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THE ROLE OF ENTEROENDOCRINE SECRETED GUT PEPTIDE HORMONES IN MODULATING IMMUNITY AND METABOLISM IN *DROSOPHILA MELANOGASTER*

BY

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ABSTRACT

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Title: The Role of Enteroendocrine Secreted Gut Peptide Hormones in Modulating Immunity and Metabolism in *Drosophila melanogaster*

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The intestine of all living organisms fosters an ecosystem of commensal microbiota that plays key roles in the maintenance of host health and pathology. Captivatingly, the influence of dysbiosis on the host has demonstrated the significance of the existing crosstalk between the gut microbiota, nutrient balance, and immune processes. Intestinal enteroendocrine cells (EE)-secreted gut peptide hormones represent an emerging area of exploration, with a gut flora-dependent role in modulating metabolism and innate immune signaling yet to be determined. In this study, we utilize the *Drosophila melanogaster* model organism to understand the systemic and/or tissue-specific roles of Tachykinin (Tk), Diuretic Hormone 31 (DH31), and Allatostatin A (AstA) EE secreted peptide hormones in maintaining metabolic homeostasis and modulating innate immune signaling. Our findings reveal significant disruptions in gut flora distribution and in several metabolic parameters including: body weight, systemic glucose and triglyceride levels, lipid transport from gut, and fat body lipid storage in three systemic mutant lines ($Tk^{EY20174}$, $Dh31^{KG09001}$ and $AstA^{MB10261}$) and in the $Tk>AstA^{RNAi}$ transgenic line. We also report altered immune status and host susceptibility profiles $Tk^{EY20174}$, $Dh31^{KG09001}$, $AstA^{MB10261}$, and $Tk>AstA^{RNAi}$ flies infected with bacteria. Consistent with these results, RNA-sequencing on the whole intestine of these systemic mutants and transgenic flies identified several differentially

expressed genes associated with the processes of metabolism and immunity. Together, the findings of this project provide further insight into the contribution of EE-secreted peptide hormones in the maintenance of immune-metabolic homeostasis in a host, a foundation that could have profound implications on the therapy of metabolic and immune illnesses as well as for metabolic ramifications of intestinal dysbiosis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

The intestinal lumen of all living being harbors a bionetwork that is demarcated by the microorganisms that inhabit it and the nutrients that traverse through it. Such a microbe-nutrient companionship subjects the intestinal ecosystem to habitual transitory changes that could affect both its immune and metabolic homeostasis. While most studies have generally focused on understanding the impact of these intestinal alterations on immunity and metabolism singly, it is becoming increasingly evident that a bi-directional "immune-metabolic" cross talk is, indeed, needed to maintain homeostatic balance post such intestinal alterations [1-6]. Provisionally, our current understanding of the intestinal players and mechanisms that orchestrates this adjacent concordance between immunity and metabolism is still at its infancy, with such a gap in knowledge partly attributed to the lack of powerful tools that could elucidate some aspects of this immune-metabolic cross talk in dictating a state of health or disease. Owing to the significant parity between the intestine of fruit flies and mammals, along with its conserved signaling pathways, and its readily available genetic tools, the fruit fly is now presented as a tool of choice to unravel such an immune-metabolic intestinal alignment. Both mammalian and fly guts are composed of intestinal stem cells (ISC) which divide and differentiate to give rise to either nutrient-absorbing enterocytes (EC) or hormone-producing EE [7]. Apart from their well-studied roles in modulating several biological processes including growth, development [8], and functions of the nervous system, [9], an emerging body of evidence highlights a potential role of EE-secreted gut peptide hormones in modulating immunity and metabolism, but their mechanism of action is yet to be determined. In this study, we employ the *Drosophila melanogaster* fruit fly as a model organism of choice to shed more light on the role of

Tk, Dh31, and AstA EE peptide hormones on maintaining metabolic homeostasis and modulating innate immune signaling.

1.1 Hypothesis

Systemic and/or EE-tissue specific knockdown (kd) of *Tk, DH31,* and *AstA* gut peptide hormones alters metabolic parameters and modulates innate immunity and pathogen susceptibility of mutant and/or transgenic *D. melanogaster*.

1.2 Study objectives

- Assess the impact of systemic and/or EE-tissue specific kd of *Tk, DH31, and AstA* on several metabolic parameters including body weight, systemic glucose and triglyceride levels, lipid transport from gut, and lipid storage in fat body.
- Assess the effect of systemic and/or EE-tissue specific kd of *Tk, DH31,* and *AstA* on different innate immune signaling pathways and immune defense mechanisms.
- Evaluate the effect of systemic and/or EE-tissue specific kd of *Tk, DH31, and AstA* on host susceptibility to pathogens and on the normal distribution of the gut microbial flora.

Chapter 2: Literature Review

2.1 Fruit fly as a model organism

Drosophila melanogaster, commonly referred to as the fruit fly, has become a highly valued model organism of paramount importance in various areas of scientific explorations. Its meritorious contribution in laboratory settings is attributable to the numerous features it possesses including its low cost, ease of rearing and maintenance, high fecundity, well-defined genome, as well as the available tools for its genetic manipulation [10, 11]. The sequencing of *Drosophila*'s genome has revealed up to 75% disease-causing genes homology with humans [12, 13]. Appealingly, the use of genetic tools like the fly's versatile GAL4-upstream activation sequence (UAS) transactivation system [14-16], for example, which allows the overexpression or knockdown of a gene of interest in a tissue-specific manner, can be used to generate "humanized flies" for an improved understanting of the function of conserved genes in disease development and progression. The bipartite expression system involves the yeast transcription factor GAL4 and its UAS, to which the GAL4 binds. UAS controls the expression of a target gene, while GAL4 is placed under the control of a gene promotor. GAL4 binds to the UAS promotor, and once activated, drives the expression of the target gene in a specific cell type in which GAL4 is also expressed [14].

The evolutionary conservation between fruit flies and mammals is not only evident at the molecular level, as analogous organs with functional similarities also exist in both [17]. While nutrients are absorbed and digested in the mammalian stomach and small intestine, these processes are mostly limited to the midgut in *Drosophila.* Dietary triacylglycerides (TAG) are metabolized in the midgut by Magro, a homolog of mammalian gastric lipase, along with dietary sugars. The products are then absorbed

by EC and eventually trafficked to the fat body [18]. The fat body is the site of lipid and carbohydrate storage and mobilization [19-21]. Lipid droplet mobilization is achieved by a homolog of human triglyceride lipase, Brummer [22]. The fat body shares properties of mammalian adipose tissue [23] and secretes factors comparable to mammalian adipokines for the regulation of metabolism [24]. It is also the site of carbohydrate and lipid storage and mobilization. In humans, acid-labile subunit (ALS) forms a complex with insulin-like growth factor (IGF) binding protein 3 and IGF-1. Correspondingly, *Drosophila* ALS (dALS) associates with *Drosophila* insulin-like peptides (Dilps) in the fat body and is thought to modulate insulin action in a manner similar to that seen in the mammalian IGF-1 pathway [25]. Moreover, the fat body is also considered to possess shared characteristics of the mammalian liver such as the presence of analogous enzymes that regulate glycogen metabolism [26]. A more recent discovery has demonstrated the presence of hepatocyte-like cells known as oenocytes in *Drosophila* larvae which were found to accumulate lipids upon food deprivation [27]. Additionally, these cells express a number of genes similar to those found in the mammalian hepatocytes including enzymes and cell surface proteins involved in lipid processing, as well as orthologs of hepatic transcription factors [28].

Colonization of the gut by commensal microbiota is a common trait of organisms with open digestive tracts. The complex relationship between the commensal microbial community, host physiology, and the environment shapes growth and development, metabolic homeostasis, and immunity [5, 29-31]. Mammals are exposed to a broad array of microbiota found in plant and animal food sources, whereas the diet of wild fruit flies is more restricted to rotting fruits and vegetables, as well as fungi [32]. The mammalian intestinal microbiota consists of more than 500 taxa as compared

to five to 30 taxa present in the gut microbiota of a wild fly [32-34]. The microbiota of laboratory-reared flies tends to be even more limited as a result of its controlled artificial environment [35]. Accordingly, *Drosophila*'s microbiota is less complex and easily manipulable, affording experimental tractability.

The initial employment of the fruit fly in research, which was predominantly genetics-based, has unraveled the basic principles of inheritance [36, 37]. Later, its use in further investigations led to the elucidation of several highly conserved signaling pathways of similar functional roles in both flies and mammals. Recently, and owing to the aforementioned organ and signaling homology, the use of *Drosophila* as a model organism of choice has also gained considerable attention in the field of immunometabolism [38].

2.2 Fruit fly gut structure

Numerous structural and functional similarities exist between the *Drosophila* and the mammalian gut. The mammalian gut encloses villi and crypts whose epithelium consists of nutrient-absorbing ECs, peptide-secreting EE, mucus-secreting goblet cells, Paneth cells, and stem cells [39-41]. On the flip side, the *Drosophila* gut is also highly specialized in organization and function and is divided into three main regions: the foregut, the midgut, and the hindgut. The foregut begins at the oral cavity and extends to the esophagus and the crop (mammalian stomach analogue). In the crop, ingested food is initially processed and subsequently digested in the midgut by proteases, carbohydrases, and lipases. The hindgut regulates the water, ions, and other nutrients released from the Malpighian tubules (mammalian kidney analogue) (Figure 1). The fruit fly gut epithelium is coated with a chitinous peritrophic matrix, the equivalence to the mucus layer in the mammalian gut [42-45]. Although less complex to its

mammalian counterpart, the *Drosophila* gut epithelium comprises similar cell types including ECs, EEs, ISCs, and immature progenitor enteroblasts (EBs) [46]. ISCs and EBs generally differentiate and give rise to ECs or EEs in a greatly similar manner to the self-renewing abilities of the mammalian gut epithelial cells [17, 47, 48]. EC are mainly known to be involved in carrying out digestive, absorptive, and innate immune processes [49, 50]. EEs, on the other hand, express cell-surface gustatory receptors [51] and bear intracellular vesicles containing peptide hormones that modulate gut peristalsis, as well as lipid and carbohydrate metabolism [52-55]. As previously touched upon, dietary lipids and carbohydrates are digested within the gut and absorbed by EC [18], after which they enter the hemolymph before being transported to the fat body which functions in energy storage and mobilization [19-21].

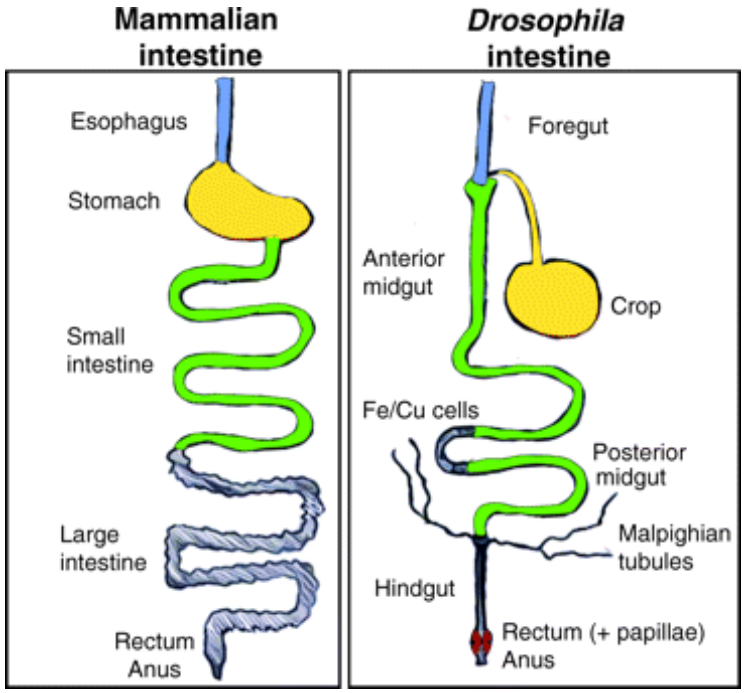


Figure 1. A comparison between the mammalian and the *Drosophila* gut structures. Similarities exist between the esophagus and foregut, the stomach and crop, the small intestine and midgut, the large intestine-rectum-anus and the hindgut-rectum-anus regions. The Malpighian tubules in the fruit fly serve as the kidneys which release water, ions and other nutrients into the gut. Reprinted from "*Drosophila melanogaster* as a model for human

intestinal infection and pathology" by Apidianakis, Y. & Rahme, L.G. (2011). *Disease Models & Mechanisms, 4*, p. 21.

2.3 Fruit fly microbiota

Drosophila's microbiota is of much lower diversity in terms of bacterial taxa as compared to the mammalian intestinal flora [56-59]. Though our exact understanding of such differences remains at its infancy, this can be correlated to many plausible host factors. The adaptive immune system in higher metazoans is thought to facilitate the greater diversity of microbes [60], compared to the fruit fly's innate immune system. Alternatively, it has been suggested that as a result of the insect gut's transient nature and its short life span, the insect gut niche more frequently experiences perturbation [61]. In addition, the foregut and hindgut are shed during molting and the entire larval gut is replaced by a new adult gut during the process of metamorphosis [32]. The difference in diversity may also be credited to the age, type of nutrient, age and development, genetic makeup, immunity and physiological response, geographical location, and environmental stimuli of the fruit fly [34, 35, 62-65]. This perhaps explains why the fruit fly may be incompatible with a gut colonization of highly diverse microbiota.

Identification of the various bacterial species and operational taxonomic units (OTUs) that make up *Drosophila*'s gut microbiota composition have principally been carried out using laboratory culture techniques and 16S rRNA gene amplicon sequencing [32]. Laboratory-reared flies are believed to have a more limited microbiota composition as a result of their restricted diets [34, 66]. Those fed a diet of composite sugars like soy flour and cornmeal have a lofty copiousness of *Lactobacillus* (Firmicutes of the order Lactobacillales), whereas those fed a sugar-rich diets have a

flora predominated by *Acetobacter and Gluconobacter* species from the family *Acterobacteraceae* (α-Proteobacteria) [34, 66]. In some fly cultures, γ-Proteobacteria or *Enterococcus* bacterial species outweigh *Acetobacteraceae* and *Lactobacillus* to the point of nearly or completely abolishing their growth. Taxonomic variations in the microbiota composition also exist among laboratory-reared and wild *Drosophila* populations whereby the latter is much more diverse [67-70]. While *Acetobacteraceae, Lactobacillales*, and γ-Proteobacteria exist in the gut of wild *Drosophila*, the number of Lactobacillus tends to be low or undetected at times. Instead, *Leuconostoc, Enterococcus, and Weissella* represent the *Lactobacillales* order [71]. Though not well-explored, wild-caught and laboratory-reared flies, especially those feeding on rotting fruits, also exhibit yeasts, namely *Hanseniaspora, Pichia*, and *Candida* as part of their normal flora [72-74].

2.3.1 Role of gut flora in immunity

The fruit fly acts as a valuable system in deciphering host-pathogen interactions. An emerging body of evidence has implicated the microbial flora of the gut in inducing host immunity and attenuating the virulence of various pathogens [11]. Germ-free (GF) or axenic flies with a diminished microbiota were reported to be more susceptible to *Serratia marcescens* and *Pseudomonas aeruginosa* infections as compared to those flies with a normal microbiota. Interestingly, this susceptibility phenotype was rescued upon supplementation of the gut flora with *Lactobacillus plantarum* which protected the flies from infection [35]. One of the chief immune responses induced upon pathogenic infection in the fruit fly is the generation of reactive oxygen species (ROS) to eliminate foreign pathogens [47, 75]. Two NADPH enzymes known as dual oxidase (Duox) and NADPH oxidase (Nox) trigger the production of ROS [76], whereby their

activation is induced by both commensals and pathogenic microorganisms [75, 77, 78]. When fed microbe-infected food, flies with a deficiency in Duox activity becomes less resistant to enteric pathogens, causing them to succumb to death faster [75]. Upon the augmentation with commensals however, particularly *Lactobacillus* spp., ROS production is stimulated through a Nox1-dependent mechanism, promoting ISC proliferation [78]. *Lactobacillus plantarum* has been also shown to serve as a strong inducer of the ROS-sensitive CncC/Nrf2 signaling pathway within EC [79]. Larvae fed *Lactobacillus plantarum* exhibit an upregulation in CncC-dependent gut expression of *upd2*, a cytokine gene product involved in regulating gut homeostasis through JAK/STAT signaling in midgut tissue [80]. Accordingly, a decrease in Upd2 levels renders *Lactobacillus plantarum* unable to stimulate the proliferation of midgut epithelial cells in the fruit fly [79]. These findings are consistent with the notion that the commensal microbial community conditions the basal level of ISC proliferation and gut epithelium renewal, possibly via elevating JAK/STAT and c-Jun N-terminal kinase (JNK) signaling [81, 82].

ROS-resistant bacteria are eliminated through the contribution of AMP production [83]. Systemic regulation of AMP production is primarily dependent on NF-κB signaling, whereas the mechanism of AMP production within the fly gut is slightly more complex [76]. The gut microbiota partakes in provoking Imd-Relish signaling within the gut. Nuclear translocation of the Rel transcription factor was evident in the intestinal cells of *Erwinia carotovora carotovora-15*-infected and uninfected conventionally raised flies, and was almost completely abolished in GF, antibiotic-treated, and Imd pathway mutant flies. The levels of gene expression of other Imd pathway components such as PGRP-SC and PGRP-LB, were also reported to be higher

in the guts of conventionally raised wild-type flies as compared to antibiotic-treated and Imd pathway mutant flies [84]. A study by Kamareddine et al. demonstrated positive regulation of NF-κB signaling in EE via the microbiota's production of the SCFA, acetate. GF flies, lacking dietary acetate production by the gut flora, display a similar phenotype to rel^{E20} mutants with disrupted Rel nuclear translocation [5].

Apart from its protective effect, the gut microbiota may augment the virulence of some pathogens in certain cases. *V. cholerae*, for example, consumes the microbiota-produced acetate, affording it a growth advantage [85]. Infected GF flies also experience a disruption in metabolic homeostasis and intestinal steatosis which is reversed by acetate supplementation [5, 85].

Fungal colonization of the gut could be also restricted by the action of the gut microbiota. The interplay between the commensal community and host immunity is thought to mediate this interference in colonization. This consideration is indeed tailored to the observation that Spätzle and Imd mutant GF larvae infected with *Candida albicans* have a reduced life span [79]. Toll signaling has long been thought of as confined to hemocytes and the fat body during systemic infection, and gut immunity was seemingly uninvolved in this defense response [86]. Yet, recent studies have demonstrated the presence of a crosstalk between constitutive activation of Toll signaling and microbiota-derived peptidoglycan which have been circulated from the gut lumen to systemic circulation. *Klf15* mutant flies lacking nephrocytes and therefore incapable of renal filtration of peptidoglycan were found to be more resistant to infections via microbiota-derived peptidoglycan activation of Toll signaling [87].

In addition to the immune response elicited in the fruit fly gut in response to bacterial and fungal pathogens, the microbiota is also considered a contributing factor

to antiviral immunity [88]. In the gut, activation of antiviral extracellular signal-regulated kinases (ERK) signaling requires the participation of two different signals. The first one is dependent on priming NF-κB signaling to produce a secreted factor known as Pvf2 upon recognition of microbiota-derived peptidoglycan, particularly that of *Acetobacter pomorum*. The second signal involves virus-initiated Cdk9 kinase-dependent signaling which is necessary for the secretion of Pvf2 and gut ERK signaling response [88].

In the tsetse fly (*Glossina* spp.), Wigglesworthia, an obligate mutualist, induces an enhanced expression of odorant binding protein (obp) six in the larvae's gut. This upregulation is adequate to provoke the systemic expression of the hematopoietic RUNX transcription factor *lozenge*, stimulating crystal cell production, a key player in the melanotic immune response [89]. Congruently, the gut microbiota of *Drosophila* larvae mediates a similar hematopoietic pathway involving obp28a, the *Drosophila* orthologue of tsetse obp6. Higher expression levels of obp28a and *lozenge* are seen in conventionally raised larvae when compared to those reared under axenic conditions. Similarly, the number of cuticular sessile crystal cells and levels of prophenoloxidase (PPO), the inactive precursor of phenoloxidase, an enzyme involved in the melanization response, are evidently higher in conventionally reared larvae [89].

2.3.2 Role of gut flora in metabolism

The gut microbial community prospers on various nutrients derived from the host's diet and gut secretions, and is shaped by the environment of the gut, the host's partiality to certain foods, and its dietary patterns [30, 41]. In succession, the gut microbiota partakes in host growth and development, immune regulation, and metabolic homeostasis through metabolite production, regulation of hormonal

signaling, secretion of vital nutrients, and amendment of nutrient obtainability [5, 31, 41, 76, 90, 91]. In the fruit fly gut, *Acetobacter pomorum* and *Lactobacillus plantarum* play key roles in growth and development via IIS [76, 91-93]. Several lines of evidence attribute dysbiosis of the gut microbiota to a disruption in IIS which translates into metabolic disorders with phenotypes similar to that seen in mammals [94]. GF flies were reported to have a prolonged development time and disruption in IIS and lipid metabolism, a phenotype that was reversed by acetate augmentation or generation of gnotobiotic flies (colonized with selected bacterial taxa) [5, 85, 92]. Interestingly, the bacterial taxon required to reinstate glucose homeostasis (*A. pomorum*, *A. tropicalis*, *L. brevis*, *L. fructivorans*, and *L. plantarum*) differed from that required to perpetuate normal rates of development and levels of triglycerides (monocolonization with *Acetobacter* species). However, both *Acetobacter* and *Lactobacillus* were necessary for the reestablishment of triglycerides to levels comparable to that of conventionally raised flies [95].

The microbiota has been also shown to impact the gut morphology by modulating the rate of epithelial renewal, epithelial cell type composition, and cellular spacing [96]. To maintain homeostasis following infectious damage to the epithelium, the gut microbiota positively regulates epithelial cell renewal through ISC proliferation and differentiation in attempts to repair damaged tissue [81, 92, 96-98]. Flies lacking such a compensatory mechanism were found to be more susceptible to infections. Not only does the microbiota regulate ISC proliferation, but its absence also skews the existing cell types in the gut, resulting in a reduction in EBs and a concurrent increase in EE [81, 92, 96]. Such a coalition between the microbiota and ISC proliferation may endorse the reason why the microbiota serves as an etiological factor for colorectal cancer.

Dysbiosis within the gut microbial community can promote hyperplasia and inflammation [99-101]. Indeed, infection with *Helicobacter pylori*, a resident bacterium of the human stomach can induce gastritis and increase the risk of gastric cancer development [102]. This bacterial species has additionally been linked to alterations in the microbiota of both the stomach and colon, though its role in tumor development is yet to be established. *H. pylori* cytotoxin-associated gene A (CagA) is a potent virulence factor involved in regulating many signaling pathways within the host, including the Ras/ERK/MAPK pathway. CagA has been reported to play a role in interfering with tyrosine kinase signaling, enhancing cellular proliferation [103], as well as increasing the expression of pro-inflammatory cytokines and provoking inflammatory processes via NF-κB signaling [104]. Interestingly, a study by Jones et al. in fruit flies described the role of the gut microbiota in *H. pylori*-induced tumor development by demonstrating that CagA promotes dybiosis of the flora, leading to uncontrolled epithelial cell proliferation [105].

