Diet-induced plasticity in the adult *Drosophila melanogaster* midgut

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Yunan Nie
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Dr. Nicolas Buchon

Abstract

Diet is a foundational factor in metabolism and development. The ratio of dietary macronutrients has significant effects on lifespan and healthspan, and the gut itself has been known to adapt to changes in diet through resizing (Lopez-Otin, et al., 2013; Mattison, et al., 2012; O'Brien et al., 2011). This thesis therefore aimed to study the effects of two diets, a high sugar-low yeast diet (HS) and a high yeast-low sugar diet (HY), on the *Drosophila* midgut and how variations in genotype affected this interaction. To that end, I studied adaptive gut growth in response to switching between these two diets and characterized the response at the cellular level. I also looked at the effect of genetic variation through analyses of the 30 Global Diversity Lines, 10 of the *Drosophila* Genetic Reference Panel (DGRP) lines, and twelve different *Drosophila* species. Finally, I evaluated the influence of specific genes on this gut growth response through a transcription factor RNAi screen of 24 lines and knockout lines of *dilp3, dilp5, dilp7, upd3,* and *upd2/3*.

I found significant but reversible differences in morphologies of *Drosophila* guts that were switched between diets, with corresponding changes at the cellular level. This phenomenon was evolutionarily conserved across twelve *Drosophila* species, but diet-by-genotype interactions were also found in the Global Diversity Lines and a sample of the DGRP. Candidate genes for closer study were identified through a transcription factor disruption screen. Of the mutant lines, only *dilp7* had a major effect on gut growth in response to diets. Together, these data show that genotype is important in adaptive gut growth, and future studies will aim to identify and further test genes that are involved in this process.

Introduction

Dietary nutrition is an integral to multiple facets of animal survival and development, from aging and metabolic disease to reproduction. A better understanding of the underlying factors that drive this interaction may have broad societal impacts as a step towards addressing the many metabolic disorders, from diabetes and obesity, that are becoming increasingly widespread in the human population (George, et al., 2005; Shaw & Zimmet, 2004).

Diet has been implicated in both metabolic health and aging (Lopez-Otin, et al., 2013). Both adjustments to the ratio of dietary nutrients and dietary restriction (DR) have significant effects on metabolic health and aging (Solon-Biet, et al., 2014; Mattison, et al., 2012; Fontana & Partridge, 2015). DR, in particular, has been found to increase the lifespan or healthspan of all eukaryote species that have been investigated thus far (Lopez-Otin, et al., 2013; Mattison, et al., 2012; Fontana & Partridge, 2015). This DR effect, however, may be related to the restriction of specific dietary nutrients, as a reduced relative availability of dietary protein has been found to have a role in human metabolic disease, as well as in animal models such as the fruit fly (Gosby, et al., 2014; Mair, et al., 2005). Amino acid ratios specifically may play role in the DR response, as reduced dietary methionine is sufficient to increase lifespan in mice (Miller, et al., 2005).

The Protein Leverage Hypothesis (PLH) suggests that a decrease in the relative ratio of dietary protein to dietary carbohydrates stimulates an increase in protein appetite that drives excess intake in order to compensate for the dilution (Simpson & Raubenheimer, 2005). This increase in intake leads to overconsumption of food and increases prevalence of downstream metabolic diseases such as type 2 diabetes and obesity. In light of this hypothesis, the longevity potentiated by DR may be due to the ratio of macronutrients rather than total caloric intake, although the interactions between dietary protein ratio and longevity are complex. The placement of a diet in

the protein:carb spectrum is important in determining how the organism will respond in terms of feeding and its subsequent effects on lifespan. Varying degrees of protein restriction could therefore lead to a variety of lifespan effects, from increased lifespan to metabolic disease. A recent study by Solon-Biet et al. (2014) found that calorie restriction through high-protein diets or dietary dilution with non-digestible cellulose did not convey beneficial effects on lifespan, supporting the idea of the ratio of macronutrients as the main contributor to lifespan effects.

While the effects of diet on feeding rate and organismal physiology are well-studied, the gut is a comparatively understudied missing link. In addition to general diet-lifespan interactions, differing gut responses to diet due to genotypic or microbiota variations between individuals may also be factors in determining responses to different diets and optimization of diets. For example, gut microbial communities can regulate expression of genes that affect fatty acid oxidation and fat deposition, affect food intake, and dictate metabolism of nutrients (Bäckhed, et al., 2004; Vijay-Kumar, et al., 2010; Wong, et al., 2014). These complex interactions affect nutrient leverage and optimization of nutrient absorption, a process that is further complicated by the potential of plasticity in the gut in response to changes in diet. The focus in nutrient studies thus far has been on feeding behavior and how different diets affect the mechanism of food intake. Preliminary studies done by the Buchon Lab have shown that the gut can adaptively grow and shrink in response to different nutrient availability, supporting the idea that the gut and its interactions with diet are variable. It is equally important, therefore, to consider how the gut, as the place of nutrient exchange and digestion, is itself a variable that can be affected by both the diet and genotype of the organism. An increased understanding of this mechanism could lead to the development of metabolic therapies aimed at changing the physiology of the gut itself.

The gut is a highly plastic organ, as it is capable of rapid turnover and regeneration, allowing it to adapt to a variety of challenges and environmental conditions ranging from bacterial infections to starvation and refeeding. The fruit fly *Drosophila melanogaster* is a key model organism in studying this gut plasticity due to the ease of manipulation of both its diet and genotype, as well the availability of genomic data. In *D. melanogaster*'s gut, bacterial infection results in a dynamic remodeling that includes synthesis of new enterocytes (large, absorptive cells which constitute the majority of the epithelium) and proliferation of intestinal stem cells (ISCs), which together replenish the damaged intestinal epithelium and maintain gut homeostasis (Buchon, et al., 2010; Jiang, et al., 2009; Buchon, et al., 2009). This response is in part mediated through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which is activated when enterocytes are subjected to stress and produce cytokines Upd1, Upd2, and Upd3 that in turn promote ISC division and gut renewal (Jiang, et al., 2009; Osman, et al., 2012). The epidermal growth factor receptor (EGFR) pathway synergizes with this JAK/STAT pathway to promote stem cell proliferation after infection and coordinates delamination and anoikis of damaged cells, leading to modified gut morphology in response to infection (Buchon, et al., 2010). After bacterial infection, guts shrink significantly but return to unchallenged levels after the infection has been cleared. While the role of these mechanisms in responding to infection is unequivocal, surprisingly there has been no parallel exploration of any role in regulating physiological responses to diet.

