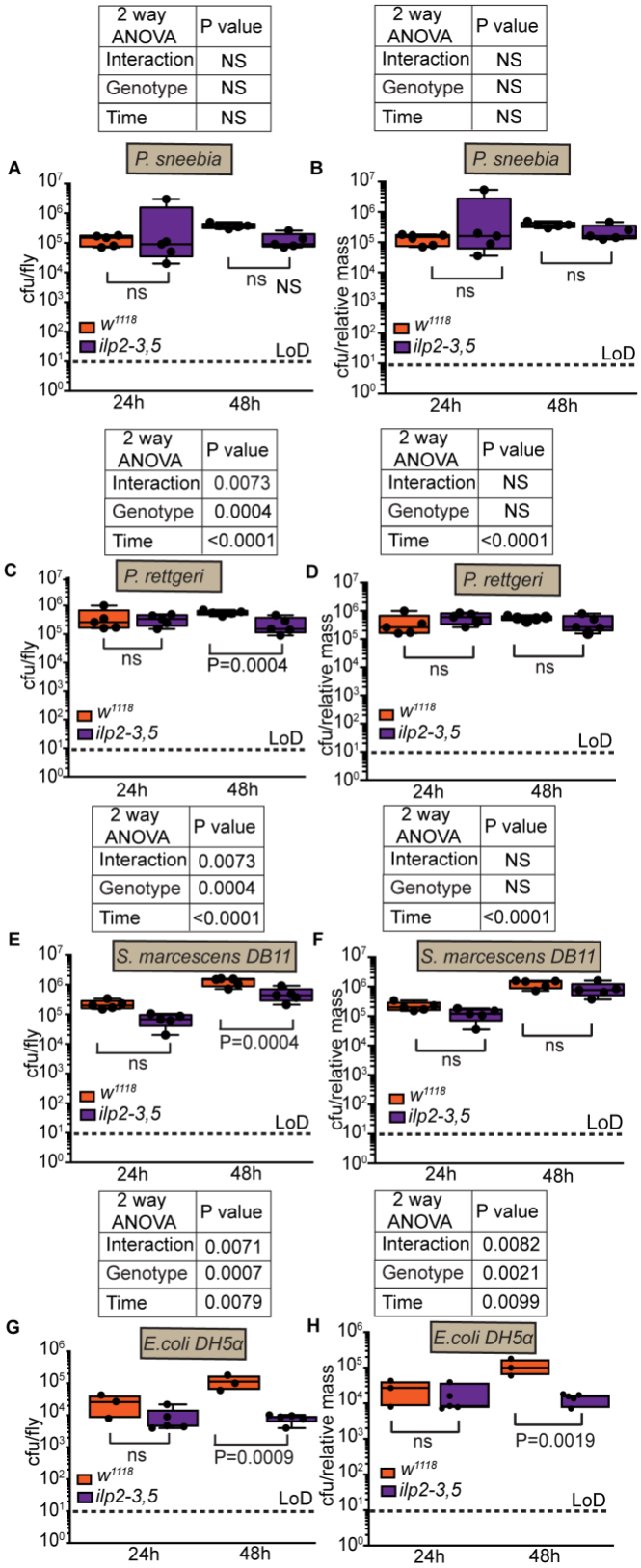


Figure 4-9 Effect of insulin deficiency on bacterial load regulation is pathogen specific

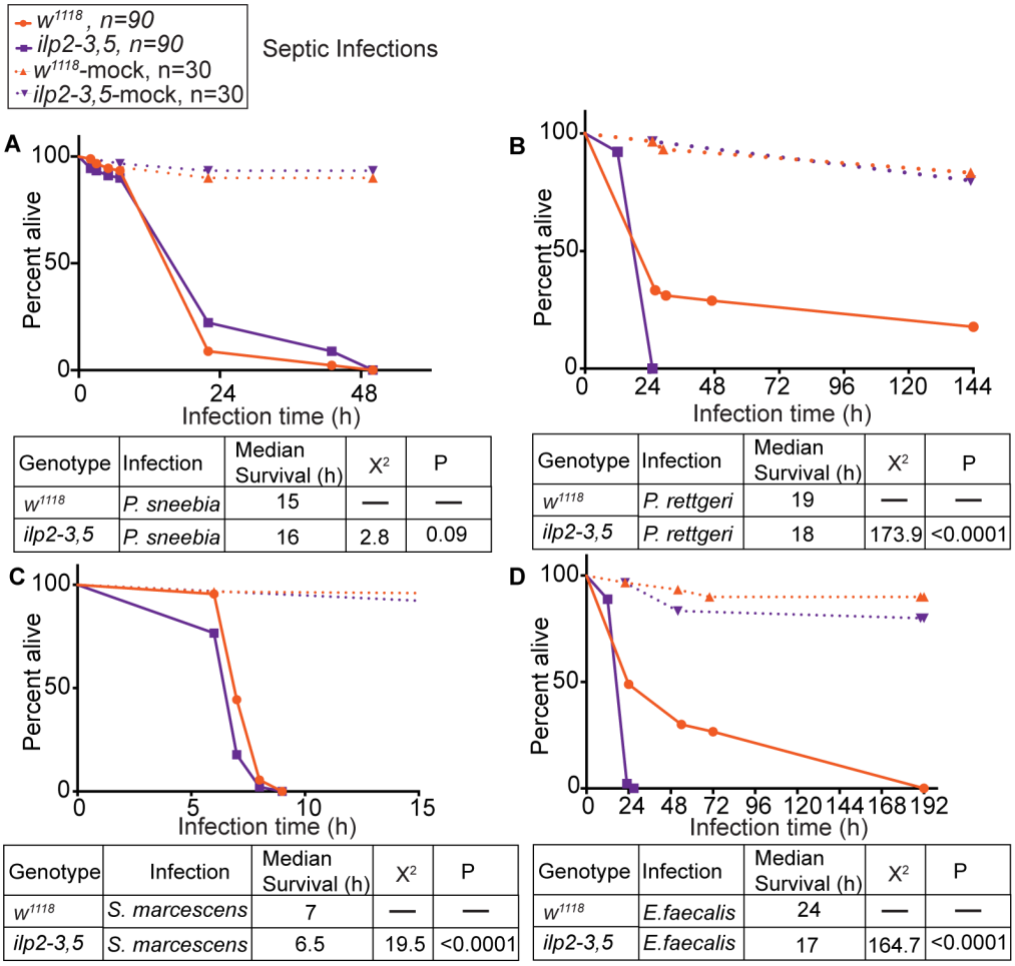
(A) Bacterial load per fly for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *P. sneebia* (B) Bacterial load per fly normalized to weight for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *P. sneebia* (C) Bacterial load per fly for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *P. rettgeri* (D) Bacterial load per fly normalized to weight for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *P. rettgeri* (E) Bacterial load per fly for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *S. marcescens Db11* (F) Bacterial load per fly normalized to weight for oral infection of w^{1118} and *ilp2-3,5* mutant flies fed with *S. marcescens Db11* (G) Bacterial load per fly normalized to weight for oral infection of w^{1118} and *ilp2-3,5* mutant flies infected with *E. coli DH5α* (H) Bacterial load per fly normalized to weight for oral infection of w^{1118} and *ilp2-3,5* mutant flies infected with *E. coli DH5α*. Each dot represents five flies that were homogenized for bacterial load comparison that were collected at 24 and 48 hours of oral infection with respective pathogen, and statistical significance was determined using two-way ANOVA with Sidak correction for multiple comparisons.

4.2.10 Route of infection alters *ilp2-3,5* mutants response to the same pathogenic microbe

Previous studies highlight the impact of route of infection for the host and disease outcomes in flies and other insects. Oral or systemic infections engage different physiological responses in the host (289). As I observed *ilp2-3,5* mutants induce a specific response to each pathogen after oral infection with a spectrum of low to high virulent microbes and given the route of infection is an important contributor of pathogenicity and host survival, therefore, I asked if *ilp2-3,5* mutants induces a general or specific response for distinct routes of pathogen entry. To answer this question, I induced a systemic infection in the *ilp2-3,5* mutant flies and wildtype controls through inoculation of pathogens by intra-thoracic pricking and monitored fly survival in 29°C. As a control, I also pricked *ilp2-3,5* and w^{1118} flies with sterile needle and monitored their survival along with the infected flies. I compared the survival curves of *ilp2-3,5* mutants injected with each pathogen with the survival curves of wildtype flies. I found that in both genotypes, pricking flies with an uncontaminated needle had minimal effect on the host survival. However, I showed *ilp2-3,5* mutants had a different survival curve for each specific pathogen. For example, septic infection with *P. sneebia* did not induce a significant difference between survival curves of *ilp2-3,5* and w^{1118} flies (Figure 4-10A).

P. sneebia has been isolated from hemolymph of wild caught *Drosophila* and avoids recognition by IMD pathway. This result suggest that *P. sneebia* cannot easily avoid immune or other humoral defence responses in *ilp2-3,5* mutant flies. In contrast, the survival of *ilp2-3,5* mutants infected with *P. rettgeri, S. marcescens Db11*, or *E. faecalis* was significantly reduced compared to the survival of control w^{1118} flies (Figure 4-10B-D). The pathogenicity of *S. marcescens Db11* through septic infection is very high as both wildtype and *ilp2-3,5* mutants

succumbed to death within 10 hours of infection and although the median survival is not significantly different between two genotypes, but in the first 7 hours of infection, more than 77% of *ilp2-3,5* mutants died compared to 55% of wildtype flies, which suggest that lack of insulin in host result in a more rapid death to septic infection with *S. marcescens Db11* compared to wildtype flies.



Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*P. sneebia*	15	—	—
ilp2-3,5	*P. sneebia*	16	2.8	0.09

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*P. rettgeri*	19	—	—
ilp2-3,5	*P. rettgeri*	18	173.9	<0.0001

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*S. marcescens*	7	—	—
ilp2-3,5	*S. marcescens*	6.5	19.5	<0.0001

Genotype	Infection	Median Survival (h)	X^2	P
w^{1118}	*E.faecalis*	24	—	—
ilp2-3,5	*E.faecalis*	17	164.7	<0.0001

Figure 4-10 Route of pathogen entry affects *ilp2-3,5* mutant flies response to each bacteria
(A) Survival curves for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. sneebia* as well as for w^{1118} and *ilp2-3,5* mutant flies stabbed with sterile needle in the thorax (B) Survival curves for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. rettgeri* (C) Survival curves for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *S. marcescens Db11* (D) Survival curves for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *E. faecalis*. Statistical significance for survival curves was determined using a Log-rank (Mantel-Cox) test that represents the survival significance between w^{1118} and *ilp2-3,5* mutant flies. For survival

experiments, 90 flies per genotype (30 flies in three vials) were used for septic infection and 30 flies per genotype for mock groups.

Overall, the difference between *ilp2-3,5* mutants survival curves after oral and septic infection with the same pathogens indicates that route of infection is an important factor in the pathogenicity of the microbes and *ilp2-3,5* mutants that survival an oral infection do not become less susceptible with the same pathogen via systemic infection.

4.2.11 Loss of insulin has pathogen specific effects on bacterial load during septic infection

After oral infection, bacterial pathogen have to bypass different layers of defence in the intestinal epithelium such as physical barriers composed of peritrophic matric and epithelial integrity (464), production of ROS (465) and secretion of AMPs through IMD pathway activation (294,299,313). However, once microbes are deposited into the body cavity of flies via septic infection, the pathogens will be directly exposed to the humoral and cellular immunity. Given the *ilp2,3,5* mutants showed different tolerance or resistance phenotypes towards each pathogens via oral infection and considering the vulnerability of host and defence strategies for fighting and reducing bacterial load is different with oral or septic route of infection, I asked how bacterial burden of *ilp2-3,5* mutants is affected by septic infection and if correcting for weight changes the impact of insulin deficiency on bacterial burden in the host. To answer this question, I collected flies at 6 and 12 hours after starting septic infection and homogenized the flies and streak them on Agar plates to count number of internal bacterial loads.

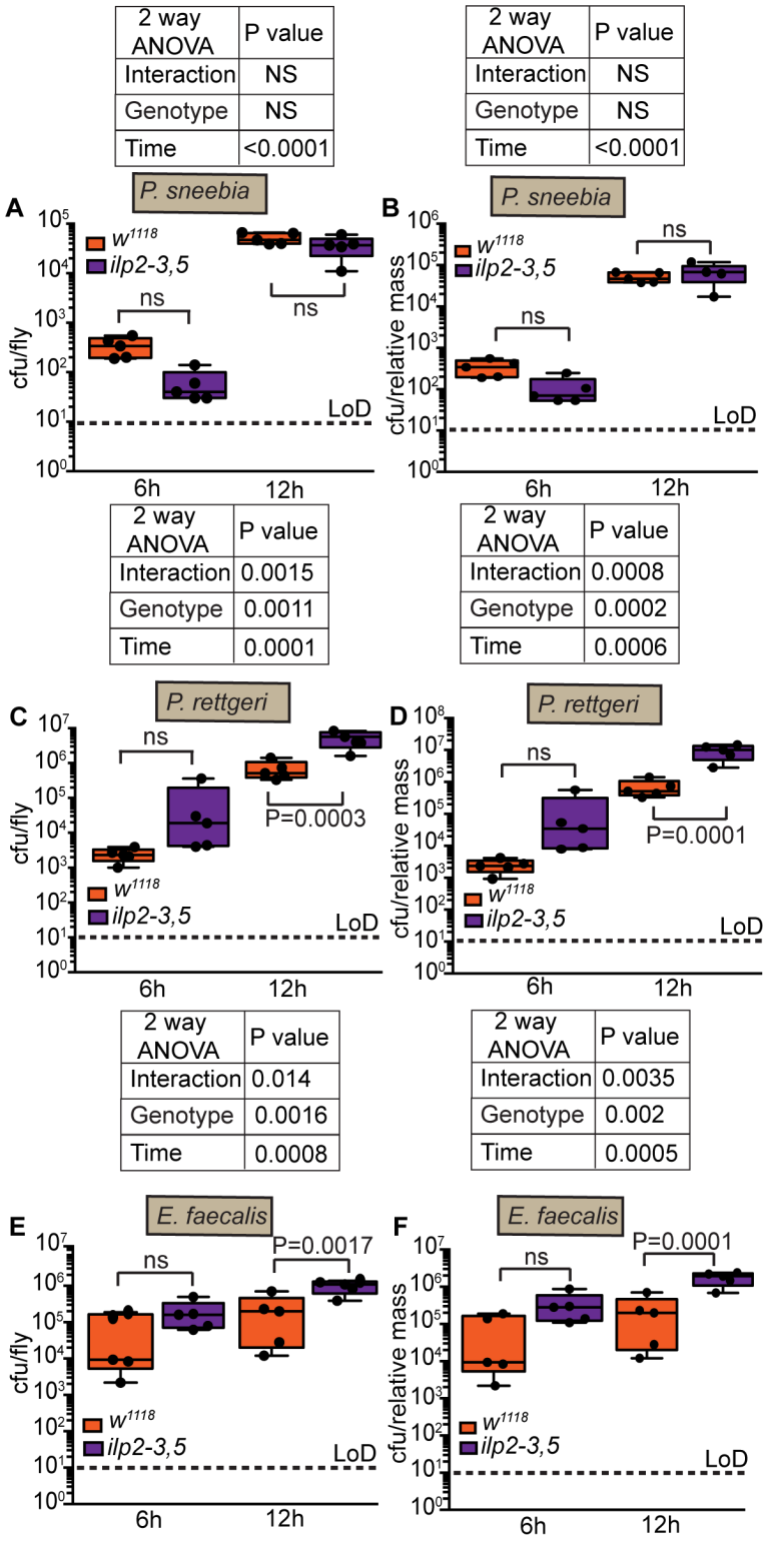


Figure 4-11 Comparison between different pathogens bacterial load following septic infection in *ilp2-3,5* mutants

(A) Bacterial load per fly for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. sneebia* (B) Bacterial load per fly normalized to weight for septic infection of w^{1118} and *ilp2-3,5*

mutant flies infected with *P. sneebia* (C) Bacterial load per fly for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. rettegri* (D) Bacterial load per fly normalized to weight for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *P. rettgeri* (E) Bacterial load per fly for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with E. faecalis (F) Bacterial load per fly normalized to weight for septic infection of w^{1118} and *ilp2-3,5* mutant flies infected with *E. faecalis*. Each dot represents five flies that were homogenized for bacterial load comparison that were collected at 6 and 12 hours of septic infection with respective pathogen, and statistical significance was determined using two-way ANOVA with Sidak correction for multiple comparisons.

Bacterial load of *ilp2-3,5* mutant and wildtype flies had no significant difference at both 6 and 12 hours of septic infection with *P. sneebia* (Figure 4-11A) and once I normalized the CFUs for weight this trend remined unchanged (Figure 4-11B). As *ilp2-3,5* mutant flies showed increased survival compared to wildtype flies after septic infection (Figure 4-10A) with *P. sneebia* while they had similar bacterial load to their wildtype counterpart, these phenotypes suggest an improved tolerance of *ilp2-3,5* mutant flies to *P. sneebia*. Bacterial load of *P. rettgeri* in *ilp2-3,5* mutants and control group was not significantly different after 6 hours of septic infection, however, insulin deficient flies showed a significant 10 fold increase in bacterial load by 12 hours of infection compared with control (Figure 4-11C). Once I corrected the CFUs for weight, there was still higher CFUs for *ilp2-3,5* mutants compared with controls (Figure 4-11D) suggesting bacterial load is not affected by *ilp2-3,5* mutants weight.

P. rettgeri septic infection resulted in reduced survival in insulin mutant flies (Figure 4-10B) while the bacterial load stayed significantly higher than w^{1118} flies which suggests an impaired resistance in *ilp2-3,5* mutants which eventually results in host death. Similar to *P. rettgeri*, septic infection with *E. faecalis* resulted in higher bacterial load in *ilp2-3,5* mutant flies after 12 hours of infection (Figure 4-11E) and normalization of CFUs to the weight did not alter significant difference between *ilp2-3,5* mutants and wildtype flies (Figure 4-11F). *ilp2-3,5* mutants succumbed to death within 24 hours of injection with *E. faecalis* (Figure 4-10D) and contained higher bacterial load compared with control flies, therefore, reduced survival and increased bacterial burden in *ilp2-3,5* mutants suggest an impaired resistance of *ilp2-3,5* mutant to *E. faecalis* systemic infection as well.