The pathogenesis of inflammatory bowel disease (IBD) has been associated with a disruption in intestinal NF-κB signaling and AMP biosynthesis [106-108]. In accordance, flies lacking the developmental control gene *Caudal* show defects in AMP level regulation and possess a gut microbiota composition enriched with the pathogenic commensal *Gluconobacter* spp. (strain EW707) accelerating gut pathology. This supports the notion that the presence of a pathogenic commensal under conditions of defective immune genotype is a tenable cause of chronic IBD [83].

2.4 Fruit fly metabolism

2.4.1 Carbohydrates mobilization and storage

Reminiscent of the metabolic regulation seen in mammals, energy homeostasis

in the fruit fly is achieved through the interplay between several organs, namely the fat body (analogous to mammalian adipose tissue), oenocytes, the gut, Malpighian tubules, and certain areas of the brain [23, 27, 109-111]. Once absorbed, nutrients are released by the gut EC and then circulate through the hemolymph. The pumping of the fly's tubular heart augments the metabolic exchange as the aorta extends into the brain with which it maintains close neuronal connections. This connection results in the production and secretion of Dilp2, Dilp-3, and Dilp-5 from a group of neurosecretory cells in the brain called insulin-producing cells (IPCs) [112]. Dilps use a single *Drosophila* insulin/IGF receptor (InR) to propagate the insulin/insulin-like growth factor signaling (IIS) system in the fat body which controls a number of physiological processes including growth and metabolism [113]. The activated InR phosphorylates a substrate known as *chico*, to stimulate a phosphorylation signal transduction cascade of phosphoinositide-3-kinase (PI3K), phosphoinositide-dependent-kinase-1, and Akt, culminating in the repression of the forkhead transcription factor dFOXO [114, 115]. This, therefore, maintains metabolic homeostasis by inhibiting gluconeogenesis and glycogenolysis while promoting glycogen and triglyceride storage. In particular, Dilp2 has been shown to regulate adult lifespan and systemic sugar levels [116, 117], whereas Dilp5 and Dilp3 are thought to modulate protein [118] and lipid [119] metabolism respectively. In a state of low carbohydrate level, release of the endocrine glucagon-like peptide known as adipokinetic hormone (AKH) from the ring gland adjacent to the IPCs induces glycogenolysis and carbohydrate mobilization in order to fulfill a steady state of energy [120, 121].

In mammals, the gut microbiota was shown to be involved in regulating systemic glucagon and insulin secretion through the short-chain fatty acids (SCFAs)

production [122, 123]. These SCFAs not only act a source of nutrition for EC but are also sensed by certain receptors on the surface of intestinal cells, ensuing the release of regulatory peptides from EE which appropriately modify food intake and its metabolism by the host [124, 125]. Although not as well-defined in *Drosophila*, several reports indicate that a similar cascade is at play. EE and plausibly other cell types in the fly gut secrete a regulatory peptide known as ecdysone-inducible gene L2 (IMPL2) which functions to block insulin signaling seemingly through binding to and inhibiting the function of Dilps [50, 126-129]. Studies have reported that repression of IMPL2 transcription by intestinal acetate increases insulin signaling [85, 92, 130], suggesting the presence of an acetate receptor on the surface of EE which moderates signaling through this pathway [41].

2.4.2 Lipid mobilization and storage

Various facets of the lipid uptake mechanism in the fruit fly gut remain largely unexplored. What has been identified to date is the role of the Magro protein in digesting TAG within the gut lumen [18, 131]. Following absorption by the EC, dietary lipids are thought to be channeled to the endoplasmic reticulum, where they are held in lipid droplets or packaged for transport through the circulation to the fat body for storage and metabolism [53]. Although the mechanism of action of this transport has not yet been elucidated, several factors have been implicated in this process. One of which is the EE secreted regulatory peptide hormone, Tk, which promotes the process of lipid mobilization from the gut to the hemolymph [53]. Three lipoproteins known as lipophorin (Lpp), the lipid transfer particle (LTP), and Crossveinless D (Cv-D) have also been associated with systemic lipid transport whereby Lpp's contribution is greatest [132]. Studies have reported the accumulation of lipid droplets in the midgut

upon knockdown of both Lpp and LTP, demonstrating the importance of their role in lipid mobilization and storage [132]. LTP is thought to facilitate the recruitment of Lpp from the gut to the Lpp receptor, as well as the uptake of lipids by distant tissues [133]. Although the mammalian proteins dedicated to lipid transport are synthesized by EC, Lpp and LTP are initially synthesized in the fat body before being transported to the intestine to carry out their intended functions [134].

2.5 Fruit fly innate immunity

2.5.1 Humoral host defense

Like all invertebrates, the fruit fly lacks an adaptive immune system and relies solely on its humoral and cell-mediated innate immune ordnance to fight off invading pathogens [11]. The humoral immune response, which mainly occurs through the evolutionary conserved Toll (Figure 2A), immune deficiency (Imd) (Figure 2B), and/or the Janus Kinase protein and the Signal Transducer and Activator of Transcription (JAK/STAT) (Figure 2C) signaling pathways [11] [135-137], chiefly culminates in the production of AMPs and other immune effectors in the fat body [135].

2.5.1.1 Toll pathway

The Toll pathway was initially recognized for its involvement in *Drosophila* development [138]. It was not until later that Toll was introduced as a key player in immune activation and defense [139], particularly against fungal and Gram-positive bacterial components [140]. For recognition of bacteria by the Toll pathway in *Drosophila*, peptidoglycan, which is the major component of bacterial cells walls, acts as the pathogen associated molecular pattern (PAMP). Alternating N-acetylmuramic acid and *N*-acetylglucoasmine subunits comprise the structure of the peptidoglycan polymer. Within this structure, short stem peptides cross-link the sugar chains, and this

gives rise to the characteristic sequence variation seen in broad groups of bacterial pathogens. In specific, the *Drosophila* Toll pathway is activated by a structure involving lysine occupying the third position of the stem peptide of the peptidoglycan, which is found in the vast majority, but not all, Gram-positive bacteria. Toll pathway related recognition involves the combined activity of two peptidoglycan recognition proteins (PGRP-SA and PGRP-SD) and Gram-negative bacteria binding protein 1 (GNBP1) [141-144]. GNBP3 serves as a circulating β-glucan receptor with specificity for fungal cell walls. Downstream of this recognition process, the signaling pathways corresponding to Lysine-type peptidoglycan and β-1,3-glucans unite to trigger the activation of the modular serine protease (ModSP) [142]. This, in turn, induces another serine protease known as Grass [145, 146], and the ongoing cascade eventually activates the Spätzle-processing enzyme (SPE) [147]. Unlike the mammalian counterpart, the activation of *Drosophila* Toll signaling is not achieved through direct recognition of microbial elements but rather through cleavage and activation of the mammalian interleukin 17 (IL-17) homolog and functional Toll ligand, Spätzle (Spz), which is achieved by SPE [148]. Once the processed Spz binds the Toll receptor, the activated receptor associates with an adaptor protein known as dMyD88 via their Toll/IL-1 receptor (TIR) domains [135, 149-151]. Recruitment of another adaptor protein known as Tube along with Pelle, a kinase, to dMyDD88 allows the formation of a complex through death domain-mediated interactions of the proteins [150, 152, 153]. The *Drosophila* IκB factor Cactus is phosphorylated by Pelle and then degraded, recruiting the bound NF-κB transcription factors Dif and Dorsal to the nucleus. The activation of the Toll pathways subsequently induces the expression of anti-microbial peptide (AMP) genes, like *Drosomycin* and *Metchnikowin* [76, 154, 155]. To prevent

an exaggerated immune response, the Toll pathway is negatively regulated by a member of the serpin superfamily protease inhibitors, Spn1, which functions upstream of SPE [156]. Interestingly, and beyond its conventional role in defense against fungi and Gram-positive bacteria, the Toll pathway was shown to also induce *Drosomycin* expression in response to *Mycobacterium abscessus*, a non-tuberculous human mycobacterium that colonizes the *Drosophila* gut [157]. Along those lines as well, The *Drosophila* Toll pathway has been shown to also take part in the fly's anti-viral immunity by efficiently inhibiting viral replication within it's the host cells [158].

2.5.1.2 Immune deficiency pathway

The *Drosophila* Imd signaling pathway is a homolog of the mammalian tumor necrosis factor (TNF) innate immune pathway [159, 160]. Imd is primarily initiated by the recognition of Gram-negative bacterial components such as diaminopimelic acid-peptidoglycan (DAP-type peptidoglycan) via surface-bound peptidoglycan recognition protein LC (PGRP-LC) and cytosolic PGRP-LE [161]. Additionally, the Imd pathway can be triggered by some Gram-positive bacterial species such as *Bacillus* and *Listeria* spp. [162, 163]. In addition to its response to bacteria, the Imd pathway has been recently involved in anti-viral immunity [164, 165]. Upon binding of the peptidoglycan ligand to the receptor, the death domain protein known as Imd, a homologous to the mammalian interacting protein kinase 1 (RIP1) [166], associates with the adaptor protein *Drosophila* Fas-Associated protein with Death Domain (dFADD) [167] and caspase-8 homolog Death-related ced-3/Nedd2-like protein (DREDD) [166-169]. DREDD cleaves Imd, which gets further activated by ubiquitination [170], and recruits and activates the TAB2/TAK1 complex responsible for phosphorylating the *Drosophila* IKK complex [170-175]. Relish is induced by the phosphorylation of

certain serine residues at its N terminus by the IKK complex [175, 176]. Rel's C-terminal segment remains in the cytoplasm while the active N-terminal portion translocates into the nucleus to initiate the expression of target effector genes encoding AMPs such as *Diptericin*, *Cecropin*, *Attacin*, and *Drosocin* [5, 161, 177-179]. It is worth noting here that Imd signaling is thought to diverge into two separate pathways following the activation of TAK1: JNK and IKK-mediated signaling [180-182]. This divergence explains the contribution of TAK1 to JNK signaling modulation as well. As such, and although the role of the JNK pathway is chiefly attributed to cytoskeleton remodeling and hemocyte activation [180], its contribution to AMP production is considered plausible [183, 184].

To avoid a constitutively active immune response, poor Imd response upon knock-in (Pirk) diminishes the level of Imd signaling downstream of PGRP-LC. A plausible mechanism by which Pirk functions is through the interaction with Imd mainly via the Pirk domain and with the cytoplasmic portion of PGRP-LC [185]. It is also suggested that amidases PGRP-LB and PGRP-SC, which limit the availability of PGRP-LC ligand, synergize with Pirk to diminish the Imd pathway response [186]. In addition, resembling PGRP-LC is PGRP-LF, a transmembrane protein lacking the intracellular signaling domain and peptidoglycan binding abilities of PGRP-LC. By binding to PGRP-LC and preventing its dimerization, PGRP-LF also serves as an inhibitor of signaling through the Imd pathway [187-189].

2.5.1.3 JAK/STAT pathway

Although the JAK/STAT pathway plays an essential role in regulating various physiological processes and tissue homeostatic in both vertebrates and invertebrates, its contribution to humoral immunity and AMP production particularly upon pathogenic

infection and/or the occurring damage following the stress response to infection has been reported [11]. JAK/STAT signaling is initiated when three IL-6-like cytokines known as Unpaired (Upd), Upd2, and Upd3 bind to and activate the receptor, Domeless (Dome). This subsequently leads to the activation of the JAK2 homolog Hopscotch (Hop) and to the phosphorylation of Dome. The phosphorylated Dome receptor acts as a docking site for the STAT dimer, STAT92E, which eventually becomes phosphorylated and activated. The resulting active STAT92E dimer then translocates to the nucleus and induces AMP gene expression [190]. The activated STAT92E dimer can also act as a transcriptional activator for regulators of the pathway. Suppressor of cytokine signaling 36E *(SOCS36E)* gene is known to form a negative feedback loop by suppressing hop activity [191, 192]. Although its mechanism of action remains elusive, *Drosophila* protein inhibitor of activated STAT (dPIAS) was reported to be another negative regulator of the JAK/STAT pathway [193]. A recent report has also highlighted a negative role of the *eye transformer (ET)* gene in regulating JAK/STAT signaling, possibly by inhibiting Dome homodimer-hop signaling [194]. Though the involvement of the JAK/STAT pathway in cell-mediated immunity is not fully understood, several studies have evidenced its contribution to hemocyte proliferation and differentiation as well [195].

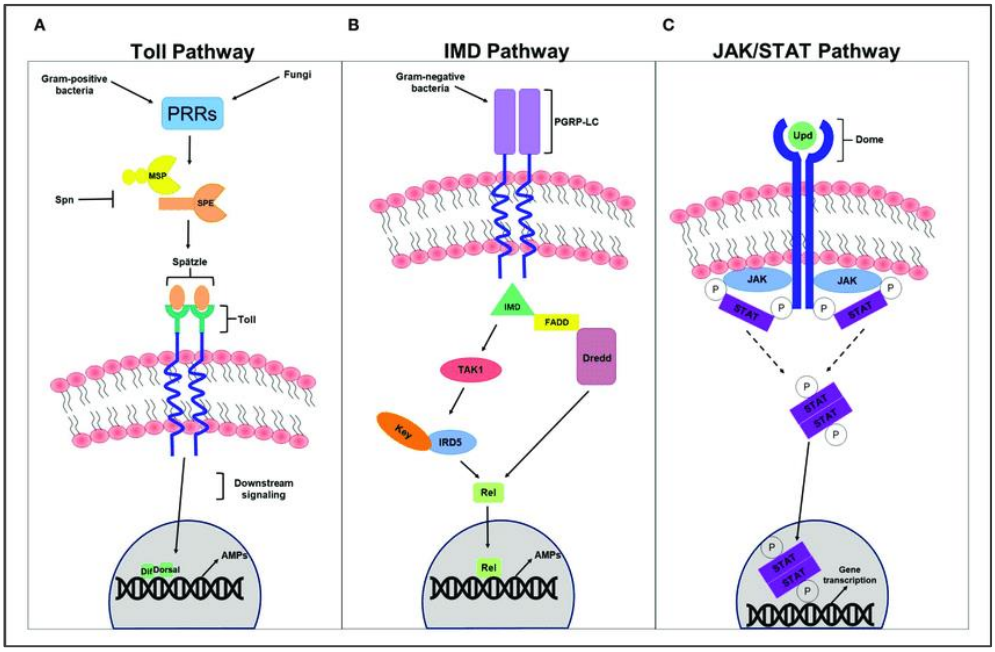


Figure 2. Humoral innate immune signaling pathways. Schematic diagram of the (A) Toll (B) Imd and (C) JAK/STAT signaling pathways. Reprinted from "*Drosophila* as a Model Organism in Host–Pathogen Interaction Studies" by Younes S., Al-Sulaiti A., Nasser E.A.A., Najjar H., and Kamareddine L. (2020). *Frontiers in Cellular and Infection Microbiology, 10*, p. 214.

2.5.2 Cell-mediated host defense

2.5.2.1 Phagocytosis

In *Drosophila*, phagocytosis, which is essentially responsible for the elimination of invading microorganisms and apoptotic cells, is carried out by plasmatocytes [135]. During this process, the plasmatocyte binds to its pathogenic target, promoting cytoskeletal rearrangements and therefore pathogenic internalization and destruction within phagosomes (Figure 3A) [135]. Several *Drosophila* receptors have been reported to be engaged in this phagocytic process. One example is the *Drosophila* scavenger receptor CI (dSR-CI) which has a specificity for polyanionic ligands similar to its mammalian homolog class A macrophage-specific scavenger receptor (SR-A) [196]. Another example is the EGF-like repeat transmembrane receptor present on the surface of *Drosophila* hemocytes, known as Eater, was shown to play a role in bacterial phagocytosis [197]. Eater also serves as a key player in the localization, attachment, and adhesion of hemocytes, as well as in efficient

phagocytosis of Gram-positive bacteria [198]. Furthermore, the integrin βν phagocytic receptor was implicated in defense against septic *Staphylococcus aureus* infections in fruit flies [199]. Dscam, a member of the Ig superfamily, was also identified as a regulator of phagocytosis, whereby hemocyte-specific silencing of the *Dscam* gene reduces bacterial engulfment and uptake [200]. Some PGRPs have been also shown to take part in phagocytosis. A study by Rämet et al., for instance, reported a role of PGRP-LC in phagocytosis of Gram-negative bacteria [201], and another by Garver et al. revealed that PGRP-SC1 acts as an opsonin, invigorating phagocytosis [202]. Interestingly, phagocytosis of apoptotic cells but not bacterial components was found to be mediated by Croquemort (CRQ), a CD36-related receptor only expressed on the surface of *Drosophila* phagocytic cells in embryos [203].

2.5.2.2 Encapsulation

Encapsulation is another cellular mechanism devoted to pathogenic elimination. During encapsulation, hemocytic capsules are formed around invading parasites whose exceptional size exceeds that of the phagocytic ability [135, 204]. Cellular encapsulation occurs in three main stages (Figure 3B). In the first stage, plasmatocytes, which normally exert continuous immune surveillance in the fruit fly's hemolymph [205], spot the parasitoid egg as foreign. This recognition permits changes in their cell membrane to expose hidden molecules and trigger downstream signaling [206]. The second stage involves a short-term increase in the number of circulating plasmatocytes, some of which differentiate into lamellocytes [207]. The activated lamellocytes travel towards the parasitoid egg and flatten to attach to it and to each other, forming a multilayered capsule [208]. The final step of encapsulation involves crystal cells which serve as PPO storage sites and are involved in the melanotic immune response. Crystal

cell lysis results in the melanization of the multilayered capsule [135, 208, 209] within which the parasitoid egg gets destroyed either by direct [210] or by the production of reactive oxygen species from the capsule content [206, 211, 212].

2.5.2.3 Melanization

Melanization is an essential immune response in *Drosophila* implicated in wound healing, blood coagulation, phagocytosis, and AMP production [213-215]. Melanization, which is activated by injury or microbial recognition via PRRs [216-219], is characterized by melanin deposition in sites of cuticular injury [135] or around invading microorganisms [135, 220]. The activation of the melanotic response ends in the cleavage of PPO to phenoloxidase (PO), the principal enzyme in melanin production, by PPO activating enzyme (PPAE) [135, 214]. To avoid excessive exposure of the host to toxic intermediates, melanization is tightly regulated by serine proteases and serpin proteins [221-225]. PGRPs, namely PGRP-LC [226] and PGRP-LE [227, 228], have been shown to play a role in driving the induction of the PPO cascade.

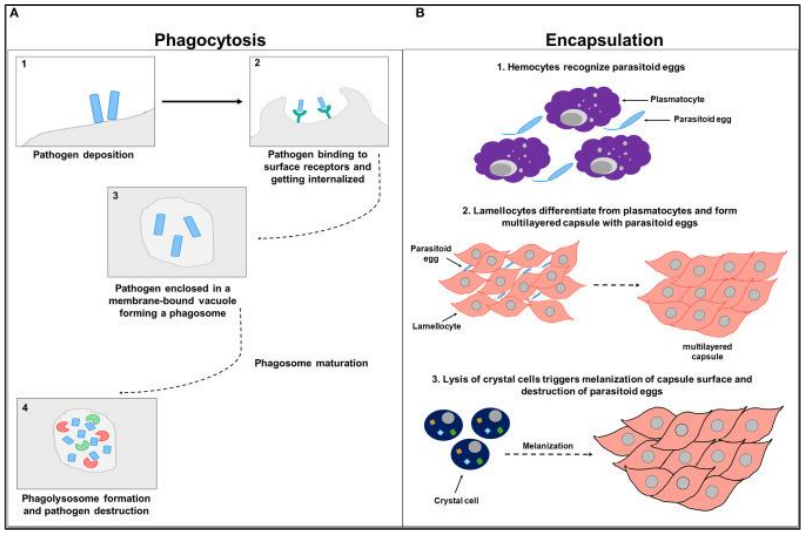


Figure 3. Cell-mediated immunity. Schematic diagram of (A) phagocytosis and (B)

encapsulation. Reprinted from "*Drosophila* as a Model Organism in Host–Pathogen Interaction Studies" by Younes S., Al-Sulaiti A., Nasser E.A.A., Najjar H., and Kamareddine L. (2020). *Frontiers in Cellular and Infection Microbiology, 10*, p. 214.

2.5.3 RNA interference

Initially described in plants, then discovered in *Drosophila*, the RNA interference (RNAi) pathway exemplifies a conserved mechanism of anti-viral immunity that induces gene-silencing through targeted RNA degradation [229-235]. Generally, the RNAi pathways (Figure 4) involve the biosynthesis of small non-coding RNAs which, depending on their origin, can be classified as endogenous or exogenous groups. The endogenous group includes small RNAs encoded and manufactured within the cell such as short hairpin RNAs, perversely expressed trans-genes, and transposons. The exogenous group, in turn, involves double-stranded RNA (dsRNA) from naturally occurring or experimentally made sources [231, 235, 236]. The execution phase of the RNAi pathway begins with the recognition and cleavage of viral dsRNA by Dicer molecules, particularly Dcr-2 (a ribonuclease III enzyme), in association with its cofactor RDR2 [237]. This forms small interfering RNAs (siRNAs) [238-240] which are incorporated into a pre-RNA-induced silencing complex (RISC) where their duplex structure is unwound, and the passenger strand is removed [241, 242]. The viral siRNA strand that remains rests in the site of catalysis of the RISC complex named Argonaute (protein component of RISC) (Ago2)/holo-RISC [241, 242]. The integrated siRNA strand can then bind viral RNA molecules with sequence complementarity resulting in targeted RNA degradation mediated by Ago2 [231]. Various studies have demonstrated that loss-of-function mutations in genes involved in the RNAi pathway increases the susceptibility of the fruit fly to viral infections [76, 235, 243].

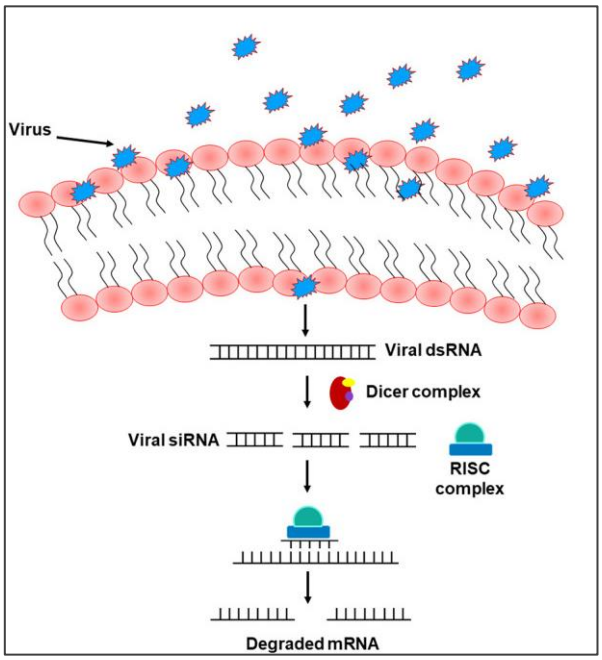


Figure 4. The RNA interference pathway. Reprinted from "*Drosophila* as a Model Organism in Host–Pathogen Interaction Studies" by Younes S., Al-Sulaiti A., Nasser E.A.A., Najjar H., and Kamareddine L. (2020). *Frontiers in Cellular and Infection Microbiology, 10,* p. 214.