Modified gut morphology is apparent in response to changes in diet, which is the main focus of this thesis. While post-developmental tissues generally maintain a constant size through homeostasis, there is adult organ plasticity through induction of growth by functional demand (O'Brien, et al., 2011). One of the best understood models for adaptive resizing is the vertebrate

small intestine, as in the case of intermittent feeders such as hibernating squirrels and ambush-hunting snakes which alternate between feasting and fasting (Carey, 1990; Secor & Diamond, 1998). This intestinal adaptation is likely to be related to altered progenitor cell populations, as there are changes in the rate of cell turnover as well as the mitotic index (Dunel-Erb, et al., 2001). O'Brien et al. performed a study on *Drosophila melanogaster* that supported this hypothesis in which they found that fed guts had a higher number of intestinal stem cells (ISC) compared to fasted guts and that this mechanism coincides with increased *Drosophila insulin-like peptide 3 (dilp3)* expression (O'Brien, et al., 2011). The eight *Drosophila* insulin-like peptides have structures similar to mammalian insulin, with eight genes located across two chromosomes (Hiu & Chalasani, 2014). In addition, midgut lengths were found to decrease and increase in response to cycles of fasting and refeeding, which fluctuated in tandem with total cell number and enteroblast number. While it has been clearly demonstrated that gut adaptive plasticity does respond to the availability of food, the adaptive response to different ratios of macronutrients has not been fully characterized.

The *Drosophila* midgut is ideal for diet due to its relative simplicity and tractability, as well as its clear separation between different functional and structural regions (Buchon, et al., 2013). The *Drosophila* gut is divided into three regions: the foregut, the midgut, and the hindgut. The foregut includes the pharynx, esophagus, and crop while the hindgut functions primarily to reabsorb water (Demerec, 1950). The Malpighian tubules, which are the functional analog of mammalian kidneys, branch at the midhindgut junction. The midgut is important for digestion and nutrition absorption and is maintained by pluripotent ISCs that are the progenitors of enteroblasts, which differentiate into either absorptive enterocytes or secretory enteroendocrine cells (Ohlstein & Spradling, 2007). The midgut has been segmented into regions R1-R5, with R1-2 representing

the anterior midgut, R3 representing the middle midgut, and R4-5 representing the posterior midgut (Buchon, et al., 2013). These regions can be discerned through histological and gene expression analyses as well as morphological analysis. All regions are comprised of four basic cell types: enterocytes (ECs), enteroendocrine cells (EECs), enteroblasts (EBs), and ISCs. ISCs divide symmetrically to maintain the stem population or asymmetrically into enteroblasts which then differentiate into either nutrient-absorbing enterocytes or secretory enteroendocrine cells (Ohlstein & Spradling, 2006). Determination of symmetric or asymmetric division of ISCs is specified by the Notch pathway, in which activation of Notch signaling promotes differentiation of ISCs (Ohlstein & Spradling, 2007).

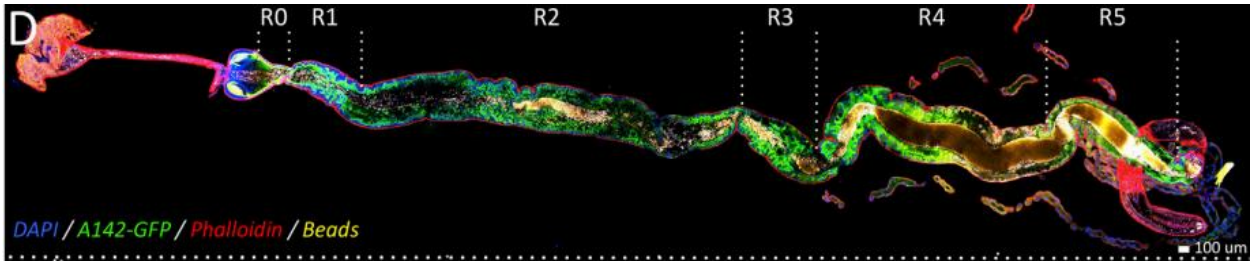

Figure 1. Fluorescent confocal imaging of the *Drosophila* gut regions. Adapted from Buchon et al. 2013. R1 and R2 include the anterior midgut, R3 is the middle midgut, and R4 and R5 encompass the posterior midgut.

This thesis aims to study the effects on the *Drosophila* midgut of two different diets, a high sugar-low yeast diet (HS) and a high yeast-low sugar diet (HY). In these diets, yeast is the sole source of protein, vitamins and trace elements, and sugar (sucrose) is the primary source of carbohydrates; therefore, these diets vary in macronutrient ratios. Since yeast and sucrose have equivalent caloric value per unit weight (Bass et al., 2007), these media were isocaloric. I studied plasticity of gut morphology in response to switching between these two diets and the corresponding response at the cellular level. In addition, I looked at the effect of genetic variation on gut interactions with diet through experiments using the Drosophila Global Diversity Lines, the *Drosophila* Genetic Reference Panel lines, and twelve different *Drosophila* species. To identify

genes that are involved in the gut resizing response, 24 transcription factor RNAi lines and three *dilp* mutants were also analyzed and screened for differential gut growth responses. Finally, as the family of *JAK-STAT* cytokines has been shown to be important in stem cell regulation and proliferation, I studied the response to two diets in *upd2*, *upd3* (*upd2/3*) double mutants and *upd3* KO flies.

Materials and Methods

Flies and diets: Mated female flies were used in all experiments as mating induces a collection of changes in the female's behavior and physiology, including lifespan and consumption of food (Chapman, 2001). Flies were reared from eggs on standard sucrose diet (1 L deionized H_2O, 7 g agar, 50 g yeast, 60 g yellow cornmeal, 40 g sucrose, 26.5 mL Moldex, and 12 mL acid mix) until they eclosed, and were placed on one of the two isocaloric diets within 24 hours of eclosion to ensure gut development primarily occurred on the experimental diets. Newly emerged flies were split between experimental diets within 24 hours. The experimental diets were cooked using the following recipe: 1 L deionized H_2O, 15 g agar, 26.5 mL Moldex (with the exception of food used for the DGRP experiments, as the food was used immediately), 12 mL acid mix, and two ratios of sucrose to yeast. The HY diet included 74.1 g of sucrose and 105.9 g of yeast. The HS diet included 168.1 g sucrose and 11.85 g of yeast.

