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Enteric pathogens modulate metabolic homeostasis in the *Drosophila melanogaster* host

Hoda Najjar [a], Sarah Al-Ashmar [b], Abeer Qush [a], Jawaher Al-Asmar [a], Sara Rashwan [a], Abdelrahman Elgamal [a], Asad Zeidan [b, c], Layla Kamareddine [a, c, d, *]

[a] Department of Biomedical Sciences, College of Health Sciences, QU Health, Qatar University, Doha, Qatar
[b] Department of Basic Medical Sciences, College of Medicine, QU Health, Qatar University, Doha, Qatar
[c] Biomedical and Pharmaceutical Research Unit, QU Health, Qatar University, Doha, Qatar
[d] Biomedical Research Center, Qatar University, Doha, Qatar

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ABSTRACT

On quotidian basis, living beings work out an armistice with their microbial flora and a scuffle with invading pathogens to maintain a normal state of health. Although producing virulence factors and escaping the host's immune machinery are the paramount tools used by pathogens in their "arm race" against the host; here, we provide insight into another facet of pathogenic embitterment by presenting evidence of the ability of enteric pathogens to exhibit pathogenicity through modulating metabolic homeostasis in *Drosophila melanogaster*. We report that *Escherichia coli* and *Shigella sonnei* orally infected flies exhibit lipid droplet deprivation from the fat body, irregular accumulation of lipid droplets in the midgut, and significant elevation of systemic glucose and triglyceride levels. Our findings indicate that these detected metabolic alterations in infected flies could be attributed to differential regulation of peptide hormones known to be crucial for lipid metabolism and insulin signaling. Gaining a proper understanding of infection-induced alterations succours in curbing the pathogenesis of enteric diseases and sets the stage for promising therapeutic approaches to quarry infection-induced metabolic disorders.

The intestinal lumen of all organisms harbors a bionetwork demarcated by the microorganisms that dwell in it and the nutrients that pass through it. This bionetwork is regularly subject to transient changes that disrupt the host immune and metabolic homeostasis. While most studies have focused on understanding the strategies used by intestinal pathogens to overcome host immunity, it is becoming increasing evident that these microbes could exhibit pathogenicity via additional or alternative approaches.

Enteric infections, which exert a major global impact on public health especially in developing countries, typically cause anorexia, malaise, and diarrhea [1,2]. Upon entry through the oral route, enteric pathogen that circumvent the proteolytic activity in the stomach breach the mucus layer and set foot into the underlying intestinal epithelium to adhere to or invade a host cell. Once invasion occurs, the pathogen initiates its survival machinery to bypass the host defense strategy [2]. Besides suppressing immunity, some enteric pathogens could adapt other virulence mechanisms, one of which is altering the host metabolic homeostasis. As an example, *Listeria monocytogenes* infection has been shown to reduce food intake in *Drosophila melanogaster* [3] and to alter energy metabolism in infected flies, promoting a gradual lose in triglycerides and glycogen stores and a decrease in the intermediate metabolites and enzymes used in the beta-oxidation and glycolysis energy pathways [4]. Such an interplay between pathogenesis and metabolism is in fact not contemporary, with bidirectional scenarios depicting a cross-talk between the host metabolic status, its immune machinery, and susceptibility to pathogens also reported. Infection-induced anorexia has been presented as an "adaptive trait" that harmonizes the host's ability to fight infection. As a case in point, Ayres and Schneider showed that infection-induced diet restriction upsurges the fly aptitude to tolerate *Salmonella typhimurium* infection while decreases its ability to resist *L. monocytogenes* infection [3]. Under the same notion, Collins et al. revealed a relation between food constituents and pathogenic virulence evidenced by the ability of dietary trehalose to contribute to *Clostridium difficile* hypervirulence when

* Corresponding author. Department of Biomedical Sciences, College of Health Sciences, QU Health, Qatar University, Doha, Qatar.
E-mail address: lkamareddine@qu.edu.qa (L. Kamareddine).