2.6 Fruit fly peptide hormones

Insects produce and secrete a number of peptide hormones implicated in modulating various physiological processes such as growth and development, immunity, and stress [244]. In such a way, the physiology of the digestive system is largely synchronized through regulatory peptides produced by endocrine cells within the gut epithelium and by neurons which function to innervate the intestinal musculature [245]. Numerous gut peptide hormones are conserved across different species. However, due to the aspect of gene redundancy along with the overlapping functions of these peptide hormones in mammalian model organisms (such as mice), conducting studies directed towards associating a knockdown of the hormone with the relevant physiological changes pose great difficulty [246]. Therefore, employing the use of other model organisms like the fruit fly to elucidate the function of these gut

peptide hormones is much favored in such a context. The regulatory peptides produced in the fruit fly midgut have extensively been characterized by previous work (Table 1) [245]. Under the control of the transcription factor Prospero (Pros), EEs in the fruit fly gut express nine prohormones, namely AstA, AstB, and AstC, neuropeptide F (NPF), short NPF, Dh31, TK, and CCHamides 1 and 2 [53, 245, 247]. These are ultimately processed into more than 24 mature peptides [245]. Though these peptide hormones have been associated with several physiological processes, their role in regulating functions such as immune signaling and metabolic homeostasis remains relatively unexplored.

Table 1. Expression of regulatory peptides in the digestive tract of adult *Drosophila*

Regulatory peptide	Brain	Thoracicoabdominal ganglion	Crop	Midgut	Tubules	Hindgut
AKH	913±52 [4]	163±56 [4]	18±5 [0]	71±6 [0]	2±0 [0]	2±0 [0]
Allatostatin A (ASA)	740±23 [4]	1,454±44 [4]	14±6 [4]	195±11 [4]	14±3 [3]	221±17 [4]
Allatostatin B (ASB)	742±39 [4]	196±6 [4]	6±0 [1]	39±3 [4]	5±1 [0]	4±1 [0]
Allatostatin C (ASC)	1,929±74 [4]	923±40 [4]	10±3 [2]	1,441±49 [4]	7±1 [1]	83±4 [4]
Allatostatin CC (ASCC)[a]	9±1 [0]	2±0 [0]	1±0 [0]	1±0 [0]	2±0 [0]	1±0 [0]
Bursicon-A	3±0 [0]	11±0 [0]	2±1 [0]	113±10 [4]	28±1 [4]	18±2 [3]
Bursicon-B	0±0 [0]	3±2 [0]	1±0 [0]	0±0 [0]	1±0 [0]	0±0 [0]
Capa	908±18 [4]	1,843±35 [4]	1±0 [0]	4±1 [0]	3±0 [0]	3±1 [0]
CCAP	33±1 [4]	102±4 [4]	2±0 [0]	4±1 [0]	4±0 [0]	4±1[0]
Corazonin	1,102±39 [4]	20±2 [3]	3±1 [0]	4±1 [0]	2±0 [0]	2±0 [0]
DH31	930±31 [4]	1,383±41 [4]	1±0 [0]	113±3 [4]	3±0 [0]	17±1 [3]
DH44	654±23 [4]	124±4 [4]	5±1 [0]	8±3 [0]	15±8 [1]	4±1 [0]
DMS	2,823±87 [4]	187±8 [4]	7±1 [0]	2±1 [0]	3±0 [0]	181±30 [4]
DSK	2,365±34 [4]	2±1 [0]	3±1 [0]	1±0 [0]	2±0 [0]	3±1 [0]
Eclosion hormone	16±7 [2]	1±0 [0]	5±2 [0]	2±1 [0]	2±0 [0]	3±1 [0]
ETH	5±0 [0]	2±1 [0]	4±1 [0]	2±1 [0]	2±0 [0]	5±1 [0]
FMRFa	317±10 [4]	3,250±100 [4]	0±0 [0]	0±0 [0]	2±0 [0]	0±0 [0]
Ilp-1	4±1 [0]	2±0 [0]	1±0 [0]	1±0 [0]	2±0 [0]	2±0 [0]
Ilp-2	1,607±15 [4]	18±1 [0]	5±1 [0]	3±1 [0]	4±0 [0]	7±0 [0]
Ilp-3	322±12 [4]	1±0 [0]	11±2 [0]	107±5 [4]	1±0 [0]	2±1 [0]
Ilp-4	0±0 [0]	1±0 [0]	4±1 [0]	1±0 [0]	1±0 [0]	1±0 [0]
Ilp-5	683±14 [4]	4±1 [0]	9±1 [1]	12±2 [4]	10±1 [2]	7±0 [2]
Ilp-6	98±5 [4]	68±3 [4]	13±1 [1]	1±0 [0]	10±0 [0]	9±1 [0]
Ilp-7	7±1 [1]	160±11 [4]	1±0 [0]	1±0 [0]	3±0 [0]	1±0 [0]
ITP	1,135±18 [4]	195±13 [4]	38±1 [4]	40±5 [4]	92±19 [4]	83±6 [4]
Leucokinin	165±6 [4]	1,537±33 [4]	8±0 [0]	18±2 [0]	6±1 [0]	8±2 [0]
NPF	805±11 [4]	3±1 [0]	0±0 [0]	338±11 [4]	0±0 [0]	5±2 [0]
Nplp1	1,019±28 [4]	3,692±134 [4]	5±1 [0]	3±1 [0]	3±0 [0]	3±1 [0]
PDF	1,365±23 [4]	539±52 [4]	1±0 [0]	1±1 [0]	1±0 [0]	1±0 [0]
Proctolin	224±10 [4]	943±84 [4]	1±0 [0]	3±1 [0]	1±0 [0]	0±0 [0]
PTTH	19±0 [4]	28±2 [4]	13±4 [2]	18±2 [4]	49±2 [4]	32±2 [4]
Pyrokinin (hugin)	1,104±30 [4]	5±1 [0]	3±1 [0]	4±1 [0]	2±0 [0]	4±1 [0]
SIFamide	443±16 [4]	13±4 [0]	14±3 [0]	4±1 [0]	9±1 [0]	5±1 [0]
sNPF	997±47 [4]	397±16 [4]	2±1 [0]	7±0 [2]	2±0 [2]	0±0 [2]
Tachykinin (DTK)	694±35 [4]	114±3 [4]	4±0 [1]	356±8 [4]	11±6 [1]	137±7 [4]

Reprinted from "Regulatory peptides in fruit fly midgut" by Veenstra, J.A., Agricola, H.J., & Sellami, A. (2008). *Cell and tissue research*, *334*, 499–516.

2.6.1 Tachykinin (Tk)

Substance P, a member of the mammalian Tk neuropeptide family, was first identified in horse brain tissue by the virtue of its influence on blood pressure regulation and smooth muscle contraction when isolated and administered to rabbits [248]. Not confined to vertebrates, the insect Tks were initially described in locusts, after which the term locustachykinin (LTK) was first coined [245]. Antisera to insect Tk-related peptides were found to exhibit immunoreactivity in both the gut and nervous system tissue of various insect species, suggesting that similar to mammalian Tks, these peptides can serve as both neuromodulators, as well as endocrine signaling molecules [249, 250]. Since then, they have demonstrated a clear involvement in various

physiological functions within neurons of the central nervous system (CNS), EE of the gut epithelium [250-254], and other cell types such as immune cells and endothelial cells [255]. The *Drosophila Tk* gene encodes five different LTKs. A Tk-related peptide prohormone gene was found to be expressed and processed in larvae and adult fly midgut EE and in the CNS [256]. Subsequent work by Veenstra et al. demonstrated that LTK-immunoreactive EE were found along the entire length of the fruit fly midgut, whereby these cells were more abundant in the anterior region and displayed stronger immunoreactivity (Figure 5) [245].

In *Drosophila*, many studies have revealed a role of the Tk neuropeptide in regulating nervous system processes and fly behaviors including odor perception [257], hyperactivity [257, 258], enhanced male-specific aggressive arousal or motivation [259], and behavioral and electrophysiological thermal nociceptive hypersensitivity [260]. However, an *in vitro* study by Siviter et al. investigating the expression and functional characterization of neuropeptide Tk, demonstrated that the treatment of adult midguts with Tk1-5 promotes hindgut contractions [256]. Interestingly, EE-secreted Tks seem to possess no influence on the fly behavior but are rather involved in regulating lipid homeostasis by mediating gut lipid production through the repression of lipogenesis in EC to prevent lipid accumulation within the midgut and maintain systemic triglyceride levels. Remarkably, nutrient deprivation was shown to augment the production of Tk in the fly's midgut whereby starved flies exhibited a repression in Tk. This repression was reversed once the flies were refed yeast whose major component is amino acids, suggesting that Tks respond to nutrient availability [53]. Recent findings of Kamareddine et al. have also revealed a gut flora-Imd dependent role of Tk in optimal lipid metabolism and insulin signaling [5]. A study by Harsh et

al. accentuated the contribution of peptide hormones, particularly Tk, to maintaining immune-metabolic homeostasis in an infected host. In this study, Harsh et al. revealed that the systemic infection of *Drosophila* adult flies with *Escherichia coli*, *Photorhabdus luminescens or Photorhabdus asymbiotica* promote intestinal steatosis depicted by lipid accumulation in the midgut, increased whole-body lipid levels, and reduced expression of EE-secreted Tk. [261]. It has also been determined that in states of desiccative, nutritional, or oxidative stress, hormonal release of Tk is promoted which in turn, acts on the Malpighian tubules to modulate Dilp5 expression, a peptide involved in activating the ISS pathway. This indicates that Dilp5 signaling initially originates in the renal tubules under the control of Tk [262].

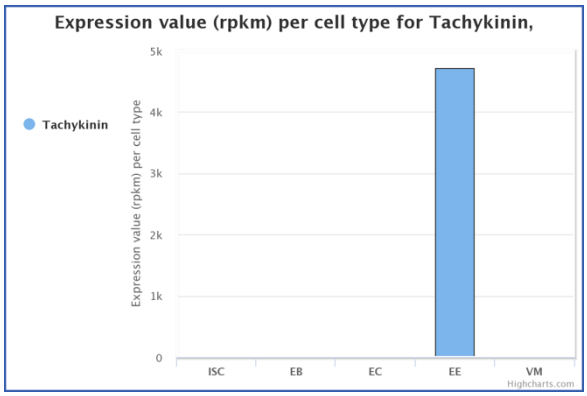


Figure 5. The expression of Tk in RPKM for midgut cell type Reprinted from Flygut-seq. http://flygutseq.buchonlab.com/. ISC: intestinal stem cell; EB: enteroblast; EC: enterocyte; EE: enteroendocrine cell; VM:visceral muscle

2.6.2 Diuretic Hormone 31 (Dh 31)

Dh31 along with its receptor, Dh31-R1, are homologs of the vertebrate calcitonin gene-related peptide (CGRP) and its receptor, respectively [263-266]. CGRP is a highly potent sensory nerve-released vasoactive peptide with a dual sensory and effector function [267]. Dh31functions to stimulate fluid secretion from the Malpighian

tubules, where its receptor was identified to be expressed [268]. Veenstra et al. has recently demonstrated that in addition to the Malpighian tubules, Dh31 receptor expression is also detected in the crop, midgut, and hindgut of the fruit fly (Figure 6) [245]. Dh31's effect on the circadian rhythm has identified it as the wake-promoting signal to promote the flies' anticipation of dawn [269]. Further studies on Dh31 have described its role in modulating locomotor behavior and night-onset temperature preference [270, 271]. In addition, the Dh31 receptor has been shown to be implicated in maintaining temperature preference rhythm (TPR), mediated by the Dh31 peptide along with pigment dispersing factor (pdf) [272], a neuropeptide responsible for regulating the activity of pacemaker neurons [273, 274]. Neuropeptides including Dh31 and Dh44 regulate fluid secretion in *Drosophila*, whereby an increase in secretion is mediated by stimulating cyclic AMP levels in the principal cells of the Malpighian tubules and therefore, activating an apical membrane V-ATPase [275]. Moreover, G protein-coupled receptor (GPCR) signaling is seemingly maintained in *Drosophila* where the Dh31 neuropeptide serves as a potent ligand for GPCRs, related to components of the calcitonin receptor-like receptor (CLR) family [268]. Interestingly, the Dh31 neuropeptide has been also shown to trigger intestinal contractions by working on its receptor located on nearby visceral muscles. Such contraction subsequently promotes gut peristalsis and culminates in the elimination of opportunistic gut bacteria [276]. Comparable to the neuropeptide counterpart, Dh31-expressing EE were also found to be essential for the larval midgut's intestinal motility, with a role of Dh31 peptide hormone in peristalsis in the midgut junction region [54, 277]. Interestingly enough, midgut preferential RNA knockdown of Dh31 was reported to increase the lifespan of adult flies, an observation correlated with a delayed midgut

senescent phenotype [278].

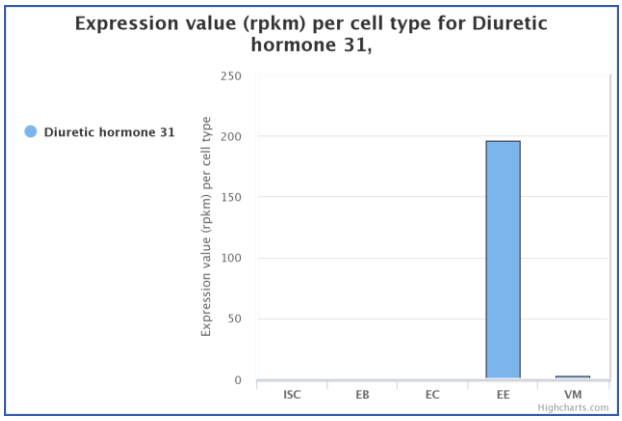


Figure 6. The expression of Sh31 in RPKM for midgut cell type Reprinted from Flygut-seq. http://flygutseq.buchonlab.com/ISC: intestinal stem cell; EB: enteroblast; EC: enterocyte; EE: enteroendocrine cell; VM:visceral muscle

2.6.3 Allatostatin A (AstA)

Allatostatins are a group of neuropeptides that were originally recognized by their ability to block the synthesis of juvenile hormone by the corpora allata. Three different families of allatostatin peptides have been identified in insects, the first being allatostatin A (AstA), also known as the FGLamides [279]. In the fruit fly, the *AstA* gene encodes a precursor that can give rise to four different peptides [280]. The allatostatins B are also known as myoinhibitory peptides (MIPs) [281] and similarly to the *AstA* gene, *AstB* encodes another precursor that produces five copies of AstB in the fruit fly [282, 283]. AstC was first identified in a moth species, and unlike the two previously discussed Ast genes, its precursor produces a single copy of the peptide [284, 285]. AstA immunoreactive EE were found in the posterior midgut (Figure 7) [245] which is a region in close proximity to some axons derived from neurons in the thoracicoabdominal ganglia [286]. Although located in the same region as the Dh31

immunoreactive EE, they represent two separate cell types of the posterior midgut [245].

AstA shares conserved structures with kisspeptin, an important contributor to puberty and sexual maturation in humans, suggesting that they may have originated from a common ancestor [287]. Indeed, silencing AstA expression in the *Drosophila* brain delays the onset of maturation, suggesting that AstA and its receptor (AstAR1) are homologous to human KISS/GPR54, a ligand-receptor complex also necessary for puberty [288]. AstA has been also shown to be involved in physiological processes relevant to food intake. Apart from endorsing sleeping, the activation of AstA-expressing neurons, for instance, promotes food aversion and induces a state of satiety [289, 290]. Owing to such a contribution to feeding behaviors, AstA is also believed to regulate AKH and Dilp signaling for maintenance of nutrient homeostasis. Along those lines, a study by Hentze et al., has demonstrated that silencing *Dar-2*, the AstA receptor gene expressed on AKH and Dilp producing cells, diminishes AKH and Dilp signaling and elevates systemic lipid levels [291]. Moreover, Hentze et al., have shown that *Drosophila* feeding decisions are mediated by neuronal AstA secretion in response to different dietary nutrients such as carbohydrates and proteins [291].

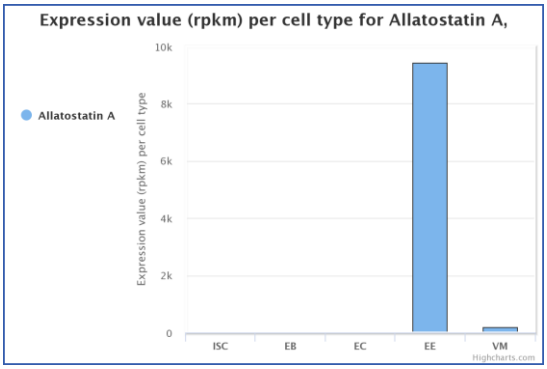


Figure 7. The expression of AstA in RPKM for midgut cell type. Reprinted from Flygut-

seq. http://flygutseq.buchonlab.com/ ISC: intestinal stem cell; EB: enteroblast; EC: enterocyte; EE: enteroendocrine cell; VM: visceral muscle

Chapter 3: Materials and Methods

3.1 Ethical compliance

All *Drosophila* experimental procedures were conducted according to international standard guidelines and were approved by Qatar University's Institutional Biosafety committee (IBC). This study was exempted from Institutional Animal Care and Use Committee (IACUC) approval as *Drosophila melanogaster* is an invertebrate model organism. The relevant ethical compliance approval document (QU-IBC-2020/044) is enclosed in the Appendix section (chapter 5) of this thesis.

3.2 *Drosophila melanogaster* rearing and maintenance

Flies reared in our laboratory were raised on standard fly food (71g/L cornmeal, 9.5g/L soy flour, 5.5 g/L agar, 16.5g/L yeast, 5.5 g/L malt, 7.5% corn syrup, and 4.42mL propionic acid) in a controlled environment (temperature 25°C, 70% humidity, 12-hour day/night cycle). In experimental set-ups encompassing bacterial infection, flies were maintained on Nutrient Broth (NA), as a source of nutrient for both bacteria and flies. For feasibility purposes, and as per many adopted protocols, all experiments were conducted on five to seven days old adult female flies. All fly lines used in the experiments (*Yw*, $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$, *Tk* driver, and $AstA^{RNAi}$) were purchased from Bloomington *Drosophila* stock center (https://bdsc.indiana.edu/).

3.3 Generation of transgenic flies

Transgenic flies with EE tissue-specific inhibition of *AstA* expression ($Tk>AstA^{RNAi}$) were generated using the Gal4-UAS transactivation system. In brief, GAL4 and UAS are found in two separate fly lines. The driver line contains the GAL4 gene (*Tk>)* which gets expressed in a tissue-specific manner under the control of a native enhancer, and the reporter line contains the UAS and the reporter gene/gene of interest (*UAS-*

AstA^{RNAi}). When these two fly lines are crossed together, the first filial (F1) generation will have both the GAL4 and the UAS gene. In F1 flies, GAL4 will be only expressed in specific tissues (in EE tissues in our case) , and will bind to the UAS gene, triggering the expression of the reporter gene/gene of interest (*AstA*^{RNAi} in our case) in those tissues only [14]. For experiments involving transgenic flies, a fly line carrying only the Gal4-driver (*Tk>)* was used as a control.

3.4 Quantification of Gene expression

3.4.1 Real-time quantitative PCR (RT-qPCR) and relative gene expression

3.4.1.1 Total nucleic acid extraction

Total nucleic acid extraction was performed on either whole fly or fly guts using the traditional phenol/chloroform traditional extraction protocol. In brief, whole bodies or guts were homogenized using the Pellet Pestle ® Motor in 500ul of TRIzol reagent. According to the supplier's recommendations, and in equivalence to a 1:5 chloform:TRIzol ratio, 100ul of chloroform was subsequently added to the TRIzol/tissue homogenate. The homogenate was then vortexed and centrifuged at 16,000 x g for 30 minutes at 4°C. The collected pellet was washed with 1mL of 70% ethanol, left to air dry at room temperature, and then re-suspended in 10ul of nuclease-free water (NFW).

3.4.1.2 DNA removal and RNA extraction

To remove contaminant Genomic DNA, nucleic acid samples were treated with DNase I (2U/L) for 1 hour, at 37°C (Table 2).

Table 2. DNase treatment reaction constituents

Reagent	Concentration	Volume
DNase I	2U/L	1ul
DNase I Buffer	10X	5ul
Total nucleic acid	10ug	X ul
NFW	-	Up to a total reaction volume of 50ul

After DNase treatment, samples were treated with phenol chloroform (Phenol:Chloroform:lsoamyl Alcohol 25:24:t, Saturated with LOmM Tris, pH 8.0,1mM EDTA) in a 1:1 phenol chloroform/sample ratio. Samples were then vortexed and centrifuged at maximum speed for 3 minutes to eliminate any traces of phenol contaminants. The aqueous layer containing total RNA was further collected and precipitated with isopropyl alcohol (1x the total volume of the aqueous layer), mixed well, incubated at room temperature for 5 minutes, then centrifuged maximum speed for 30 minutes at 4°C. The collected pellet was subsequently washed with 1mL of 70% ethanol, left to air dry at room temperature, and then re-suspended in 8ul of NFW. The nanodrop-1000 spectrophotometer [Thermo Scientific, USA] was used to quantify the total RNA concentration by measuring absorbance at 260 nm, and RNA purity was evaluated by the 260/280nm ratio. An RNA sample with a ratio of about 2 was considered pure.

3.4.1.3 Reverse transcription and complementary DNA (cDNA) synthesis

To reverse transcribe the RNA samples and synthesize cDNA, the SuperScript III First Strand Synthesis Super Mix for qRT-PCR kit was used to prepare a 20ul reaction volume according to supplier's recommendations (Table 3). The reaction was then placed in a thermal cycler for 30 minutes at 42°C, followed by 10 minutes

incubation at 85°C.

Table 3. Reverse transcription and cDNA synthesis reaction constituents

Reagent	Concentration	Volume
cDNA Synthesis Mix	5X	4ul
Reverse Transcriptase	20X	1ul
Total RNA	500ng-1ug	X ul
NFW	-	Up to a total reaction volume of 20ul

3.4.1.4 RT-qPCR

RT-qPCR was performed using the qPCRBIO SyGreen Mix Kit in a StepOnePlus real-time PCR system (Applied Biosystems). In brief, and per replicate, a total reaction volume of 10ul was prepared as per the supplier's recommendations in a 96-well plate (Table 4). Samples were run in duplicates. Table 5 represents the forward and reverse primer sequences used.