***Samples Sizes*:** All experiments analyzed a minimum of three guts per replicate, with three replicates per experiment. Variations in number of guts resulted from differences in health of lines and ease of dissection. In the majority of replicates, five guts were analyzed.

***Quantification of adaptive gut growth*:** The gut growth phenotype was tested using wild-type CantonS (CS) flies that were fed the standard sucrose diet (S food) at room temperature for five days. One-third of the flies on S food were dissected to measure for gut length. The remaining flies were randomly split into two groups and placed on either HS or HY food. After an additional 5 days, all flies were dissected. Reversibility of the gut growth phenotype was tested using CS flies that were fed the HY/HS diets at room temperature for 5 days. On day 5, half of the flies on each diet were dissected and measured for gut length. The remaining half were switched to the other experimental food (HS to HY and vice versa). Experimental procedure is pictured in Figure 2. Data were analyzed using an unpaired two-tailed t-test.

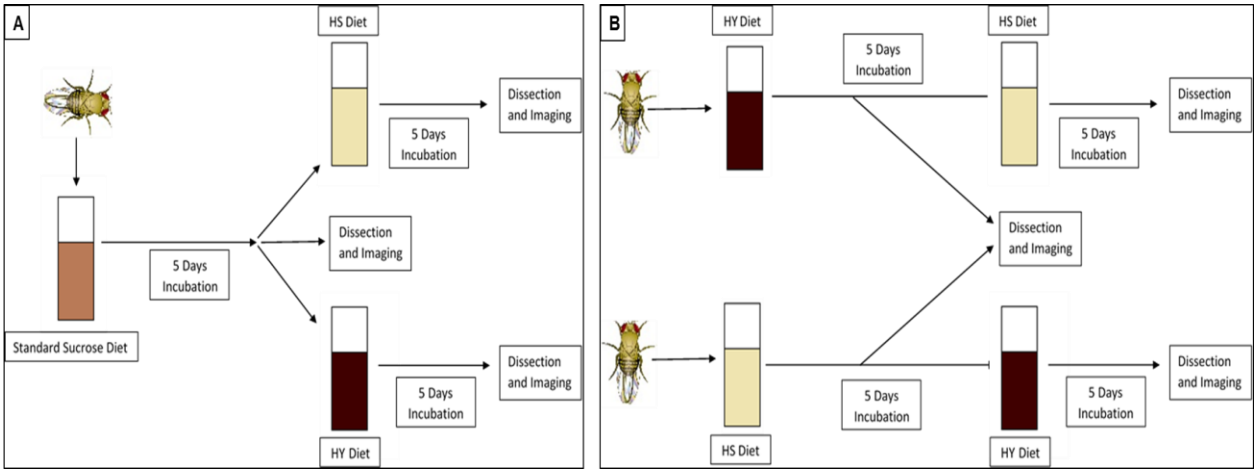

Figure 2. Adaptive gut growth experimental procedure. A) Flies were initially placed on standard sucrose food (S food) and switched to the HY and HS diets. B) Flies were initially placed on either HY or HS food and switched to HS food or HY food.

Cellular characterization: Changes in the number of enteroendocrine cells (EEC), number of enterocytes (EC), number of intestinal stem cells (ISC), and surface area on HY and HS food were characterized using the *prosperov1 (w UAS-GFP tubGal80ts prosv1-Gal4 / TM6B)*, *ubi-DE-cad-GFP*, *esg*^{ts} (*w esgGal4/CyOlacZ UAS-GFP, tubGal80ts*), and *A142-GFP* lines, respectively (Full genotypes also available in Figure 3 and Supplementary Data Table 1). *Prosperov1* is a temperature sensitive line with the *prosv1-Gal4* transgene, which drives GFP expression in

enteroendocrine cells after incubation for a minimum of three days at 29 °C. *Ubi-DE-cad-GFP* is a line with a ubiquitin promoter region followed by an e-cadherin gene on the second chromosome fused with GFP. GFP is expressed in the cell-cell junctions and was used to quantify enterocytes. The *esg*^{ts} line is a temperature sensitive line with the transgene *esgGal4*, which expresses GFP in intestinal stem cells after incubation for a minimum of three days at 29 °C. Cell types of interest exhibited GFP fluorescence after fixation and imaging (Figure 4). Absorption area was measured through fluorescence confocal imaging of the guts from the fly line *A142-GFP* with the *A142-GFP* transgene, which expresses a GFP fusion that localizes to the brush borders of enterocytes.

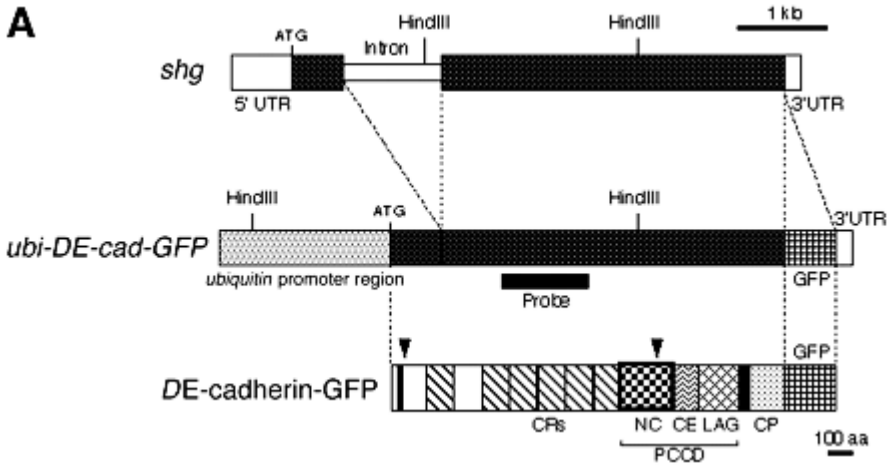

Figure 3. *ubi-DE-cad-GFP* genes. Adapted from Oda & Tsukita, 2000.