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supplemented with human diet [5]. It is worth noting here that laying down a defined alliance, whether unidirectional or bidirectional, between immunity, pathogenesis, and metabolism is not a straight forward deed, but rather a complex chore that should take into account the pathogen used, the immune output scored for, and the different key players involved. For the nonce, our current understanding of these alliances and the molecular mechanisms orchestrating the adjacent concordance between a host's nutritional status, its immune system, and pathogenic virulence is still not fully elucidated. Being the first barrier to combat non-commensal pathogens ingested along with food; however, it would not be surprising to have the gut, particularly through its enteroendocrine cells and the peptide hormones they produce, contribute to this metabolic-immune-pathogenic alignment dictating a state of health or disease in an infected host. Classically, regulatory enteroendocrine peptides are known to control satiety, intestinal peristalsis, and carbohydrate and lipid metabolism [1,6–8]. Recently; however, their role in modulating the metabolic-immune-pathogenic axis in mammals has proffered [9–12]. Yet, and plausibly due to gene superfluity and overlapping functions, loss-of-function studies of mammalian gut hormones have floundered to fully associate them with severe immune and metabolic changes in both infection and non-infection settings, and to decipher the exact mechanisms underlying their roles in immune, metabolic, and pathogenic alterations [7]. Owing to the significant similarity between a fruit fly and a mammalian gut, along with the simplicity of a fly's signaling system as compared to that of mammals, and to its readily available genetic tools [13,14], several studies including those from our group have employed the *D. melanogaster* as the model organism of choice to unravel the role of regulatory enteroendocrine peptides in host metabolism, immunity, and susceptibility to infection. A study by Benguettat et al. reported a role of the Diuretic hormone 31 (DH31) enteroendocrine peptide in eliciting intestinal visceral muscles contractions favoring opportunistic bacterial elimination from the fly gut [15]. By the same token, we have shown that the IMD innate immune pathway signaling specifically in Tachykinin (Tk)- expressing enteroendocrine cells is not only vital for defense against gut pathogens, but also lays out a landline for metabolic relays between the microbe and the host [16,17]. Along those lines, we provide evidence herein that *Escherichia coli* (*E.coli*) and *Shigella sonnei* (*S. sonnei*) infection modulate peptide hormone expression and metabolic homeostasis in flies as a plausible pathogen virulence mechanism. Our findings serve as a foundation for profound implications to control the pathogenesis of enteric pathogen-caused gastrointestinal tract disorders, and opens up for promising treatment regimens for infection-induced metabolic alterations.

1. Materials and methods

1.1. Drosophila *husbandry and infection*

Drosophila procedures were conducted as per national and international regulatory and safety guidelines and all experimental protocols were approved by Qatar University's Institutional Biosafety Committee (Approval #: QU-IBC-2019/004). The wild-type (Oregon-R) fly strain used in all experiments was purchased from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/) and maintained on regular fly food (71 g/L cornmeal, 9.5 g/L soy flour, 5.5 g/L agar, 16.5 g/L yeast, 5.5 g/L malt, 7.5% corn syrup, 0.4% propionic acid) in a 25 °C, 70% humidity, 12 h day/night cycle incubator. Adult female flies five to seven days old were used in all experiments. For oral infections, flies were randomly distributed into three vials containing a cellulose acetate plug infiltrated with either 2.5 ml of Nutrient Broth alone (control) or 2.5 ml of 1:10 overnight bacterial culture suspension to Nutrient Broth, as previously described [18,21,22].

1.2. Survival assay and bacterial burden quantification

For survival assays, adult female flies were randomly selected, co-housed, and maintained on either Nutrient Broth alone or Nutrient Broth inoculated with either *E. coli* or *S. sonnei* as described above. On a daily basis, fly death/survival was scored twice. Three cohorts of vials represent an independent biological replicate and the reproducibility of all survival curves was confirmed in at least three independent experiments.

To assess bacterial growth and localization in the fly, five cohorts including five flies per cohort (for each independent replicate) fed on either Nutrient Broth alone or Nutrient Broth inoculated with bacteria were surface sterilized with 70% ethanol, washed with 1x phosphate buffered saline (1xPBS), and homogenized in 1xPBS 96 h post infection or feeding on Nutrient Broth. The resultant homogenate was then diluted, plated on Nutrient Broth agar, and incubated at 37 °C, as previously described [18,22,42]. Colony forming unit/Fly was assessed 48 h post plating and three independent experiments were performed.

1.3. Quantification of body weight

Groups of eight to ten adult female flies/vial were initially weighed using a microbalance and transferred to fly vials containing Nutrient Broth alone or Nutrient Broth with either *E. coli* or *S. sonnei*. 24 h post transfer, and over a course of three days, the weight of Nutrient Broth or Nutrient Broth/bacteria fed flies was recorded. Three vials per group were used for each independent repeat and measurements were recorded for two independent experimental repeats.

1.4. Metabolic assays

Five female flies per group maintained on either Nutrient Broth or orally infected with either *E. coli* or *S. sonnei* for four days were homogenized in 100 μl TE buffer (500 mM Tris pH 7.5, 50 mM EDTA with 0.1% Triton X-100) and heat-treated at 95 °C for 20 min to inactivate endogenous enzymatic activity. Metabolic assays including systemic glucose and triglyceride quantification were conducted using commercially available kits according to the manufacturer's recommendations, as previously described [16]. All metabolic assay experiments were repeated at least three independent times.

1.5. Organ dissection and fluorescence microscopy

The guts and abdomen-containing fat bodies of adult female flies maintained on either Nutrient Broth or orally infected with either *E. coli* or *S. sonnei* for four days were dissected in 1xPBS, fixed in 4% paraformaldehyde/1x PBS, washed with 1xPBS/0.1% Trition X-100 (PBST), and incubated with 1:1000 DAPI (4',6-diamidino-2phenylindole, Dihydrochloride) and 1 mg/ml BODIPY (4,4 Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) for 1 h at room temperature. Tissues were again washed and then mounted in VECTASHIELD Antifade mounting medium and images were taken using the Olympus SF 3000 confocal microscope. To assess lipid accumulation in the dissected organs, total BODIPY fluorescence was quantified and normalized to the total organ area, as previously described [16]. At least 10 flies per repeat from each group were dissected and at least 5 images per group from each experimental repeat were quantified.