Table 4. RT-qPCR reaction constituents

Reagent	Concentration	Volume
qPCRBIO SyGreen Mix	2X	5ul
Forward primer	10uM	0.5ul
Reverse primer	10uM	0.5ul
cDNA template	1:5 diluted	1ul
NFW	-	3ul
		Up to a total reaction volume of 10ul

Table 5. Primer sequences

Gene	Primer sequence
Tachykinin	**Fw** TACAAGCGTGCAGCTCTCTC **Rv** CTCCAGATCGCTCTTCTTGC
Diuretic Hormone 31	**Fw** TCTTCTGCCTCTTGGCCATC **Rv** CGTTTCGAGCCCGTATGATG
Allatostatin A	**Fw** ATATGCCAGCCCAGGCAATC **Rv** CGGGCAGCCGATAAAGTTCA
Cecropin	**Fw** CTCTCATTCTGGCCATCACC **Rv** CTTGTTGAGCGATTCCCAGT
Diptericin	**Fw** AGGTGTGGACCAGCGACAA **Rv** TGCTGTCCATATCCTCCATTCA
Metchnikowin	**Fw** GAGCCACATGCGACCTACTC **Rv** CAGTAGCCGCCTTTGAACC
Defensin	**Fw** GAGCCACATGCGACCTACTC **Rv** CAGTAGCCGCCTTTGAACC
Rp49	**Fw** TACAGGCCCAAGATCGTGAAG **Rv** GACGCACTCTGTTGTCGATACC

3.4.2 RNA sequencing: capture, library preparation, and sequencing of fruit fly guts

The guts of 5-to-7-day old female flies with *Tk, DH31,* and *AstA* systemic mutation were dissected and stored in Lysis RLT Buffer. Sample processing, cDNA library preparation, and RNA sequencing procedures were performed at Sidra Medicine under the direct supervision of Dr. Luis Saraiva. In brief, guts were individually homogenized in Lysis RLT Buffer and total RNA was extracted using the RNeasy Mini kit (QIAGEN) along with genomic DNA eliminator (QIAGEN) according to manufacturer's protocol. The SMARTer PCR cDNA Synthesis kit (Clontech) and the Advantage 2 PCR kit (Clontech) were used to reverse transcribe pre-amplified cDNA according to the Fluidigm manual's instructions. The Bioanalyzer DNA High-

Sensitivity kit (Agilent Technologies) was then used to quantify the obtained cDNA and the Nextera XT DNA Sample Preparation Kit and the Nextera Index Kit (Illumina) were used to prepare Nextera libraries. Multiplexed libraries were subsequently pooled and paired-end 150-bp sequencing was performed using the Illumina HiSeq4000 platform. Preparation of the mRNA for sequencing was carried out using the TruSeq protocol [292].

3.5 Immunity-related experiments

3.5.1 Bacterial culture and oral *Drosophila* infection

Staphylococcus aureus (*S. aureus*) and *Shigella sonnei* (*S. sonnei*) bacteria were cultured overnight on NB agar plates, and bacterial colonies were inoculated the second day into NB liquid medium and incubated overnight at 37°C. To infect flies, *S. aureus* and *S. sonnei* overnight broth cultures were diluted in a 1:10 ratio of bacterial suspension to NB fresh broth. In an arthropod containment level 2 facility, 5 to 7 days old adult female flies were randomly selected and distributed into three cellulose-plug containing vials infiltrated with 2.5 ml of the diluted bacterial suspension. 2.5 ml of NB with no bacterial suspension served as the control group. Flies were then incubated at 27°C over the needed period of time, depending on the type of the experiment performed. Relevant time of bacterial infection is indicated in pertinent sections of this materials and methods chapter.

3.5.2 Survival and bacterial colony forming units (CFUs) assays

For survival assays, and for each biological replicate, a total of 30 adult female flies per experimental or control group were used. Fly survival was monitored daily by scoring for the survival/death rate of infected and non-infected flies twice a day (with 12-hour intervals between each count), on daily basis, for 8-10 days. At-least two

independent biological repeats were conducted.

To assess bacterial colonization within the fly gut, flies were collected four days post-infection, surface sterilized by washing three times with ethanol followed by three washes in 1X phosphate buffered saline (PBS), then homogenized using the Pellet Pestle ® Motor in 1000ul of 1X PBS. Fly homogenates were then serially diluted, plated on NB agar, and incubated at 37°C. 48 hours post-plating, colony forming units/fly were calculated by enumerating *S. aureus* and *S. sonnei* formed colonies. To assess the intestinal burden of the gut flora (*Acetobacter* sp., *Lactobacillus* sp., and other bacterial species), the same procedure described above was followed; yet, the serial dilutions of the homogenates were plated on deMan, Rogosa and Sharpe (MRS) selective agar plates and colony forming units/fly were enumerated 4-5 days post plating. Five independent replicates (with five flies per replicate) were carried out.

3.6 Metabolism-related experiments

3.6.1 Body weight measurements

From a total of 30 female flies (10 flies per repeat), 5-7 days old individual females were weighed (to the nearest microgram) using a microbalance. To prudently control fly density and to avoid weight variability due to food resources and space competition, newly eclosed female offspring were saved daily and transferred into new vials containing fresh fly food prior to weight measurement.

3.6.2 Organ dissection and fluorescence microscopy

The gut and fat body of 5 to 7 days old female flies with *Tk, DH31,* and *AstA* systemic mutation or EE-specific *AstA* knockdown were dissected in 1X PBS, fixed in 4% paraformaldehyde (PFA)/1X PBS for 30 minutes, washed three times with 1X PBS/0.1% Triton X-100 (PBST), blocked in a blocking solution (1-2%

BSA/1xPBS/0.1% Triton X-100) at room temperature for 1 hour, stained with 1:1000 DAPI (4',6-diamidino-2 phenylindole, Dihydrochloride) and 1mg/ml BODIPY (4,4 Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-sIndacene) in blocking buffer at room temperature for 1 hour and washed three times with 1X PBST. The Vectashield Antifade mounting medium was used to mount fat body and gut tissues and images were captured using Olympus SF3000 confocal microscope. To measure lipid storage in the fat body and deposition in gut tissues, the green fluorescence of BODIPY was quantified relative to the total imaged area (by deducting background fluorescence per unit area from this measurement). A minimum of 10 flies per control and experimental group were score and at-least five tissues were quantified for in each independent biological replicate.

3.6.3 Metabolic assays

3.6.3.1 Glucose assay

Five adult female flies 5-7 days old with *Tk, DH31*, and *AstA* systemic mutation or EE-specific *AstA* knockdown were starved for 1 hour, washed with 1mL of 1X PBS to remove possible traces of food contaminants, homogenized using the Pellet Pestle ® Motor in 100ul 1X PBS, and incubated at 95°C for 15 minutes to inactivate endogenous enzymatic activity. Using a commercially available kit and according to supplier's recommendations, the glucose assay was carried out. Briefly, 0.01 to 0.16 mg/mL glucose standards, 30ul of 1X PBS (blank), and 1:5 diluted samples were loaded in a clear-bottom 96-well plate. 100ul GO reagent was then dispensed into each well and the plate was incubated at 37°C for 1 hour. To terminate the chemical reaction, 100ul of 12N sulfuric acid (H_2SO_4) was added to all wells. Absorbance was measured at 540nm using a plate reader. Free glucose concentration was calculated by comparing

the free glucose measurement of each sample to the plotted glucose standard curve. At-least three independent biological repeats were conducted.

3.6.3.2 Triglyceride assay

The preparation of the homogenate part is similar to that described above in the glucose assay section. As for triglycerides measurements, the assay was carried out using a commercially available kit and according to the supplier's guidelines. Briefly, 0.125 to 0.5mg/mL glycerol standards, 20ul of 1X PBS (blank), and 20ul samples were loaded in Eppendorf tubes and incubated at 37°C for 1 hour with either 20ul triglyceride reagent (used to measure total glycerol) or with 20ul 1X PBS (used to measure free glycerol). Samples were then centrifuged at maximum speed for 30 minutes and 20ul of each sample was subsequently loaded into a clear-bottom 96-well plate. 100ul free glycerol reagent was further dispensed in each of the wells and the plate was incubated at 37°C for 5 minutes. Absorbance was measured at 540nm using a plate reader. The triglyceride content in each sample was calculated based on the standard curve by subtracting free glycerol concentration in the untreated samples from the total glycerol concentration in the triglyceride reagent-treated samples. At-least three independent biological repeats were conducted.

3.7 Statistical analysis

The GraphPad Prism 8 statistical analysis software was used to analyze all collected data. For the survival assay, the Kaplan-Meier survival test was used to calculate percent survival, and statistical significance between compared groups was calculated using the log-rank test. As appropriate per data type and experimental groups compared, unpaired t-test with Welch's correction or one-way ANOVA with Dunnett's multiple comparison test was used to calculate significance between compared groups

in all experiments. A p-value ≤ 0.05 was considered statistically significant. The statistical test used for data analysis in each experiment is indicated in the figure legend, with ns dictating no statistical significance between compared groups, and asterisks reflecting the calculated p-value. In all conducted experiments, none of the collected data was excluded from statistical analysis. The researcher who carried out the experiments was not blinded.

Chapter 4: Results and Discussion

4.1 Results

4.1.1 Knockdown status of systemic and transgenic flies

4.1.1.1 Confirmed knockdown of Tk, Dh31, and AstA genes in whole body of purchased systemic mutants

To confirm the knockdown of *Tk*, *Dh31*, and *AstA* genes in the whole body of purchased systemic mutant female flies, the transcript levels of *Tk* (Figure 8A), *Dh31* (Figure 8B), and *AstA* (Figure 8C) were measured and compared to *yw* (control) flies. The transcription levels of all three peptide hormones were down-regulated in systemic mutant flies as compared to *yw* (control) flies.

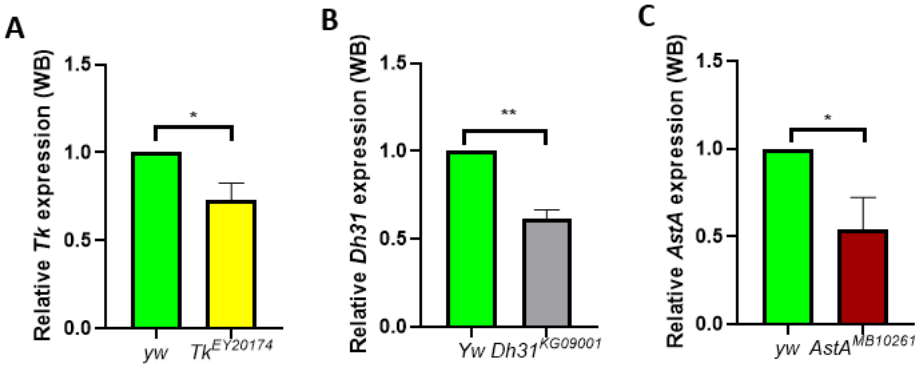


Figure 8. Reduction in peptide hormone expression in the whole body of systemic mutants Quantification of (A) *Tk* (B) *Dh31* and (C) *AstA* in the whole body (WB) of adult *yw* (control), $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants. Measurements representing the mean of three independent repeats were normalized to the transcript levels of *yw*. *Rp49* was used as the reference gene. Error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. *: $p< 0.05$; **: $p< 0.01$.

4.1.1.2 Confirmed knockdown status of **AstA** *gene in the gut of* **Tk>AstA^{RNAi}** *generated transgenic flies*

To confirm the knockdown of the *AstA* gene in the gut of the generated transgenic female flies, the transcript levels of *AstA* (Figure 9) were measured and compared to *Tk>* (control) flies. The transcription level of the *AstA* peptide hormone was down-regulated in transgenic flies as compared to *Tk>* (control) flies.

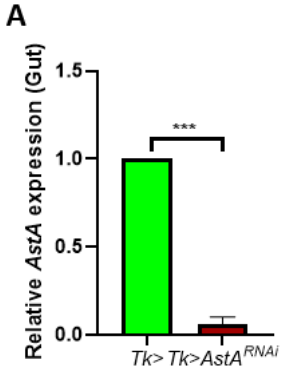
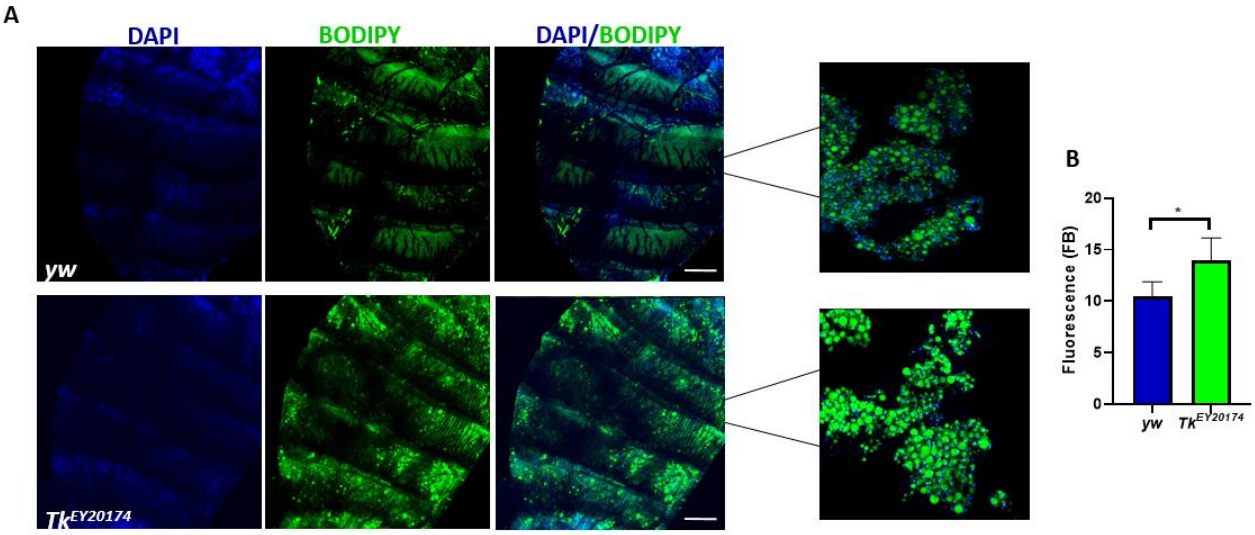


Figure 9. Reduction in *AstA* expression in enteroendocrine gut tissues of *Tk>AstA^{RNAi}* transgenic flies. Quantification of (A) *AstA* expression in the gut of *Tk*-driver only (control) adult female flies and those with EE-gut specific knockdown of *AstA* expression (*Tk>AstA^{RNAi}*). Measurements indicate the mean of three independent repeats (with 10 flies in each repeat) were normalized to the transcript levels of *Tk*-driver only control. *Rp49* was used as the reference gene. Error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. ***: $p < 0.001$.

4.1.2 Metabolism-relevant experiments

4.1.2.1 Lipid profiles of the fat body and gut metabolic organs in $Tk^{EY20174}$ mutants

To detect the effect of Tk peptide hormone knockdown on normal lipid storage and mobilization, lipid droplet storage in the fat body and accumulation in the gut of $Tk^{EY20174}$ mutants was evaluated using BODIPY, a hydrophobic fluorescent dye, and total BODIPY fluorescence was measured (Figure 10B and D). $Tk^{EY20174}$ mutant flies consistently demonstrated an elevation in lipid storage in the fat body (Figure 10A-B) (main lipid storage organ in *Drosophila*) and abnormal accumulation of lipid droplets in the gut (Figure 10C-D) as compared to *yw* (control) flies.



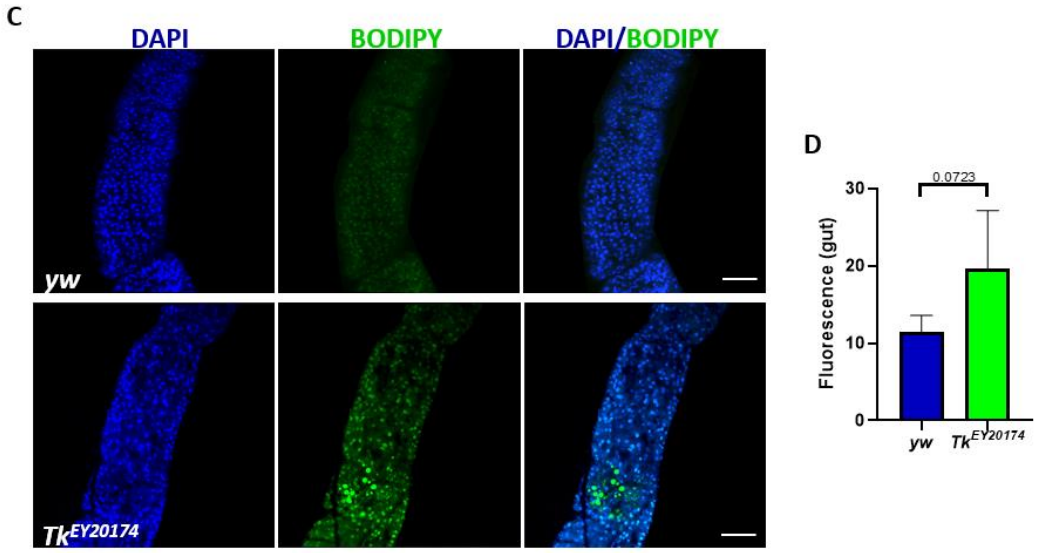


Figure 10. Enhanced lipid storage in the fat body and irregular accumulation of lipid droplets in the gut of *Tk^{EY20174}* flies. Representative fluorescence images of (A) fat body and (C) gut tissues of *yw* (control) and *Tk^{EY20174}* mutants, with nucleus stained with DAPI (blue) on the left, lipid stained with BODIPY (green) in the middle, and both merged on the right. Scale bar, 50µm. (B and D) Quantification of the normalized total florescence of BODIPY in fat body and gut tissues in the fly genotypes indicated in A and C, respectively. Measurements indicate the mean; error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. *: $p < 0.05$.

4.1.2.2 Lipid profiles of the fat body and gut metabolic organs in Dh31^{KG09001} mutants

To detect the effect of Dh31 peptide hormone knockdown on normal lipid storage and mobilization, lipid droplet storage in the fat body and accumulation in the gut of *Dh31^{KG09001}* mutants was evaluated using BODIPY, a hydrophobic fluorescent dye (Figure 11A and C), and total BODIPY fluorescence was measured (Figure 11B and D). *Dh31^{KG09001}* mutant flies consistently demonstrated an elevation in lipid storage in the fat body (Figure 11A-B) (main lipid storage organ in *Drosophila*), with normal mobilization pattern of lipid from the gut (Figure 11C-D) as compared to *yw* (control) flies.

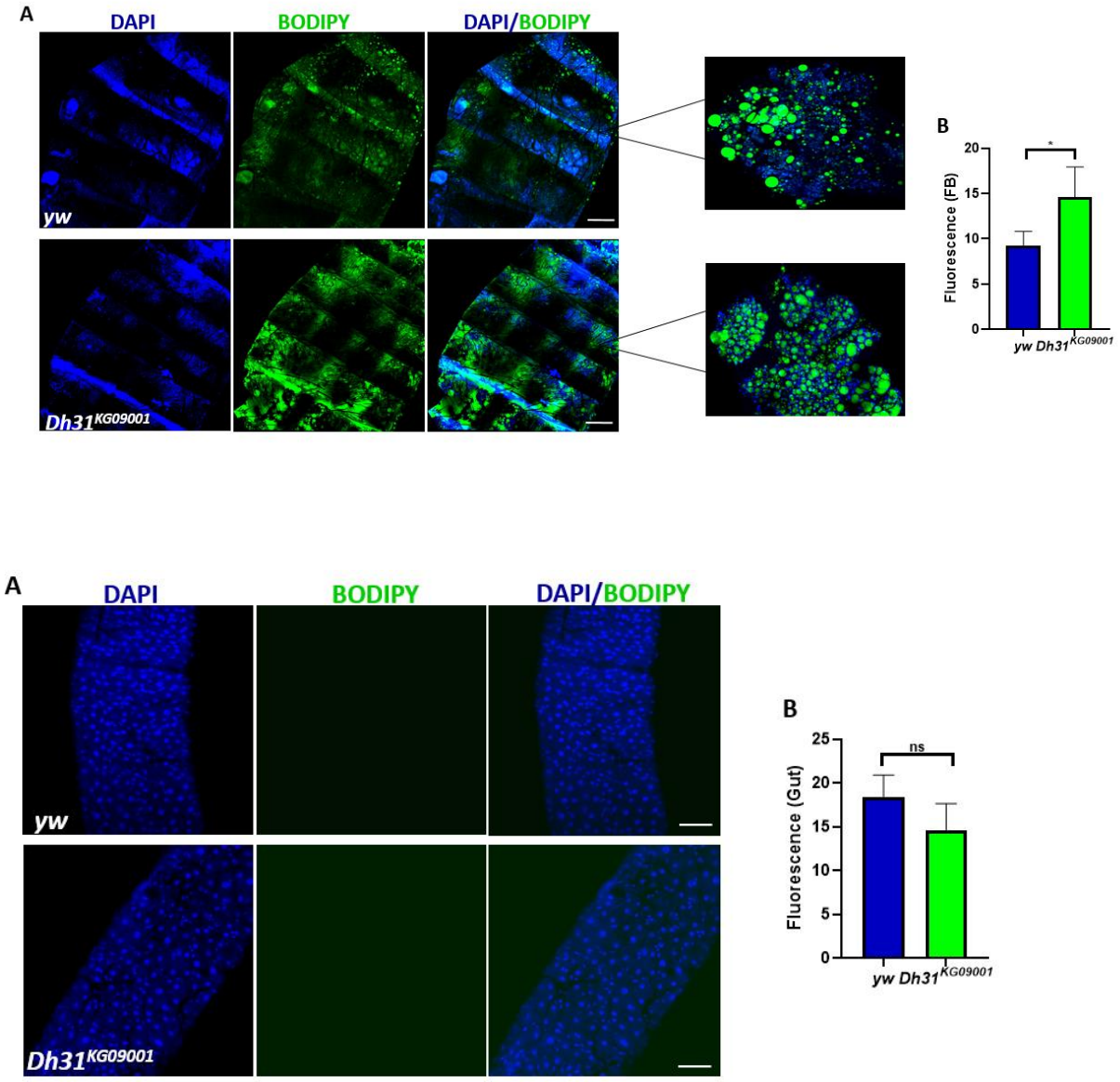
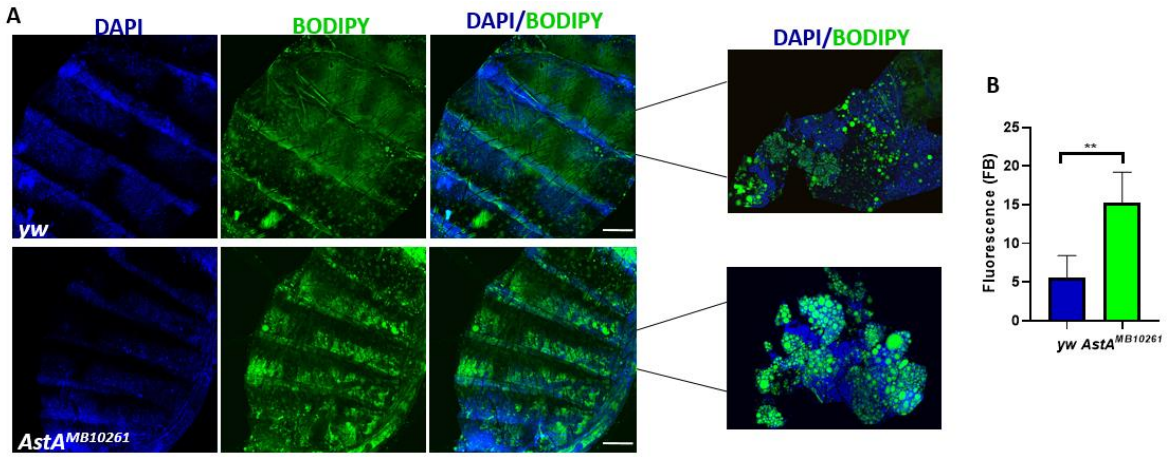


Figure 11. Enhanced lipid storage in the fat body and irregular accumulation of lipid droplets in the gut of *Dh31*^{KG09001} flies. Representative fluorescence images of (A) fat body and (C) gut tissues of *yw* (control) and *Dh31*^{KG09001} mutants, with nucleus stained with DAPI (blue) on the left, lipid stained with BODIPY (green) in the middle, and both merged on the right. Scale bar, 50µm. (B and D) Quantification of the normalized total florescence of BODIPY in fat body and gut tissues in the fly genotypes indicated in A and C, respectively. Measurements indicate the mean; error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. *: $p < 0.05$.