These observations suggest that route of infection is an important factor to consider while evaluating the effect of host insulin for resistance or tolerance for any given pathogens. I observed a different bacterial load levels in *ilp2-3,5* mutants for the same pathogen with two different route of microbe entry. This observation suggests that *ilp2-3,5* mutant flies use specific defence strategies

to remove or tolerate a microbial pathogen and these approaches are specific for each pathogenic bacteria meaning protection to an infection through one route does not lead to protection against the same pathogen via different route of entry.

4.3 Summary

V. cholerae is an intestinal pathogens that has become a public health threat (342). This pathogen attacks intestinal epithelial cells, inverts the host metabolism for its own advantages, and competes with the gut resident symbionts for space and nutrients to progress its colonization. As infection progresses, *V. cholerae* dynamically alters virulence gene expression to facilitate its own metabolic needs. As there was no study characterizing the effects of *V. cholerae* El Tor strains, causing the ongoing cholera 7th pandemic, on host metabolism in the *Drosophila* model system, therefore, I asked how modulation of the insulin signaling affects host survival against *V. cholerae C6706,* an El Tor biotype. I found that flies that lack insulin peptides have an increased survival after oral infection with *V. cholerae*, while flies with increased systemic insulin activity showed a reduced survival in respond to oral infection with *V. cholerae*. I showed that total glucose levels is diminished and adult flies have higher levels of circulatory glucose after oral infection with *C6706*. I then asked if a high glucose diet improves survival of wild type flies infected with *V. cholerae* and showed holidic diet supplemented with glucose extends host survival upon oral infection with *V. cholerae.*

It has been shown that IMD signaling contributes to the pathogenesis of the *V. cholerae* during oral infection but the role of AMPs which are produced downstream of IMD signaling was not clear during enteric infection with *V. cholerae*. I used a mutant fly line that lacks the AMPs (Δ*AMPs)* along with isogenic wildtype flies and measured the survival after oral and septic infection with *V. cholerae.* I found that Δ*AMPs* flies show no significant difference in the survival after oral infection, however, upon septic infection Δ*AMPs* die significantly faster compared to control flies. These results suggest AMPs are not involved in survival of flies after intestinal infection with *V. cholerae* while they are important to combat systemic infection with *V. cholerae.* Then, I asked if insulin deficiency was protective against different pathogens as well or the increased survival of *ilp2-3,5* mutants flies upon oral infection with *V. cholerae* was specific to this pathogen.

To answer this question, I challenged *ilp2-3,5* mutants flies with a panel of high to low virulent bacteria and measured survival and bacterial load after oral and septic infection with each

pathogen. I found that *ilp2-3,5* mutants have a pathogen specific respond towards different pathogens, and protection to infection through one route does not lead to protection against the same pathogen via different route of entry. In summary, results from this chapter uncovers effects of oral infection with *V. cholerae C6706* on carbohydrate metabolism in *Drosophila*. Lack of insulin peptides in *Drosophila* improves host survival against *V. cholerae* while increased insulin signaling contributes to the pathogenesis of *V. cholerae* after oral infection. This chapter in in line with previous observation showing a connection between insulin and immune activity. These results indicate that impact of insulin on host responses to infection is a function of the infectious microbe and the route of infection. Future studies are required to understand mechanism of how insulin modifies immune response to contain microbes.

Chapter 5:

Suppression of insulin and IMD signaling in the fat body has protective effects against enteric *V. cholerae* infection

5.1 Introduction

Once a pathogen invades the host, a collection of defence strategies take place to maintain the health and fitness of the host and control the pathogen burden. Resistance are control strategies that reduce the pathogen load while tolerance are defence processes that limit the damages induces by the pathogens or disruption derived from increased host immune activation without affecting pathogen load (254). In chapter 4, I showed that insulin modifies host immunity to *V. cholerae*. I found that lack of insulin peptides in *Drosophila* protects the host from oral infection with *V. cholerae* with limiting bacterial burden which suggest a resistance strategy. However, systemic increase in insulin signaling activity reduces the host survival to *V. cholerae* and bacterial loads are significantly higher in flies with increased insulin signaling activity which suggest an impaired resistance in the host. I also showed that raising flies on holidic diet supplemented with glucose extends host survival to oral infection with *V. cholerae* while the bacterial load between flies raised on holidic diet and holidic diet supplemented with glucose remained similar. This observation suggests that glucose supplemented diet increases host tolerance to oral infection with *V. cholerae*.

Insulin signaling activation in the fat body promotes lipid and glycogen synthesis. A previous study showed that oral infection with *V. cholerae* MO10 biotype depletes lipid stores in the *Drosophila* fat body (419). Suppression of the insulin receptor from the fat body increases the expression of immune response genes and alters sensitivity to infection (406). Given that the *Drosophila* fat body is a multifunctional tissue and integrates both immune and metabolic signaling, I was interested to understand if modulation of insulin signaling in the fat body affects host response to intestinal infection with *V. cholerae*. In this chapter, I asked if tissue-specific inhibition of insulin signaling in the fat body affects the defense response towards *V. cholerae* oral infection. I examined the alteration in macronutrients in the intestine of flies with suppressed insulin signaling in the fat body before and after enteric infection with *V. cholerae*. I also looked at the effects of blocking insulin signaling in the fat body on the intestinal stem cell division as well as enteroendocrine population before and after infection with *V. cholerae*. I asked these questions to understand if suppression of insulin signaling in the fat body improves tolerance or resistance towards *V. cholerae* intestinal infection and if it does what are the possible mechanisms that contributes to these defence strategies.

Our lab showed that cell specific inhibition of IMD signaling in enterocyte and progenitor cells results in improved or reduced survival against oral infection with *V. cholerae*, respectively (381). Infection in the *Drosophila* intestine triggers a systemic anti-microbial responds in the fat body that emphasizes an immunological crosstalk between distant organs (383). Given that the *Drosophila* fat body is responsible for production of AMPs against pathogens via activation of IMD pathway and previous data regarding protection of *imd* mutants against *V. cholerae* (382,420,454), I asked if inhibition of IMD in an immune-metabolic tissue such as the fat body modifies immunity against *V. cholerae* enteric infection. First, I looked at the survival and bacterial load as outcomes of the host immune response in the flies with suppressed IMD signaling in the fat body and control. As I observed improved tolerance in flies with suppressed IMD signaling activity in the fat body upon oral infection with *V. cholerae*, I investigated further to understand the possible mechanisms involved in improved tolerance of flies with blocked IMD signaling in the fat body. Impaired intestinal barriers results in translocation of pathogen to the hemolymph, therefore, I compared the bacterial load in the hemolymph of flies with suppressed IMD signalling in the fat body and controls. In addition to that, as one of the tolerance mechanisms in the intestine is increasing antioxidant levels to protect the host from ROS damage induced by pathogen, I measured the expression level of genes involved in transcription of antioxidants in the intestine of flies with suppressed IMD signaling in the fat body fed with *V. cholerae* or with LB media. Combined, in this chapter, I investigated the effects of modulation of insulin and IMD signaling in the fat body upon oral infection with *V. cholerae*.

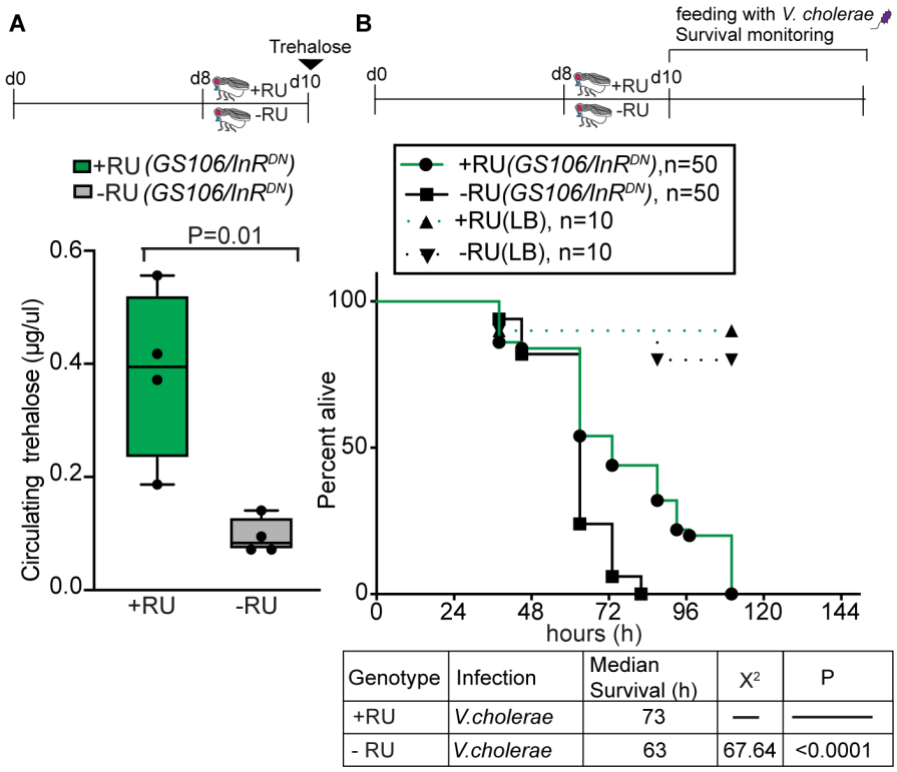
5.2 Results

5.2.1 Suppression of insulin signaling in the fat body extends host survival against enteric *V. cholerae*

In multicellular organisms, inter-organ communications are complex, and critical for the maintenance of homeostasis. Once a host is invaded by pathogens, the crosstalk between different organs becomes more important to coordinate immune responses with metabolic adaptations to protect the host from fatal damages induced by microbes. For example, stress stimulation by ROS or infection in the *Drosophila* intestine triggers production of AMPs in the fat body which emphasizes an immunological crosstalk from the gut to the fat body (466). Another study showed that activation of IMD signaling in the *Drosophila* intestine increases the sorbitol and galactitol

levels in the hemolymph and these sugar molecules relay a signal from gut to the fat body to activate IMD in the fat body (383). Therefore, different organs in an organism rely on each other to maintain homeostasis, however, how distant organs in the host communicate in the context of infection is not clear yet. As one of the main responsibilities of the fat body in *Drosophila* is to respond to the metabolic status of the fly by modulating metabolic signals such as the insulin signaling pathway, I was curious to understand if a tissue specific inhibition of insulin signaling in a metabolic organ such as the fat body affects host respond to enteric infection with *V. cholerae*.

After each meal, activation of the evolutionary conserved insulin pathway results in increased glucose transport to cells and synthesis of trehalose or glycogen from extra glucose in the fat body of *Drosophila*. During starvation in *Bombyx mori* (467), degradation of glycogen stores in the fat body and conversion to trehalose results in increased hemolymph trehalose levels. This study suggested that reduced insulin signaling results in increased degradation of energy supplies to provide energy units to other tissues. Trehalose is a non-reducing disaccharide and the dominant sugar present in the hemolymph of *Drosophila* (468). To confirm inhibition of insulin signaling in the fat body dysregulates sugar metabolism in *Drosophila*, I measured the circulatory trehalose levels in flies with suppressed insulin signaling in the fat body and compared it with a control group. Specifically, I used transgenic flies expressing a dominant negative insulin receptor (*UAS-InR^{DN}*) with a fat body specific GeneSwitch 108 driver. This system consists of a GAL4-progesterone receptor fusion protein that induces expression of the gene of interest once the activator RU486 (mifepristone) is added to the food (78). Therefore, using this system I avoid negative effects of insulin signaling inhibition during development. I first raised *GS106>InR^{DN}* female flies for 8 days on Bloomington normal food and then transferred them to food vials containing either RU486 on the food surface (+RU486) or ethanol (-RU486). I kept flies on the +RU486 or -RU486 food for 48 hours to allow the expression of *UAS-InR^{DN}* in the fat body of RU486-fed flies.



Genotype	Infection	Median Survival (h)	X^2	P
+RU	*V.cholerae*	73	—	———
- RU	*V.cholerae*	63	67.64	<0.0001

Figure 5-1 Inhibition of insulin signaling in the fat body protects the host from *V. cholerae* infection (A) Comparison between circulatory trehalose levels of flies with suppressed insulin signaling in the fat body, +RU486 (*GS106>InR^{DN}*) and control flies, -RU486 (*GS106>InR^{DN}*). Schematic representation of experimental design shows that *GS106>InR^{DN}* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of insulin signaling in the fat body and flies were collected for trehalose measurement. Each dot represents hemolymph collected from 25 flies. Statistical significance was determined using an unpaired Student t test (B) Survival curves of ±RU486 (*GS106>InR^{DN}*) adult female flies fed with *C6706* or LB. Schematic representation of experimental design shows that *GS106>InR^{DN}* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of insulin signaling in the fat body, then both +RU486 and -RU486 flies were fed with *C6706* and their survival was monitored overtime. The significant difference between +RU486 (*GS106>InR^{DN}*) and control flies, -RU486 (*GS106>InR^{DN}*) was determined using log-rank (Mantel-Cox) test.

I found that after 48 hours of feeding *GS106>InR^{DN}* flies with RU486, the average circulatory trehalose levels was four times higher than control flies (Figure 5-1A). This observation confirms that inhibition of insulin signaling via a GeneSwitch system results in disruption of metabolic homeostasis in the host and increases circulatory trehalose levels. To test if suppression of insulin signaling in the fat body alters host response to intestinal infection of *V. cholerae*, I raised *GS106>InR^{DN}* flies for 8 days at 25°C and then transferred flies to vials containing +RU486 or -RU486 for 48 hours to induce UAS-*InR^{DN}* expression in the fat body. Then, I fed ±RU486

(*GS106>InR^{DN}*) flies *C6706*, or LB media as a control group, and monitored survival. LB media did not show any lethal effect on survival of ±RU486 (*GS106>InR^{DN}*) flies. In contrast, I found that flies with suppressed insulin signaling in the fat body had a 16% extension of survival compared with control group (Figure 5-1B). This result suggests that inhibition of insulin signaling in the fat body protects the flies against *V. cholerae* intestinal infection. This observation was quite interesting as I noticed modulation of insulin signaling in the fat body results in survival improvement in response to an infection that occurs in a distant tissue such as the intestine. Overall, I found that suppression of insulin signaling in the adult *Drosophila* fat body results in increased circulatory trehalose levels and protects the host from an enteric infection with *C6706*.

5.2.2 Inhibition of insulin signaling in the fat body alters *V. cholerae* bacterial load upon oral infection

I showed that inhibition of insulin signaling in the fat body extends survival of the host during oral infection with *V. cholerae* in adult *Drosophila*. During a tolerance defence strategy, host is able to limit the damages induced by pathogen or by increased immune system activation of the host without affecting the pathogen load, while resistance is the ability of the host to limit the pathogen burden (469,470). Measuring the ability of the host to clear pathogens combined with survival outcome suggest if host defence strategies toward infection is through tolerance or resistance. Therefore, I asked how modulation of metabolic signalling in the fat body affects host defence responses and specifically the bacterial burden of *V. cholerae* in the host. To answer this question, I showed in (Figure 5-1B) that suppression of insulin signaling in the fat body extends survival of the host upon oral infection with *C6706* and I decided to measure the bacterial load of ±RU486 (*GS106>InR^{DN})* at 12 and 24 hours of oral infection.

As oral infection of wildtype flies results in a lethal infection for the host, I chosed 12 and 24 hours for bacterial load measurements as by 48 hours of oral infection with *C6706*, 50% of flies succumb to death. To count the number of CFUs from flies infected with *C6706*, flies collected at each timepoints were homogenized, and next day I counted the CFUs on the LB-Agar plates supplemented with Streptomycin. If the bacterial load of RU486-treated flies is similar to untreated controls, these data may suggest improved tolerance of *V. cholerae* infection in flies with suppressed insulin signaling in the fat body. However, if intestinal bacterial loads of flies with inhibition of insulin pathway in the fat body is lower than control group, the data may indicate improved resistance of the host to enteric *V. cholerae* infection. I found that in both timepoints

there was no significant difference between bacterial loads of flies with suppressed insulin signaling in the fat body and control counterparts (Figure 5-2A). These results suggest that the dynamics of *V. cholerae* bacterial burden is not affected in the host while insulin signaling is modulated in the fat body. Overall, stable bacterial burden in +RU486 (*GS106>InR^{DN})* and increased survival upon intestinal infection with *C6706* suggests an improved tolerance of the host towards intestinal infection with *V. cholerae*.

Measuring the kinetics of *V. cholerae* load once flies are consistently fed with pathogen showed no significant difference between flies with suppressed insulin signaling and controls. However, a previous study by the Watnick lab showed that in the absence of continuous ingestion of *V. cholerae*, flies were still colonized by the pathogen (378). As +RU486 (*GS106>InR^{DN})* showed increased tolerance to *C6706* infection through extension of host survival and a similar bacterial burden compared with control flies, I asked if flies with suppressed insulin signaling activity in the fat body will have a slower rate of bacterial clearance in the absence of continuous ingestion of *V. cholerae*. To answer this question, I measured the bacterial burden of *V. cholerae* in the ±RU486 (*GS106>InR^{DN})* flies after 24 and 48 hours of withdrawal from oral infection of *V. cholerae*.