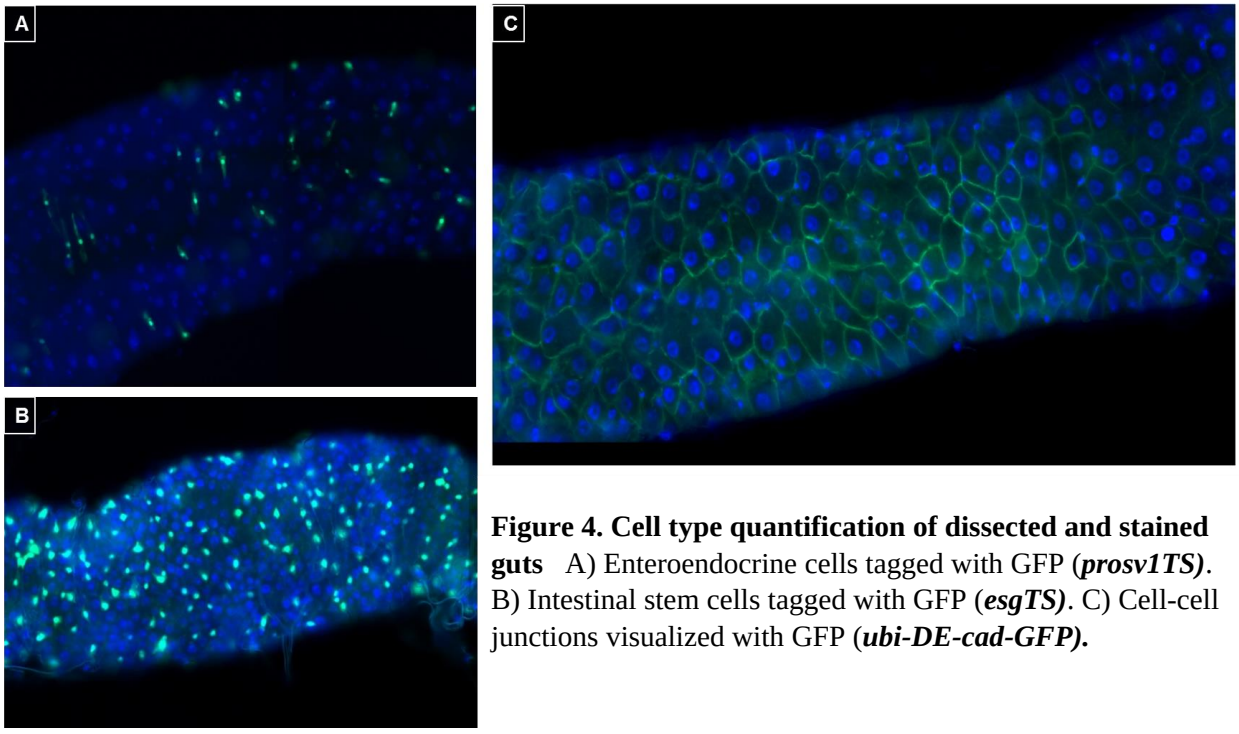

Figure 4. Cell type quantification of dissected and stained guts A) Enteroendocrine cells tagged with GFP (***prosv1TS)***. B) Intestinal stem cells tagged with GFP (***esgTS)***. C) Cell-cell junctions visualized with GFP (***ubi-DE-cad-GFP).***

Flies from each line were placed on the HY/HS diets at room temperature for 7 days and dissected. Dissected guts were fixed with 4% PFA in a PBT solution for an hour, washed 3X with PBT, and stained with DAPI before being mounted. Images were taken using a Zeiss LSM 700 confocal microscope and the Zeiss Zen Blue imaging program.

Using the fluorescent markers detailed above, numbers of ECs, ISCs, and EEs were quantified in the R4 region of the midgut, as initial experiments showed that this is the most variable region in response to diet. Surface area of a cross-section of the R4 region was measured and cell counts were taken within a smaller sample area. The total surface area was then divided by the sample surface area and multiplied by the sample cell count to calculate the total R4 region cell count. Cell counts were analyzed using an unpaired two-tailed t-test.

To measure absorption area, *A142-GFP* guts were fixed and stained with DAPI before mounting and heights and radii of the folds in the epithelial lining were collected. The surface area

of a dome formula, $2\pi rh$, was used to calculate surface area of brush border per enterocyte. Shape of the fold was assumed to be most similar to a dome as microvilli extend from the apical side of enterocytes in a parallel manner and follow the shape of the enterocyte, which is approximated to be circular based on imaging shown in Figure 7 (Apidianakis & Rahme, 2011). The total length was also measured to find length per enterocyte. Values were analyzed using an unpaired two-tailed t-test.

Variation of diet by genotype interaction:

Both a) inter- and b) intraspecific variation in gut growth responses were monitored through the study of a) twelve *Drosophila* species and b) the Global Diversity and DGRP lines.

12 species

To assess evolutionary conservation of *D. melanogaster* phenotypes, 12 congeneric species (*Drosophila pseudoobscura, D. sechella, D. simulaus, D. suzukii, D. willistoni, D. mojavensis, D. yakuba, D. heydei, D. virilis, D. erecta, D. persimilis, and D. iso*.) were phenotyped, in addition to two strains of *Drosophila melanogaster* (Canton-S and Oregon-R). After 7 days on the HS/HY diets, flies were dissected and gut length was measured from the dense mass within the proventriculus of the fly to the midgut-hindgut junction, which were delineated by the Malpighian tubules. An ANOVA was performed to evaluate variables for effect on gut length.

Global Diversity Lines

The 30 Global Diversity Lines, generated in the neighboring laboratory of Andrew Clark, are a set of genetically diverse lines that were placed on HS and HY food (Grenier, et al., 2015). After 5 days on the two experimental diets, flies were dissected and gut surface areas as well as

posterior and anterior midgut lengths were measured. An ANOVA was performed to test for significance of interaction between genotype and diet.

Drosophila Genetic Reference Panel

The *Drosophila* Genetic Reference Panel comprises 205 variable inbred isofemale *Drosophila melanogaster* lines whose genomes have been sequenced (Huang, et al., 2014; Mackay, et al., 2012). For this thesis, 10 of the DGRP lines were measured to find an initial correlation between genotypes and variations in response to different diets.