4.1.2.3 Lipid profiles of the fat body and gut metabolic organs in AstA^{MB10261} mutants

To detect the effect of AstA peptide hormone knockdown on normal lipid storage and mobilization, lipid droplet storage in the fat body and accumulation in the gut of *AstA*^{MB10261} mutants was evaluated using BODIPY, a hydrophobic fluorescent dye (Figure 12A and C), and total BODIPY fluorescence was measured (Figure 12B and D). *AstA*^{MB10261} mutant flies consistently demonstrated an elevation in lipid storage in the fat body (Figure 12A-B) (main lipid storage organ in *Drosophila*) and abnormal accumulation of lipid droplets in the gut (Figure 12C-D) as compared to *yw* (control) flies.



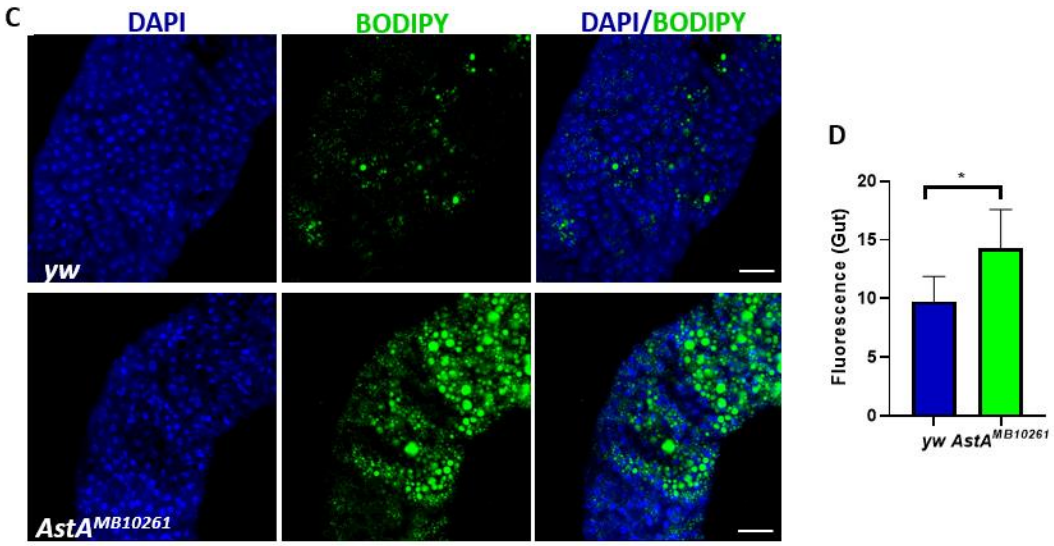


Figure 12. Enhanced lipid storage in the fat body and irregular accumulation of lipid droplets in the gut of *AstA*^{MB10261} flies. Representative fluorescence images of (A) fat body and (C) gut tissues of *yw* (control) and *AstA*^{MB10261} mutants, with nucleus stained with DAPI (blue) on the left, lipid stained with BODIPY (green) in the middle, and both merged on the right. Scale bar, 50µm. (B and D) Quantification of the normalized total florescence of BODIPY in fat body and gut tissues in the fly genotypes indicated in A and C, respectively. Measurements indicate the mean; error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. *: $p < 0.05$.

4.1.2.4 Lipid profiles in the fat body and gut metabolic organs of Tk>AstA^{RNAi} *transgenic flies*

To detect the effect of localized EE-specific AstA peptide hormone knockdown on normal lipid storage and mobilization, lipid droplet storage in the fat body and accumulation in the gut of *AstA*^{RNAi} transgenic flies was evaluated using BODIPY, a hydrophobic fluorescent dye (Figure 13A and C), and total BODIPY fluorescence was measured (Figure 13B and D). *AstA*^{RNAi} transgenic flies consistently demonstrated an elevation in lipid storage in the fat body (Figure 13A-B) (main lipid storage organ in *Drosophila*) and abnormal accumulation of lipid droplets in the gut (Figure 13C-D) as compared to *Tk>* (control) flies.

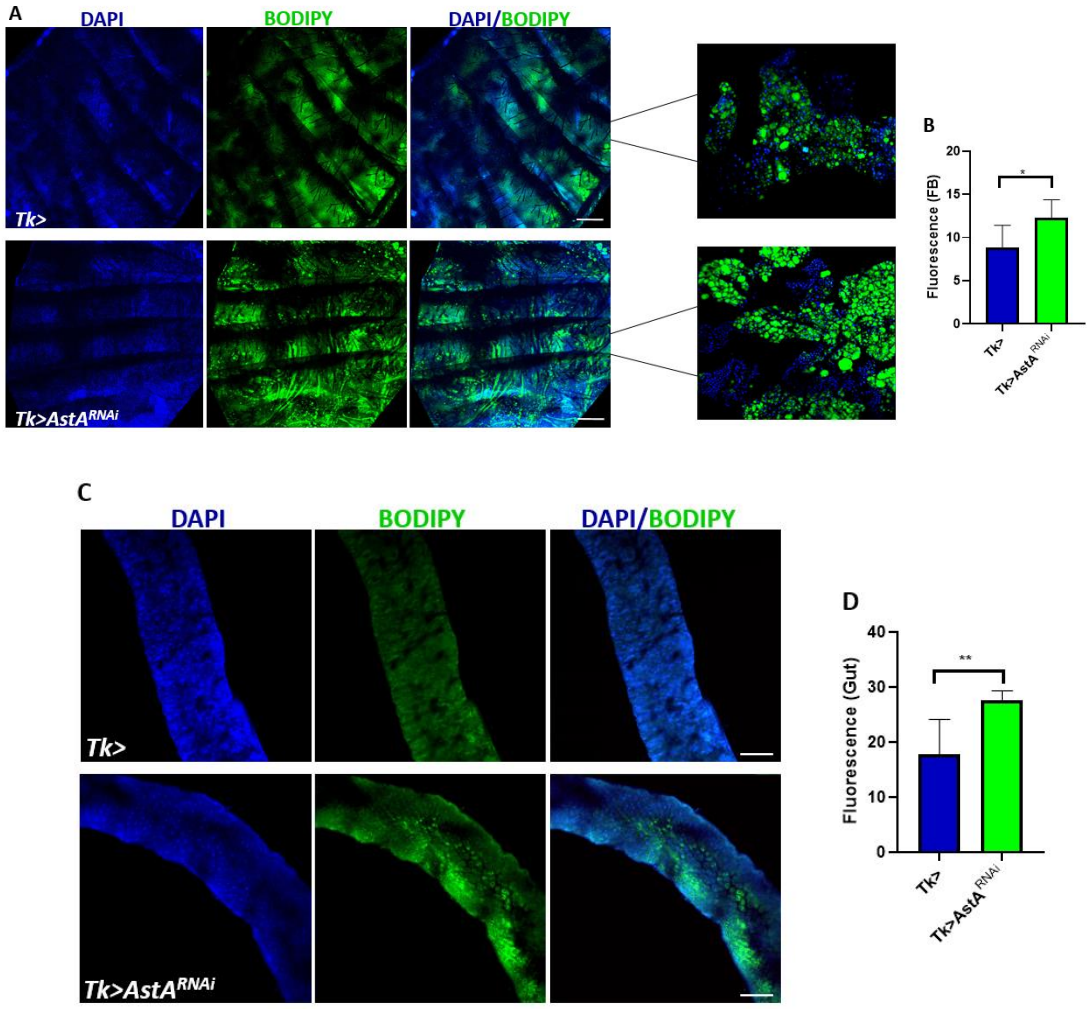


Figure 13. Enhanced lipid storage in the fat body and irregular accumulation of lipid droplets in the gut of *Tk>AstA^{RNAi}* transgenic flies. Representative fluorescence images of (A) fat body and (C) gut tissues of *Tk>* (control) and *Tk>AstA^{RNAi}* transgenic flies, with nucleus stained with DAPI (blue) on the left, lipid stained with BODIPY (green) in the middle, and both merged on the right. Scale bar, 50µm. (B and D) Quantification of the normalized total florescence of BODIPY in fat body and gut tissues in the fly genotypes indicated in A and C, respectively. Measurements indicate the mean; error bars indicate the standard deviation. Unpaired t-test with Welch's correction was used to calculate significant difference. *: $p < 0.05$.

4.1.2.5 Systemic glucose and triglycerides levels and body weights in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants

To determine whether the abnormal lipid droplet accumulation in the gut of $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant female flies is acquainted by a disruption in other metabolic parameters, systemic glucose and triglyceride levels, as well as body weights were measured. As expected, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant female flies had a significant elevation in glucose levels (Figure 14A), triglyceride levels (Figure 14B), and body weights (Figure 14C) as compared to *yw* (control) flies. Surprisingly however, these measured metabolic parameters and body weight in $Tk^{EY20174}$ mutant females were indifferent from those reported in *yw* (control) flies (Figure 14A-C).

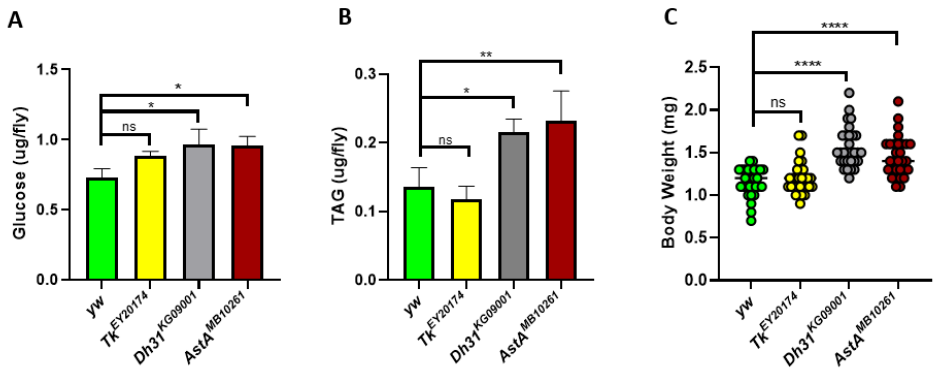


Figure 14. Elevated glucose levels, triglyceride levels, and body weights in $Dh31^{KG09001}$ and $AstA^{MB10261}$ mutant flies. Quantification of systemic (A) glucose (B) triglyceride levels and (C) measurements of body weights in *yw* (control), $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants. In (A) and (B), measurements indicate the mean of three independent biological repeats and error bars indicate the standard deviation. *yw* was used as the reference for comparison. In (C), vertical bars represent the mean measurement. Ordinary One-way ANOVA with Dunnett's multiple comparison test was used to calculate significant difference. ns: not significant; *: $p< 0.05$; **: $p< 0.01$; ****: $p< 0.0001$.

4.1.2.6 Systemic glucose and triglycerides levels and body weights in Tk>AstA^{RNAi} *transgenic flies*

To determine whether the abnormal lipid droplet accumulation in the gut of *Tk>AstA*^{RNAi} mutant female flies is acquainted by a disruption in other metabolic parameters, systemic glucose and triglyceride levels, as well as body weights were measured. As expected, *Tk>AstA*^{RNAi} mutant female flies had a significant elevation in glucose (Figure 15A) and triglyceride levels (Figure 15B) as compared to *Tk>* (control) flies. The body weight of 5 *Tk>AstA*^{RNAi} transgenic female flies; however, demonstrated no difference in body weight as compared to *Tk>* (control) flies (Figure 15C).

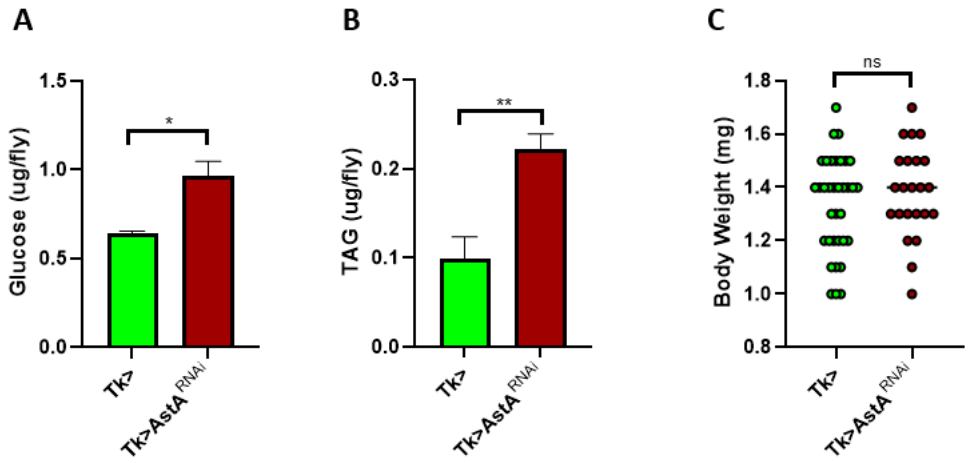


Figure 15. Elevated glucose levels, triglyceride levels, but not body weights in *Tk>AstA*^{RNAi} transgenic flies. Quantification of systemic (A) glucose (B) triglyceride levels and (C) measurements of body weights in *Tk>* (control) and *Tk>AstA*^{RNAi} transgenic flies. In (A) and (B), measurements indicate the mean of three independent biological repeats and error bars indicate the standard deviation. *Tk>* was used as the references for comparison as indicated. In (C), vertical bars represent the mean measurement. Unpaired t-test with Welch's correction was used to calculate significant difference. ns: not significant; *: $p < 0.05$; **: $p < 0.01$.

4.1.3 Immunity-relevant experiments

4.1.3.1 Survival profiles of Shigella sonnei infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants

To address the effect of enteric *S. sonnei* pathogenic infection on the life span of an infected host lacking the expression of $Tk^{EY20174}$, $Dh31^{KG09001}$, or $AstA^{MB10261}$ peptide hormones, the survival/death rates of orally infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant flies were followed over a period of 8-10 days. *S. sonnei* infected mutant flies exhibited a reduced life span as compared to *yw* (control) infected flies (Figure 16A-D).

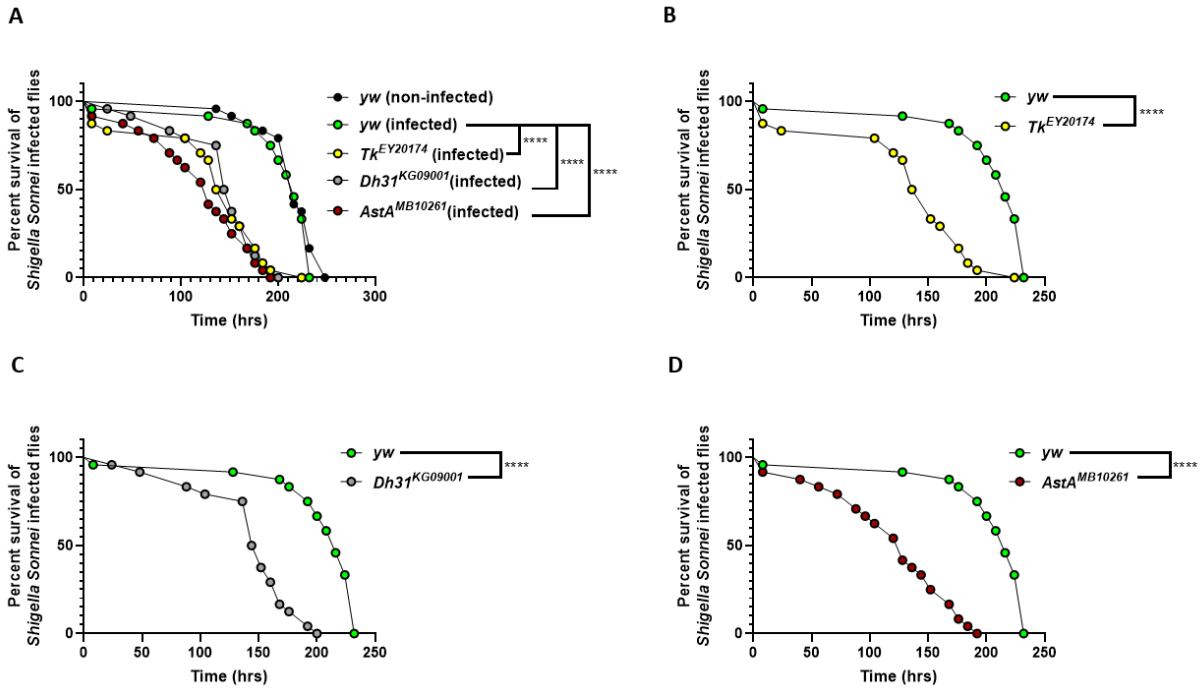


Figure 16. Reduced life span of *Shigella sonnei* infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ flies. (A) Overlaid survival curves of *yw* (non-infected), *yw* infected (control), and $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ infected mutants. Compared survival curves of (A) yw and $Tk^{EY20174}$ infected flies (B) yw and $Dh31^{KG09001}$ infected flies (C) yw and $AstA^{MB10261}$ infected flies. Kaplan-Meier survival test was used to calculate percent survival. The statistical significance of the observed differences was calculated using the log-rank test. ****: $p < 0.0001$.

4.1.3.2 Survival profiles of Staphylococcus aureus infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants

To address the effect of enteric *S. aureus* pathogenic infection on the life span of an infected host lacking the expression of $Tk^{EY20174}$, $Dh31^{KG09001}$, or $AstA^{MB10261}$ peptide hormones, the survival/death rates of orally infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant flies were followed over a period of 8-10 days. *S. aureus* infected flies exhibited a longer life span as compared to *yw* (control) infected flies (Figure 17A-D).

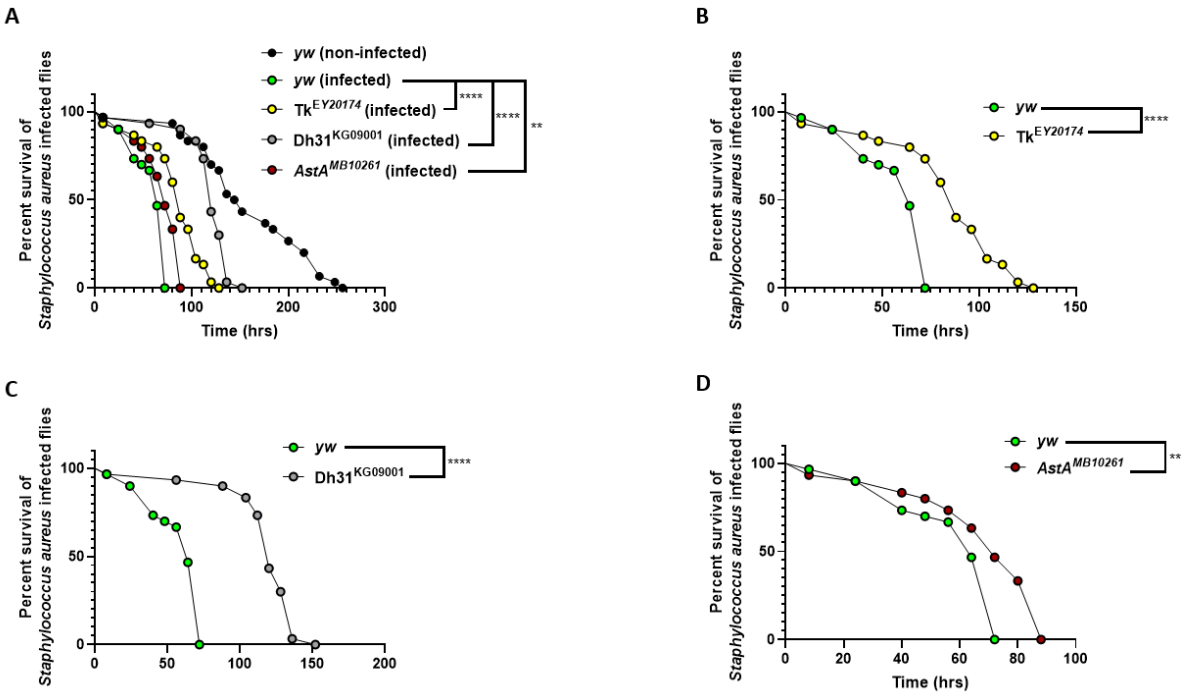


Figure 17. Increased life span of *Staphylococcus aureus* infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ flies. (A) Overlaid survival curves of *yw* (non-infected) *yw* infected (control), and $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ infected flies. Compared survival curves of (A) yw and $Tk^{EY20174}$ infected flies (B) yw and $Dh31^{KG09001}$ infected flies (C) yw and $AstA^{MB10261}$ infected flies. Kaplan-Meier survival test was used to calculate percent survival. The statistical significance of the observed differences was calculated using the log-rank test. ****: $p < 0.0001$.