1.6. Quantification of peptide hormones gene expression

To evaluate the transcript levels of peptide hormones, cDNA was generated from 10 whole female flies per group four days post either feeding on Nutrient Broth alone, Nutrient Broth/*E. coli*, or Nutrient Broth/*S. sonnei*. Accordingly to the suppliers' instructions, total RNA was extracted using TRIzol, chloroform, and phenol-chloroform reagents, contaminant genomic DNA was removed using DNase I, and 250–500 ng of total RNA was used to generate first-strand cDNA using the Superscript III cDNA synthesis kit. qRT-PCR was performed using qPCRBIO SyGreen Mix kit on a StepOnePlus real-time PCR system. The comparative Ct method was used to calculate relative gene expression values. *Rp49* was used as an internal control for normalization. qRT-PCR primer sequences used are listed in Table 1.

1.7. Quantification and statistical analysis

The GraphPad Prism 8 statistical analysis software was used to analyze all collected data. For the survival assays, the Kaplan–Meier survival test was used to compute percent survival and statistical significance was calculated using log-rank analysis. For other experiments, a one-way ANOVA with Dunnett's multiple comparison test was used to determine significant differences between compared groups. A p-value ≤ 0.05 was considered statistically significant. The statistical tests used for each experimental analysis are indicated in the figure legend, with ns implying no statistical significance between compared groups, and asterisks reflecting the calculated p-value. For all experiments, none of the collected data was excluded from statistical analysis.

2. Results

2.1. E. coli *and* S. sonnei *localize in the fly and reduce the life span of the infected host*

Previous work has presented *D. melanogaster* as a model host to study oral *Vibrio cholerae* (*V. cholerae*) enteric infection and understand the mechanistic nature of the human Cholera disease [18]. As such, we reasoned that this model organism could also serve as a host for other enteric bacteria that cause human gastrointestinal tract diseases inaugurating through the oral route. To determine whether *E. coli* and *S. sonnei*, two enteric pathogens, grow and localize in the fly following oral ingestion, the colony-forming units (CFUs)/Fly of *E. coli* and *S. sonnei* in wild-type flies fed on either sterile Nutrient broth (NB) alone (control), *E. coli*/NB mixture, or *S. sonnei*/NB mixture were measured 96 h post infection. As shown in Fig. 1A, *E. coli* and *S. sonnei* localized in orally infected flies. To assess whether this bacterial localization affected the life span of colonized flies, we also followed the survival rates of wild-type flies fed with NB alone, or NB inoculated with either *E. coli* or *S. sonnei*. As shown in Fig. 1B–D, flies infected with *E. coli* and *S. sonnei* succumbed to death significantly faster than non-infected flies, complementing the bacterial colonization consequence in the fly.

Table 1
Sequence of primers used for qRT-PCR.

Gene	Sequence
AstA	**F:** CGCCTGCCGGTCTATAACT; **R:** CGGCACTCTGTAGTCGATCTC
DH31	**F:** TCTTCTGCCTCTTGGCCATC; **R:** CGTTTCGAGCCCGTATGATG
Tk	**F:** TACAAGCGTGCAGCTCTCTC; **R:** CTCCAGATCGCTCTTCTTGC
Rp49	**F:** TACAGGCCCAAGATCGTGAAG; **R:** GACGCACTCTGTTGTCGATACC

2.2. E. coli *and* S. sonnei *infected flies exhibit altered systemic glucose and triglyceride levels with no significant change in body weight*

In an enteric infection setting, the nutritional consequence of infectious diseases is usually evaluated, with intermediary metabolic disorders scored for and malnutrition/body weight chiefly surveyed [19]. Of common enteric diseases delineated outcomes, the enterotoxigenic *E. coli*-caused diarrhea has been reported to negatively affect the bimonthly weight gain of infected children in a studied community [20]. Likewise, shigellosis caused by Shigella infection also exhibited a very strong negative effect on the bimonthly and annual linear growth of infected children [20]. If these enteric pathogens-causing diarrhea act via a similar mechanism in *Drosophila*, weight loss is also expected to occur in infected flies. To test this, the weight of flies fed either NB alone, *E. coli*/NB mixture, or *S. sonnei*/NB mixture was recorded daily over a course of three days. Surprisingly, and as shown in Fig. 2A, flies challenged with *E. coli* and *S. sonnei* showed no significant change in their body weights 24, 48, and 72 h post infection as compared to the non-infected control group. Although these findings raised dubiety in the similarity of the pathogenesis mechanism used by diarrhea-causing enteric pathogens in both flies and humans and questioned the congruency of dehydration caused weight loss consequence in both models, the similitude of infection-induced alterations of other metabolic parameters was not ruled out. As such, we scored for the effect of *E. coli* and *S. sonnei* infection on inducing hyperglycemia and hyperlipidemia by measuring systemic glucose and triglyceride metabolic parameters in control and infected flies four days post infection. As expected, flies infected with *E. coli* and *S. sonnei* exhibited an elevation in their glucose (Fig. 2B) and triglyceride (Fig. 2C) levels as compared to non-infected control flies.