Flies were placed on either HS or HY food. After 5 days on the experimental food at 25°C, flies were dissected and imaged in order to measure gut lengths. Wings from each fly were also mounted and imaged. Each gut was measured for anterior, middle, and posterior midgut lengths. Associated wings were also measured for area.

To correlate weight with gut length, each of the lines were placed on either HS or HY food. After 5 days at 25°C, flies were placed into pre-tared Eppendorf tubes, frozen at -80 C for 5 minutes to ensure death, and thawed before weight was measured. The individual weight of the fly was calculated by subtracting the empty Eppendorf weight from the total weight and dividing by the number of flies in the tube. Data were analyzed through a MANCOVA test.

Functional Genetics: The GAL4-UAS system was used to induce RNAi-mediated knockdown of 24 genes (listed in Table 8), with mbGFP (membrane GFP) and CS crosses as controls. Lines were selected for binding to the promoter of *Drosophila insulin-like peptide 3* (*dilp3*), which has been implicated in both stem cell proliferation as well as the gut resizing response. Each UAS-RNAi line was crossed with the driver line Gal4 and maintained at 18°C until eclosion and induced at

29°C for seven days. In addition, 3 *dilp* (*dilp3, dilp5, dilp7*) KO lines were also analyzed (Hiu & Chalasani, 2014).

Each line was placed on HS and HY food for 5 days, after which flies were dissected and anterior, middle, and posterior midgut lengths were measured. As there was one repeat, statistical analysis was not possible at this time. An unpaired two-tailed t-test was used to analyze significance of data from the *dilp* KO lines and controls specifically.

Wild-type (CS) flies, *upd3* KO mutants, and *upd2*, *upd3* (*upd2/3*) double mutants were placed on HS and HY food and dissected after 7 days at room temperature. Gut lengths were measured and an unpaired unequal two-tailed t-test was used to compare the gut lengths of the *upd3* KO mutants and *upd2/3* double mutants to wild-type CS flies.

Results

Changes in macronutrient ratio alter gut size in a reversible manner

To test plasticity of the *D. melanogaster* gut, flies were exposed to different ratios of nutrients (HY and HS) after initial rearing on S food (Figure 5A). Guts were significantly longer in flies that were placed on HY food (Student's t-test, $p=0.0003$, Table 2). Guts on the HS diet however, were held at the same length as when they were on the standard sucrose food (Student's t-test, $p>0.05$, Table 2). To test reversibility of this phenotype, therefore, flies were placed on either HY or HS food and subsequently switched to the other diet after five days. Gut lengths were significantly longer when switched from the HS food to the HY food (Student's t-test, $p < 0.0001$, Table 2), and shorter when switched from the HY food to the HS food (Student's t-test, $p<0.0001$, Table 2). In general, guts showed plastic gut growth and reversible gut resizing when switched between different diets (Figure 5A and 5B).

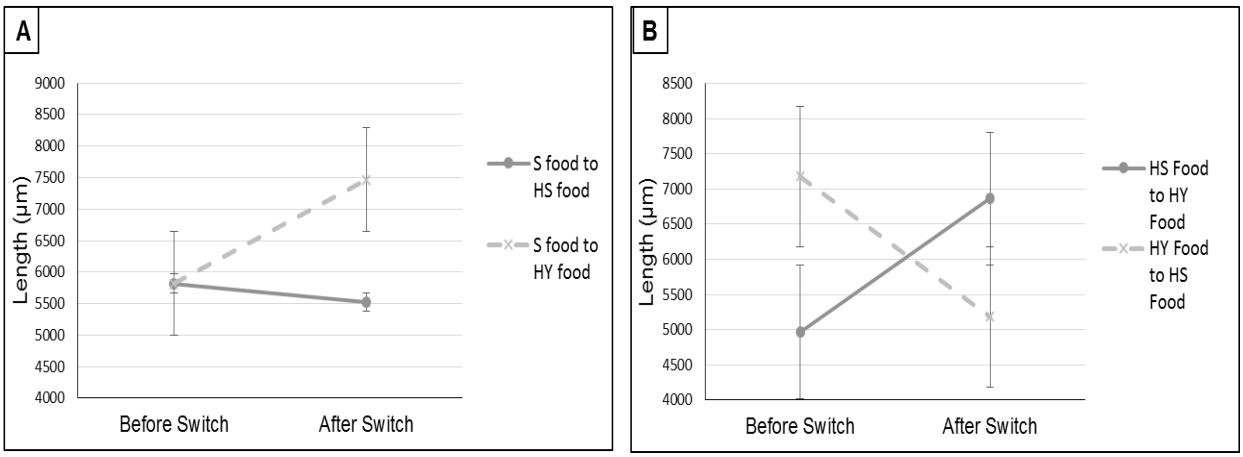

Figure 5. Adult *Drosophila* midguts show adaptive growth after switch to different diets. Error bars represent standard error. A) Flies were placed on either HS food or HY food after 5 days. Gut lengths of flies placed on HY food, but not HS food, were significantly different from gut lengths of flies dissected before transfer (Table 2). B) Both flies switched from HS food to HS food and vice versa had significantly different gut lengths to flies dissected prior to the switch (Table 2).

Diet changes absorption area as well as number and density of enterocytes, intestinal stem cells, and enteroendocrine cells in the R4 midgut region

Once it was determined that diet could induce changes in gut length, cell populations in the midgut were analyzed after exposure to different diets. Different regions of the gut are involved in specific functions and gut growth responses in each region could differ based on their role in absorption and digestion (Buchon, et al., 2013). I therefore limited my analysis to the R4 region of the midgut due to evidence from preliminary studies done by the Buchon Lab that the *Drosophila* gut is allometric in its response, i.e. midgut regions respond by varying degrees to different diets. To that end, the total numbers of cells of each cell type (EC, ISC, and EEC) in the R4 region were estimated to determine if changes in gut size were due to a change in the actual number of cells.