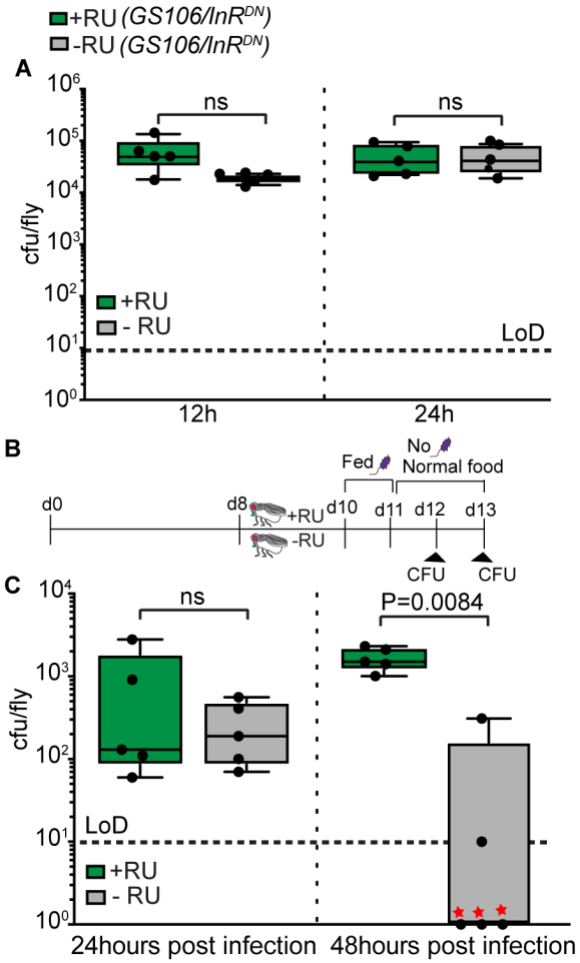


Figure 5-2 *V. cholerae* bacterial growth in flies with reduced insulin signaling in the fat body

(A) Bacterial load of ±RU486 (*GS106>InR^{DN}*) adult female flies at 12 and 24 hours during oral infection with C6706 (B) Schematic representation of experimental design shows that *GS106>InR^{DN}* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of insulin signaling in the fat body and then flies were fed with *V. cholerae* for 24 hours, moved to vials with no pathogen and samples at 24 and 48 hours of transferring to normal food was collected to measure bacterial load (C) Bacterial load of ±RU486 (*GS106>InR^{DN}*) adult female flies measured post 24 and 48h of oral infection with C6706. ±RU486 (*GS106>InR^{DN}*) flies were fed with C6706 for 24 hours and then moved to food vials without any pathogen. At each timepoints, 5 flies per replicate were collected and homogenized to plate on the LB agar plates with Streptomycin. A one-way ANOVA was used to compare statistical significance for CFUs, and the Sidak correction method was used for multiple comparisons. Red asterisk (*) indicates measurements below the limit of detection.

I predicted that if flies with reduced insulin signaling activity in the fat body are more tolerant of *V. cholerae* compared to control flies, I would observe a slower bacterial clearance once flies are moved from oral infection with *V. cholerae* to normal food vials. I first raised

GS106>InR^{DN} flies for 8 days and then treated them with, or without, RU486 for 48 hours for transgene expression followed by oral infection with *C6706* for 24 hours. I then transferred both groups of flies into pathogen-free vials with normal food, and measured the bacterial load 24 and 48 hours after transfer to normal food. I found that 24 hours after transfer, the CFUs of flies with suppressed insulin signaling in the fat body were similar to control flies. However, after 48 hours, the bacterial load in the -RU486 group was 10-fold lower than flies with inhibition of insulin signaling in the fat body (Figure 5-2B). In contrast, the bacterial load of flies with suppressed insulin signaling in the fat body remained steady 48 hours after infection. These observations suggest that after pathogen ingestion has been terminated, bacterial load of *V. cholerae* in the control flies is reduced over time, whereas suppression of insulin signaling in the fat body supports persistence of *V. cholerae* burden in the intestine.

5.2.3 Inhibition of insulin signaling in the fat body alters intestinal epithelium physiology

The *Drosophila* intestine has different levels of defense against pathogens. For example, the peritrophic matrix acts as a physical barrier to protect the intestinal epithelium from microbes (313). Activation of IMD pathway in enterocytes results in production of AMPs that are important immune effectors to inhibit pathogen growth. Enterocytes undergo thinning through extrusion of damaged organelles into the intestinal lumen in response to microbial toxins in order to protect the intestine from other virulence factors produced by the invaded pathogen (269). Intestinal stem cell regeneration replaces damaged enterocytes and maintains the integrity of epithelium in response to pathogens. Although each cell type in the intestinal epithelium has a specific role in the maintenance of metabolic and proliferative homeostasis against stress stimuli or pathogen invasion, coordination between intestine and other peripheral organs has important regulatory effects on growth and metabolic homeostasis (238,323,471–473). For example, Enteroendocrine (EE) cells recognize metabolites in the intestinal lumen and secrete hormone peptides that regulate host lipid and carbohydrate metabolism (323). EE cells maintain metabolic homeostasis in response to infection as well (474). As I showed earlier that insulin signaling suppression in the fat body improves host tolerance to oral infection with *V. cholerae*, I asked if inhibition of insulin signaling in the fat body alters host intestinal epithelium growth and metabolism to protects against *V. cholerae* infection. To answer this question, I counted the number of EEs in the +RU486

(*GS106>InR*^{DN}) and -RU486 (*GS106>InR*^{DN}) flies before and after oral infection with *C6706* using a Prospero antibody as an EE cell marker.

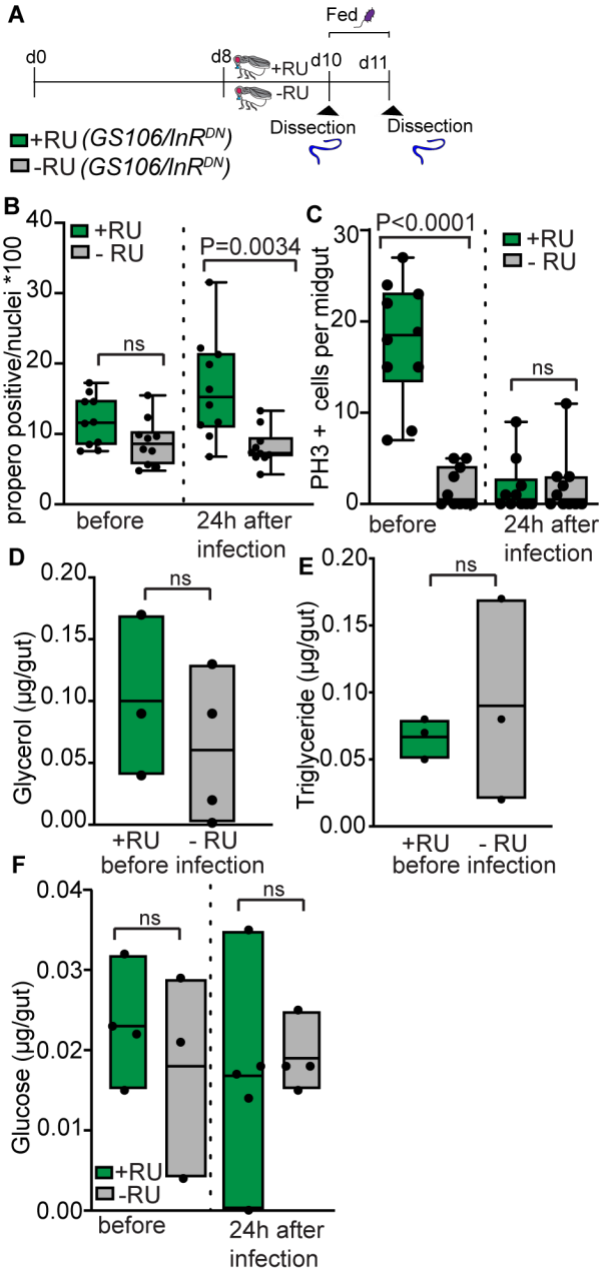


Figure 5-3 Effect of insulin signaling inhibition in the fat body on intestinal physiology and macronutrient levels

(A) Schematic representation of experimental design shows that *GS106>InR*^{DN} flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of insulin signaling in the fat body and then flies were fed with *V. cholerae* for 24 hours, and intestine was dissected after 24 hours of oral infection with *V. cholerae* (B) Number of enteroendocrince cells stained with Prospero antibody was counted per nuclei from dissected guts of ±RU486 (*GS106>InR*^{DN}) flies before and after oral infection with *V. cholerae* (C) Number of PH3 positive

cells per midgut in ±RU486 (*GS106>InR^{DN}*) flies before and after oral infection with *V. cholerae* (D) Total intestinal glycerol levels of ±RU486 (*GS106>InR^{DN}*) flies before oral infection with *V. cholerae* (E) Total intestinal triglyceride levels of ±RU486 (*GS106>InR^{DN}*) flies before oral infection with *V. cholerae* (F) Total intestinal glucose levels of ±RU486 (*GS106>InR^{DN}*) flies before and after oral infection with *V. cholerae*. Statistical significance was determined using an unpaired Student t test.

I raised *GS106>InR^{DN}* female flies for 8 days on Bloomington cornmeal food at 25°C and then transferred to vials containing +RU486 or -RU486 for 48 hours to allow for activation of *UAS-InR^{DN}* in the fat body (Figure 5-3A). By the end of 48 hours, I dissected the intestine from +RU486 (*GS106>InR^{DN}*) and -RU486 (*GS106>InR^{DN}*) flies and stained with Prospero antibody to detect EE cells before infection. I fed the rest of the +RU486 (*GS106>InR^{DN}*) and -RU486 (*GS106>InR^{DN}*) flies *C6706* for 24 hours, followed by dissection of the intestine and staining with the Prospero antibody. I found no significant difference in EE numbers between +RU486 (*GS106>InR^{DN})* and -RU486 (*GS106>InR^{DN})* flies before infection (Figure 5-3B). This observation suggests that suppression of insulin signaling in the fat body does not affect EE cells in the intestine. In contrast, there was a significant increase in EE proportions in the +RU486 (*GS106>InR^{DN})* flies 24 hours after infection (Figure 5-3B). This observation suggests that inhibition of insulin signaling in the fat body increases the EE population in the intestine after oral infection of *V. cholerae.* As EE cells produce secretory hormones that facilitate communication of the gut with the brain or fat body, and it has been shown that in response to infection with *P. entomophila*, EE cells produce a prosecretory transcription factor important for production of peptide hormones and induction of AMPs (475), it is possible that increased EE cells in flies with suppressed insulin signaling after intestinal infection could facilitate communication between gut and distant organs or improve respond to metabolites produced by commensal microbes to protect host from enteric *V. cholerae* infection.

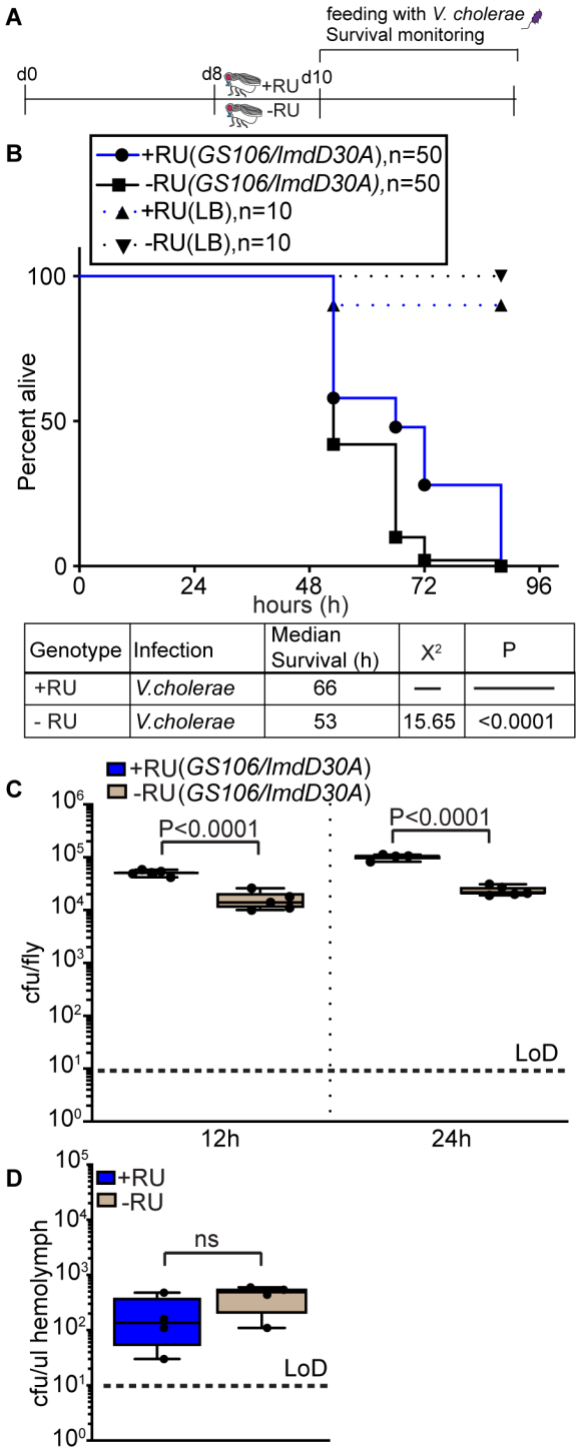
Recent studies in our lab showed that *V. cholerae* intestinal infection induces severe damage to the intestinal epithelium and inhibits tissue renewal in the fly (453,454). Intestinal tissue destruction affects the fly lifespan, therefore, I asked if inhibition of insulin in the fat body improves survival of the flies to enteric infection of *C6706* by increasing the intestinal stem cell proliferation before flies are infected with *V. cholerae*. To answer this question, I stained the intestine of +RU486 (*GS106>InR^{DN})* and -RU486 (*GS106>InR^{DN})* flies with the anti-phospho histone H3

antibody (PH3), which marks dividing stem cells (476). I found that flies with suppressed insulin signaling in the fat body have a significant higher PH3 positive cells compared to control flies before oral infection with *V. cholerae*, however, after infection there was no significant difference between +RU486 (*GS106>InR^{DN}*) and -RU486 (*GS106>InR^{DN}*) flies (Figure 5-3C). This result raises the possibility that increased stem cell division in flies with suppressed fat body insulin signaling before infection improves the ability of the host to maintain intestinal epithelium integrity once host is exposed to pathogens.

To understand if modulation of insulin signaling in the fat body affects macronutrient levels in the intestine, I measured intestinal levels of triglyceride, glycerol and glucose in +RU486 (*GS106>InR^{DN})* and -RU486 (*GS106>InR^{DN})* flies. I found that there was no significant difference between glycerol or triglyceride levels in the intestine of +RU486 (*GS106>InR^{DN}) and* -RU486 (*GS106>InR^{DN})* flies (Figure 5-3E&F). As I previously showed that infection of wildtype flies with *V. cholerae* hemolymph glucose, and given that suppression of insulin in the fat body increased circulating trehalose levels, I asked if increased glucose supplies to peripheral tissues such as the intestine led to the extended survival of +RU486 (*GS106>InR^{DN})* flies with enteric *V. cholerae* infection. To answer this question, I measured total intestinal level of glucose in the ±RU486 (*GS106>InR^{DN})* flies after 24 hours of oral infection with *C6706* but found no significant difference between glucose levels in the intestine of flies with suppressed insulin signaling and the control group (Figure 5-3F). Taken together, these results suggest that insulin signaling inhibition in the fat body alters intestinal stem cells and EE proliferation via communication to the intestine, which improves host tolerance to *V. cholerae* intestinal infection. Although the intestinal macronutrient levels in the host is not altered by insulin signaling suppression in the fat body, it is possible that blocking insulin signaling in the fat body alters absorption of macronutrients in intestinal epithelium cells and possibly affecting growth and differentiation of these cells via alteration in metabolism. However, further experiments are required to investigate if suppression of insulin signaling in the fat body affects signaling molecules that enable communication between fat and gut in the host during infection with *V. cholerae* as well as affects in the epithelial cells uptake of carbohydrates before and after infection with *V. cholerae.*

5.2.4 Inhibition of IMD signaling in the fat body improves host survival against enteric *V. cholerae*

Previously, our lab showed that cell specific inhibition of IMD signaling in enterocyte and progenitor cells results in improved or reduced survival against oral infection with *V. cholerae*, respectively (381). Given that during a systemic infection, the *Drosophila* fat body produces AMPs via activation of IMD pathway, and our previous data regarding protection of *imd* mutants against *V. cholerae* infection (420,454), I asked if inhibition of IMD in an immune-metabolic tissue such as the fat body protects the host from oral infection against *V. cholerae*. To answer this question, I used transgenic flies in our lab that express a dominant negative IMD (*UAS-ImdD30A*) to inhibit IMD signaling in the fat body using the GeneSwitch system. I measured survival and bacterial load as outcomes of the host immune response in the flies with IMD inhibition in the fat body (*GS106>ImdD30A*, fed with RU486, transgene expression is on) along with their control group (*GS106>ImdD30A*, fed with no RU486, transgene expression is off). First I raised *GS106>ImdD30A* female flies at 25°C for 8 days and transferred them to food with either RU486 or no RU486 for 48 hours to induce expression of *ImdD30A* in the fat body. Then I started oral infection with *C6706* for both ±RU486 (*GS106>ImdD30A)* flies and monitored their survival at 29°C. I also fed ±RU486 (*GS106>ImdD30A)* flies with LB media to serve as a control, uninfected group (Figure 5-4A).



Genotype	Infection	Median Survival (h)	X^2	P
+RU	*V.cholerae*	66	—	———
- RU	*V.cholerae*	53	15.65	<0.0001

Figure 5-4 IMD signaling inhibition in the Drosophila fat body extends survival of host against enteric *V. cholerae* infection

(A) Schematic representation of experimental design shows that *GS106>ImdD30A* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of IMD signaling in the fat body and then flies were fed with *V. cholerae* or LB and their survival was monitored overtime. Survival curves of ±RU486 (*GS106>ImdD30A*) adult female flies fed

with *C6706* or LB. The significant difference between +RU486 (*GS106>ImdD30A*) and control flies, -RU486 (*GS106>ImdD30A*) was determined using log-rank (Mantel-Cox) test (B) Bacterial load of ±RU486 (*GS106>ImdD30A*) adult female flies at 12 and 24 hours during oral infection with *C6706*. A one-way ANOVA was used to compare statistical significance for CFUs, and the Sidak correction method was used for multiple comparisons (C) Bacterial load of ±RU486 (*GS106>ImdD30A*) adult female flies from hemolymph collected at 24 hours of intestinal infection with *C6706*. Statistical significance was determined using an unpaired Student t test.