2.3. E. coli *and* S. sonnei *infection promote disrupted lipid metabolism and intestinal steatosis*

Recent studies from our group have presented an interplay between *V. cholerae* enteric infection and the nutritional and metabolic status of the *Drosophila* host. Flies infected with wild-type *V. cholerae* exhibited systemic insulin signaling deactivation, deprivation of lipid storage from the fat body, and intestinal steatosis attributed to the consumption of the short chain fatty acid acetate by *V. cholerae* in the fly gut [21]. Activation of *V. cholerae* quorum sensing; however, reverted this scenario into a favorable interaction between the fly host and *V. cholerae* by repressing *V. cholerae* succinate uptake and therefore reducing the nutritional burden of intestinal colonization [22]. Similar to the *V. cholerae* infection diegesis, and taken the elevation in systemic glucose (Fig. 2B) and triglyceride levels (Fig. 2C) detected in *E. coli* and *S. sonnei* infected flies into consideration, we anticipated a comparable plot of disrupted lipid metabolism and intestinal steatosis in *E. coli* and *S. sonnei* infected flies. As such, we assessed normal accumulation of lipids in the fat bodies of control, *E. coli*, and *S. sonnei* infected flies four days post infection. As expected, the fat bodies of *E. coli* and *S. sonnei* infected flies were abnormally depleted from lipid droplets as compared to non-infected controls that had normal accumulation of lipid in their adipose tissues (Fig. 3). We reasoned that such a depletion of lipid storage from fat bodies could be either due to a diminish in lipid uptake and synthesis in the fat body or to an increase in lipid mobilization from the fat body to other non-adipose tissues such as the gut. To test whether this disrupted normal storage of lipids in the fat body was in fact associated with intestinal steatosis, we scored for lipid droplet accumulation in the intestine of those fly groups. In congruity with lipid deprivation from fat bodies, irregular lipid