4.1.3.3 Survival profiles of Shigella sonnei and Staphylococcus aureus infected Tk>AstA^{RNAi} transgenic flies

To address the effect of enteric *S. sonnei* and S. *aureus* pathogenic infection on the life span of an infected host, the survival/death rates of orally infected *Tk>AstA^{RNAi}* transgenic flies were followed over a period of 8-10 days. Flies infected with *S. sonnei* survived similarly to their control counterpart (*yw*), whereas flies infected with *S. aureus* exhibited a longer life span compared to *yw* (control) flies (Figure 18 A and B).

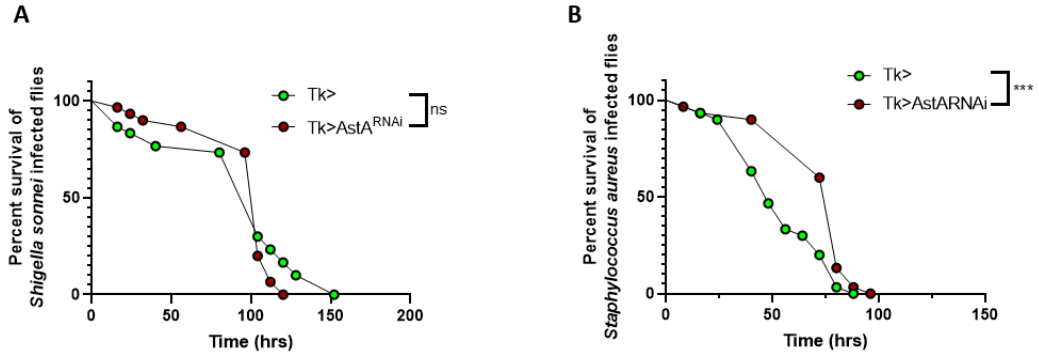


Figure 18. Increased life span of *Staphylococcus aureus* but not *Shigella sonnei* infected *Tk>AstA^{RNAi}* transgenic flies. Compared survival curves of *Tk>* (control) and *Tk>AstA^{RNAi}* transgenic flies infected with (A) *Shigella sonnei* and (B) *Staphylococcus aureus* (C). Kaplan-Meier survival test was used to calculate percent survival. The statistical significance of the observed differences was calculated using the log-rank test. ****: p< 0.0001.

4.1.3.4 Gut microbiota in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants

To identify whether $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant female flies exhibit alterations in their gut microbiota composition, $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant flies were assessed for changes in numbers and types of gut flora bacterial species by scoring for normal flora colony forming units and types of bacterial genus/species present. As expected, $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants exhibited a decrease *Lactobacillus* and *Acetobacter* counts, the two predominant bacterial species in laboratory reared fly guts (Figure 19A and B), as compared to *yw* (control). Interestingly however, additional bacterial species, not present in the *yw* (control) group, were also identified in the three systemic mutants (Figure 19C-E) as determined by colony morphology identification (Figure 19F) and Gram-staining (Figure 19G).

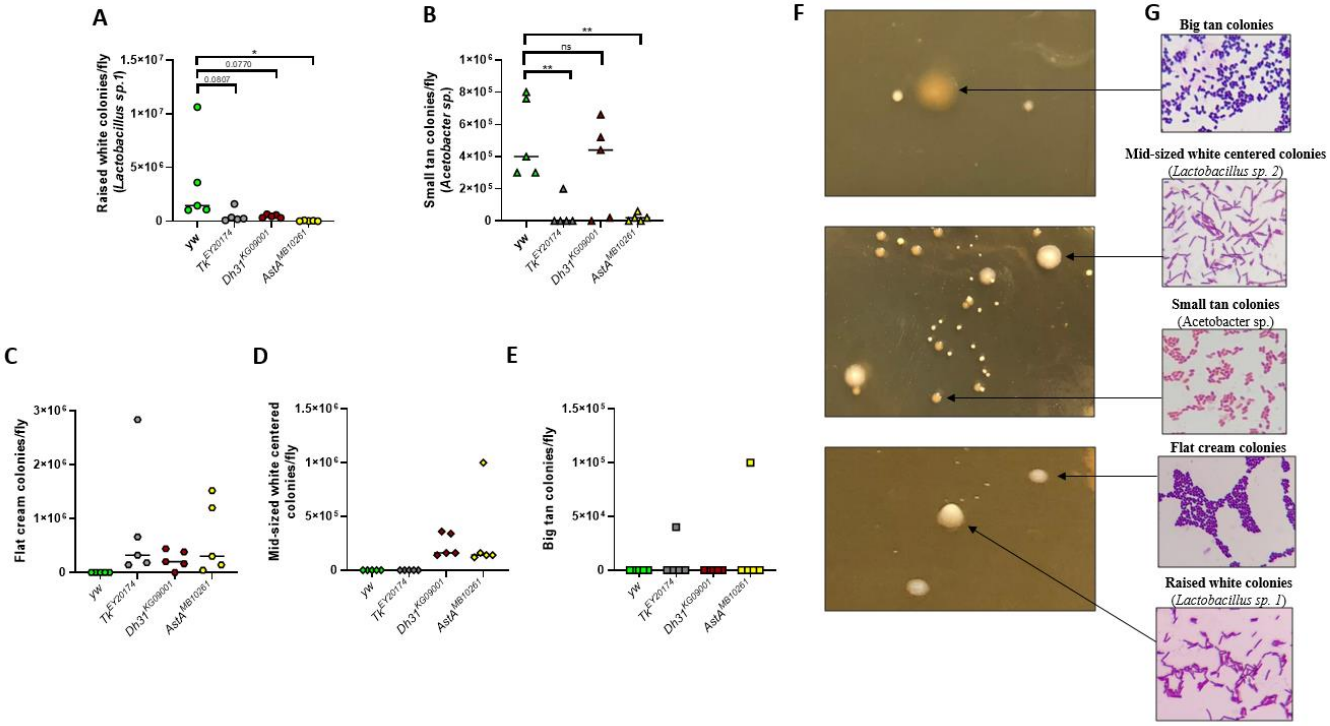


Figure 19. Altered numbers and types of gut flora bacterial species in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ flies. (A-E) Quantification of colony forming units per fly (CFUs/fly) from five independent replicates in *yw* (control), $Tk^{EY20174}$, $DH31^{KG09001}$, and $AstA^{MB10261}$ non-infected mutant flies. (F) Representative colony morphology and (E) Gram-stain of identified gut flora bacterial species in indicated fly groups. One-way ANOVA with Dunnett's multiple comparison test was used to evaluate statistical significance between *yw*, $Tk^{EY20174}$, $DH31^{KG09001}$, and $AstA^{MB10261}$ groups. ns: not significant; *: $p< 0.05$; **: $p< 0.01$.

4.1.3.5 Antimicrobial peptides expression in Shigella sonnei and Staphylococcus aureus infected $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutants

To correlate antimicrobial peptide expression with the survival profiles seen in *S. sonnei* and *S. aureus* infected systemic mutant flies, the transcript levels of *Cec*, *DiptA*, *Met*, and *Def* antimicrobial peptides were measured in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant flies, relative to *yw* (control) flies. As anticipated, *Cec* and *DiptA* were down-regulated in *S. sonnei* infected systemic mutants (Figure 20A and B), whereas *Mtk* and *Def* were up-regulated in *S. aureus* infected $Tk^{EY20174}$ and $Dh31^{KG09001}$ mutants, and only *Def* but not *Mtk* was up-regulated in $AstA^{MB10261}$ mutants (Figure 20C and D).

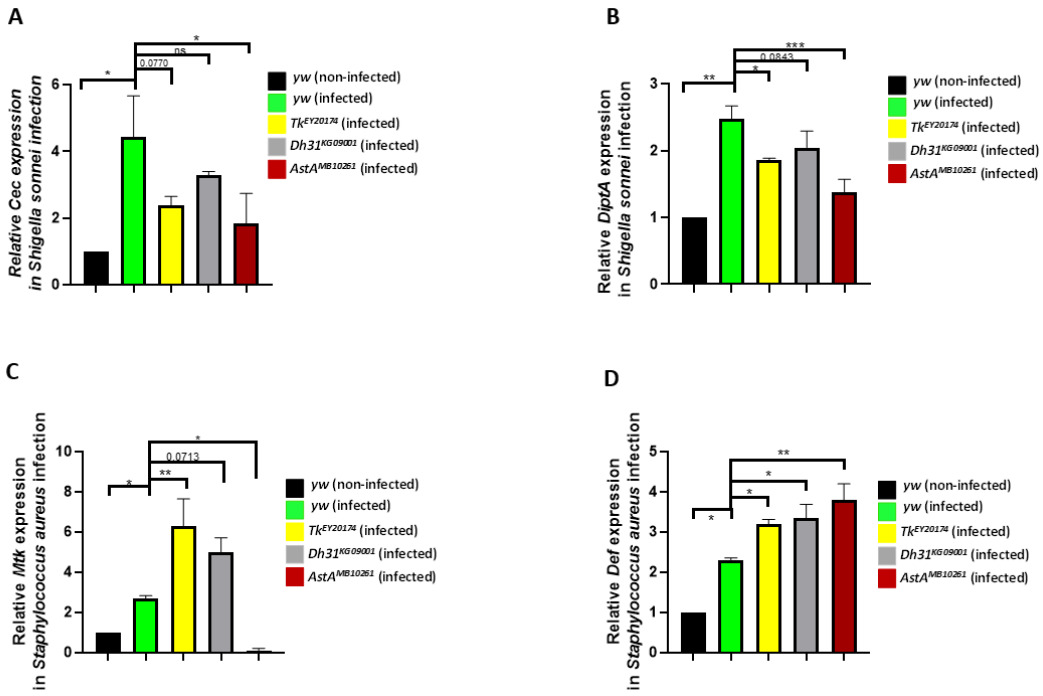
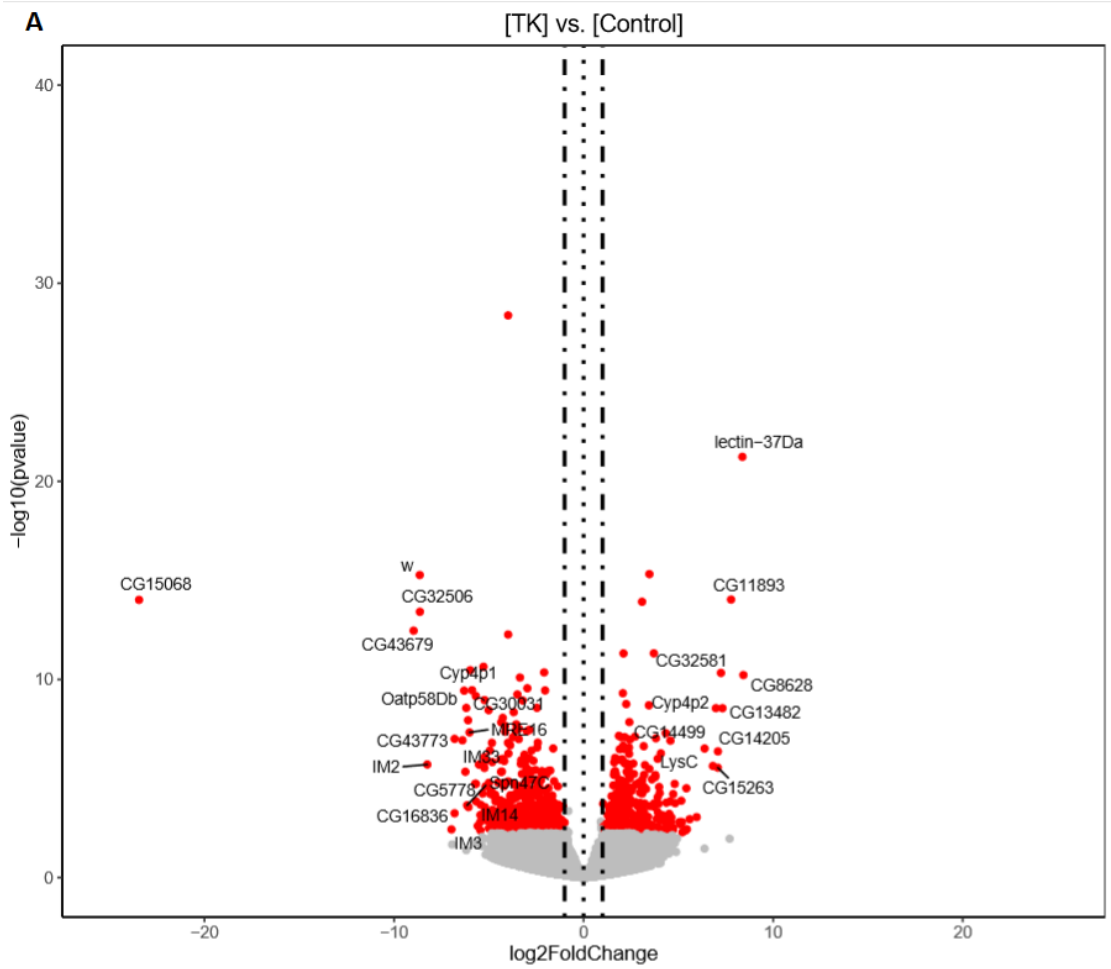
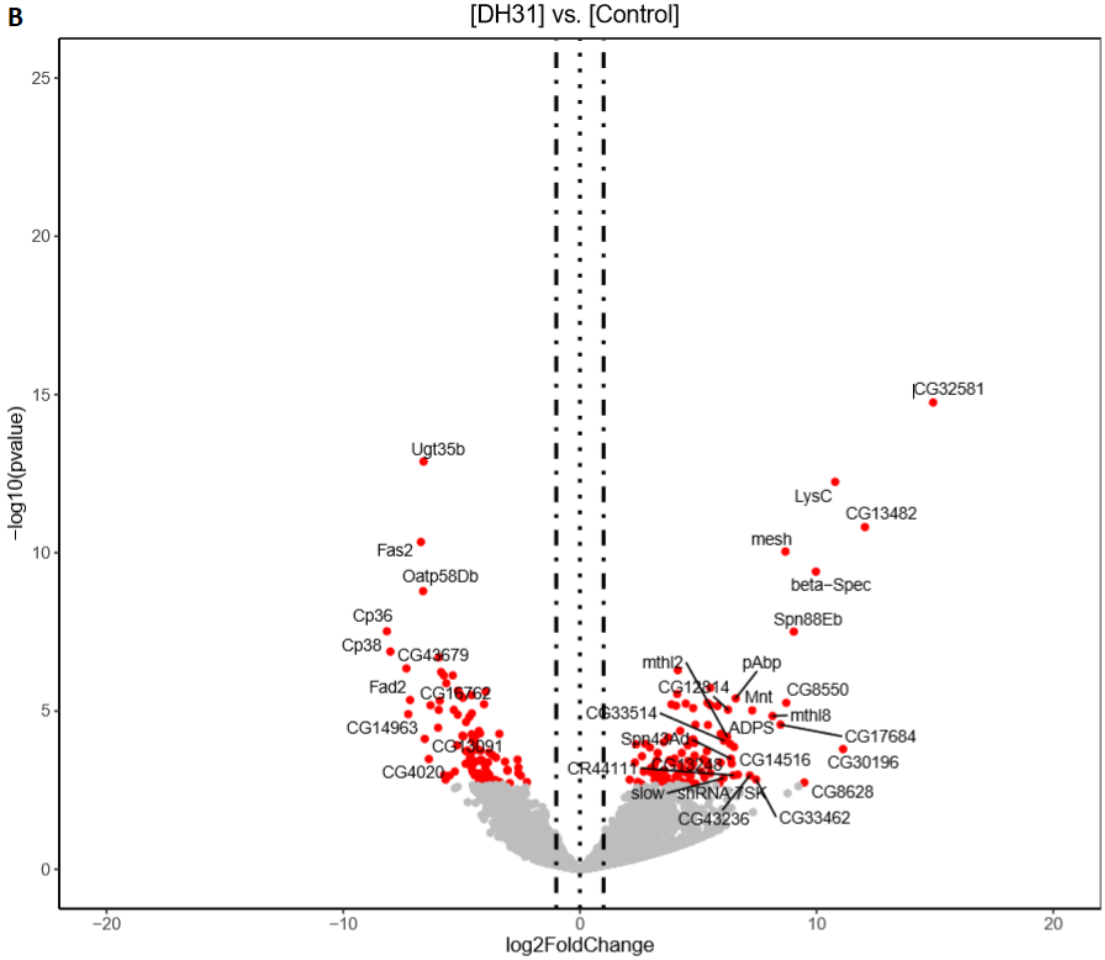


Figure 20. Differential down regulation of *Cecropin* (*Cec*) and *Diptericin A* (*DiptA*) and upregulation of *Metchnikowin* (*Mtk*) and *Defensin* (*Def*) in *Shigella sonnei* and *Staphylococcus aureus* infected flies, respectively. Quantification of (A) *Cec* (B) *DiptA* in *yw* (non-infected) and *yw* (control), $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ *Shigella sonnei* infected flies. Quantification of (C) *Mtk* (D) *Def* in *yw* (non-infected) and *yw* (control), $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ *Staphylococcus aureus* infected flies. Measurements in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ indicating the mean of three independent repeats (with 10 flies per repeat) were normalized to the transcript levels of *yw* (infected) flies. Measurements in *yw* (infected) were normalized to the transcript levels of *yw* (non-infected) flies. Error bars indicate the standard deviation. *Rp49* was used as the housekeeping gene. Unpaired t-test with Welch's correction was used to calculate significant difference between *yw* non-infected and infected groups. Ordinary One-way ANOVA with Dunnett's multiple comparison test was used to calculate significant difference between *yw* infected (control) and $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ infected groups. ns: not significant; *: $p< 0.05$; **: $p< 0.01$; ***: $p< 0.001$.

4.1.4 RNA sequencing and relative gene expression

To score for a broad spectrum of differentially expressed genes in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ mutant female flies and correlated this expression pattern with metabolic and immune-related detected phenotypes, RNA sequencing was performed on individual fly guts of systemic mutants in Sidra Medicine under the direct supervision of Dr. Luis Saraiva. In the $Tk^{EY20174}$ mutant guts, 361 genes were significantly up-regulated and 456 genes were significantly down-regulated (Figure 21A). In the $Dh31^{KG09001}$ mutant guts 103 genes were significantly up-regulated and 96 genes were significantly down-regulated (Figure 21B). In $AstA^{MB10261}$ mutant guts 499 genes were significantly up-regulated and 103 genes were significantly down-regulated (Figure 21C). Functionally, these differentially expressed genes in the three different mutants were further categorized as per their involvement in distinct physiological processes (Figure 22A-C) and Reactome pathways (Figure 23A-C). The genes that were of most relevance to this project, particularly those engaged in metabolism or immune-related facets were extrapolated, functionally identified, tabulated herein (Tables 6-11), and used interpret the reported findings.


A
[TK] vs. [Control]
−log10(pvalue)
log2FoldChange
lectin−37Da
CG15068
w
CG32506
CG43679
CG11893
CG32581
CG8628
Cyp4p1
Oatp58Db
CG30031
Cyp4p2
CG13482
CG43773
IM18
CG14499
CG14205
IM2
IM23
LysC
CG15263
CG5778
Spn47C
CG16836
IM14
IM3


B
[DH31] vs. [Control]
−log10(pvalue)
log2FoldChange
25
20
15
10
5
0
−20
−10
0
10
20
CG32581
Ugt35b
LysC
CG13482
mesh
Fas2
beta−Spec
Oatp58Db
Spn88Eb
Cp36
Cp38
CG43679
mthl2
pAbp
Fad2
CG12814
Mnt
CG8550
CG33514
mthl8
CG14963
ADPS
CG17684
CG30196
CG4020
CR44111
CG14516
slow
shRNA:7SK
CG8628
CG43236
CG33462