Diet affected the numbers and densities of cells in a cell-type specific manner. While only the number of enterocytes was significantly different between the two experimental diets, with a higher total number of ECs in the R4 of guts fed on the HY diet (Student's t-test, p=0.0098, Figure 6A and Table 3), the reverse was true of the density of cells. The ISCs and EEs, but not ECs, were

significantly more dense in the R4 midgut region on the HS diet than the HY diet (Student's t-test, p=0.0188 and p=0.0014, Figure 6B and Table 3). Figure 6C represents this difference through the ratio of cell number and cell density on HY food and HS food. While the ratios of total number of ISCs and enteroendocrine cells on the two diets approach 1.0, the ratios of number of cells per μm^2 are under 1.0, signifying more cells per μm^2 on the HS diet (Figure 6C). This implies that not only does the number of ECs increase on the HY diet, but also the size, leading to a lower density of nuclei.

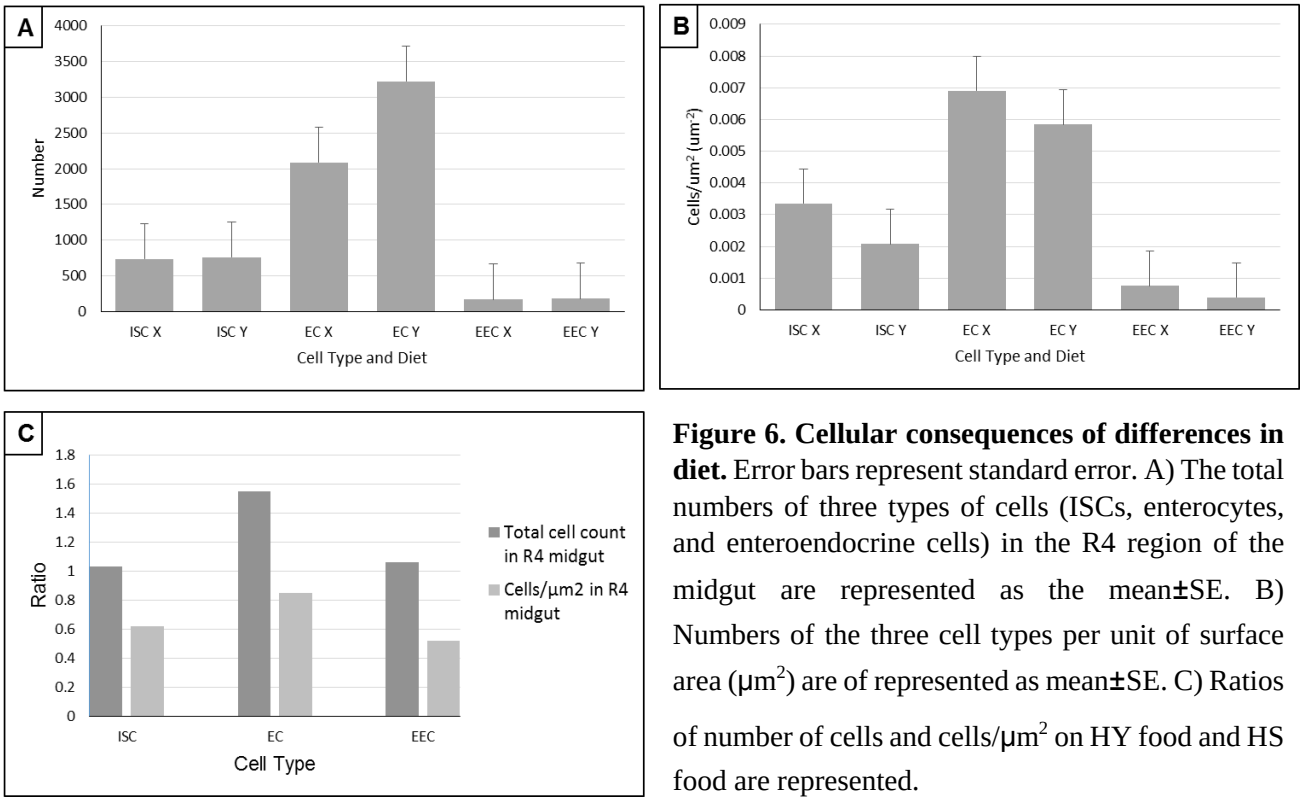

Figure 6. Cellular consequences of differences in diet. Error bars represent standard error. A) The total numbers of three types of cells (ISCs, enterocytes, and enteroendocrine cells) in the R4 region of the midgut are represented as the mean±SE. B) Numbers of the three cell types per unit of surface area (μm^2) are of represented as mean±SE. C) Ratios of number of cells and cells/μm^2 on HY food and HS food are represented.

To investigate this possible enterocyte size difference and how it might interact with absorption area, I evaluated the differences in the brush border of guts raised on two different diets. Surface area of the brush border, height of brush border folds, and length per enterocyte varied significantly between the two diets (Table 4). The *Drosophila* brush border does not have the

extensive folding that mammalian small intestines exhibit, but does extend into the lumen to increase absorption area. The height of these folds varied significantly between the HS and HY diets, with flies on HY diets exhibiting greater folding (Student's t-test, $p<0.0001$, Figure 7A, 7C, 7E, 7F, and Table 4). The surface area per fold, as estimated by the height and the radius of the fold, also varied significantly between the two experimental groups, with flies raised on the HY diet showing greater gut surface areas (Student's t-test, $p<0.0001$, Figure 7G, 7C and Table 4). The length of brush border per enterocyte as measured in a cross-sectional image (Figure 7D) was also found to be significantly different between the two diets, again with guts on the HY diets exhibiting greater brush border lengths per enterocyte (Student's t-test, $p=0.0047$, Figure 7B and Table 4).

There was a higher fold height of the brush body in flies on the HY food, with a difference of 56.2%, and a 65.3% difference in surface area per fold per enterocyte, with far greater absorption surface areas per enterocyte available in the guts of flies fed on the HY diet. These data support a size difference in the enterocyte itself as a result of differences in diet.