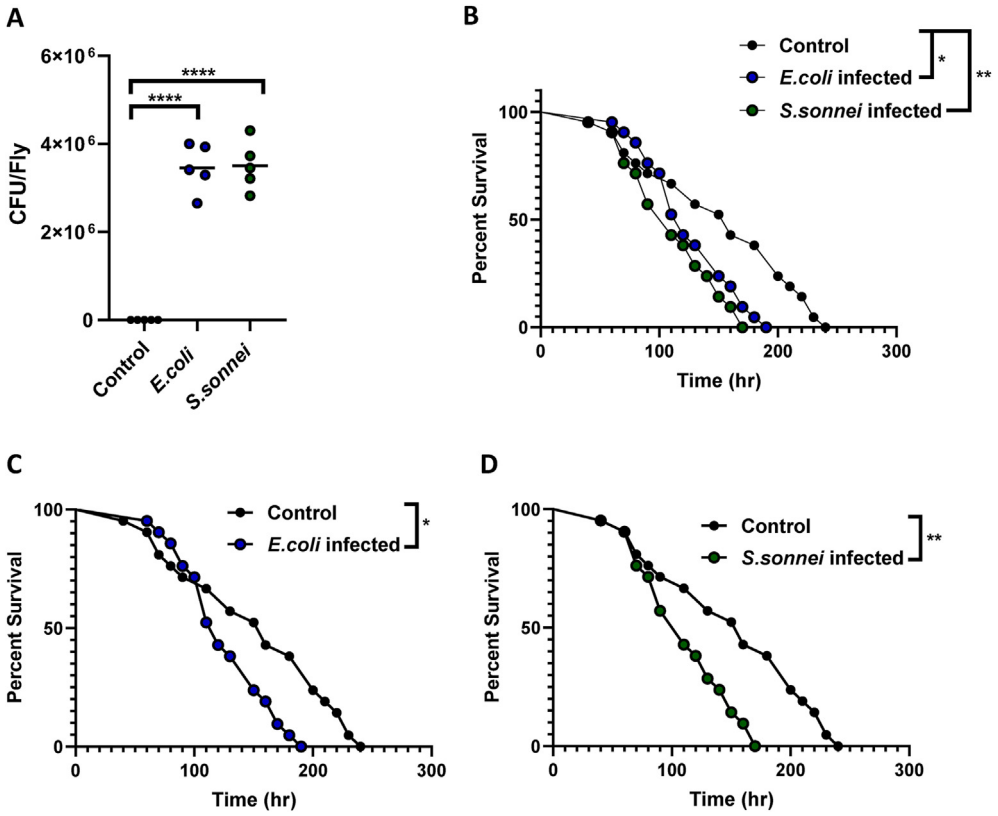

Fig. 1. *E. coli* and *S. sonnei* localize in the fly and reduce the life span of the infected host. (A) Quantification of CFU/Fly of *E. coli* and *S. sonnei* in control (non-infected), *E. coli* or *S. sonnei*. **(B)** Overlaid survival curves of fly groups indicated in (A). Survival curves of **(C)** *E. coli* and **(D)** *S. sonnei* infected flies as compared to a control (non-infected) group. A one-way ANOVA with Dunnett's multiple comparison test was used to evaluate statistical significance in (A) with control (non-infected) being the reference used. Percent survival was computed using Kaplan–Meier survival analysis and statistical significance was calculated using log rank analysis in (B), (C) and (D). *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$.

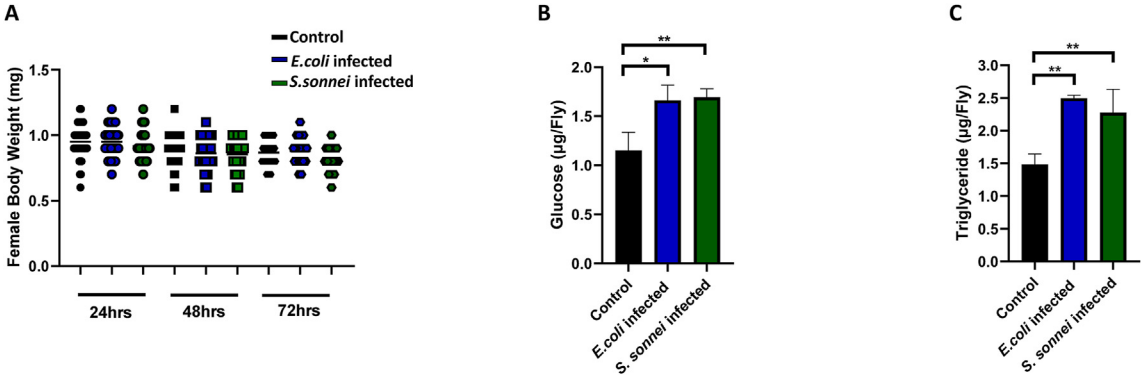

Fig. 2. *E. coli* and *S. sonnei* infection induce elevation in glucose and triglyceride levels without causing changes in body weight of infected flies. (A) Quantification of the body weight of control (non infected), *E. coli*, and *S. sonnei* infected flies over a course of three days. Horizontal bars represent the mean measurement. Quantification of systemic **(B)** glucose and **(C)** triglyceride levels in fly groups denoted in (A). Measurements of three independent biological repeats in (B) and (C) represent the mean and error bars signify the standard deviation. A one-way ANOVA with Dunnett's multiple comparison test was used to calculate statistical significance in (A), (B) and (C), with control (non-infected) being the reference used. The absence of (*) indicates no significance between compared groups; *: $p < 0.05$; **: $p < 0.01$.

droplets accumulated in the anterior midgut of *E. coli* and *S. sonnei* infected flies, a pattern not observed in control non-infected flies (Fig. 4).

2.4. E. coli *and* S. sonnei *infection alter the expression of genes encoding peptide hormones*

The role of enteroendocrine secreted gut hormones in regulating systemic lipid homeostasis has been well buttressed in rodents [23–26]. In an adult *Drosophila*, midgut enteroendocrine cells express nine major gut prohormones including Allatostatin (Ast) A, AstB, AstC, Neuropeptide F (NPF), short neuropeptide F precursor (sNPF), Tachykinin (Tk), Diuretic hormone 31 (DH31), and CCHamides-1 and 2, which are processed into over twenty-four mature peptides [27,28]. Among these hormones, the physiological role of Tk, AstA, and DH31 in gut-specific functions, including those pertinent to intestinal and fat body lipid metabolism, have been reported [15–17,29–36]. As such, we hypothesized that the metabolic alteration detected in the