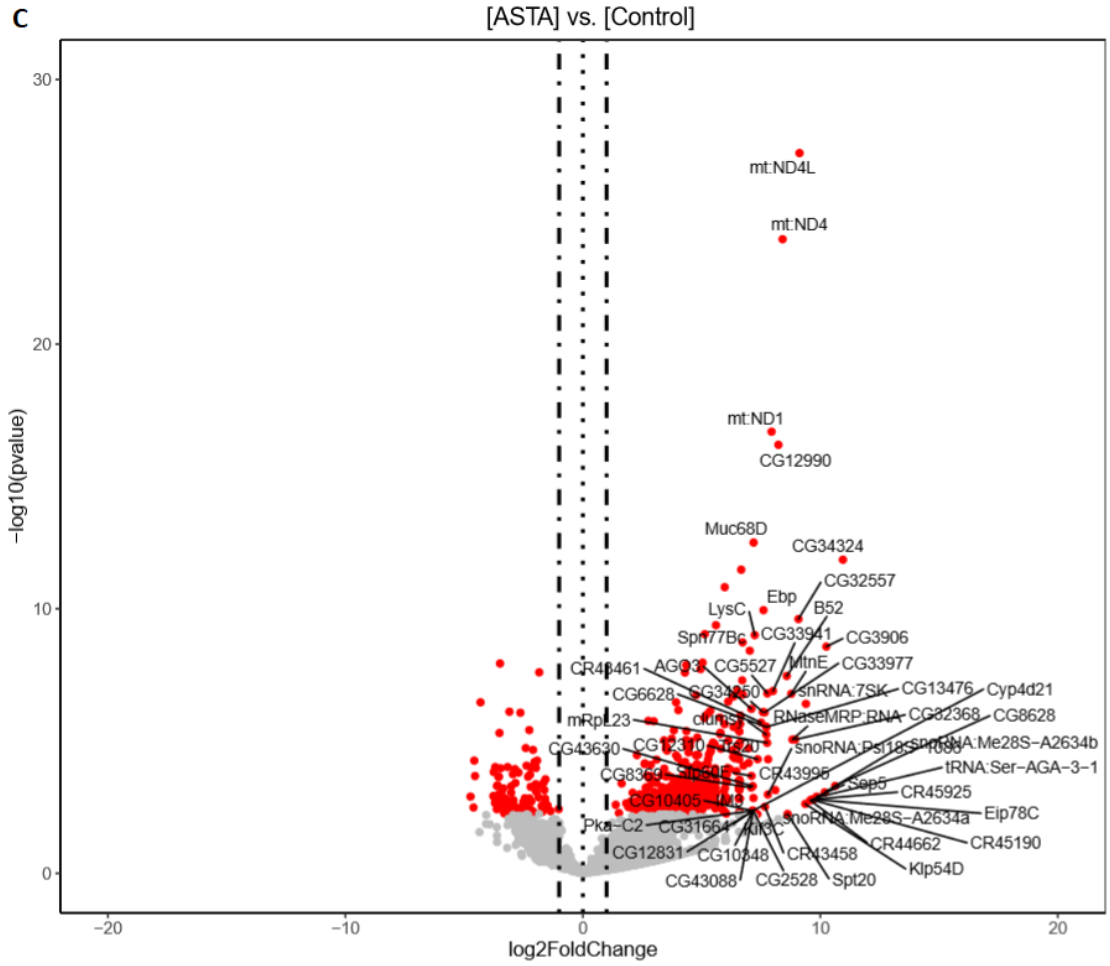
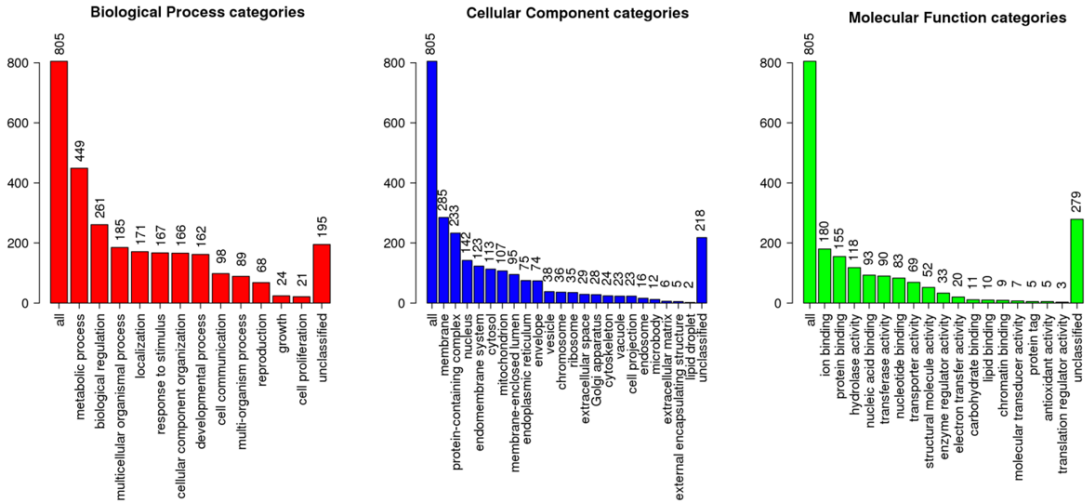
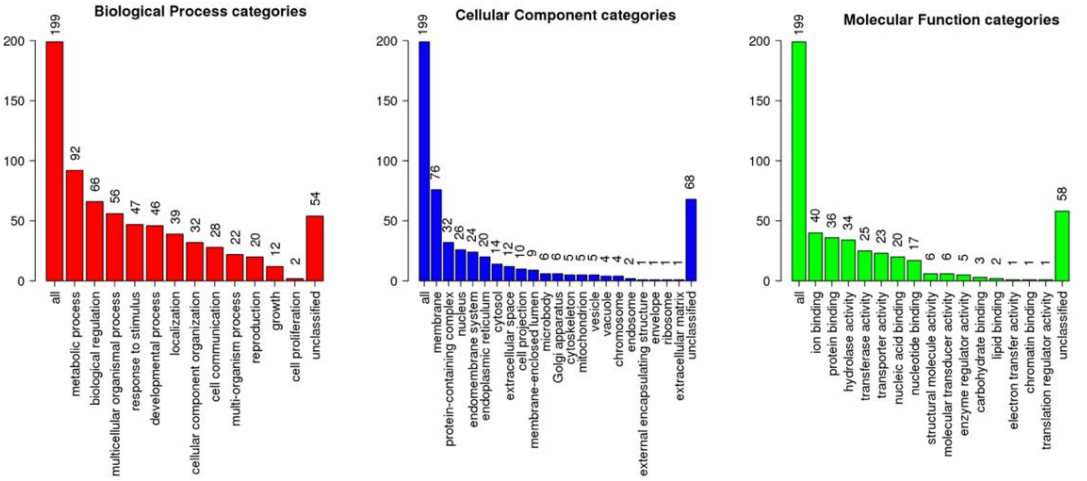


Figure 21. Volcano plot of differentially expressed genes in non-infected *Tk*^{EY20174}, *Dh31*^{KG09001}, and *AstA*^{MB10261} mutant guts as compared to *yw* control guts. Volcano plot for DEA result visualization of (A) *yw* vs *Tk*^{EY20174} (B) *yw* vs *Dh31*^{KG09001}, and (C) *yw* vs *AstA*^{MB10261}. The vertical axis (y-axis) represents the mean expression value of log-transformed false discovery rate-adjusted p-values and the horizontal axis (x-axis) displays the $\log_2$ fold change value. Positive x-values represent up-regulation and negative x-values represent down-regulation. In (A) and (B), genes having p-adjusted value >0.05 and|log2FC| >1 are colored in red and genes having p-adjusted value >0.05 and |log2FC| >6 are tagged. In (C), genes having p-adjusted value >0.05 and |log2FC| >1 are colored in red and genes having p-adjusted value >0.05 and |log2FC| >7 are tagged.

A


Biological Process categories
805
449
261
185
171
167
166
162
98
89
68
24
21
195
all
metabolic process
biological regulation
multicellular organismal process
localization
response to stimulus
cellular component organization
developmental process
cell communication
multi-organism process
reproduction
growth
cell proliferation
unclassified
Cellular Component categories
805
285
233
142
123
113
107
95
75
74
38
36
35
29
28
24
23
23
16
12
6
5
2
218
all
membrane
protein-containing complex
nucleus
endomembrane system
cytosol
mitochondrion
membrane-enclosed lumen
endoplasmic reticulum
envelope
vesicle
chromosome
ribosome
extracellular space
Golgi apparatus
cytoskeleton
vacuole
cell projection
endosome
microbody
extracellular matrix
external encapsulating structure
lipid droplet
unclassified
Molecular Function categories
805
180
155
118
93
90
83
69
52
33
20
11
10
9
7
5
5
3
279
all
ion binding
protein binding
hydrolase activity
nucleic acid binding
transferase activity
nucleotide binding
transporter activity
structural molecule activity
enzyme regulator activity
electron transfer activity
carbohydrate binding
lipid binding
chromatin binding
molecular transducer activity
protein tag
antioxidant activity
translation regulator activity
unclassified

B


Biological Process categories
199
92
66
56
47
46
39
32
28
22
20
12
2
54
all
metabolic process
biological regulation
multicellular organismal process
response to stimulus
developmental process
localization
cellular component organization
cell communication
multi-organism process
reproduction
growth
cell proliferation
unclassified
Cellular Component categories
199
76
32
26
24
20
14
12
10
9
6
6
5
5
5
4
4
2
1
1
1
68
all
membrane
protein-containing complex
nucleus
endomembrane system
endoplasmic reticulum
cytosol
extracellular space
cell projection
membrane-enclosed lumen
microbody
Golgi apparatus
cytoskeleton
mitochondrion
vesicle
vacuole
chromosome
endosome
external encapsulating structure
envelope
ribosome
extracellular matrix
unclassified
Molecular Function categories
199
40
36
34
25
23
20
17
6
6
6
5
3
2
1
1
1
58
all
ion binding
protein binding
hydrolase activity
transferase activity
transporter activity
nucleic acid binding
nucleotide binding
structural molecule activity
molecular transducer activity
enzyme regulator activity
carbohydrate binding
lipid binding
electron transfer activity
chromatin binding
translation regulator activity
unclassified

C

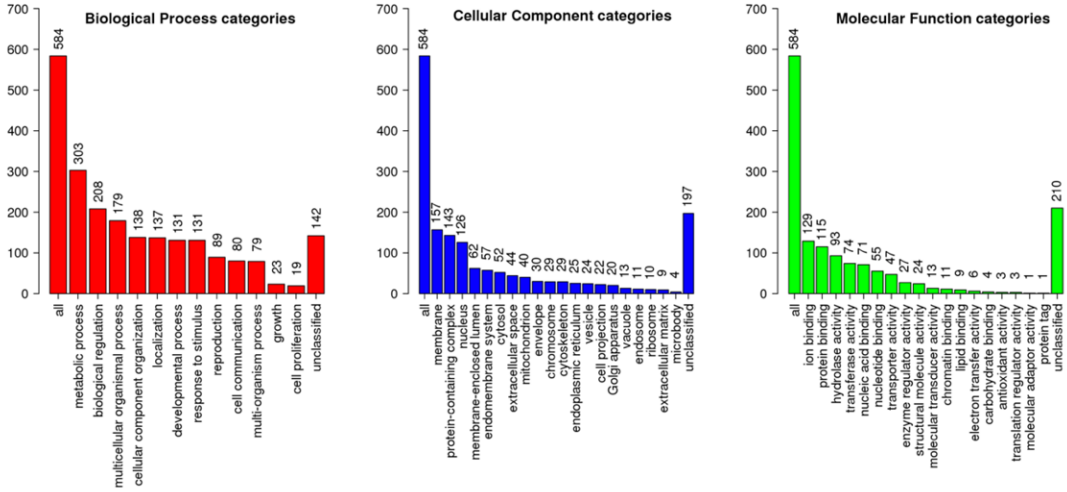
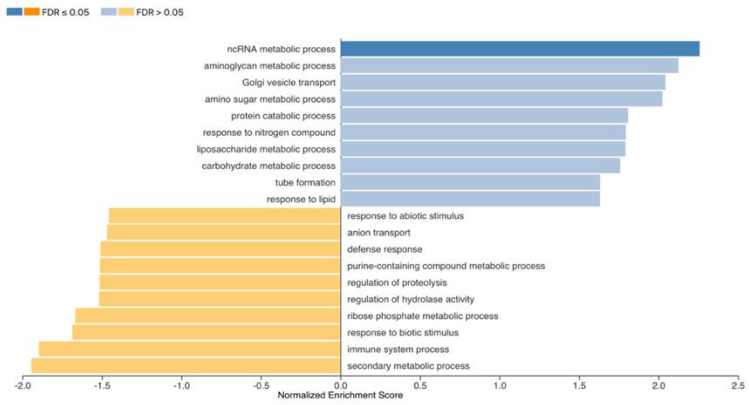
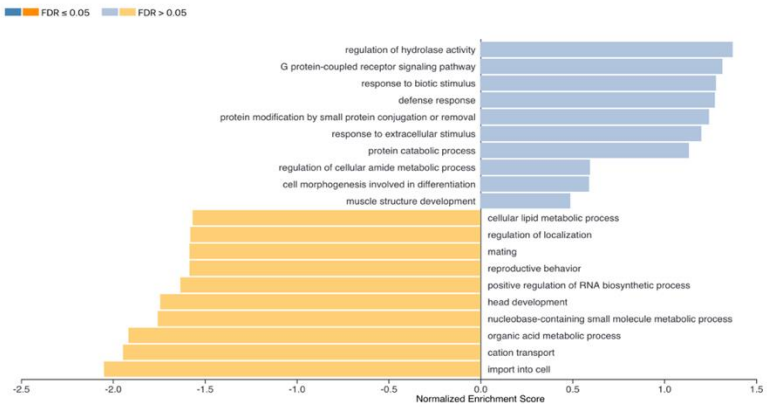


Figure 22. Gene ontology enrichment analysis in *Tk*^{EY20174}, *Dh31*^{KG09001}, and *AstA*^{MB10261} mutant gut. Bar charts of biological process categories (red), cellular components categories (blue), and molecular function categories (green) of differentially regulated genes detected in (A) *Tk*^{EY20174} (B) *Dh31*^{KG09001}, and (C) *AstA*^{MB10261} mutant guts as compared to *yw* control guts. The number on each bar indicates the count of enriched genes annotated with the corresponding gene ontology term. Graphs were generated using WebGestalt gene set analysis toolkit.

(A)



(B)



(C)

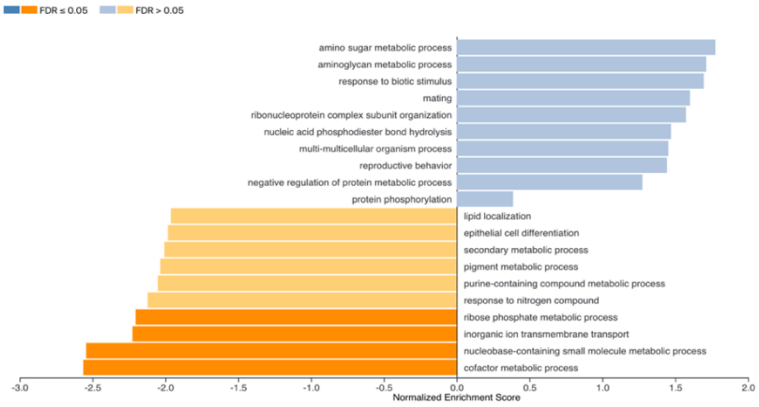


Figure 23. Enriched Reactome pathways in *Tk*^{EY20174}, *Dh31*^{KG09001}, and *AstA*^{MB10261} mutant guts. Bar chart displaying enriched reactome pathways of differentially regulated genes detected in (A) *Tk*^{EY20174} (B) *Dh31*^{KG09001}, and (C) *AstA*^{MB10261} mutant guts as compared to *yw* control guts. Positive normalized enrichment scores (NES) indicate that genes from the pathway are at the top of the ranked list (mostly upregulated). Negative NES indicate that genes from the pathway are at the bottom of the ranked list (mostly down-regulated). FDR≤0.05 indicates significance. Graphs were generated using WebGestalt gene set analysis toolkit.

Table 6. Differential expression of genes involved in metabolic processes in *Tk*^{EY20174} as compared to *yw* (control)

Gene name Upregulated	Physiological function	Reference of gene indicated function
Mal-A7	Involved in hydrolysis of maltose to glucose	[293, 294]
Mal-A8	Involved in hydrolysis of maltose to glucose	[293, 294]
Mal-A1	Involved in hydrolysis of maltose to glucose	[293, 294]
CG3552	Involved in glucose metabolism, enables GDP-D-glucose phosphorylase activity	[295]
CG8475	Involved in glycogen metabolism	[294]

Atg9	Involved in glycogen metabolism	[296]
deltaCOP	Regulation of lipid storage	[297]
Mfe2	Involved in fatty acid beta-oxidation	[295]
betaCOP	Regulation of lipid storage	[297]
Hexo1	Involved in carbohydrate metabolism	[294]
iPLA2-VIA	Involved in triglyceride homeostasis	[298]
Pmm45A	Involved in carbohydrate metabolism	[294]
Sap-r	Involved in lipid homeostasis	[299]
nst	Involved in carbohydrate metabolism	[294]
trbl	Involved in glucose homeostasis Negative regulation of lipid storage	[300] [300]
Uba1	Involved in glucose homeostasis Involved in regulation of lipid storage	[301] [297]
SERCA	Involved in lipid biosynthetic process	[302]
alphaCOP	Involved in regulation of lipid storage	[297]
gammaCOP	Involved in regulation of lipid storage	[297]
ATPCL	Involved in glucose homeostasis	[301]

	Involved in fatty acid biosynthetic process	[295]
Mtpalpha	Involved in fatty acid beta-oxidation	[303]
Gfat1	Involved in carbohydrate derivative biosynthetic process	[294]
LPCAT	Involved in lipid modification	[304]
Snmp1	Involved in lipid homeostasis	[305]
Ilp3	Involved in insulin receptor signaling pathway	[306]
Npc1b	Involved in intestinal cholesterol absorption	[307]
CG8834	Involved in fatty acid biosynthetic process	[308]
dob	Involved in lipid homeostasis, triglyceride catabolism	[295, 309]
Pask	Involved in negative regulation of glycogen biosynthetic process	[295]
Down-regulated		
CG6296	Enables lipase activity, involved in lipid catabolism	[295]
IA-2	Involved in gut development Regulation of insulin and hexokinase secretion	[310] [310]
CG7910	Enables acylglycerol lipase activity	[293]

Fad2	Enables stearoyl-CoA 9-desaturase activity, involved in unsaturated fatty acid biosynthetic process	[295]
bwa	Involved in regulation of lipid metabolism	[311]
Yp3	Enables lipase activity, involved in lipid catabolism	[295]
Yp1	Enables lipase activity, involved in lipid catabolism	[295]
rdgA	Enables diacylglycerol activity, involved in lipid phosphorylation	[295, 312-314]
Yp2	Enables lipase activity, involved in lipid catabolism	[295]
Pdp1	Regulation of lipid metabolism	[315]
Lst	Involved in glucose homeostasis Involved in triglyceride homeostasis Negative regulation of peptide hormone secretion	[316] [316] [316]
Gabat	Regulation of carbohydrate metabolism	[317]
CG3961	Involved in long-chain fatty acid metabolism	[308]
Thor	Involved in insulin receptor signaling pathway Involved in triglyceride metabolism	[318] [319]

CG8993	Involved in glycerol ether metabolism	[294]
GLaz	Involved in lipid catabolism	[295, 320]
Roc1a	Negative regulation of insulin receptor signaling pathway	[321]
bigmax	Involved in response to glucose	[322]
Sod2	Involved in regulation of rate of metabolism	[323]

Table 7. Differential expression of genes involved in immune-related processes in *Tk^{EY20174}* as compared to *yw* (control)

Gene name	Physiological function	Reference of gene indicated function
Upregulated		
Eb1	Involved in melanotic encapsulation of foreign target	[324]
cathD	Involved in defense response against Gram negative bacterium	[325]
Rm62	Involved in defense response again viruses	[326]
betaCOP	Positive regulation of innate immune response against Gram negative bacterium	[97]
	Negative regulation of viral entry into host cell	[327]
Sec5	Involved in defense response against virus	[328]
Npl4	Involved in cellular response to viruses	[329]
AP-1-2beta	Negative regulation of viral entry into host cell	[327]
kay	Positive regulation of biosynthetic process of AMPs active against Gram negative bacteria	[330]
	Involved in peptidoglycan recognition protein signaling pathway	[330, 331]

Diap2	Positive regulation of biosynthetic process of AMPs active against Gram negative bacteria	[330, 332-334]
	Involved in peptidoglycan recognition protein signaling pathway	[176, 330, 331, 333-336]
Cyp6a13	Involved in defense response against bacterium	[337]
ClC-b	Positive regulation of phagocytosis	[338]
mys	Involved in hemocyte migration	[339]
pic	Involved in defense response to fungus	[340]
Hsc70-4	Involved in RNA interference pathway	[341]
Snmp1	Enables scavenger receptor activity	[295]
Uba2	Positive regulation of AMP production through Toll signaling pathway	[342]
	Positive regulation of NF-κB transcription factor	[343]
	Negative regulation of melanotic encapsulation of foreign target	[344]
IKKbeta	Regulation of innate immune/antibacterial humoral response	[345, 346]
	Positive regulation of NIK/NF-κB signaling	[347]
	Involved in peptidoglycan recognition protein signaling pathway	[176, 348]
	Positive regulation of AMP biosynthetic process	[349, 350]
	Involved in defense response to viruses	[351, 352]
Pi3K59F	Involved in RNA interference pathway	[353]
Mtr4	Involved in defense response to viruses	[354]
heix	Negative regulation of hemocyte proliferation & differentiation	[355]
pes	Enables scavenger receptor activity	[295]
mbo	Involved in antimicrobial humoral response	[356]

Charon	Regulation of innate immune response against bacterium & fungus	[357, 358]
	Involved in positive regulation of NF-κB transcription factor activity & peptidoglycan recognition protein signaling pathway	[357]
	Involved in negative regulation of NF-κB transcription factor activity & peptidoglycan recognition protein signaling pathway	[358]
pirk	Involved in negative regulation of peptidoglycan recognition protein signaling pathway (Imd pathway)	[185, 359-361]
Dis3	Involved in defense response to viruses	[354]
Down-regulated		
IM2	Peptide induced by Toll signaling for resistance to infection	[362-364]
IM3	Peptide induced by Toll signaling for resistance to infection	[363, 364]
IM14	Peptide induced by Toll signaling for resistance to infection	[363-365]
TotM	Provides immunity against Gram positive bacterium & fungus	[366, 367]
DptB	Involved in defense response against Gram positive bacterium	[367, 368]
TotA	Activated by JAK/STAT & Imd signaling	[363, 366, 369, 370]
Npc2h	Binds to LPS, Lipid A & peptidoglycan of bacteria	[371]
ubl	Regulation of Toll signaling	[342]
PPO1	Involved in melanization defense response	[146, 222, 372-375]
	Involved in defense response against gram positive bacterium	[372, 373]
	Involved in defense response against fungus	[222, 372]
PPO2	Involved in melanization defense response	[372, 373, 375]

	Involved in defense response against gram positive bacterium	[372, 373]
	Involved in defense response against fungus	[372]
RNASEK	Involved in defense response against viruses	[376]
IM33	Serine protease inhibitor, suspected to be involved in Toll signaling	[363]
Yp3	Precursor of DIM 30 gene product, implicated in immune mechanisms due to structural similarities with other proteins	[363]
vvl	Positive regulation of AMP biosynthetic process in a manner independent of Toll & Imd signaling	[377]
DnaJ-1	Positive regulation of crystal cell differentiation	[378]
Sls	Regulation of hemocyte proliferation	[379]
Sty	Regulation of lamellocyte differentiation	[380]
nplp2	Relatively unexplored - believed to be involved in innate immune response	[364, 368]
Cpo	Regulation of hemocyte proliferation	[379]
CG6426	Involved in defense response against Gram negative bacterium	[293]
Rap1	Involved in hemocyte migration	[339, 381]
Antp	Involved in lymph gland development	[382]
St3	Involved in defense response to bacterium	[337]
CG16799	Involved in defense response against Gram negative bacterium	[293]
CG13551	Involved in defense response against Gram positive bacterium	[383]
lolal	Involved in lymph gland hemopoiesis	[384]
Thor	Involved in antibacterial humoral response	[385]
ben	Involved in peptidoglycan recognition protein signaling pathway	[176, 335, 336, 386]

	Positive regulation of TNF-mediated signaling pathway	[387]
smt3	Involved in positive regulation of AMP production	[342, 343]
	Positive regulation of Toll signaling pathway	[342]
Crk	Involved in phagocytosis & engulfment	[388]
Uev1A	Involved in peptidoglycan recognition protein signaling pathway	[176, 335, 336, 386]
	Positive regulation of TNF-mediated signaling pathway	[387]
RhoL	Involved in melanotic encapsulation of foreign target	[324]
	Involved in hemocyte migration	[381]
Sod2	Involved in hemocyte proliferation	[379]
Sar1	Negative regulation of viral entry into host cell	[327]

Table 8. Differential expression of genes involved in metabolic processes in *Dh31^{KG09001}* as compared to *yw* (control)

Gene name Upregulated	Physiological function	Reference of gene indicated in function
SERCA	Positive regulation of lipid storage	[302]
	Involved in ISC homeostasis	[389]
Pfk	Involved in glycolytic process	[295, 390]
	Involved in glucose homeostasis	[301]
	Involved in response to sucrose	[391]
Mtpalpha	Involved in fatty acid oxidation	[295, 303]

Snmp1	Involved in lipid metabolism	[305]
Mal-A5	Involved in carbohydrate metabolism, enables maltose alpha-glucosidase activity	[293, 294]
Ilp3	Involved in insulin signaling	[306, 392]
ADPS	Involved in lipid biosynthetic process	[393]
Down-regulated		
CG2772	Involved in lipid metabolism, enables lipase & sterol esterase activity	[293]
Pdp1	Involved in lipid metabolism	[315]
PhKgamma	Involved in glucose catabolism & glycogen biosynthesis	[394]