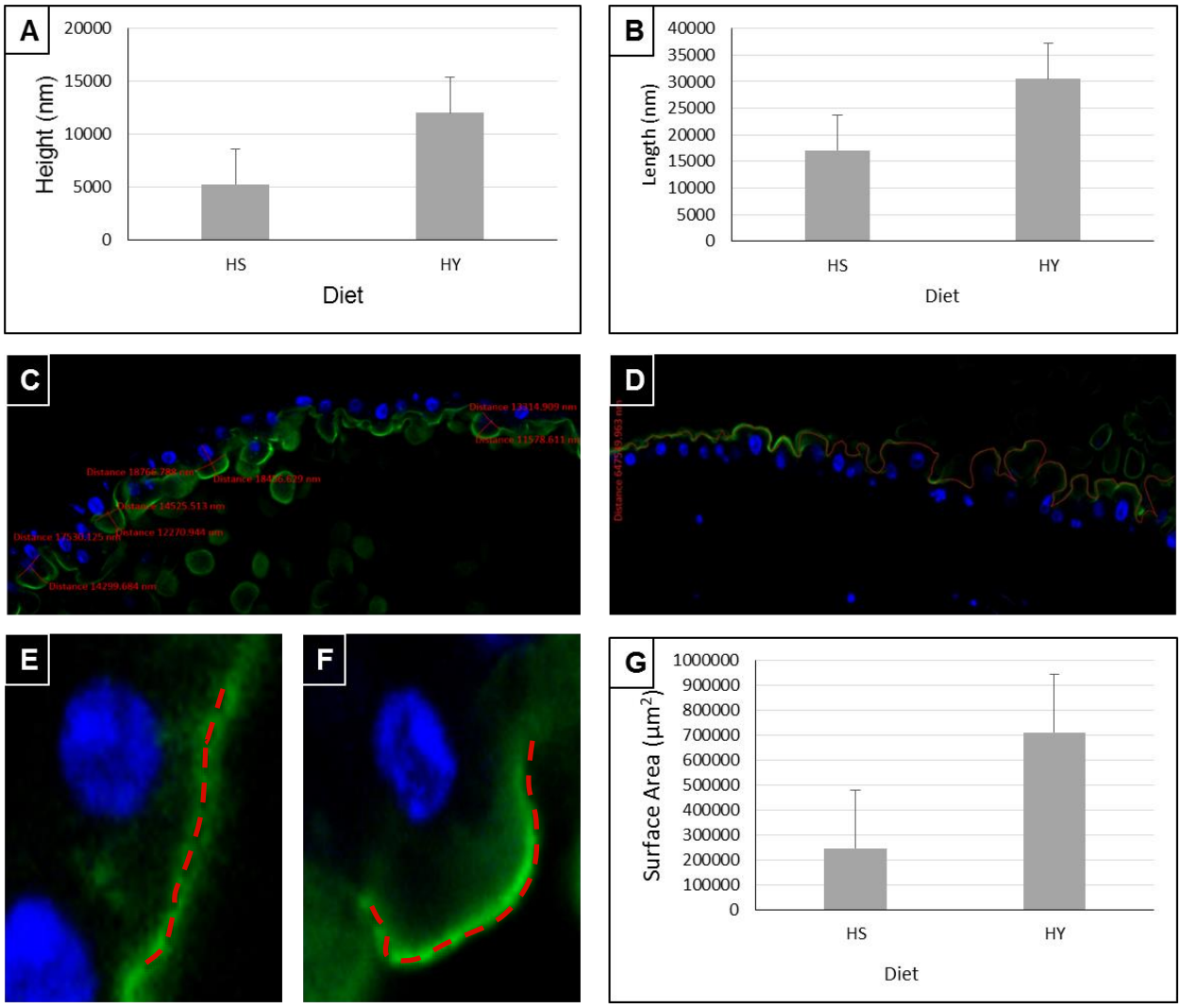

Figure 7. Changes in surface area and shape of absorption surface in the *Drosophila* midgut on two diets. Error bars represent SE. A) Height per fold of the brush border was measured in four randomly selected folds. B) Length of the brush border was measured in a cross-section to estimate length of absorption surface per enterocyte. C) Measurements of height and base length (which was further divided by 2 to calculate radius of the dome) were used to calculate surface area. D) Measurement of the length of absorption surface per enterocyte. E) Brush border of an enterocyte in gut of fly on HS diet with limits of cell denoted with dotted line F) Brush of an enterocyte in gut of fly on HY diet with limits of cell denoted with dotted line G) Calculated surface area per fold of the brush border on two diets.

Differences in gut size can be attributed to genetic variation, diet, and the interaction between the two

Having established a cellular basis to diet-dependent gut resizing in *D. melanogaster*, the next step was to establish generalizability of gut resizing. To evaluate the role of genotypic

differences in the gut plasticity response to diet, effects of genetic variation on gut responses to diet between species and within a species were studied.

Diet-species interactions were studied through variable gut length responses to different diets between 12 species of *Drosophila*. The total gut length was affected by *Drosophila* species, diet, and the interaction between species and diet (ANOVA, *Species* $F = 17.8779$, $p < 0.0001$; *Diet* $F = 22.3981$, $p < 0.0001$; *Species:Diet* $F = 8.2638$, $p < 0.0001$, Table 10). The proportion of variance explained by diet, species, and diet-species interaction was calculated as the sums of squares for each factor in the ANOVA divided by the total sums of squares. The majority of explained variance was attributed to the main effects of species and diet (Table 11). In addition, two lines, *D. mojavensis* and *D. willistoni*, showed an opposite response to the rest of the species, with a longer gut length on the HS food than the HY food (Figure 8). The interaction between diet and species is clearly a variable in the gut growth response and different species react differently to variations in diet.