I found that feeding LB did not have any lethal effect on the survival of plus RU486 (*GS106>ImdD30A)* or minus RU486 (*GS106>ImdD30A)* flies. However, I showed a 24.5% survival extension in infected flies with suppressed IMD activity in the fat body compared to control flies (Figure 5-B). This observation suggests that modulation of IMD signaling in the fat body improves host response to enteric infection with *C6706*. To understand how bacterial load of *V. cholerae* is changed by inhibition of IMD signaling in the host fat body, I collected flies from ±RU486 (*GS106>ImdD30A)* groups at 12 and 24 hours of oral infection and counted the CFUs from whole flies. I found at both 12 and 24 hours timepoints, the bacterial load in +RU486 (*GS106>ImdD30A)* flies was significantly higher than control flies (Figure 5-4C). The higher bacterial load in the flies with reduced IMD signaling activity combined with the survival extension of these flies after intestinal infection with *C6706* suggests an improved tolerance to *V. cholerae* once IMD signaling is inhibited in the fat body.

During infection, loss of the intestinal epithelial barrier results in translocation of microbes to the hemolymph, inducing a systemic immune response. I asked if modulation of IMD in the fat body leads to an increased intestinal integrity of *Drosophila*, preventing the pathogen from crossing the midgut barrier, thereby improving the tolerance of host against enteric infection of *V. cholerae.* To answer this question, I investigated if there are detectable bacterial colonies in the hemolymph of ±RU486 (*GS106>ImdD30A*), and if increased tolerance of ±RU486 (*GS106>ImdD30A*) is associated with reduced numbers of *V. cholerae* escaping into the hemolymph. I fed ±RU486 (*GS106>ImdD30A*) flies *C6706* for 24 hours and collected the hemolymph from both +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) flies and plated the hemolymph on LB Agar to count CFUs. I found no significant difference between the bacterial load of flies with suppressed IMD signaling in the fat body and control counterparts (Figure 5-4D). This result suggests that in both control and flies with IMD suppression in the fat body detectable CFUs were observed in the hemolymph after oral infection with *C6706* although the absence of any significant difference between +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) CFUs indicates IMD

suppression in the fat body increase host tolerance to *V. cholerae* oral infection via a mechanism independent of preventing pathogen breach into the hemolymph .

5.2.5 Flies with blocked IMD signaling in the fat body have reduced glucose levels after enteric *V. cholerae* infection

Previously, I showed that oral infection of wildtype flies with *C6706* results in reduced total glucose levels, and increased circulatory glucose (Figure 4-1). The lab of Dr. Paula Watnick also showed that oral infection with the MO10 biotype of *V. cholerae* impairs metabolic homeostasis and suppresses systemic insulin signaling (419). Therefore, I asked how suppression of IMD in the fat body affects ILPs expression and glucose levels once flies are exposed to *V. cholerae* oral infection. To understand how total glucose levels is affected in flies with suppression of IMD signaling in the fat body after oral infection with *V. cholerae*, I first fed flies *C6706*, or LB as a control group, for 24 hours and then measured the total glucose levels in +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) flies. I previously observed a higher bacterial load at both 12 and 24 hours in the flies with suppressed IMD signaling in the fat body after oral infection with *C6706* (Figure 5-4). Therefore, I asked if glucose consumption by *V. cholerae* positively contributes to the increased bacterial burden, then what would happen to the total levels of glucose in the flies with suppressed IMD signaling in the fat body after oral infection with *V. cholerae*. As both genotype and infection were variables in this experiment, I used a two way analysis of variance (ANOVA) to examine the contribution of each variable on the total glucose levels. 2-way ANOVA found significant effects from RU486 meaning suppression of IMD signaling in the fat body suppress total glucose levels and the statistical analysis showed significant effects from interaction of infection and genotype on the glucose levels, however, infection alone did not show to be a significant variable affecting glucose levels (Figure 5-5-B). This result suggests that host total glucose levels are reduced in the flies with inhibition of IMD signaling in the fat body upon *V. cholerae* enteric infection.

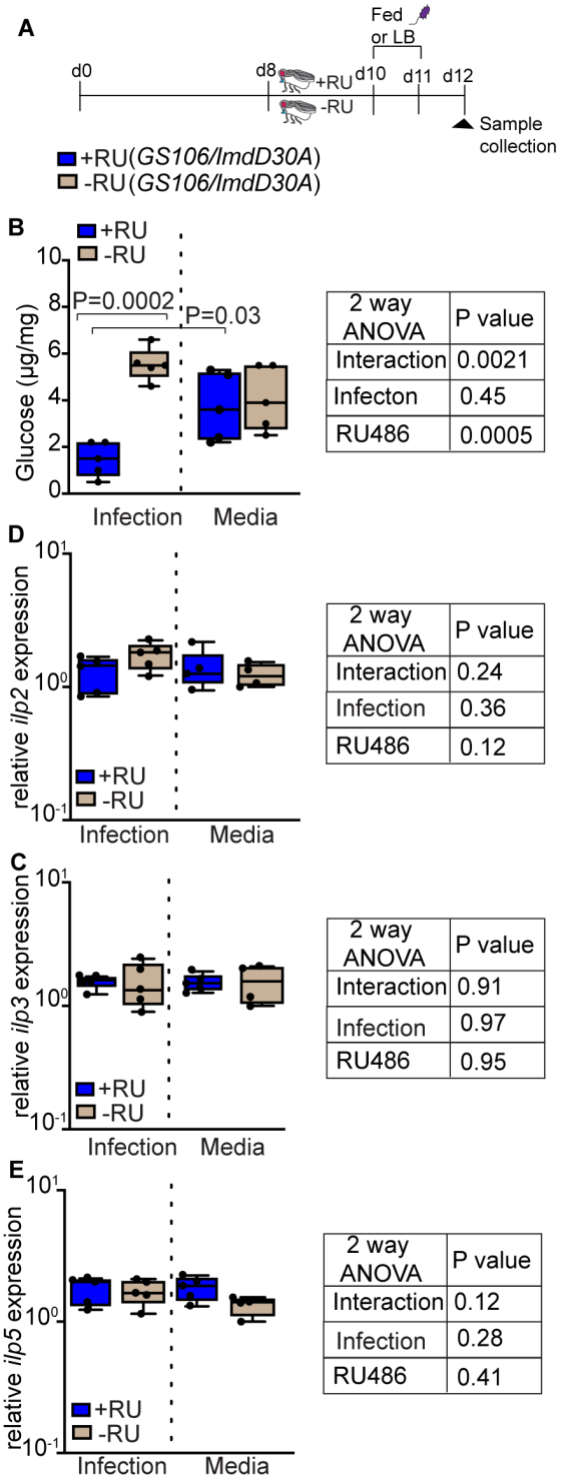


Figure 5-5 Suppression of IMD pathway in the Drosophila fat body alters glucose homeostasis during infection with *V. cholerae*

(A) Schematic representation of experimental design shows that *GS106>ImdD30A* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of IMD signaling in the fat body and then flies were fed with *V. cholerae* or LB for 24 hours and after 24 hours of oral infection with *V. cholerae*, samples were collected for total glucose measurements,

or heads were dissected to measure insulin peptide expression (B) Total glucose levels of ±RU486 (*GS106>ImdD30A*) flies after 24 hours of oral infection with *V. cholerae* (C) Relative expression of *ilp2* (D) *ilp3* (E) *ilp5* from dissected heads of ±RU486 (*GS106>ImdD30A*) after 24 hours of oral infection with *V. cholerae* or fed with only LB media. Statistical significance was determined using two way ANOVA.

As I previously showed that *insulin 2-3,5* mutant flies have improved survival against oral infection with *V. cholerae*, and *imd* mutants have low expression of *ilp3* compared to controls (Figure 4-3, Figure 3-13), I asked if tolerance of flies with IMD inhibition in the fat body against *V. cholerae* is mediated through suppression of insulin peptide expression. To test this question, I measured the expression of *ilp2*, *ilp3* and *ilp5* from the heads of +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) flies after 24 hours of oral infection with *C6706*. IPCs, which are located in the brain of *Drosophila*, produce and secrete ILP2, ILP3 and ILP5 peptides, therefore, I measured the mRNA levels of *ilp2*, *ilp3* and *ilp5* from dissected heads. I also fed +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) flies with LB media to serve as a control group. I found no significant difference between *ilp2*, *ilp3* and *ilp5* expression in the flies with suppressed IMD signaling in the fat body and control flies and 2-way ANOVA showed no significant effect from infection or suppression of IMD signaling in the fat body on insulin peptide expression (Figure 5-5C-E). Previous data from Kamareddine et al supports this observation as they showed inhibition of Relish in the fat body does not affect AKT phosphorylation in whole flies (477). However, visceral muscle in *Drosophila* midgut produce ILP3 which is nutritionally regulated and its expression is maintained by the EE peptide tachykinin (Tk) (478). Therefore, future experiment to measure the expression of *ilp3* from intestine of flies with suppressed IMD signaling in the fat body before and after infection with *V. cholerae* will provide more information regarding the role of IMD signaling in the fat body and effect on alteration of local insulin production in the intestine. Overall, this observation suggests that IMD signaling inhibition in the fat body does not affect expression of insulin peptides in neuronal insulin-producing cells upon feeding with *V. cholerae* or LB media.

5.2.6 Suppression of IMD signaling in the fat body does not affect expression of antioxidant genes in the intestine

Production of ROS in the intestine is an important defence strategy to control intestinal microbes in *Drosophila*. For example, microbiocidal ROS is produced via dual oxidase DUOX in response to uracil released by pathogens (479). Exposure to non-pathogenic bacteria such as

commensal microbe *L. plantarum* results in production of ROS via NADPH oxidase pathway (480). Homeostasis of redox balances is critical as impaired removal of H_2O_2 results in ROS-induced damages such as lipid and protein oxidation as well as DNA damage, which eventually accelerates host death (481,482). As the intestine is constantly in contact with pathogenic and non-pathogenic microbes through food ingestion, regulatory mechanisms have been developed to protect the host from increased ROS production and to maintain host fitness. One of the tolerance mechanisms in the intestine is increasing antioxidant levels to protect the host from ROS damage resulting from pathogen invasion or increased host ROS production against microbes. For example, an immune regulated catalase is essential to maintain redox balance, and extend host survival, after intestinal infection with *E. coli* in *Drosophila* (270). As I showed IMD suppression in the fat body improves host tolerance of *V. cholerae* intestinal infection, I asked if increased expression of antioxidant in the intestine of these flies will contributes to host tolerance.

To answer this question, I measured expression of antioxidants genes belong to metallothioneins, catalase, superoxidase dismutase, thioredoxin peroxidase, and glutathione S-transferase groups which are part of the intracellular defence systems and protect the damage induced by ROS (483). I dissected guts from both ±RU486 (*GS106>ImdD30A*) flies after 24 hours of oral infection with *C6706* or feeding on LB media as control, and I looked at the expression of antioxidants genes such as *irc* (270), *MtnB* (484), *sod1* (483), *gstd1* (485) and *jafrac1* (486) (Figure 5-6A). If increased antioxidants levels in the intestine suppress infection-mediated damage by *V. cholerae*, I expected increased level of antioxidant expression in flies with IMD inhibition in the fat body. Instead, I found that there was no significant increase in the expression of antioxidants genes between +RU486 (*GS106>ImdD30A*) and -RU486 (*GS106>ImdD30A*) flies after oral infection with *C6706* or in ±RU486 (*GS106>ImdD30A*) flies fed on LB (Figure 5-6B-E).

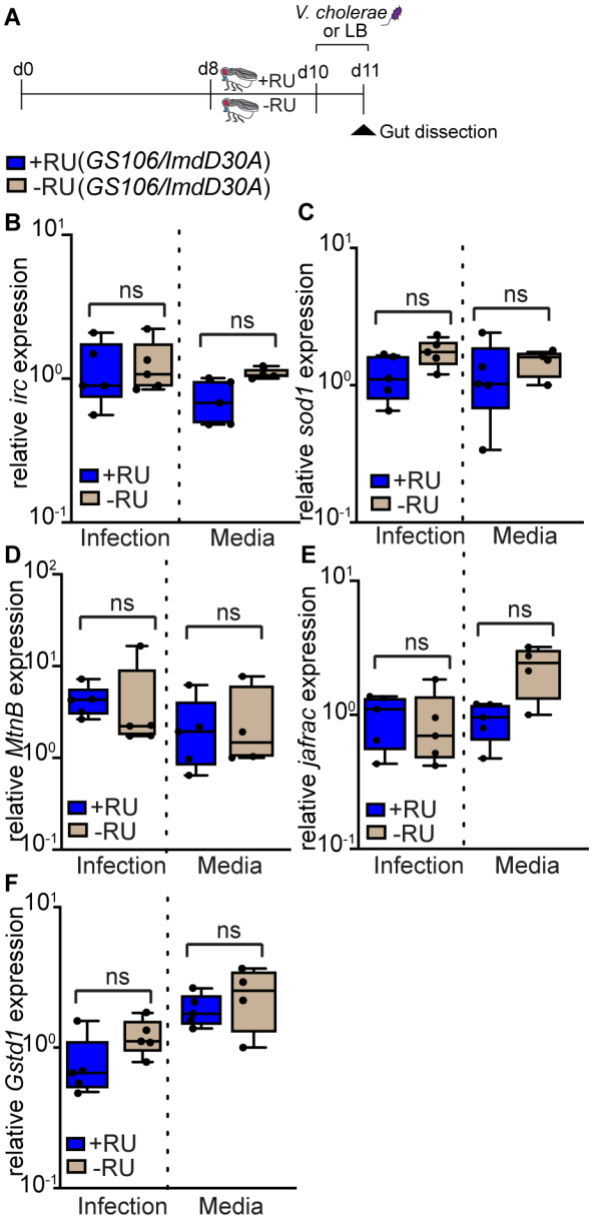


Figure 5-6 Inhibition of IMD signaling in the fat body does not affect antioxidants level in the intestine towards *V. cholerae* oral infection

(A) Schematic representation of experimental design shows that *GS106>ImdD30A* flies were raised for 7-8 days, then fed with either +RU486 or -RU486 for 48 hours to induce suppression of IMD signaling in the fat body and then flies were fed with *V. cholerae* or LB for 24 hours and finally intestine was dissected for measuring gene expression. Quantification of relative gene expression from dissected guts of +RU486 (*GS106>ImdD30A*) versus -RU486 (*GS106>ImdD30A*) infected with *V. cholerae* or fed with only LB media (B) *irc* (C) *sod1*(D) *MtnB* (E) *jafrac1* (F) *gstd1*. Statistical significance was determined using an unpaired Student t test.

Combined, these data suggest that improved tolerance of flies with suppressed IMD activity in the fat body is independent of the increased antioxidant expression at least in the transcriptional level.

However, as I only measured the transcription levels of antioxidants genes in the intestine, further experiments measuring ROS activity will inform us if inhibition of IMD in the fat body improves host survival against *V. cholerae* via suppression of intestinal ROS production.

5.3 Summary

In this chapter, I asked if suppression of immune or metabolic signaling in the fat body affects host response to intestinal infection with *V. cholerae*. I found that suppression of insulin signaling in the fat body extends survival against oral *V. cholerae* infection, however the bacterial load is not affected by modulation of insulin signaling in the fat body. Increased survival and steady bacterial load suggest tolerance towards infection. To further investigate this phenotype in flies with suppressed insulin signaling in the fat body, I tested if blocking insulin signaling in the fat body affects rate of pathogen clearance in the host after discontinuation of exposure with *V. cholerae*. I found that control flies completely cleared *V. cholerae* within 48 hours post infection, however, bacterial load of flies with suppressed insulin signaling in the fat body remained steady after 48 hours post infection. These observation further supports blocking insulin signaling in the fat body increased tolerance of host to *V. cholerae* intestinal infection. Then, I asked how insulin signaling suppression in the fat body affects intestinal morphology before and after oral infection with *V. cholerae*. I found that flies with suppressed insulin signaling in the fat body have an increased proliferation of intestinal stem cells before infection with *V. cholerae*. I also found an increased number of EEs in the flies with suppressed insulin signaling in the fat body. These observation suggests that perhaps suppression of insulin signaling in the fat body increases ISCs division which results in reduced damage after exposure to *V. cholerae* possibly via increased epithelial integrity.

I found an extension of survival along with higher bacterial load in the flies with suppressed IMD in the fat body after oral infection with *V. cholerae* which is an indication of improved tolerance in these flies. I also showed that that there was no significant change in the expression of intestinal antioxidants genes between flies with suppressed IMD signaling in the fat body and fed on *V. cholerae* or LB media. Combined, these observations indicate that modulation of IMD and insulin signaling in the fat body improves the tolerance of flies upon intestinal infection with *V. cholerae*. These results also suggest a possible communication between the fat body and intestine of *Drosophila* upon blocking IMD or insulin signaling in the fat body as well as during infection with *V. cholerae*. Results from this chapter indicate how modulation of immune and metabolic

signaling in the *Drosophila* fat body affects host-pathogen interaction using intestinal model of *V. cholerae*. Studying the contribution of immune and metabolic signaling in promoting tolerance or resistance in *Drosophila* against pathogen will provide a better understanding of the host-pathogen interaction and the impact of host defence mechanisms that improve host fitness upon infection.

Chapter 6:

Discussion

6.1 Summary

Despite the importance of immune-metabolism crosstalk for metabolic and developmental homeostasis, we know very little about the consequences of dysregulation of immune-metabolic interactions for overall host fitness and also when the animal is fighting a microbial infection. In this thesis, I used *Drosophila melanogaster* model to explore how immune signaling affects metabolism, growth and development and how metabolically compromised flies combat non-pathogenic and pathogenic infections and if modulation of immune and metabolic signaling in an endocrine organ such as the fat body impacts host survival in a distant tissue. Pathogenic infections induce metabolic adaptation in the host which is often costly. In chapter 3, I used a genetic approach to elevate IMD activity, which resembles a natural infection, in the fat body during larval development and explored how IMD signaling activation in the *Drosophila* fat body affects macronutrients, larval development, and energy stores. I also looked at the regulatory effect of systemic IMD on the host metabolic homeostasis.