Table 9. Differential expression of genes involved in immune-related processes in *Dh31*^{KG09001} as compared to *yw* (control)

Gene name	Physiological function	Reference of gene indicated in function
Upregulated		
Diap2	Positive regulation of biosynthetic process of AMPs active against Gram negative bacteria	[330, 332-334]
	Involved in peptidoglycan recognition protein signaling pathway	[176, 330, 331, 333-336]
TM9SF4	Phagocytosis & encapsulation	[395]
	Negative regulation of peptidoglycan recognition protein signaling pathway	[396]
Gbp2	Induction of AMP expression via JNK signaling	[397]

CG10433	Predicted to be an AMP	[389]
LysB	Involved in defense response against Gram negative bacterium	[293]
CG31741	Enables scavenger receptor activity	[295]
Spn88Eb	Involved in defense response against fungus	[363]
Snmp1	Enables scavenger receptor activity	[363]
Down-regulated		[295]
tim	Positive regulation of phagocytosis	[398]
lolal	Involved in larval lymph gland hemopoiesis	[384]

Table 10. Differential expression of genes involved in metabolic processes in *AstA*^{MB10261} as compared to *yw* (control)

Gene name	Physiological function	Reference of gene indicated in function
Upregulated		
CG17097	Involved in lipid metabolism, enables triglyceride lipase & sterol esterase activity	[389]
Mal-A7	Involved in carbohydrate metabolism, enables maltose alpha-glucosidase activity	[294]
Mal-A8	Involved in carbohydrate metabolism, enables maltose alpha-glucosidase activity	[294]
Imp	Regulation of insulin expression Involved in sugar metabolism Regulation of dilp2 & dilp3 expression	[399]

CG18284	Involved in lipid metabolism, enables lipase & sterol esterase activity	[389]
Amy-p	Involved in carbohydrate metabolism	[400]
Pfrx	Enables 6-phosphofructo-2-kinase & fructose-2,6-bisphosphate 2-phosphatase activity	[295]
trbl	Antagonizes insulin signaling Negative regulation of lipid storage Involved in glucose homeostasis	[300]
CG10202	Involved in carbohydrate metabolism	[294]
CG18301	Involved in lipid metabolism, enables lipase & sterol esterase activity	[293]
CG17841	Involved in lipid homeostasis	[295]
CG5326	Involved in fatty acid biosynthetic process	[295]
srp	Involved in fat body development	[401, 402]
Down-regulated		
CG17192	Involved in lipid metabolism, enables lipase activity	[295]
Taldo	Involved in carbohydrate metabolism	[294]
Arf79F	Regulation of lipid storage	[297]
Thor	Involved in lipid metabolism	[403]

Table 11. Differential expression of genes involved in immune-related processes in *AstA*^{MB10261} as compared to *yw* (control)

Gene name	Physiological function

Upregulated		Reference of gene indicated in function
B52	Involved in defense response against viruses	[329]
Dcr-2	Involved in detection of & defense against viruses	[404-410]
	Involved in RNAi pathway	[411, 412]
	Positively modulates Toll signaling	[409]
Etl1	Positive regulation of innate immune response against Gram negative bacterium	[97]
Spn38F	Positive regulation of innate immune response against Gram negative bacterium	[408]
Tab2	Involved in peptidoglycan recognition protein signaling pathway	[176, 330, 331]
	Positive regulation of biosynthetic process of AMPs active against Gram negative bacteria	[330]
	Positive regulation of defense response against viruses	[164]
tim	Positive regulation of phagocytosis	[398]
Crag	Positive regulation of innate immune response against Gram negative bacterium	[97]
Pxn	Involved in phagocytosis	[413]
MED25	Positive regulation of biosynthetic process of AMPs, involved in IMD pathway	[414]
CG6168	Positive regulation of innate immune response against Gram negative bacterium	[408]
CG9029	Predicted to be an AMP	[389]

CG12780	Involved in defense response against viruses	[415]
Exn	Involved in melanotic encapsulation of foreign target	[324]
IM3 (BomS3)	Involved in antibacterial humoral response	[364]
	Involved in response against bacterium	[363]
IM14	Involved in defense response	[364]
	Involved in response to bacterium	[363]
	Positive regulation of antifungal innate immune response	[365]
	Involved in Toll signaling pathway	[365]
cher	Negative regulation of lamellocyte differentiation	[416]
pnt	Involved in plasmatocyte differentiation	[380]
dia	Involved in melanotic encapsulation of foreign target	[324]
pyr	Regulation of crystal cell & plasmatocyte differentiation	[380]
DCTN1-p150	Involved in melanotic encapsulation of foreign target	[324]
ECSIT	Intermediate in Toll signaling	[417]
srp	Negative regulation of crystal cell differentiation	[402, 418-420]
Down-regulated		
Arf79F	Negative regulation of Imd signaling AMP targets	[421]
	Positive regulation of Toll signaling AMP targets	[421]
	Regulation of hematopoietic stem cell homeostasis	[422]
Thor	Positive regulation of innate immune response against Gram negative bacterium	[385, 423]
Cdc42	Involved in phagocytosis & encapsulation	[324, 424]

4.2 Discussion

The intestinal epithelium represents one of the most complex interfaces with the outer environment. As such, intestinal epithelial cells, which are chiefly known to be involved in maintaining metabolic homeostasis, are also thought to play a defensive role against invading pathogens, a failure of which leads to severe enduring infections and inflammatory disorders [425]. This role of some intestinal epithelial cells as both innate immune sensors and metabolic modulators, is supported by the notion that ingested nutrients often contain colossal numbers of microorganisms, a condition that necessitates the presence of a protective gastrointestinal innate immune machinery. Even in the absence of these dietary microorganisms, the intestinal epithelial lining still forms a defensive barricade to avert the direct exposure of immune cells to the commensal bacteria of the gut [426]. In the absence of such a safeguarding barrier, the gut microbiota can cross the bowel wall, promoting the eventuality of inflammatory immune responses [427, 428]. Among different intestinal epithelial cells, the contribution of EEs to this immune-metabolic axis has been considered censorious. This anticipated interplay between EEs and innate immunity has been advocated by a collection of observations like those highlighting the involvement of EEs in regulating host metabolism in response to inflammation [429, 430], and others manifesting the expression of Toll-like receptors on the surface EEs and their response to activation by elevating the transcription of genes encoding cytokines and EE peptides [431-434]. Other studies have also shown that metabolic byproducts, particularly SCFAs arising from intestinal bacterial processing of the host diet, are recognized by specific GPCR on EEs [435]. This promotes EEs to secrete small peptides to moderate both local and systemic lipid and carbohydrate metabolism and therefore maintain host homeostasis

[436-438]. Although such observations present a salient contribution of EEs to the gut immune-metabolic homeostasis, our understanding of the key players and the molecular mechanisms orchestrating this adjacent concordance between a host's nutritional status, its immune system, and pathogenic virulence is not fully understood. Recently, the regulatory role of EE-secreted peptide hormones in this metabolic-immune-pathogenic axis has proffered. The mammalian Glucagon-like peptide-1 (GLP-1), for example, seems to exhibit anti-inflammatory effects against a broad spectrum of chronic inflammatory diseases [439]. Likewise, GLP-1 receptor signaling was also shown to ameliorate impaired immunity in Chronic Obstructive Pulmonary Disease [440]. In this context as well, mammalian GLP-2 was also shown to regulate mucosal growth and immune responses in burned rats [441] and mammalian Peptide YY (PYY) to stimulate the function of murine peritoneal macrophages in a protein Kinase C dependent manner [442]. Thus far, mainly due to gene redundancy and overlapping functions, loss-of-function studies of mammalian gut hormones have failed to fully associate them with severe immune and metabolic changes and to decipher the exact mechanisms underlying their roles in immune and metabolic alteration [443]. As such, we employed in this study the *Drosophila melanogaster* model organism to unravel the role of selected EE-secreted peptide hormones in gut immune-metabolic homeostasis and to present novel roles of EE-secreted peptide hormones previously known for their neuro and behavioral rather than immune and metabolically related regulatory functions.

An emerging body of evidence including studies from our group presents a role of EE-secreted Tk in regulating carbohydrates and lipid metabolism [5, 53, 444]. Likewise, findings of Hentze et al., revealed a role of the neuropeptide AstA in

regulating fat body metabolism and feeding decisions in *Drosophila* [291]. Herein, we provide further evidence on the role of EE-secreted Tk in glucose and lipid metabolism and introduce novel roles of EE-secreted Dh31 and AstA in maintaining metabolic homeostasis in gut and fat body, two metabolic fly organs. Our findings reveal a significant elevation in fat body storage in $Tk^{EY20174}$, $Dh31^{KG09001}$, and $AstA^{MB10261}$ systemic mutant flies (Figures 10A-B, 11A-B, 12A-12B). Interestingly, this increase in lipid storage is acquainted by irregular accumulation of lipid droplets in the anterior midguts of $Tk^{EY20174}$, $AstA^{MB10261}$, but not $Dh31^{KG09001}$ mutant flies (Figures 10C-D 11C-D, 12C-D).

Glucose intolerance is a chief underlying mechanism of metabolic syndrome, where blocking insulin signaling is generally associated with hyperlipidemia and hyperglycemia. As such, we expected an elevation in systemic glucose and triglyceride levels in $Tk^{EY20174}$, $AstA^{MB10261}$, and $Dh31^{KG09001}$ mutant flies with altered metabolic profiles. As expected, total glucose and triglyceride levels were significantly elevated in $AstA^{MB10261}$ and $Dh31^{KG09001}$, but not in $Tk^{EY20174}$ mutants (Figures 14A-B). Since hepatic steatosis is a facet of obesity and type-2 diabetes, we also anticipated an increase in body of weight of mutant flies with disrupted metabolism. As hypothesized, $AstA^{MB10261}$ and $Dh31^{KG09001}$, but not Tk^{EY2017} mutants exhibited an increase in their body weights (Figure 14C). Some of the $Tk^{EY20174}$ mutant relevant phenotypes were a bit surprising to us as similar phenotypes to those seen in $Dh31^{KG09001}$ and $AstA^{MB10261}$mutants were expected. This trend discrepancy could be attributed to the ~ 25% *Tk* gene expression knockdown only in the $Tk^{EY20174}$mutant flies (Figure 8A) unlike the ~40% and ~50% gene knockdown status of *Dh31* and *AstA* in $Dh31^{KG09001}$ and $AstA^{MB10261}$ mutants, respectively (Figures 8B-C). The detected metabolic

phenotypes as well as the phenotypic similarities and differences between the three compared EE-peptide mutants could be also attributed to the network of genes regulated by these peptide hormones (Figures 21A-C, 23A-C, Tables 6, 8, 10) and to the involvement of these EE-peptide regulated gene in different biological, cellular, and molecular processes (Figures 22A-C).

To validate the detected metabolic regulation to EE-secreted peptides and rule out the contribution of brain neuropeptides in this regulatory process, we decided to generate transgenic flies with EE-tissue specific knockdown of peptide hormones. Owing to the time constrains for completing this project and to the fact that *AstA* systemic mutation exhibited consistent and sturdy metabolic disrupted phenotypes in both gut and fat body as compared to those seen in $Dh31^{KG09001}$ and$Tk^{EY20174}$mutants, we proceeded with generating transgenic flies with EE-specific *AstA* knockdown ($Tk>AstA^{RNAi}$) only (Figure 9). Consistent with the findings detected in $AstA^{MB10261}$ mutants, $Tk>AstA^{RNAi}$ transgenic flies revealed a significant increase in fat body storage (Figures 13A-B), irregular lipid droplets accumulation in the gut (Figures 13C-D), and elevated systemic glucose and glyceride levels (Figures 15A-B), supporting the role of gut, and not brain, derived AstA peptide hormone in modulating gut and fat body metabolic homeostasis. Because EEs account for only 1% of *Drosophila* cells [445], and to the possibility of receiving other body-weight dependent modulatory signals, the failure of achieving significant difference in the body weight measurements between *Tk>* and $Tk>AstA^{RNAi}$ transgenic flies could be explained (Figure 15C).

The impact of intestinal microbes on host metabolism has been described in key studies. In laboratory raised *Drosophila,* the gut flora predominantly comprised of *Lactobacillus* and *Acetobacter* species was shown to promote growth and development

[92, 93]. Interestingly, germ-free flies deprived of gut flora exhibit disrupted insulin signaling and lipid metabolism patterns [5, 85, 92]. As such, we hypothesized that the altered metabolic patterns detected in $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$ mutant flies and in $Tk>AstA^{RNAi}$ transgenic flies could be, at least partly, ascribed to an alteration in the gut flora of these flies. As expected, $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$ mutants exhibited a decrease in the predominant *Lactobacillus* and *Acetobacter* species normally present in a control fly. Surprisingly however, additional bacterial species, not detected in the control group, have been identified in groups with systemic peptide mutations (Figures 19A-E). As we lack the needed microbiology and molecular tools to categorize the peculiarly identified bacteria in the mutant groups at the genus and species level, we differentiated these bacterial groups based on colony morphology (Figures 19F) and Gram-stain (Figures 19G).

The digestive tract is the primary body organ influenced by ingested foodborne microbes. While normal flora are usual residents of that track, invading microbes are generally eliminated by localize gut immune responses. Recently, novel immune-independent clearance mechanisms of gut pathogens have been proposed. A finding by Benguettat at al. presents a role of DH31/CGRP in triggering gut contractions promoting the clearance of opportunistic bacteria (Gram-positive *Bacillus thuringiensis* or Gram-negative *Erwinia carotovora carotovora*) from the fly gut [276]. Likewise, Siviter et al., reported the ability of TK1-5 treatment to promote contractions in adult midguts [256]. Harsh et al. has also correlated EE-secreted Tk levels with systemic *Drosophila* infections [261]. Collectively, these studies accentuate a role of peptide hormones in host defensive strategy and support the notion of a dual role of peptide hormones in maintaining both metabolic and immune homeostasis in a fly gut. As such,

we hypothesized that the absence of Tk, Dh31, and AstA alters a host's susceptibility to pathogenic infection. As anticipated, $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$ mutants and $Tk>AstA^{RNAi}$ transgenic flies infected with either Gram-negative *Shigella sonnei* or Gram-positive *Staphylococcus aureus,* revealed an altered susceptibility profiles as compared to infected controls. Surprisingly however, the susceptibility pattern in a Gram-negative infection setting revealed a divergent phenotype from that detected in a Gram-positive infection setting. *Shigella sonnei* infected $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$, but not $Tk>AstA^{RNAi}$, exhibited a shorter life span as compared to their control groups (Figures 16A-D, 18A), while *Staphylococcus aureus* infected $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$, and $Tk>AstA^{RNAi}$ flies displayed a longer life span (Figures 17A-D, 18B). This bacteria-dependent trend discrepancy could be attributed to multi-factors. The fact that Gram-negative bacteria generally trigger the Imd pathway and Gram-positive bacteria generally activate the Toll pathway could be one possibility [76]. Han and Ip have previously shown that the transcription of the genes encoding *Cecropin* (*Cec*), *Attacin (Att),* and *Diptericin (Dipt)* can be induced by Rel homodimers (Imd pathway), whereas *Defensin* (*Def*) is best stimulated by Rel/Dorsal or Rel/Dif heterodimers (Imd and Toll pathways) [446]. *Metchnikowin* (*Mtk*) was also shown to be regulated by Imd and Toll pathways [447]. By comparing the differential expression patterns of *Cec* and *DiptA* in a *Shigella* infection context and that of *Def* and *Mtk* in a *Staphylococcus* infection context, we report opposing patterns that correlate well with the detected survival drifts. The overall trend of *Cec* and *DiptA* was reduced in $Tk^{EY20174}$, $AstA^{MB10261}$, $Dh31^{KG09001}$ mutants (Figures 20A-B), while the overall expression of *Mtk* and *Def* was increased in all three mutants (except for *Mtk* expression in $AstA^{MB10261}$) (Figures 20C-D). It is worth noting here that although the trend was

obviously increased or decreased in relevant groups, the failure to detect significant differences between some compared groups is likely due to interindividual and infection-related variability, which are commonly known variables in these experiments. The bacteria-dependent trend discrepancy could be further attributed to various immune-related genes differentially up-regulated or down regulated in different EE-peptide hormone mutants (Tables 7, 9, 11). In $Tk^{EY20174}$ mutants for instance, the significant down regulation in the expression of *immune induced peptides IM2, IM3,* and *IM14* and the *ventral veins lacking* (*vvl*) genes (Table 7) could possibly explain the host susceptibility to *Shigella* infection. In $Dh31^{KG09001}$ mutants, a plausible explanation of the extended survival rates of *Staphylococcus* infected flies as compared to control groups could be, at least partly, due to the significant upregulation of the *Growth-blocking peptide 2* (*Gbp2)* gene involved in induction of AMP expression via JNK signaling [397] and to the increase in *CG10433* expression, a plausible AMP [389] (Table 9). In $AstA^{MB10261}$ mutants, the significant up-regulation of a number of immune-related genes including *Dcr-2* (a positive modulator of Toll signaling) [216, 404-411], *CG9029* (a predicted AMP), *IM3* and *IM14* (involved in anti-bacterial humoral response) [363-365], *ECSIT* (intermediate in Toll signaling) [417] could also potentially explain the fly's increased life span in a *Staphylococcus* infection setting. On the other hand, the reduced survival span of *Shigella* infected $AstA^{MB10261}$ mutants could be plausibly tailored to the detected down-regulation of *Thor* (a positive regulator of innate immune response against Gram-negative bacteria) [385, 423] (Table 11). Numerous genes involved in cell-mediated immune responses including phagocytosis, melanization, and encapsulation also appear to be up-regulated or down-regulated in different EE-peptide mutants (Tables 7, 11). As such, further detailed mechanistic

studies are needed for conclusive identification of the specific genes involved in the detected host susceptibility patterns. It should also be mentioned here that the virulence mechanism of the pathogen itself might also contribute to the divergence of the host-pathogen detected interactions.

4.3 Conclusion and future directions

Though several studies have begun revealing the involvement of Tk, Dh31, and AstA EE-secreted peptide hormones in several physiological processes, much of our understanding of their exact role in processes such as immunity and metabolism remains at its infancy. In this project, we describe a unique role of these EE-secreted peptide hormones in modulating numerous metabolic and immune parameters. Our findings serve as a solid foundation for future studies directed towards elucidating the exact mechanisms of function of these EE-secreted peptides. Moreover, addressing whether the role of these peptide hormones is tissue-specific and occurs in a localized manner within EE of the midgut warrants future investigation. Addressing these raised questions is essential prior to drawing a clear causal relationship between peptide hormones and relevant immune and metabolic disorders in higher organisms, including humans.

4.4 Limitations

Though serving as a useful and practical model system, conducting fruit fly experiments requires expertise and delicacy, particularly in fly rearing, when separating males and females, as well as when carrying out techniques such as organ dissection and tissue staining. Therefore, a period of training was required to familiarize myself with the fly model before performing the experimental techniques. As such, time constraint was a major limitation in this project as if time permitted, several additional

experiments would have been carried out to further support the study's findings and aid in unraveling the mechanisms behind the detected phenotypes.

4.5 Proposed diagram

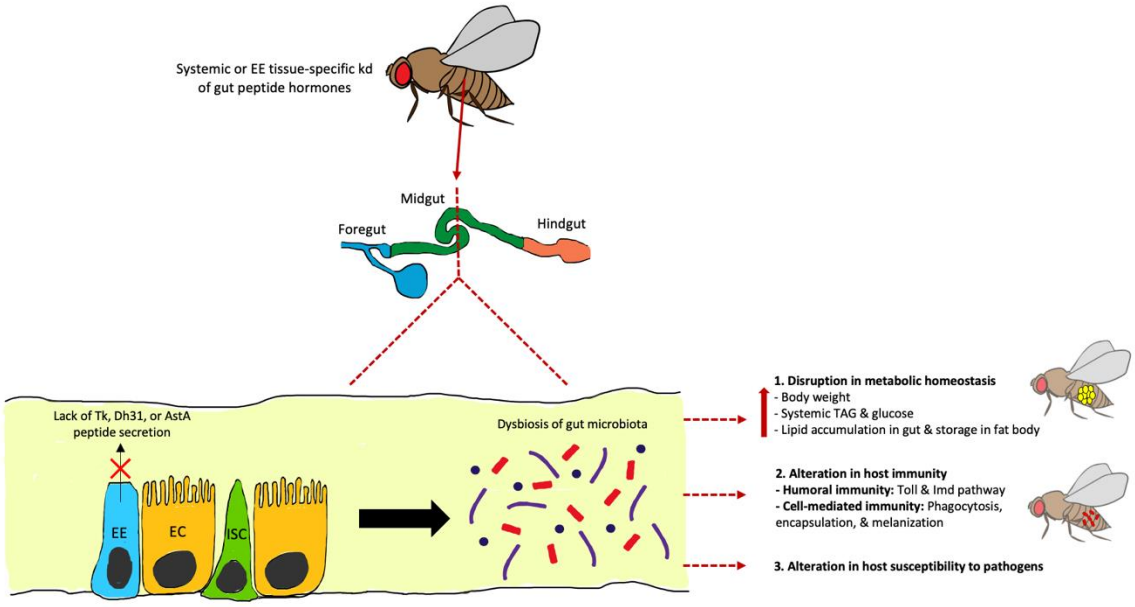


Figure 24. A proposed visual representation of the overall findings of the study. Systemic or localized EE tissue-specific kd of the gut peptide hormones results in microbial dysbiosis within the gut. This, in turn, culminates in (1) dysregulation of metabolic homeostasis, (2) alterations in host immunity, and (3) alterations in susceptibility to pathogens. Diagram was created using Adobe Illustrator 2021.

Chapter 5: Appendix

5.1 QU-IBC approval

Qatar University
Institutional Bio-safety Committee

To: Dr. Layla Y. Kamareddine
Biomedical Sciences Department
Qatar University

30th September 2020

Ref: Project Titled "The role of enteroendocrine secreted gut peptide hormones in modulating immunity and metabolism in Drosophila melanogaster"
Grant: QUST-2-CHS-2020-4

Dear Dr. Layla,

We would like to inform you that your application along with supporting documents provided for the above proposal have been reviewed by QU-IBC, and having met all the requirements, has been granted approval. The approval is for a period of one year and renewable for each year thereafter, should be sought and approved by QU-IBC period to continue.

Please note that QU-IBC approval is contingent upon your adherence to the following QU-IBC Guidelines:

- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of QU Safety Plans and applicable regulations/guidelines, or any significant research-related accidents and illnesses to the QU-IBC. Also, ensuring personnel receive appropriate orientation and specific training for the safe performance of the work.

Your research approval No. is: **QU-IBC-2020/044**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes,

Hadi M. Yassine, M.Sc., Ph.D
Chairperson, QU-IBC
Section Head of Research
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Institutional Bio-safety Committee (IBC)
Office Of Academic Research

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