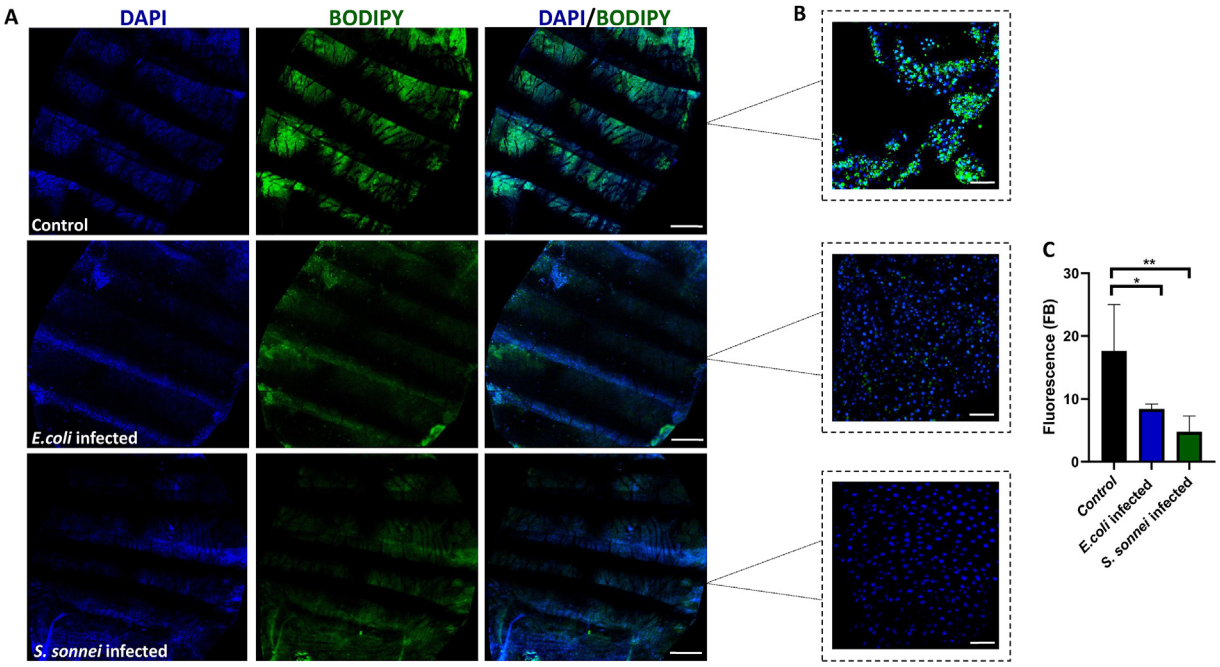

Fig. 3. ***E. coli* and *S. sonnei* infection promote lipid deprivation from the fly fat body. (A)** Representative fluorescence images of abdomens in control (non-infected), *E. coli*, and *S. sonnei* infected fly, with **(B)** fat storage in fly fat body (FB) tissues displayed. DAPI (blue) staining nucleus is shown on the left, BODIPY (green) staining lipid droplets in the middle, and an overlay of both on the right. **(C)** Quantification of the normalized total BODIPY fluorescence in the fly fat body (FB) of indicate groups in (A). A one-way ANOVA with Dunnett's multiple comparison test was used to evaluate statistical significance in (C). *: $p < 0.05$; **: $p < 0.01$.

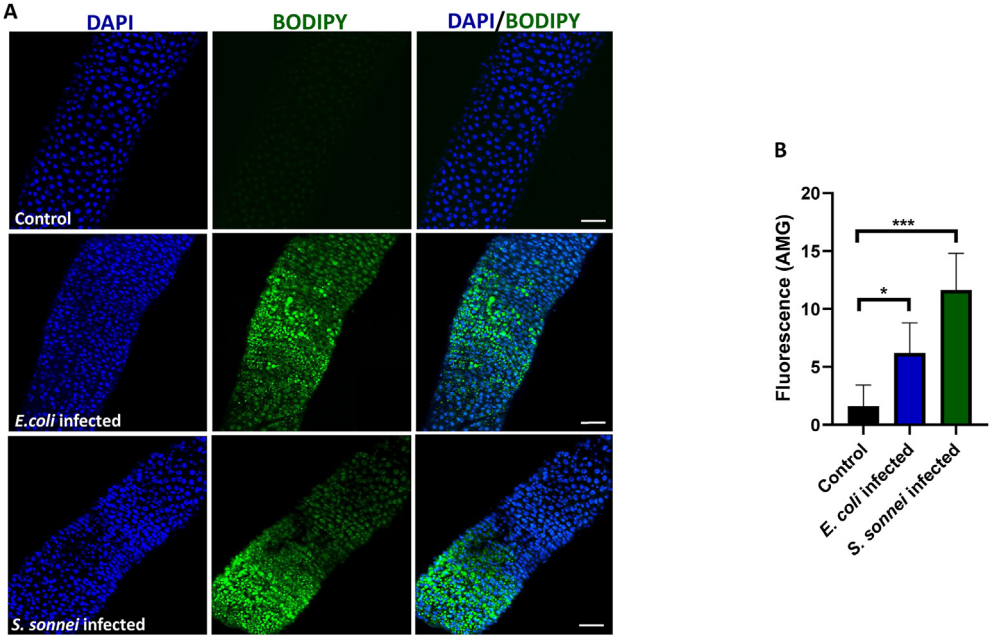

Fig. 4. ***E. coli* and *S. sonnei* infection promote irregular accumulation of lipid droplets in the fly gut. (A)** Representative fluorescence images and **(B)** Quantification of the normalized total BODIPY fluorescence in the anterior midgut of control (non-infected), *E. coli*, and *S. sonnei* infected flies, with DAPI (blue) staining nucleus on the left, BODIPY (green) staining lipid droplets in the middle, and an overlay of both on the right. A one-way ANOVA with Dunnett's multiple comparison test was used to evaluate statistical significance in (B). *: $p < 0.05$; ***: $p < 0.001$.