In chapter 4, I explored the effects of systemic depletion of insulin peptides on the host response to a panel of pathogens with different virulence capacity. I asked if route of infection alters the host response to the same pathogen and explored the effect of *V. cholerae* intestinal infection on host metabolic and immune responses. In chapter 5, I investigated the effects of immune and metabolic modulation in the fat body on the host response to the enteric pathogen *V. cholerae*. In combination, the data in this thesis investigate the immunity and metabolism crosstalk in the host development and uncovers the impact of IMD signaling in the metabolic regulation of *Drosophila*. I also provide answers regarding the role of insulin peptides in the oral and septic infection models of *Drosophila* and highlight the impact of IMD and insulin signaling modulations in the fat body on the host survival against intestinal infection with *V. cholerae.*

6.2 Effects of increased IMD activity in the fat body on host physiology

6.2.1 Elevated IMD activity in the fat body suppress insulin signaling and impairs growth and development

In addition to its role as a mater regulator of inflammatory cytokine production, chronic expression of TNFα has been linked to pathogenesis in metabolic disease. Blocking TNF activity increased insulin sensitivity in mice (487,488) and down-regulation of the insulin-regulated glucose transporter (Glut4) might be a mechanism of TNFα-mediated insulin resistance (24,25).

Adipocytes treated with TNFα showed a dose-dependent reduction in insulin receptor substrate (IRS) phosphorylation due to suppression of insulin receptor kinase activity (24), which indicates that TNFα directly disrupts insulin signaling pathway in adipose tissue. Expression of TNFα in hepatoma cells leads to a decrease in insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 (30). The anti-diabetic drug, thiazolidinediones increases insulin sensitivity in the adipocytes by blocking lipolytic activity of TNFα (489). These results indicate that TNFα is an important mediator of insulin resistance in obesity through its effects on several important sites of insulin action (21).

As the IMD pathway shares remarkable similarities with the mammalian Tumor Necrosis Factor Receptor pathway (92,163), I asked if modulation of IMD signaling affects the insulin pathway in *Drosophila*. I found that larvae with increased IMD activity showed reduced expression of *ilp3* and increased expression of negative regulators of insulin signaling such as *ilp6* and *ImpL2* (Figure 3-2). Consistent with reduced insulin signaling at the transcriptional level, I also showed that at the protein level, phosphorylation of Akt which is downstream of insulin signaling was reduced in larvae with increased IMD activation in the fat body (Figure 3-12). Combined, these results suggest systemic insulin signaling suppression in response to elevated immune activity in the fat body. I also showed that systemic ablation of IMD disrupts insulin signaling as circulating ILP2 levels in *imd* mutant flies was significantly higher and *imd* mutants showed a slower glucose clearance compared to control (Figure 3-13). These findings suggest that lack of IMD in *Drosophila* negatively impacts insulin function.

Although I found suppression of *ilp3* expression in larvae with increased IMD activity in the fat body, transcription of ILPs does not necessarily reflect the protein level in the hemolymph or the total ILPs stored in the IPCs (415). Another important factor that needs to be considered in measuring ILP expression is possible redundancy or compensation effects between ILPs. For example, in *ilp2* null mutants, *ilp3* and *ilp5* expression was increased and *ilp2-3* double mutants showed increased expression of *ilp5* (94). Another study showed suppression of *ilp2* expression in IPCs was followed by increased expression of *ilp3* and *ilp5* which suggests compensatory effects between ILPs (115). Therefore, in my study, a compensation effect could be responsible for not detecting reduced expression of *ilp5* or *ilp2*. Although I showed reduced phosphorylation of Akt

in larvae with increased IMD activity in the fat body, I did not measure circulating ILPs in the larval hemolymph. Therefore it is still possible that IMD activation in the fat body induces insulin resistance which could be tested by stimulating fat body with exogenous insulin and measuring the levels of phospho-Akt in the fat body and peripheral tissues. If IMD activation in the fat body induces an insulin resistance phenotype, I would expect absence of Akt phosphorylation after insulin stimulation. The observation that *imd* mutants have higher circulating ILP2 peptide, increased weight and had a higher glucose levels following feeding and a slower rate of glucose clearance suggest an insulin resistance phenotype (Figure 3-12). As I showed that *imd* mutants at both day one and day twenty had impaired insulin function to clear glucose, these data suggests that IMD is important for insulin function and insulin resistance is not a consequence of aging in *imd* mutants.

Toll signaling is required for the innate immune response against Gram positive and fungi. Phosphorylation of Akt by pdk1 is inhibited once Toll is constitutively active in the larval fat body. The authors showed a cell autonomous effect of Toll activation in the fat body on suppression of insulin signaling and non-autonomously which resulted in suppression of the larval growth (414). Hemolymph ILP2 levels were not altered by Toll signaling, suggesting that systemic growth defects could be due to peripheral insulin resistance. In contrast to my observation, activation of IMD pathway in the fat body by expression of Relish did not affect Akt phosphorylation (400). The difference between my observation and data reported by DiAngelo could be explained by Relish-independent effects of IMD on insulin signalling or by differences in the experimental design, as they performed their study with adult *Drosophila*. Nonetheless, these studies suggest that elevated innate immune signaling in the fat body suppress insulin signaling locally, as well as systemically. Comparison between genes altered by IMD activation and suppression of insulin receptor in the fat body showed 29.8% overlap (Figure 3-6) which suggests a crosstalk between IMD and insulin signaling in the fat body (406). In *Drosophila*, natural infection with *M. marinum* results in suppression of insulin signaling and Akt phosphorylation as well (64). The suppression of systemic insulin signaling by increased innate immune responses via genetic approach or natural infection suggest that host reduces insulin sensitivity in tissues that are not essential for immune responses to reduce the cost of infection.

I showed that IMD activation in the fat body of *Drosophila* larvae delayed development, reduced pupal volume and also had a semi-lethal effect on pupal development as number of adults eclosed from this group were reduced (Figure 3-10). These results are consistent with reduced growth and developmental delay along with reduction of pupal and adult size in flies with ablation of IPCs (104). Activation of Toll in the fat body also restricts growth of the larvae (64,400,414). Overall, these studies indicate the activation of innate immune signaling in the fat body non-autonomously affects host growth and development and results in similar phenotypes observed in the insulin mutants. Interestingly, similar observations have been reported for the negative effect of chronic enteropathogen infection on the growth in the first two years of childhood (490). Activation of insulin and target of rapamycin signaling (TOR) signaling in the larval fat body is essential for systemic growth and proper coordination of pupal and adult size (123,147,160,491). Both in mammals and *Drosophila,* Akt stimulates growth by phosphorylating the tuberous sclerosis complex 2 (TSC2) and inhibiting formation of a TSC1–TSC2 complex, which is responsible to inhibit TOR signaling (150). In addition to that, TOR complex is also able to phosphorylate Akt at S505 site to provide maximal level of Akt activity (492). I showed in the larvae with increased IMD activity in the fat body, that TOR activity was suppressed (Figure 3-11). Reduced TOR signaling results in activation of the fly 4EBP ortholog, Thor, and I found an increases expression of *Thor* in larvae with consistent IMD activity in the fat body (Log_2 foldchange:0.88) (Figure 3-2). These observations suggest that IMD activity in the fat body systemically impairs growth possibly through a IMD-TOR-IIS axis.

The IMD pathway branches into two arms, Relish and JNK pathway at the transforming growth factor β-activated kinase 1 level (213). The JNK pathway, belong to the mitogen-activated protein kinase family and is activated by inflammatory cytokines and environmental stress (222). Increased JNK pathway activation has been reported in insulin resistance, obesity and pancreatic beta cell dysfunction (493). Different mechanisms such as inhibition of insulin receptor substrate phosphorylation (494), metabolic inflammation (495,496) and negative effect on liver fatty acid oxidation (497) have been reported for JNK-driven metabolic disorders. Inhibition of JNK1 in obese mice, protected them from obesity-induced insulin resistance (498). In *Drosophila*, activation of JNK in fat body antagonizes systemic insulin signaling and suppress insulin peptide expression through activation of FOXO (228). As I showed that expression of a JNK target gene

lipocalin Neural Lazarillo was upregulated in the larvae with consistent IMD activation in the fat body (Log_2 foldchange:1.52)(Figure 3-2), it is possible that IMD supress systemic insulin signaling through a IMD-JNK axis. This hypothesis is supported by a study showing suppression of *lipocalin Neural Lazarillo* expression in the *Drosophila* fat body results in improved insulin sensitivity in peripheral tissue (127).

6.2.2 Does consistent IMD activation in the fat body affect apoptosis?

Drosophila IMD has a death domain which is similar to the death domain of mammalian receptor interacting protein (RIP). RIP associates with TNFα receptor 1 for NF-κB activation. Overexpression of RIP has been shown to induce morphological changes that are indicative of apoptosis (499). Cell death is an important regulatory mechanism during *Drosophila* embryogenesis. A group of pro-apoptotic genes such as *reaper*, *hid*, and *grim* share an inhibitor of apoptosis (Iap) binding motif which binds to Iap and antagonizes its inhibitory function, resulting in activation of downstream apoptotic caspases *Dronc* and *Drice* (500–503). In my microarray, I found upregulation of genes involved in apoptosis in larvae with increased IMD activity in the fat body (Figure 3-2). For example, *reaper* (Log_2 foldchange:1.21), *Dronc* (Log_2 foldchange:0.95) and *drice* (Log_2 foldchange:0.74) were upregulated in larvae with consistent IMD activity in the fat body compared to control larvae. In addition to that, I showed that IMD activation in the larval fat body had semi-lethal effects on larval development as a significant number of larvae died at the pupal stage and a significantly reduced number of adults eclosed from larvae (Figure 3-10).

These observations suggest that IMD activation in the fat body induces semi-lethal effects on developing tissues via increased apoptotic activity. Previous studies confirm the apoptotic effect of increased IMD pathway activation in the fat body as IMD overexpression in the adult *Drosophila* fat body increased apoptosis through induction of the *reaper* transcript (197). In line with apoptotic roles of IMD, overexpression of AMPs in the fat body also showed increased apoptosis via impairment of mitochondrial function and depolarization of its membrane (504). Disruption of mitochondrial proteostasis negatively affects its function and accelerates aging in *Drosophila* (505,506). I also observed suppression of genes involved in mitochondrial protein synthesis and function in larvae with increased IMD activity in the fat body (Figure 3-2), which suggests that suppression of mitochondrial function could contributes to the IMD-induced apoptosis in larvae with elevated IMD activity in the fat body. The apoptotic stimulatory ability of AMPs seems to be conserved as mammalian AMPs also induce apoptosis in cancer cells via mitochondrial

depolarization (507). Conversely, *imd* mutants flies were more resistant to UV radiation that indicates IMD could be involved in the host apoptotic responses to DNA damage (508).

IMD signaling leads to the activation of the JNK pathway (220). JNK is involved in both cell death (509) and pro-survival cues (510) and its activity is inhibited by a negative feedback loop induced by puckered (511). JNK activation also promotes nuclear translocation of FOXO and results in FOXO-induced transcription of stress response genes (228). FOXO is a transcriptional factor and a signal transducer that acts downstream of IIS (512). FOXO target genes are involved in different cellular processes such as oxidative stress (513), cell cycle arrest (514), DNA damage (515), apoptosis (516) and metabolism (517). As I observed suppression of insulin signaling in larvae with increased IMD activity in the fat body, it is possible that FOXO is activated downstream of either IMD-JNK axis or insulin signaling. As JNK acts downstream of IMD, and both JNK and FOXO activation results in activation of apoptotic genes such as *hid* (518) and *bim* (516), it is possible that IMD activation in the fat body results in FOXO nuclear translocation and activation of downstream cellular pathways that leads to apoptosis. In support of this hypothesis, I found an upregulation of FOXO-target genes such as *ImpL2*, *thor* and *ilp6* (Figure 3-2) in larvae with increased IMD activity in the fat body. Previous research supports this hypothesis as increased IMD and JNK pathway activation in *Drosophila* results in increased apoptosis (519). The authors showed that presence of PGRP-LF which is a membrane associated receptor protein and a negative regulator of IMD signaling is required in the absence of infection to inhibit the constitutive activation of IMD and JNK pathway and to prevent the developmental defects resulted from increased apoptosis (519). Future work needs to test this hypothesis via using assays such as Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for apoptosis quantification specifically during pupal stages as most of the larvae with increased IMD activity in the fat body did not develop further to adulthood and died at the pupal stage.

6.2.3 IMD signaling has a regulatory role on lipid homeostasis

The balance between lipolysis and lipogenesis is essential for lipid regulation (520). Increased expression of inflammatory molecules such as interleukin 1β in the adipose tissue of obese mice is associated with the development of insulin resistance (521). Cytokine production in the adipocytes increases lipolysis and suppresses triglyceride synthesis leading to increased

circulatory free fatty acid concentration which then is absorbed by the skeletal muscle and induces insulin resistance in this tissue (522). TNFα has a regulatory role in both physiological and pathological regulation of lipid metabolism (523,524). For example, overactivation of TNFR in adipocytes is associated with obesity and metabolic diseases (525), however, acute expression of TNF signaling is required for a functional adipose tissue (526). Lipid droplets (LDs) stores triglycerides and are important organelles for lipid synthesis and lipolysis and they are reported to be involved in the pathophysiological processes in the host as well (527,528). Impaired lipid biogenesis and mobilization in the LDs results in increase free fatty acid concentrations leading to lipotoxicity (529,530) and elevation of inflammation which consequently disrupts metabolism.

I found that increased IMD activity in the fat body of larvae results in depletion of LDs in the fat body (Figure 3-10). In addition to that, total triglyceride and glycerol levels of larvae with consistent IMD activity in the fat body were reduced (Figure 3-9). Comparing transcription profile of larvae with IMD activity in the fat body with control larvae showed suppression of genes involved in lipid biogenesis (Figure 3-2). I also showed that *imd* mutants weigh more and have a significantly higher level of total triglyceride levels compared with wildtype flies (Figure 3-13). Overall, my observations suggest that basal levels of IMD activity is required for the lipid homeostasis in *Drosophila* while an overactivation of IMD activity in the fat body negatively affects lipogenesis in the larvae.

I found that increased IMD activity in the fat body suppressed expression of lipid droplet storage (*Lsd*) 1 and *Lsd2 (*Figure 3-2). Lsd1 and Lsd2 are homologues of mammalian PLIN1 and PLIN2, respectively and reside on the surface of LDs (531,532). Lsd2 promotes lipid storage and LD growth and shields them from lipolysis (533–535), while PLIN1 modulates protein transportation on LDs (408,536). In line with my observation, *Lsd2* mutants showed a reduced level of neutral lipid content compared to wild type which suggests *Lsd2* function is required for normal lipid storage (533). Similarly, *plin1* deficiency was associated with smaller adipocytes, macrophage infiltration and insulin resistant diabetes in both mice and humans (537,538). These findings overall highlight the impact of impaired LDs formation in the development of metabolic diseases. A recent study showed that chronic IMD signaling in the adult fat body resulted in a significant decrease in triglyceride levels in the fat body and in whole fly via a reduction in both size and area of LDs accompanied by a reduction in the expression of *plin1* in the fat body (539).

This observation combined with my results also matches clinical studies showing TNFα induces lipolysis and downregulates expression of perilipins (540). Once TNFα suppresses perilipin, the lipase activity on the LD surface increases which results in enhance concentration of fatty acid in the circulation as is reported in *plin1-/-* mice (541).

The *Drosophila* homologue of mammalian adipose TAG lipase is Brummer which mediates LD lipolysis (542,543) and is directly controlled by FOXO (544). I showed that IMD activation in the fat body suppresses insulin signaling activity (Figure 3-12) and as FOXO is downstream of insulin signaling, therefore, I hypothesize that persistent IMD activation in the larval fat body increases lipolysis in the fat body via IIS-FOXO axis. This hypothesis could be tested by co-expression of a constitutively active insulin receptor and ImdCA in the fat body to test if depletion of lipid stores could be rescued. Consistent with this hypothesis, increased insulin signaling activity in the fat body results in accumulation of lipids and increased triglyceride levels (143,545). Other studies have also shown IMD activation by fat body-specific overexpression of Relish significantly increase *brummer* expression on the surface of LDs (539). Another study showed that FOXO activation during a necrosis-induced systemic immune activation resulted in increased lipolysis in the fat body and wasting phenotype that suggests FOXO integrates immune-metabolic signals to increase host adaptability in infection or increased immune responses (546).

Previous studies that looked at the effect of innate immune signaling such as Toll pathway activation in the fat body support my observations regarding a reduction in lipid and triglyceride storage in response to increased immune responses in *Drosophila*. These studies showed expression of a constitutively active Toll receptor in the larval fat body inhibits triglyceride storage (400,547,548). In a recent study, Toll activation in the fat body, or infection with *E. faecalis* caused a suppression of *de novo* lipogenesis by reduction in expression of genes responsible for the triglyceride synthesis such as *lipin* and *midway* (547). Similar to this study, I also found suppression of *midway* expression in larvae with increased IMD activation in the fat body (Figure 3-2), which suggests that IMD activation in the fat body suppress de novo lipid synthesis as well. In other infection models in *Drosophila*, such as infection with the intracellular bacterial pathogen *M. marinarum* resulted in depletion of triglyceride stores (64). Different pathogens such as *Tubulinosema ratisbonensis* which is an intracellular parasites also showed reduced triglyceride storage. Authors suggest the free fatty acid released form triglyceride lipolysis is used towards parasite growth (549).

Systemic infection of *Drosophila* with *L. monocytogenes* results in a reduced level of total triglyceride level (550). A recent study showed during poor nutrient condition, an adipokine secreted from fat body called NimB5 results in increased hemocyte number at the expense of triglyceride storage in the fat body (57). This study showed that hemocyte production has a metabolic cost and under poor nutrition, excessive hemocyte number is lethal for the host as prevents lipid deposition in the fat body. It is possible that innate immune activation in the fat body, which mimics a systemic infection, is mobilizing the lipids in the fat body to provide energy for increased AMPs production or synthesis of negative regulatory peptides of IMD pathway. These studies combined with my observations suggest increased immune responses during an infection or genetic activation of innate immune responses negatively regulate lipid metabolism and triglyceride storage in the host and induce a trade-off between increased immune activity and fat body energy storage.