fat body (Fig. 3) and midgut (Fig. 4) of *E. coli* and *S. sonnei* infected flies may relate to an effect of the enteric infections on the expression of these peptide hormones. To test this, we measured the transcript abundance of *Tk*, *DH31,* and *AstA*, in flies fed with NB alone, or NB inoculated with either *E. coli*or *S. sonnei*. As presented in Fig. 5, the transcript expression of *Tk* (Fig. 5A), *DH31* (Fig. 5B), and *AstA* (Fig. 5C) was differentially down regulated in *E. coli* and *S. sonnei* infected flies relative to that of the non-infected control group.

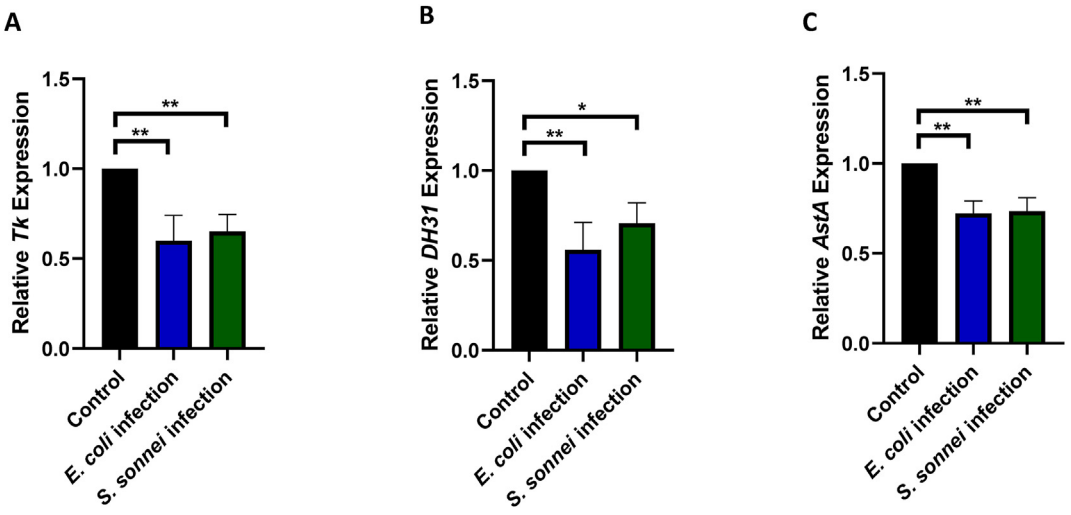

Fig. 5. *E. coli* and *S. sonnei* infection alter the transcript levels of peptide hormones involved in regulating metabolic homeostasis. Quantification of **(A)** *Tk* **(B)** *DH31* and **(C)** *AstA* transcript levels in control, *E. coli*, and *S. sonnei* infected flies. Measurements, which represent the mean of three independent biological repeats, were normalized to the transcript levels of control flies. Error bars indicate the standard deviation. A one-way ANOVA with Dunnett's multiple comparison test was used to evaluate statistical significance. *: $p < 0.05$; **: $p < 0.01$.