I showed that *imd* mutants have increased level of triglycerides compared with wildtype controls. In line with my observation, downregulation of the IMD pathway in the fat body increased weight and lipid stores (552). A recent study in our lab showed that IMD suppression in the intestinal enterocytes leads to increase in weight and whole fly triglyceride levels (381). These observations suggest that IMD regulates lipid metabolism at the tissue and systemic level. Mutants in the IMD pathway such as *kenny*, *Relish*, and *Dredd* showed increased triglycerides compared to controls. In this study authors showed that the fat body of *imd* mutants flies had reduced lipids while LDs were accumulated in the enterocytes of *imd* mutants (477).

These observations suggest that systemic ablation of IMD results in reallocation of fat stores to the intestine probably due to higher levels of pathogen exposure to microbes during *Drosophila* feeding. This speculation is supported by this study showing large LDs reduces the ability of pathogens to use free fatty acids for their growth (553). However, others have reported decreased triglycerides in *relish* mutants (554). In their study, during starvation condition, regulatory role of Relish was necessary in the fat body to inhibit excessive lipolysis. Authors showed that increased lipid degradation was inhibited through suppression of *brummer* expression in the fat body which resulted in increased fitness of the host during metabolic stress. In contrast to my study, Diangelo et al. found no significant effect on Relish overexpression in the fat body on the triglyceride levels (400). The difference between my observation and Diangelo may be that

regulation of lipid metabolism by immune signaling is upstream of Relish possibly though JNK arm of IMD. As JNK can activate FOXO and FOXO induces lipolysis, it is possible that IMD increase lipolysis through JNK. In conclusion, I propose a model in which larvae with increased IMD activity in the fat body show an IMD-dependent suppression of insulin signaling which results in FOXO nuclear translocation and enhanced lipolysis in the fat body. As I showed reduction of *midway* expression in larvae with increased IMD activity in the fat body, I speculate that IMD could decrease lipogenesis through direct or indirect suppression of *midway* in the fat body (Figure 6-1).

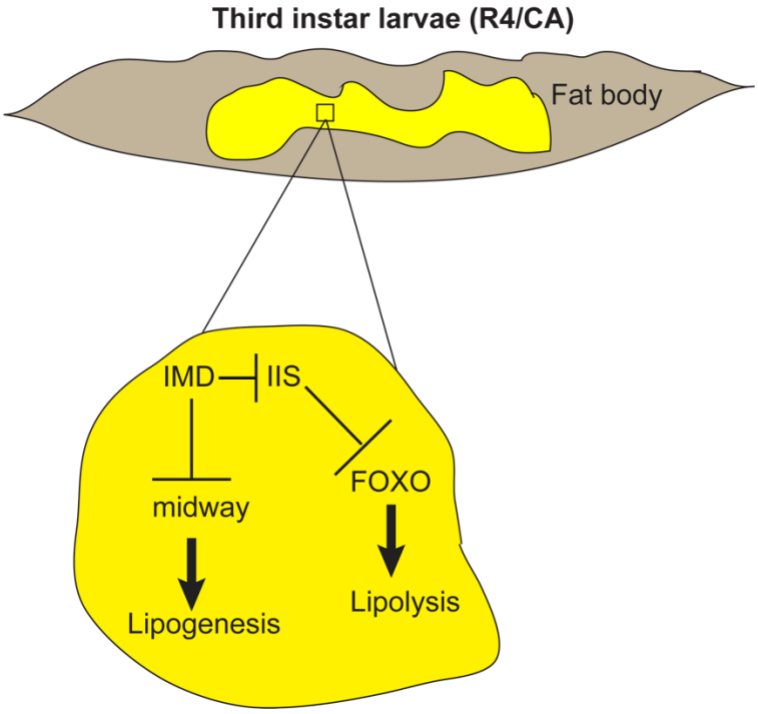


Figure 6-1 Proposed model for disruption of lipid metabolism in larvae with elevated IMD activity in the fat body
The following is a hypothetical model of how increased IMD activity in the larval fat body disrupts lipid homoeostasis. IMD suppresses insulin signaling activity leading to FOXO nuclear translocation. FOXO target genes which are involved in lipolysis accelerates the lipid degradation in the fat body. Increased IMD activity could also suppresses expression of midway which regulates lipogenesis in the fat body therefore lipid synthesis is reduced.

6.2.4 IMD activity in the fat body and its effects on survival and starvation resistance

I showed that activation of IMD in the fat body during the adult stage was only beneficial for male flies and no significant difference for female was observed compared with control flies

(Figure 3-14). This was an unexpected result as chronic inflammation is usually linked to reduced lifespans in mammals (555). Similarly in *Drosophila*, increased immune activation in the fat body via over expression of PGRP-LC results in reduced survival in both male and female flies (417). However, PGRP-LC was activated during larval development which might explain the severe lethality in the adults. In line with negative effects of chronic activation of immune signaling, inhibition of negative regulators of IMD signaling such as *pirk* and *diedel* reduces lifespan (556,557). In contrast to negative effect of PGRP-LC activation in the fat body in both male and female flies, activation of PGRP-LE in the fat body reduced survival of males flies more significantly compared to females (416). However, the effect of increased IMD expression on lifespan is tissue-specific as expression of a constitutively active IMD in the progenitor cells did not affects adult lifespan (380). As I showed IMD activation in the larval fat body suppresses insulin signaling (Figure 3-12) and given the survival extension in insulin deficient flies, the increased survival of males with consistent IMD activity in the fat body is reminiscent of the increased survival in flies with depletion of cells expressing *ilp2*, *ilp3* and *ilp5* in the IPCs (108). The difference between extended lifespan in males compared with females with increased IMD activation in the fat body could be due to the sexual dimorphism that exists in response to infection.

The observation that male and female *Drosophila* behave differently in response to infections have been previously recorded and emphasizes the importance of including both sexes in immune function studies. For example, a fungal infection model showed that males had a 80% survival compared to 40% survival of females after infection with *Beauveria bassiana* which was shown to be Toll-dependent (452). However, this sexual dimorphism in immune responses seems to be pathogen-dependent as females showed an increased survival compared with male flies upon systemic infection with *E. faecalis* (558) and in some studies from different laboratories looking at the same pathogen, different sexual biases have been observed as was the case for *P. aeruginosa* (559–561). Production of AMPs downstream of IMD are found to be dimorphic at the expression level and at higher levels for females compared to males (562). As females have different reproductive requirements and requires energy for egg production during adulthood, and given the suppression of metabolic homeostasis observed in larval with IMD activity in the fat body, it is possible that depletion of energy storage is more vital for females with IMD activation in the fat body and therefore does not results in survival extension.

Another possible explanation for the difference between my observation that IMD activation in the adult fat body increases lifespan in male flies while PGRP-LC and PGRP-LE activation in the fat body showed reduced lifespan in adults could be differences between the transgene activation method employed. I used the temperature-sensitive $GAL80^{ts}$ expression system to activate IMD pathway in the fat body at 29°C while PGRP-LC was activated in the fat body using GAL4-UAS system during larval development at 25°C and for PGRP-LE acute overexpression in the adult fat body, the GeneSwitch system was used to examine the effect of increased IMD signaling on lifespan at 25°C (416,417). Another issue could be the genetic background effect therefore repeating this lifespan study using the control flies backcrossed to the wildtype could solve this issue.

I found that for both male and female flies with consistent IMD activation in the fat body during adult stage, sensitivity to starvation was increased and flies succumbed to death faster than the control group (Figure 3-14). This observation suggests that consistent innate immune pathway activation in metabolic tissue depletes stored resources in the flies and therefore makes the adult more vulnerable to a poor diet. This observation contradicts the increased starvation resistance in *insulin 2-3,5* mutant flies. However, in *insulin 2-3,5* mutants flies, triglycerides and glycogen levels were increased which could explain the ability of insulin mutants to survive the starvation (108). In line with my observation, previous studies using bumblebee workers, *Bombus terrestris,* showed a 70% reduced survival after they were challenged with lipopolysaccharides to stimulate their immune system under starvation condition (563). Therefore, my observation of reduced survival for flies with IMD activity in the fat body on 1% agar food combined with these studies suggest that increased immune signaling induces a metabolically compromised state in the host. This is supported by a previous study showing *relish* mutant flies survived longer under starvation (564).

An increase in energy stores such as lipid and glycogen was associated with the increased resistance to starvation (565), which support my observations of increased lipid and glucose storage in *imd* mutants (Figure 3-13) and the depletion of triglyceride storage in larvae with increased IMD activity in the fat body (Figure 3-9, Figure 3-10). Suppression of Relish in the adult *Drosophila* by crossing NF-κB/Rel deficient fly line with fat body specific driver reduces starvation resistance of flies due to reduced triglyceride levels which suggests that basal IMD activity is required in the fat body of *Drosophila* for proper regulation of energy stores (554).

Previous studies have revealed that low protein to carbohydrate ratios extend longevity of mice and flies in which the most beneficial effects were observed for 1:16 protein: carbohydrate ratio in flies (566,567). Future studies examining the effects of protein to carbohydrates ratio in the *imd* mutants and flies with increased activity of IMD signaling in the fat body diet would uncover the relationship between IMD activity, diet and lifespan.

6.2.5 Elevated IMD activity in the fat body disrupts sugar homeostasis in *Drosophila*

During an infection, both host and pathogen have metabolic demands to survive. Immune defence is costly for the host, therefore host undergoes metabolic adaptations to reallocate energy from physiological processes such as storage and growth towards energy requirements for immune system activation (306,554,568–572). The integration of immune and metabolic pathways is particularly evident in *Drosophila*, where the fat body simultaneously regulates energy storage and humoral immunity (88,92,163). I used the *Drosophila* model to characterize the contributions of IMD to immune-metabolic homeostasis. I found that activation of IMD in the fat body impacts host metabolism at molecular, genetic, and phenotypic levels. On the transcription level, the main difference that I found comparing the transcriptional profile of larvae with increased IMD activity in the fat body and control was suppression of genes involved in metabolic pathways including, glycolysis, gluconeogenesis, TCA cycle, fatty acid beta oxidation, lipid metabolic processes, cellular respiration. Conversely, genes involved in the metabolic pathways such as glycogenolysis, catabolic processes, proteolysis and autophagy were upregulated (Figure 3-2).

At the macronutrients levels, I found IMD activation caused a depletion of lipid stores in the larval fat body, hyperglycemia, and reduced level of triglyceride (Figure 3-9, Figure 3-10). I also found that loss of function *imd* mutants weigh more, have hyperlipidemia, and impaired glucose tolerance (Figure 3-13). These observations suggest that IMD activity in the fat body systemically regulates metabolic homeostasis in *Drosophila.* My findings are consistent with other studies showing septic infections with *L. monocytogenes* or *M. marinum* results in depletion of lipids and glycogen stores as well as suppression of glycolysis pathway (64,550). Adaptations of the host to infection frequently involve redistribution of energy reservoirs. In my study, reduced expression of genes involved in metabolism and energy production in larvae with activated IMD in the fat body suggests that larvae switched from glycolysis to glycogenolysis. Upregulation of genes involved in amylase and maltase production suggests that larvae with elevated IMD activation in the fat body use different sources of energy to avoid a glucose shortage.

My finding that increase immune activity affects the overall metabolic process in the host is in line with recent studies showing a transcription factor in the fat body suppresses anabolic processes in response to infection (573). Phosphorylation of MEF2 under normal physiological condition induces lipogenesis and glycogenesis, however, bacterial infection leads to a loss of MEF2 phosphorylation that shifts fat body activity from the accumulation of energy stores to the release of antimicrobial peptides. Therefore, it is possible that IMD activity affects expression of core metabolic genes or transcription factors that regulate metabolic responses to infection through suppression of energy recourses or disruption of trehalose and glucose homeostasis. This hypothesis is supported by previous studies finding core metabolic genes such as *Mef2* (573), *lime* (574), *CrebA* (306) and *seven up* (575) expression in response to pathogenic bacteria in the fat body that switch metabolic responses from anabolic metabolism to increasing immune activity for the production of AMPs. This hypothesis could be tested by performing RNA-sequencing on the dissected fat body of larvae with increased IMD activity in the fat body and compared with control so that we will have a comprehensive knowledge of the fat body transcriptional profile altered by IMD elevation in the adipose tissue. I showed that IMD activation in the fat body and host infection with a panel of pathogens share similar effects on biological processes with a prominent impact on suppression of host genes involved in the regulation of metabolism (Figure 3-5). This observation is important as it suggests that a genetic approach for elevation of IMD activity reflect natural infection in *Drosophila*. Increased activation of intestinal IMD activity also controls expression of genes involved in developmental and metabolic processes (380,402,576). However, I found minimal overlap between genes altered by IMD activation in the fat body and those affected by elevated IMD activation in the progenitor cells (Figure 3-6) which suggest that IMD has tissue-specific impacts on metabolic regulation.

My observation regarding depletion of lipid stores and increased expression of genes involved in glycogenolysis match previous observations regarding modulation of insulin signaling in *Drosophila*. In these studies, increased insulin signaling results in increased lipid and glycogen storage (143) and this is reversed by ablation of insulin producing cells (104). Therefore, suggesting consistent IMD activation in the larval fat body results in switching from glycolysis to glycogenolysis and emphasizes the role of efficient insulin signaling function in regulating carbohydrate metabolism in the host. Sugar homeostasis in *Drosophila* is regulated via endocrine

systems same as mammalians via evolutionary conserved pathways. Both glucose and trehalose homeostasis are regulated by insulin and AKH which is the *Drosophila* homologue of mammalian glucagon. AKH elevates hemolymph trehalose titers by increasing glycogen degradation in the fat body (577,578) and insulin reduces circulating sugar by increasing glucose uptake in the peripheral tissues. I observed significant increases in circulating trehalose in larvae with persistent IMD activity in the fat body (Figure 3-9). Given the role of trehalose as main circulating carbohydrate in the fly hemolymph (579), and increased expression of genes involved in glycogenolysis, these results suggest that increased glycogenolysis in the fat body results in elevated level of circulating trehalose. However, it is possible that due to a disruption in glucose transporter expression in peripheral tissue to uptake trehalose from the hemolymph, larvae with increased IMD activity in the fat body have higher levels of trehalose in the hemolymph.

In addition to the AKH regulatory effect on glycogen mobilization in the fat body, crosstalk between AKH-IIS is necessary to maintain carbohydrate balance in the hemolymph. In *Drosophila* larvae, trehalose levels in the hemolymph stimulates ILP3 secretion from IPCs through AKH-producing cells of the corpora cardiaca (580). Given my results showing increased trehalose levels in the circulation and suppression of *ilp3* expression in larvae with increased IMD activity in the fat body (Figure 3-2, Figure 3-9), it is possible that IMD activation in the fat body impairs AKH and insulin signaling crosstalk which results in dysregulation of circulatory carbohydrates. I found in my microarray that *akhr* expression was significantly downregulated in larvae with increased IMD activity in the fat body (Figure 3-2) which could suggest suppression of *akhr* expression in IPCs results in inhibition of ILP3 secretion into the hemolymph resulting in elevate trehalose levels. The impact of inter-organ communication in regulation of carbohydrate metabolism is crucial in all organism and will be discussed in more detail in another section.

6.2.6 Consistent IMD activation in the fat body induces a systemic effect in larvae

Multicellular organisms have developed inter-organ communication systems to coordinate and maintain homeostasis. In *Drosophila*, communications between the fat body, brain, muscle, imaginal discs and intestine have been extensively investigated (122,581–583). However, even though we have learned a lot about interorgan communication during infection there are still important knowledge gaps. The fat body communicates with the brain through mediators such as CCHamide2 (130), the TGF/activin-like ligand dawdle (129), the leptin-like cytokine upd2 (128),

ILP6 (105), and stunted (124) to maintain metabolic homeostasis. For example, inhibition of *upd2* expression in the fat body prevents release of ILPs into the hemolymph, negatively affecting systemic growth and energy metabolism (128). My data suggest that IMD activation in the fat body affects cross-talk between fat body and brain through inhibition of systemic insulin signaling. This hypothesis is consistent with the phenotypic overlaps between larvae with elevated IMD activity in the fat body and insulin deficient flies. Once IMD is activated, two possible downstream pathways are potentially activated: Relish and JNK-pathway. In *Drosophila*, increased activation of JNK, downstream of IMD in the fat body results in a systemic reduction in insulin signaling through a FOXO-induced suppression of ILPs expression (228). The interaction between IIS and JNK signaling is conserved (584,585). For example, in *C. elegans*, JNK activation results in nuclear translocation of DAF-16 which is a FOXO homologue (586–588). In vertebrates JNK signaling antagonizes IIS by suppression of insulin production from pancreatic β cells (589). FOXO expression in the adult *Drosophila* fat body induces ILP6 secretion that consequently decreases ILPs secretion in IPCs (133). As JNK acts downstream of IMD and given my observation that increased IMD activity in the fat body suppresses systemic insulin signaling and existing connections between insulin, FOXO, IMD and JNK pathways, I hypothesize that elevated IMD activity in the fat body remotely suppresses systemic insulin signaling through a JNK-FOXO-ILP6 axis (Figure 6-2). In support of this hypothesis, I found increased expression of FOXO target genes such as *tobi*, *thor, ilp6* in larvae with consistent IMD activity in the fat body (Figure 3-2). In this proposed model, consistent IMD activity in the larval fat body signals through JNK and activates FOXO resulting in increased expression of FOXO target genes such as *ilp6*, which then ILP6 is released into the hemolymph and suppresses ILPs secretion from IPCs.

In insects, the ecdysone hormone regulates the developmental transition, timing of larval molting, pupariation and growth duration (590). For a proper larval-pupal transition during larval development, circulating ecdysone antagonizes insulin function resulting in suppression of peripheral insulin signaling and reduced larval growth (107,591,592). In my microarray, expression of *Ecdysone-induced protein 63E* (*Eip63E*) and *Ecdysone-induced protein 93F* (*Eip93F*) as well as matrix metalloproteinase 1 and 2 which both respond to ecdysone signaling to dissociate adipocyte cells (593) were significantly upregulated in larvae with increased IMD activity in the fat body (Table 6-1). In addition to that, I found increased *ImpL2* (Figure 3-2) expression in larvae

with consistent IMD activity in the fat body. It is possible that in larvae with increased IMD activity in the fat body, over activation of ecdysone results in increased *ImpL2* expression in the fat body which suppresses peripheral insulin signaling (Figure 6-2). This hypothesis is supported by a study showing poor nutrition in third instar larvae increases ecdysone production in the prothoracic gland which results in elevating ecdysone titration in circulation which increases ImpL2 production from the fat body; and consequently ImpL2 attenuates peripheral IIS and body growth (594).

Gene	Log_2FC
Epi63E	0.64
Epi93F	1.77
Mmp1	0.96
Mmp2	0.79

Table 6-1 Comparison of gene expression between larvae with increased MD activity in the fat body and control larvae

As I observed increased *ImpL2* expression in larvae with IMD activity in the fat body, it is possible that ImpL2 inhibits ILPs binding to the insulin receptor in peripheral tissue therefore suppresses systemic insulin signaling. ImpL2 inhibits insulin signaling by forming a protein complex with circulating ILPs (131,132). For example, activation of an oncogene orthologue in the intestinal stem cells results in a wasting phenotype via increased ImpL2 secretion that results in suppression of insulin signaling and hyperglycemia (5 95). Another study showed mitochondrial disruption in the muscle of *Drosophila* reduces systemic insulin signaling via release of ImpL2 from atrophic muscles (596). In clinical context, diabetic patients develop muscle atrophy which indicate the disruption of distant tissues (597). As crosstalk between insulin and immune signaling is evolutionary conserved, identifying the components of the inter-organ communication will contribute to our understanding of pathology of the systemic disease in humans.

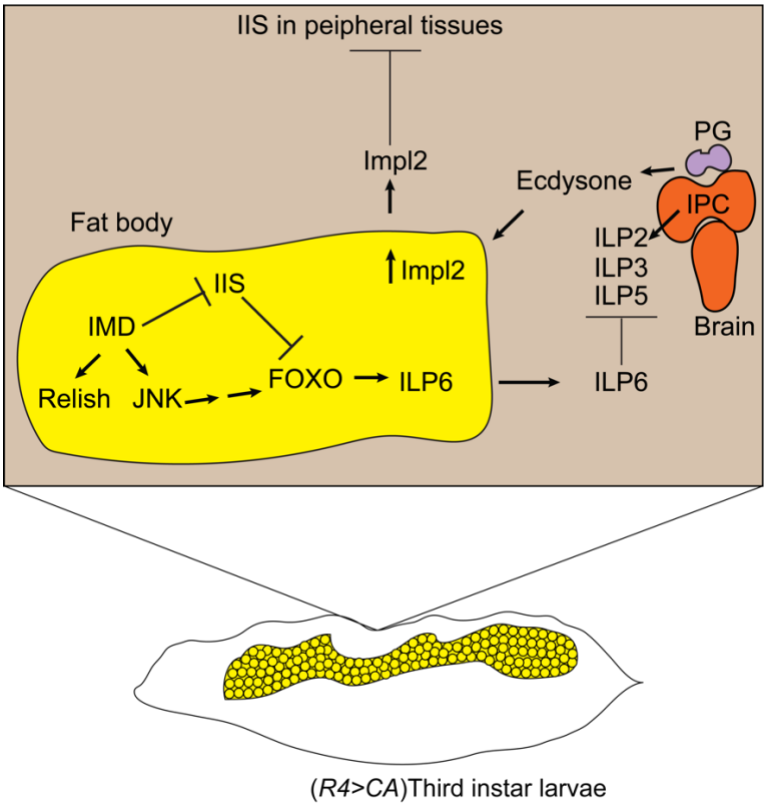


Figure 6-2 Proposed model for systemic effects of consistent IMD activation in the fat body
In this model, I propose that increase IMD activity in the larval fat body suppresses insulin signaling which results in nuclear translocation of FOXO and expression of FOXO-target genes such as ilp6. On the other hand, JNK has been shown to induce FOXO nuclear translocation which could also results in increased expression of FOXO target genes. ILP6 that is released into the hemolymph supresses release of ILP2, ILP3 and ILP5 from IPCs which results in systemic suppression of insulin signaling. As I found increased expression of *ImpL2* in larvae with increased IMD activity in the fat body as well as increased expression of ecdysone inducible genes, I also propose that increased ecdysone secretion from prothoracic gland (PG) results in in elevated expression of *ImpL2* in the fat body which then binds to insulin peptides and reduces peripheral insulin signaling.

6.3 Immune-metabolic axis impacts host-pathogen interaction

6.3.1 Host insulin signaling interaction with pathogens depends on the identity of the infectious microbe

Tolerance and resistance are defence mechanisms that are used by the host to optimize their fitness upon pathogen attack (434). In tolerance, different mechanisms are employed to reduce the damage caused by immunopathology or pathogen itself through increased tissue repair systems and metabolic adaptation to the new environment that has to be shared with a pathogen without any effect on pathogen burden. In resistance, increased antimicrobial responses limits pathogen

proliferation and improves host survival. In some diseases the increased immune activation to clear the pathogens induces more damages than pathologies caused by the microbes. In tuberculosis infection, the immune responses that clears bacteria also induces accumulation of fluid in the lung (598) and patients can suffer from wasting and glucose intolerance as well (599). In *Drosophila*, resistance mechanisms such as cellular (600) and humoral immunity (196,205,261,262) and strategies such as altered energy use by host, anorexia (275), diet restriction (435) or genetic variation (266) are recognized as the contributing factors to increased tolerance of the host. For example, *M. marinum* infection causes a reduction in AKT activity downstream of insulin signaling, which results in increased FOXO transcriptional activity, enhancing the wasting process in flies and consequently leading to death. As tolerance is defined by processes that improve host fitness through tissue damage control mechanism, this study showed that FOXO suppression improves survival of *Drosophila* with no effect on microbial load therefore, improves *Drosophila* tolerance against *M. marinum* infection (64).

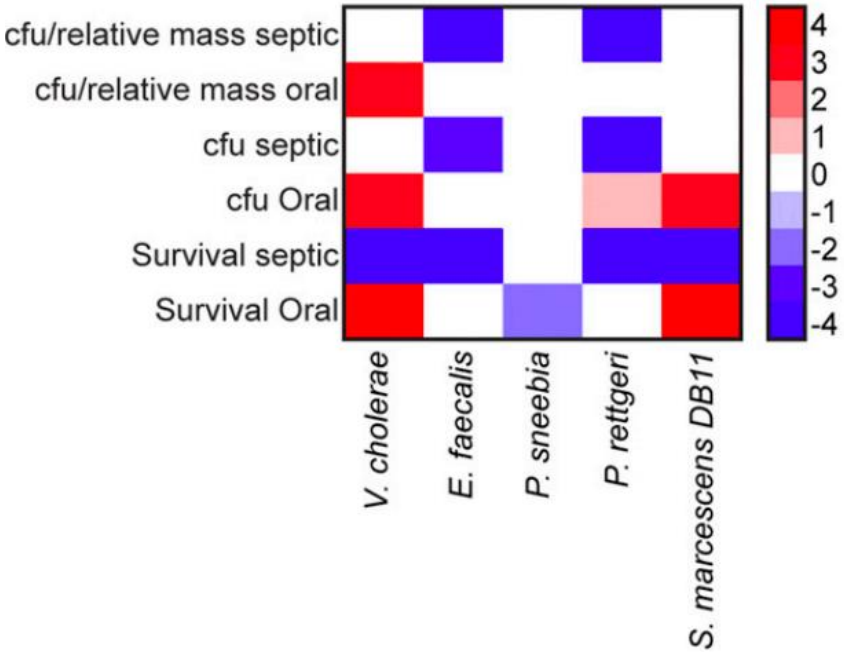


Figure 6-3. Heat map summarizing the results of all infections performed in chapter 4. Positive scores indicate experiments in which insulin mutants had enhanced survival or lower bacterial load. Negative scores indicate experiments in which insulin mutants had diminished survival or increased bacterial load. Each experiment was binned according to the degree of significance of the observed phenotype: scores of 1 or 21 indicate experiments in which p , 0.05; scores of 2 or 22 indicate experiments in which p , 0.01; scores of 3 or 23 indicate experiments in which p , 0.001; and scores of 4 or 24 indicate experiments in which p , 0.0001.

In chapter 4, I showed that suppression of insulin in the host protects from oral infection with *V. cholerae* while increased insulin signaling reduces the survival of flies infected with *V. cholerae* (Figure 4-3, Figure 4-4). To understand the complex relationship between host insulin signaling and pathogenic microbes, I asked how insulin affects host immune responses to a variety of pathogens. As I wanted to have a comprehensive overview of insulin peptides involvement in the host response to infection, I used a variety of pathogens with a spectrum of virulence, including Gram-positive and negative pathogens. I showed that insulin deficient flies have a unique response to each pathogen and that ILP 2,3 and 5 affects both tolerance and resistance of the host or induce no change on tolerance and resistance phenotypes depending on the bacteria (Figure 4-8, Figure 4-9, Figure 4-10, Figure 4-11). After correcting for size differences, I found that only during oral infection with *S. marcescens DB11* and *P. rettgeri,* the difference between bacterial load disappeared however size correction for the rest of pathogens had no effects on bacterial load difference between *ilp2,3,5* mutants and control *(*Figure 4-9). These observations suggest that reduced levels of bacteria in *S. marcescens DB11* and *P. rettgeri* is not a result of host ability to clear the infection but only due to a small size of the *ilp2-3,5* mutants (Figure 4-5).

One limitation of my study is that I only measured bacterial load during two timepoints which makes it difficult to precisely predict or define the tolerance curve shape which is an important approach to understand tolerance during host-pathogen interactions (1,305,601). In line with my observations that *insulin 2-3,5* mutants have a specific response to each pathogen, mutation of the Insulin Receptor Substrate orthologue, *chico* resulted in improved survival against *P. aueroginosa* and *E. faecalis* (438). However, another study showed that although *chico* mutants did not show improved survival towards *P. luminescens* or *E. coli*, an increased cellular immunity was observed for these flies (441). The crosstalk between insulin signaling and impact on host survival upon infection is conserved through evolution as in *C. elegans*, mutation in the insulin-like receptor encoded by *daf-2* and phosphatidylinositol 3-kinase (PI3–kinase) encoded by *age-1* results in survival extension against *E. faecalis, S. aureus*, and *P. aeruginosa* (602). In *Drosophila*, Forkhead box transcription (FKH) factor family which is a FOXO homologue is activated in response to reduced insulin signaling and acts in parallel to FOXO to regulates immune response. In adult *Drosophila*, intestinal FKH extends host survival to systemic infection with *Ecc15* (603). Conversely, flies deficient in intestinal FOXO signaling showed impaired resistance to oral

infection with *S. marcescens*. The authors showed that increased ectopic FOXO expression in the enterocytes improves resistance of the host via increased AMP production (440).

Consistent with previous studies that route of infection impacts diseases outcome (287), I found that route of infection was an important factor contributing to the survival of *ilp2-3,5* mutants to different pathogens. Deposition of *S. marcescens DB11*, *P. rettgeri*, and *E. faecalis* through thorax to the body cavity of *Drosophila* resulted in reduced survival in *ilp2-3,5* mutants. In contrast, *P. sneebia* septic infection did not affects survival or bacterial load in *ilp2-3,5* mutants flies compared with control (Figure 4-10). These results suggest that lack of insulin is lethal for host during septic infection with *S. marcescens DB11*, *P. rettgeri*, and *E. faecalis* while insulin deficiency does not impact host ability to combat systemic infection with *P. sneebia*. As *insulin 2,3,5* mutants have increased circulatory levels of trehalose and glucose (104,119), it is possible that high levels of carbohydrates in the hemolymph positively contributes to the pathogenesis of *S. marcescens DB11*, *P. rettgeri*, and *E. faecalis* possibly through increased expression of virulence factors. However, high levels of circulatory trehalose could be limiting bacterial load upon septic infection in *P.* sneebia as I showed there was no difference between bacterial load at both 6 and 12 hours post septic infection in *ilp2-3,5* mutants and control flies (Figure 4-11). Galac and Lazzaro showed systemic infection of wildtype flies with *P. sneebia* does not induce AMPs production and they suggest *P. sneebia* is able to avoid IMD signaling activation, as I found no difference between bacterial load of *ilp2-3,5* mutants and control flies in *P. sneebia* infection, therefore it is also possible that a balanced combination of tolerance and resistance mechanisms have contained the bacterial burden in a metabolically compromised host (304).

E. faecalis has been shown to natively colonize the *Drosophila* intestine (309) but inoculation of *E. faecalis* in the hemolymph results in induction of virulence factors such as proteases that are able to degrade cecropin, an antimicrobial peptide (604). Therefore, it is possible that systemic suppression of insulin signaling in *ilp2-3,5* mutants during the septic infection increases expression of proteases in the *E. faecalis* which results in increased degradation of AMPs and increased bacterial burden in the *ilp2-3,5* mutants. AMPs production, melanization , phenoloxidase activity and phagocytosis of pathogen by *Drosophila* hemocytes are defence mechanisms which are required in the hemolymph against specific pathogens (605–607). I hypothesize that *ilp2-3,5* mutant flies are more vulnerable to septic infection of *P. rettgeri* or *E. faecalis* due to reduced functional hemocytes or reduced proliferation of hemocytes. This

hypothesis is supported by a recent study showing during starvation, a fat body derived signal suppressed hemocytes proliferation and forces hemocyte adhesion to cuticle to minimize energy utilization (551). I also speculate that insulin has a spatial regulatory role in *Drosophila* immunity against each pathogen. For example, in intestine, increased FOXO contributes to host survival against *S. marcescens* but in systemic infection lack of insulin could be detrimental for the host (Figure 4-10).

It is also possible that insulin influences disease progression by additional mechanisms such as avoidance behaviour. For example, in *C. elegans* attenuation of an insulin like neuropeptide increased avoidance behavior in response to pathogenic microbe *P. auerogínosa* (445). I showed that *ilp2-3,5* mutants consume significantly less food compared to the controls before and after infection with *V. cholerae* (Figure 4-5). However, as I measured food consumption using CAFÉ assay after 24 hours of infection and the food in the capillary tubes was a yeast-sucrose solution with no pathogens, I could not test if *ilp2-3,5* mutants had less appetite towards pathogens using this experimental approach. Also the CAFE assay is useful to measure liquid food uptake over long period of time but it does not test if *Drosophila* alters its preference to feeding non-pathogenic food as opposed to feed on food containing pathogenic microbes. Therefore, using the FlyPad assay will give a more precise understanding about the contribution of taste or post-ingestion effects on the reduced bacterial load during oral infection in *ilp2-3,5* mutants. Combined my observations along with other studies emphasizes the impact of insulin on host immune responses depends on the identity of the infectious microbe, contribution of AMPs and the route of bacterial introduction to the fly. To understand how lack of insulin affects host response toward these different pathogens and the mechanisms of pathogenesis for each microbe, I propose a transcriptome study using RNA sequencing on the *ilp2-3,5* mutants as well as pathogens after infection at early and later stage of infection. Using this approach we will be able to identify enrichments in biological processes in *ilp2-3,5* mutants and understand the contribution of virulence gene expression at earlier or later stage of pathogenesis.

6.3.2 The crosstalk between IMD pathway, AMPs and *V. cholerae*

The *Drosophila* intestine is exposed to pathogenic and non-pathogenic bacteria through feeding as the most relevant route of infection. Intestinal immunity is a critical part of the fly defense system and activation of the IMD pathway is a defence mechanism against pathogens though production of AMPs (608). For example, activation of IMD in response to a *Pseudomonas*

entomophila oral infection protects the host against infection and extends survival (299). During systemic infection, IMD and Toll signaling activation in the *Drosophila* fat body are mainly responsible for a humoral immune response via increased expression and secretion of AMPs into the hemolymph (334). In chapter 4, I asked if oral infection of wildtype flies with *V. cholerae* affects expression of systemic AMPs. I showed that enteric *V. cholerae* infection results in increased systemic expression of *diptericin* but had no effects on expression of *drosocmycin* or *attacin* (Figure 4-6). This observation suggests that intestinal *V. cholerae* infection could induce a humoral immune response. A previous study using a different biotype of *V. cholerae O139 MO10* supports this results as they showed a significant increase in *diptericin* mRNA extracted from whole flies after feeding flies with *V. cholerae* (420). If intestinal defence mechanisms fail to contain the pathogen in the epithelium, entry of pathogens into the hemolymph is lethal for the host. For example, the human opportunistic pathogen, *P. aeruginosa* is able to cross the intestinal epithelium of *Drosophila* which results in a septic infection and host death (609).

As *V. cholerae* causes extensive damage to the midgut epithelium (382,453,454), and usually results in 50% death within 48 hours of intestinal infection, it is possible that *V. cholerae* intestinal infection induces a systemic humoral immune response which accelerates the pathogenicity of *V. cholerae*. In addition to systemic effects of AMP on pathogen clearance, production of AMPs by epithelial immune responses is important to contain pathogens. To understand the contribution of AMPs in the host response during both oral and systemic infection, I used a null mutant fly line that lacks 10 out of 14 known AMPs (195) and exposed them to *V. cholerae*. I found that *ΔAMPs* have a reduced survival upon septic infection with *C6706* and have a higher bacterial load compared with isogenic wildtype flies (Figure 4-6). This observation suggests that lack of AMPs in the host results in reduced resistance to *V. cholerae* septic infection which is consistent with this study showing reduced survival for *dredd* mutants upon septic infection with *V. cholerae* (420). Conversely, I found no difference between survival of the *ΔAMPs* mutants and control flies upon intestinal infection with *C6706* and the bacterial load was similar to isogenic control flies (Figure 4-6). This observation suggests that lack of AMPs does not affect host response to enteric infection of *V. cholerae* and perhaps other intestinal defence mechanisms such as ROS production or physical or microbial barriers are contributing to the host defence. Measuring AMPs at different timepoints and correlating it to the microbe loads will be more

informative in future studies to identify if increased bacterial load correlates with the reduced resistance via suppression of AMP production.