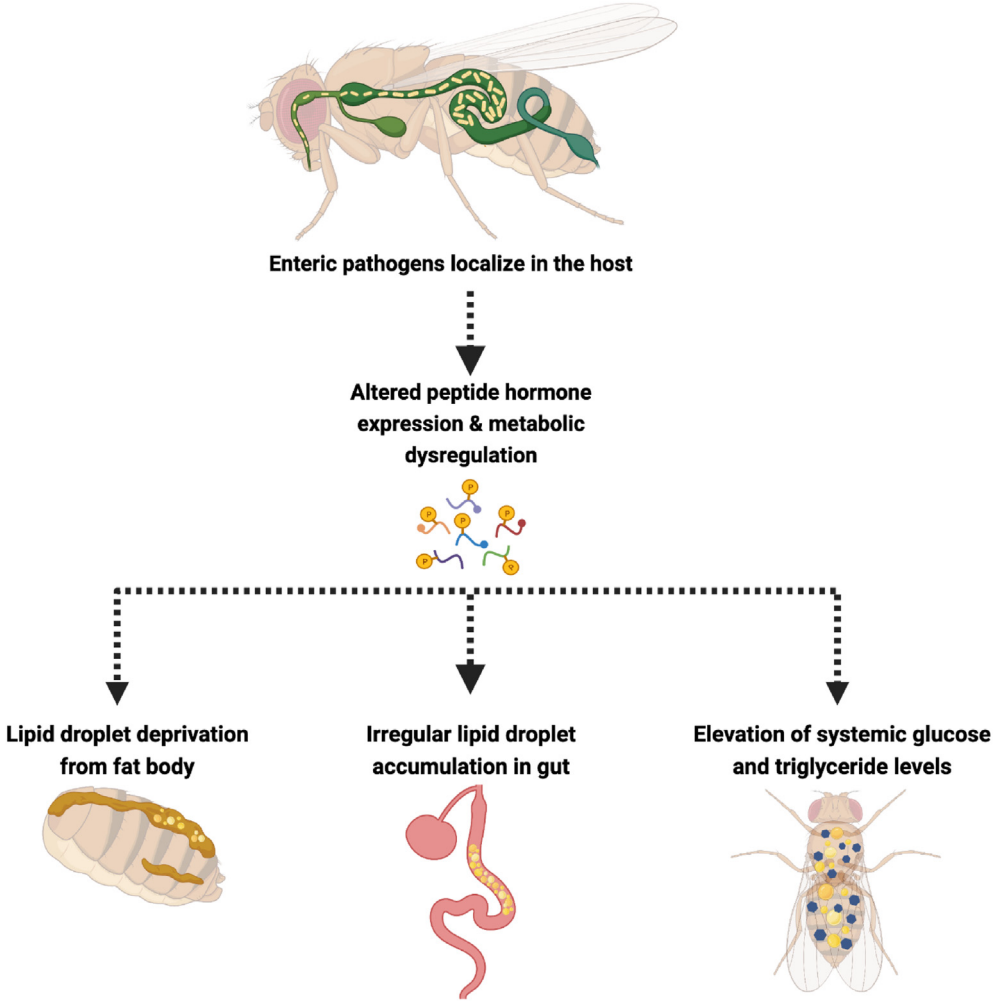

Fig. 6. Proposed Model. Oral infection and colonization of the host with enteric pathogens contribute to an altered expression of peptide hormones known to play key roles in regulating lipid metabolism and insulin signaling. This alteration could plausibly explain the infection-induced metabolic dysregulation alleged in infected flies demarcated by lipid droplet deprivation from the fat body, irregular accumulation of lipid droplets in the midgut, and significant elevation of systemic glucose and triglyceride levels. The proposed model diagram was created with BioRender.com.

3. Discussion

Enteric pathogens have developed a pre-eminent array of virulence facets to colonize the host gut and cause disease predominantly manifested as anorexia, malaise and diarrhea. Canonically, the mechanisms by which enteric bacteria colonize the intestine and disrupt its normal function causing malabsorption and diarrhea are mainly attributed to microbial adherence, epithelial effacement, production of secretory enterotoxins and cell-destroying cytotoxins, and/or direct epithelial invasion [37]. Recently; however, a broad spectrum of studies have elegantly presented a pathogen's ability to impact the host metabolism, as another course of virulence action. Apropos, and given that several enteric bacterial species are closely concomitant with arthropods that could serve as reservoirs or vectors for these enteric pathogens in natural habitats, many studies including ours adopted insects to investigate the impact of enteric infection on host metabolism. To mimic the natural route utilized by enteric pathogens to gain access to the gastrointestinal tract of humans, we infected flies orally with either *E. coli* or *S. sonnei*, two enteric bacterial species. The results of our study revealed that both pathogens localize in the fly post feeding, resulting in a reduction in the life span and in an alteration in the metabolic profile of the infected host. These finding align with similar studies uncovering the role of gut microbes, whether pathogenic or commensal, on dipteran metabolism. Germ-free flies, for instance, manifest altered insulin signaling and lipid metabolism profiles [16,21,38], and *Drosophila* subject to a systemic infection with *E. coli*, *P. asymbiotica* or *P. luminescens* exhibit intestinal steatosis demarcated by lipid accumulation in the gut, and an increase in lipogenesis and systemic lipid levels [39]. Similarly, *Francisella novicida* infection leads to metabolic dysregulation [40] and *L. monocytogenes* infection triggers a gradual loss in triglycerides and glycogen stores in infected flies [4].

Peptide hormones have been reported to be involved in lipid metabolism during several occurrences including flight, reproduction, diapause, malnourishment, infections and immunity [41]. Fundamentally, it would not be surprising; therefore, to have pathogens alter a host's metabolic homeostasis in a peptide hormones-dependent manner. In fact, we have shown in a previous study the ability of a low-virulence pathogen such as a quorum sensing-competent *V. cholerae* strain to increase *Tk* transcription [22]. Within the same notion, a recent study by Harsh et al. demonstrated a decrease in *Tk* expression in a systemic bacterial infection scenario [39]. In accordance with these findings, we provide evidence in this study of differential down-regulation in the expression of *Tk*, *DH31*, and *AstA* peptide hormones in *E. coli* and *S. sonnei* infected flies, an alteration that could plausibly explain the infection-induced metabolic dysregulation detected in these flies (Fig. 6). The findings of our study provide ancillary insight into the mechanistic nature of infection-induced metabolic alterations, an apprehension that will open up for approaches to modulate the pathogenesis of infection-induced gastrointestinal tract diseases.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

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