Consistent with the improved survival of *imd* mutants upon enteric infection with *V. cholerae ,* a recent study from our group examined the consequences of IMD inhibition in specific intestinal cell types for host survival after intestinal infection with *V. cholerae* (381). We found that inhibition of IMD in enterocytes significantly extended the survival of infected flies, whereas inhibition of IMD in the progenitor cells results in reduced survival of the host. These observations suggest that the activity of IMD in enterocytes is sufficient to enhance *V. cholerae* pathogenesis. Interestingly, I showed that IMD inhibition in fat body increases survival of the host to intestinal infection with *V. cholerae* while having higher bacterial load, which suggests increased host tolerance (Figure 5-4). As *V. cholerae* competes with commensal bacteria in the intestine for colonization and nutrients, I speculate that suppression of IMD in the fat body results in alteration of intestinal metabolites or residential microbes through a fat-gut or fat-brain axis which results in higher *V. cholerae* proliferation or colonization in the intestine, however, reduces the virulence expression of *V. cholerae* therefore higher loads of *V. cholerae* is not lethal for the host. It will be interesting to visually scan the midgut in the flies with IMD suppression in the fat body using GFP-tagged *V. cholerae* to examine the colonization of the *V. cholerae* in these flies.

6.3.3 *V. cholerae* infection disrupts host metabolism

In chapter 4, I showed that intestinal infection of wildtype flies with *V. cholerae C6706* strain mainly affects carbohydrate metabolism in the host leading to increased circulating glucose, reduction in circulating trehalose and reduced total glucose levels after 24 hours of oral infection (Figure 4-1). However, I did not observe any difference between weight or total triglyceride levels of infected and control flies which could be due to the timing of sample collection after 24 hours by which the effects on weight is too early to be noticed. These observations suggest that *C6706* oral infection negatively affects carbohydrate metabolism in the host. A previous study by Watnick lab supports my results as they showed oral infection with *V. cholerae MO10* strain which belongs to O139 serogroup suppresses metabolic homeostasis via reduced glycogen and glucose levels, however, they also observed reduced triglyceride levels in whole flies and depletion of lipid stores from the fat body and accumulation of lipid droplets in the enterocytes (419). The difference between this study and my results showing no difference in triglyceride levels could have different

explanations such as different serogroups of *V. cholerae* and timeline for the collection of samples as I only measured the macronutrient levels after 24 hours of infection, while Hang et al. measured the macronutrient levels at different timepoints until 72 hours of infection. Although *V. cholerae* O139 serogroup induces similarity in clinical severity to El Tor biotype it has a different antigen structure and produces a polysaccharide capsule (610) which could also induces different pathology compared to O1 El Tor strains. For example, encapsulation is associated with resistance to the bactericidal activity of normal human serum and increased virulence in animal models (611).

V. cholerae O1 El Tor strains grow extremely well in a glucose-rich environment as they avoid acidification of their growth medium by production of the neutral fermentation end products acetoin and 2,3-butanediol (431). Acetoin production through glucose fermentation offers *V. cholerae* cells a survival advantage during infection by downregulating the host innate immune responses (612). Infecting zebrafish with *V. cholerae* El Tor strain after 12 hours of glucose feeding resulted in a five-fold increase in colonization compared to *V. cholerae* colonization of fish without glucose feeding (613). These studies suggest that glucose provide a favorable environment for *V. cholerae* growth. In line with these studies that glucose benefits *V. cholerae* colonization, I found that holidic diet supplemented with high glucose was beneficial for the host during oral infection with *V. cholerae* (Figure 4-2). I showed that bacterial load of *V. cholerae* on the high glucose diet was similar to the control group that suggests a high glucose diet confers tolerance to the host against *V. cholerae* infection. This result suggests that glucose facilitates host tolerance to *V. cholerae* and on the other hand increases *V. cholerae* fitness through extending host-pathogen interaction to extend the host-pathogen interaction and a prolong environmental dissemination.

As I showed increased circulatory levels of glucose and reduced total glucose levels, it is possible that increased glucose influx alters metabolites consumption by commensals and suggests a symbiont-induced signal suppresses virulence gene expression in *V. cholerae* which results in containment of *V. cholerae* proliferation. Another possible hypothesis is that glucose increases intestinal integrity therefore reducing the chances of bacteremia resulted from escape of *V. cholerae* to the hemolymph. This hypothesis is supported by a recent study from our group showing increased intestinal barrier integrity though improved localization of Coracle to septate junctions in flies fed a holidic diet supplemented with glucose and exposed to intestinal infection with C6706 (614).

The insulin-glucagon hormonal circuit is the main metabolic pathway responsible for maintaining carbohydrate homeostasis and is conserved in *Drosophila* (98). Similar to mammalian pancreatic β-cells, IPCs in adult *Drosophila* directly responds to high levels of circulatory sugars and secret ILPs into the hemolymph (118). As I observed increased survival of flies raised on high glucose diet against intestinal infection with *V. cholerae* and given a study by Hang et al showed suppression of systemic insulin signaling by intestinal infection with *V. cholerae* (419), I hypothesized that lack of insulin in the host is beneficial for fighting *V. cholerae*. I used a specific *Drosophila* null mutant for *ilp2-3,5* to investigate the relationship between host insulin signaling and *V. cholerae* pathogenesis. I found increased survival along with reduced bacterial load after 48 hours of infection in *ilp2-3,5* mutants upon enteric infection with *C6706* (Figure 4-3). This result suggests insulin deficiency protects the host against *V. cholerae* via increased resistance of the host towards *V. cholerae*. Conversely, I showed increased systemic insulin signalling reduced resistance to *V. cholerae* as *ImpL2* ^{def20} mutants had a reduced survival and increased bacterial load after 48 hours of oral infection with *C6706* (Figure 4-4). These observations suggest that modulation of host insulin signaling alters host response to *V. cholerae* infection. It is possible that *V. cholerae* suppresses insulin signaling directly as it has been shown for *Vibrio vulnificus* that contain an insulin degrading enzyme called sidC (615). The SidC homolog in *V. cholerae* is also up-regulated during the late stages of infection in mice (616). The authors suggest that once *V. vulnificus* infects a host, it secrets SidC to degrade insulin and consequently increasing blood glucose levels which results in improving pathogenicity and survival of the pathogen. As *Drosophila* visceral muscle in in the intestine produces ILP3, it is possible that *V. cholerae* could suppresses local production of *ilp3* at the mRNA level or degrading the ILP3 peptide, however, future experiments are needed to test this possibilities.

Protective effects of insulin mutations are not limited to *V. cholerae* and previous studies have reported the impact of mutation in different components of insulin signaling on different pathogens (438,440,441), however, the impact of insulin signaling in response to each individual pathogen on the host is complex and has been discussed in section 6-8. Consistent with increased survival of insulin-deficient flies after an intestinal *V. cholerae* infection, I showed that suppression of insulin signaling in the fat body extends host survival after *V. cholerae* intestinal infection. I showed that bacterial load of flies with suppressed insulin signaling in the fat body was similar to control flies and flies with suppressed insulin signaling in the fat body had a slower rate of pathogen

clearance after withdrawal of the host from *V. cholerae* oral infection (Figure 5-2). These results suggest increased tolerance of flies with suppressed insulin signaling in the fat body to *V. cholerae* infection and indicate an inter-organ communication between the fat body and intestine as inhibition of insulin signaling in the fat body alters host response to an infection in a distant tissue. As I was interested in understanding how insulin inhibition in the fat body is beneficial for the host against *V. cholerae*, I examined intestinal progenitor division and enteroendocrine (EE) cells before and after infection in flies. Progenitor cells proliferation is important for maintenance of epithelial repair programs (617). EE cells are involved in regulating local stem cell division (478,618), lipid metabolism (323), and feeding (619). Metabolites released in the intestinal lumen by food consumption or by-products of intestinal microbes are sensed by EE cells and EE cells secrete peptides to maintain local and systemic metabolic homeostasis (620).

I found increased PH3 positive mitotic cells in the progenitor cells before infection with *V. cholerae* in flies with suppressed insulin signaling in the fat body and increased number of Prospero positive cells indicating increases in population of EE cells after oral infection with *V. cholerae* (Figure 5-3). I propose that suppression of insulin signaling in the fat body results in increased circulatory trehalose, increased progenitor division which probably provides an environment more capable of tolerating pathogens possibly by increasing the epithelial barrier integrity. I speculate that increased enteroendocrine cells after infection with *V. cholerae* protects progenitor cells from *V. cholerae* which results in increased survival of flies with suppressed insulin signaling in the fat body (Figure 6-3). I showed that suppression of insulin signaling in the fat body increases circulatory trehalose and holidic diet supplemented with glucose improves host tolerance against enteric *V. cholerae* as well (Figure 5-1, Figure 4-2). A previous study showed that upon chronic high sugar diet, EE cells signals to the fat body via Activin β to increase AKH signaling resulting in hyperglycemia (238). Therefore, it is possible that increased EE cell in the flies with suppressed insulin signaling in the fat body after intestinal *V. cholerae* infection improves host tolerance to *V. cholerae* via communicating with the fat body to increase circulatory glucose and therefore providing an environment which facilitate improved tolerance to *V. cholerae.* Follow up studies should investigate the molecular mechanisms of host insulin signaling in the pathogenesis of *V. cholerae* and identify the signaling molecules probably releasing from fat body that improves host survival to enteric infection of *V. cholerae*. For example, we could extract hemolymph of flies with suppressed insulin signaling in the fat body before and after infection with *V. cholerae* and identify

the alteration in the metabolites or peptides released in the hemolymph that might be involved in the increased tolerance of host to *V. cholerae*.

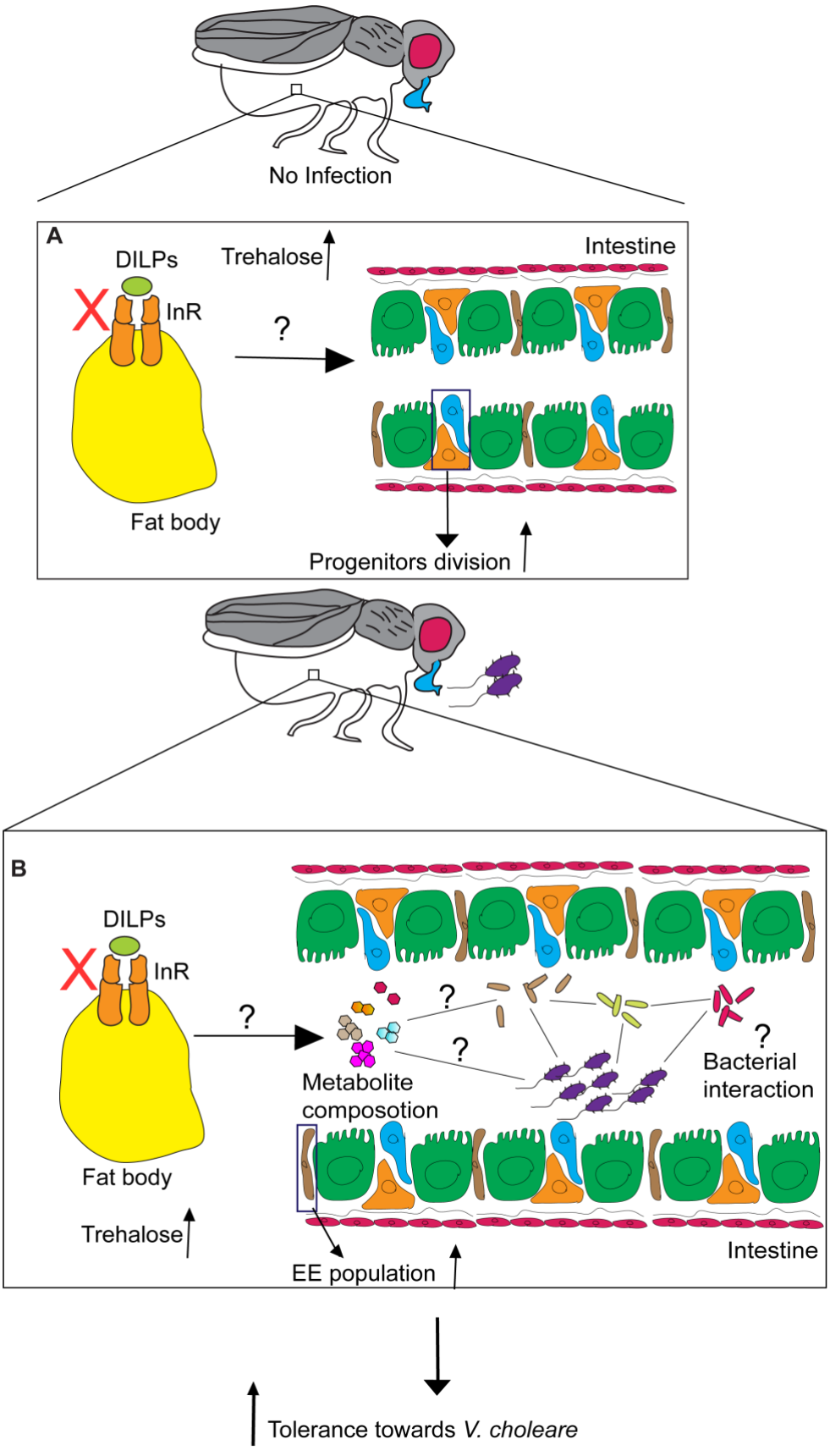
Once *V. cholerae* attacks intestinal epithelial cells, it subverts host metabolism for its own advantages, and competes with the gut resident symbionts for space and nutrients to progress its colonization. *V. cholerae* challenge impacts intestinal levels of acetate (419), succinate (429), and methionine sulfoxide (621) which results in suppression of host insulin signaling, lipid homeostasis, and epithelial renewal. The type six secretion system (T6SS) is an apparatus that punctures adjacent cells and delivers toxic effector proteins that disrupt lipid membranes and cell walls of the bacterial envelope (622,623). *V. cholerae* competes with other Gram negative bacteria including commensals for colonization of a niche using its T6SS, resulting in fewer competitors (624–626). Inactivation of the T6SS, or removal of commensal bacteria, attenuates disease severity. Reintroduction of the commensal, *Acetobacter pasteurianus*, into a germ-free host is sufficient to restore T6SS-dependent pathogenesis (454,625,627).

Given the role of the T6SS in pathogenesis *of C6706* in *Drosophila* (453,454), it is possible that suppression of insulin signaling systemically or locally in the fat body alters metabolites in the intestine that provides protection for commensals against toxic effects of T6SS and therefore results in inhibiting *V.* cholerae growth or limiting the damaged induced by *V. cholerae* which consequently results in survival extension. In support of this hypothesis, a co-culture of *E. coli* and *V. cholerae* in the presence of glucose resulted in reduced growth of El Tor strains *in vitro* (628). *E. coli* fermentation of glucose produces an acidic environment therefore reduces *V. cholerae* growth. Another study showed exogenous glucose protects *E. coli* strains from T6SS-mediated pathogenesis by *V. cholerae* which is probably through alterations to the *E. coli* cell envelope (629). Combined these studies with my results suggest a dynamic interaction between *V. cholerae*, host metabolism, fat body, intestinal physiology which collectively affect host survival. In the future, it may be valuable to investigate the mechanisms of fat to gut interaction and impact on commensal population, metabolite availability in the intestine as well as colonization of *V. cholerae* in flies.

6.4 Concluding remarks

Growing metabolic disorders due to chronic inflammation is concerning for public health. How host metabolism adapts to an infection or increased inflammation requires more investigation. Therefore, understanding the mechanisms of the crosstalk between immune and metabolic

responses by using different animal models empowers us to better understand the complex biology of the immune-metabolic interactions that also exists in metabolic and infectious diseases. This thesis highlights the values of using *Drosophila* model which shares conserved metabolic and immune signaling with mammals to study the immune-metabolism. I believe my findings in this thesis contributed to the scientific community by uncovering the impact of immune-metabolic regulations for host fitness during development and upon facing pathogenic microbes. Overall, these results indicate imbalance between immunity and metabolism crosstalk has negative consequences for host such as suppression of metabolism, delay in development and reduction of body size in the larvae. The results also indicate a systemic role for IMD in the regulation of host metabolic homeostasis in the adult stage and emphasizes the impact of a conserved metabolic signaling on the host response to pathogenic microbes. Finally, this thesis uncovers an interesting possible communication between *Drosophila* organs by showing modulation of immune-metabolic pathway in the fat body alters host responses to infection in a distant tissue such as the intestine. Future work in the immune-metabolism fields needs to focus on the molecular and cellular mechanisms that host is adapting to regulate metabolic signaling as well as uncovering the pathogens virulence factor contribution that alters host metabolic adaptation.


No Infection
A
DILPs
InR
Fat body
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?
Intestine
Progenitors division
B
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InR
Fat body
Trehalose
?
Metabolite
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?
?
?
Bacterial
interaction
Intestine
EE population
Tolerance towards V. choleare

Figure 6-4 Proposed model for impact of insulin signaling in the fat body in protection of host against intestinal *V. cholerae* infection

In this proposed model, suppression of insulin signaling in the fat body in the absence of infection results in elevation of circulatory trehalose and increased division rate of progenitor cells. As *V. cholerae* suppresses progenitor cell proliferation, increased division rate before infection in flies with suppressed insulin pathway in the fat body could enhance survival of flies against *V. cholerae*. The mechanisms or signals that increases stem cells division in flies with blocked insulin signaling in the fat body remained to be investigated. Once flies are fed with *V. cholerae*, in flies with suppressed insulin signaling in the fat body, number of enteroendocrine cells in the midgut epithelium increases which could possibly communicates with the fat body or other organs to improve host fitness against *V. cholerae* infection. On the other hand, unknown signals from the fat body to the intestine in flies with blocked insulin signaling in the fat body alters metabolites available in the intestinal lumen or composition of commensals microbes in which *V. cholerae*, bacterial burden is contained and *V. cholerae* tolerance is improved in flies with a deficient insulin signaling in the fat body.

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