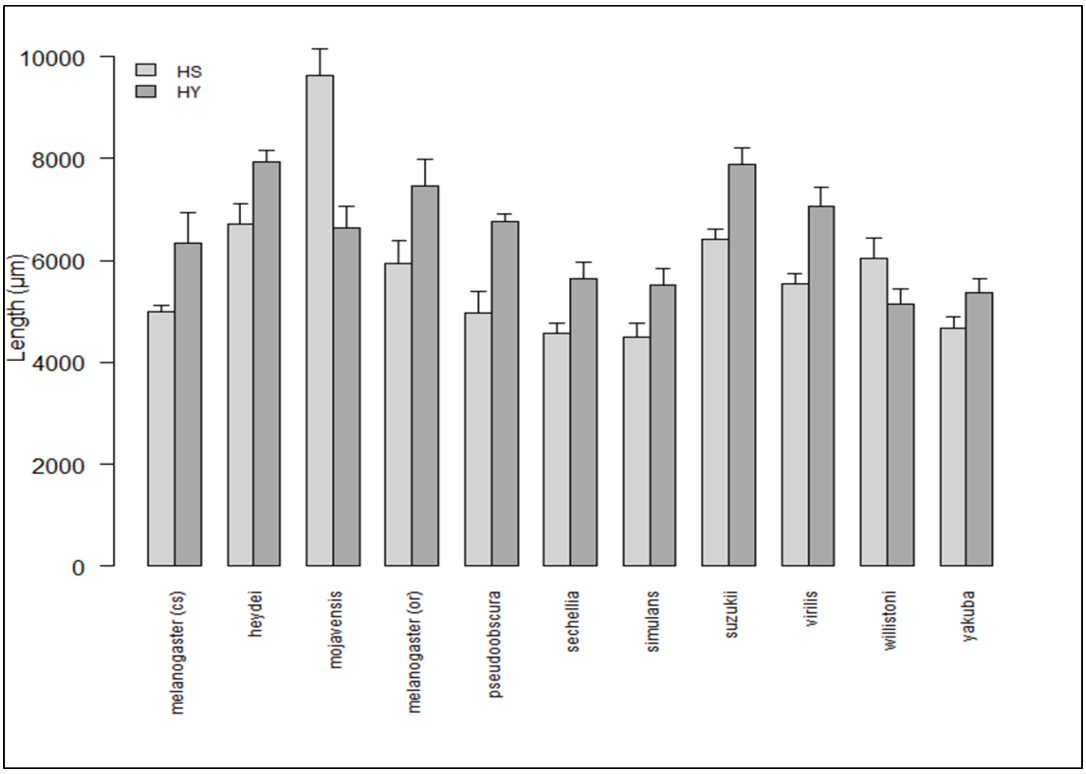

Figure 8. 12 species of *Drosophila* reared on HS and HY diets. Total mean gut lengths of 12 *Drosophila* species on HS and HY diets. Error bars represent standard deviation.

To evaluate diet-genotype interaction within a species, I performed gut growth experiments with two sets of *D. melanogaster* lines, the Global Diversity lines and the *Drosophila* Genetic Reference Panel lines.

The Global Diversity lines served as an initial investigation into the presence of diet-by-genotype interactions. Analysis of 30 *Drosophila* lines (Global Diversity Lines) showed that response to diet was genetically variable and ranged from almost no adaptive gut growth to a two-fold increase in gut surface area and length (ANOVA, $p<0.0001$, Figure 9). With evidence that supported the importance of these interactions, I aimed to study a larger set of lines through experiments with the DGRP lines, which have been fully genotyped and presented the opportunity to perform a genome wide association study (GWAS). For this thesis, I randomly selected ten of

the DGRP lines to analyze for the effect of genotype-by-diet interactions on the gut growth response, as well as diet and genotype individually.

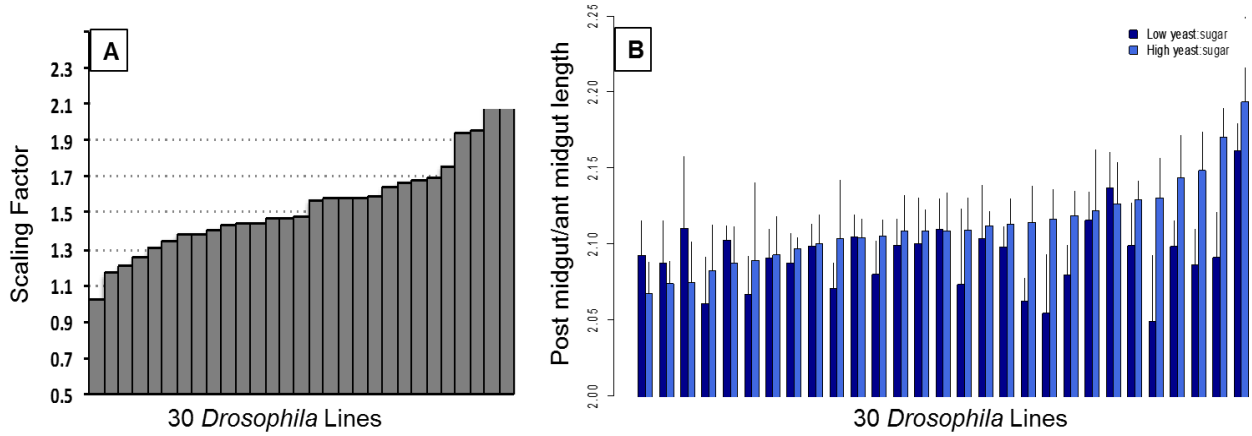

Figure 9. Diet-genotype interactions determine adult *Drosophila* midgut allometry on two diets. A) Scaling factor is calculated through a ratio of gut surface area on HY diet and HS diet. B) Pairs of bars represent one line and show mean proportions of the middle and posterior midgut midgut (R3-5) to the anterior midgut (R1-2). Error bars show standard deviation.

A sample of 10 of the approximately 200 DGRP lines were reared on the HS and HY diets and measured for body weight and anterior, middle, and posterior midgut length (Figure 10). The sizes of all regions were affected by genotype, diet and the interaction of line and diet, but not by body weight, demonstrating quantitative genetic variation in both gut size and the capacity to resize the gut on different diets, independent of general gain of mass (MANCOVA, *Fly line* $F_{24,705}$=10.4, p<0.0001; *Diet* $F_{3,233}$=51.9, p<0.0001; *Fly line:Diet* $F_{21,705}$=2.6, p<0.0005; *body mass* $F_{3,233}$=0.6, p=0.6, Table 5). The proportion of variance explained by diet, fly line, body weight and line-weight interaction was calculated for the anterior, middle and posterior midgut, as the sums of squares for each factor in the MANCOVA divided by the total sums of squares (Table 6). The majority of explained variance was attributed to the main effects of genotype and diet. Compared to the middle and anterior midgut, the posterior midgut was more strongly affected by diet-independent variation and by diet than by their interaction, indicating that the size of this region is strongly affected by

standing genetic variation and nutrition, but the effect of diet on resizing does not depend as strongly on standing variation.

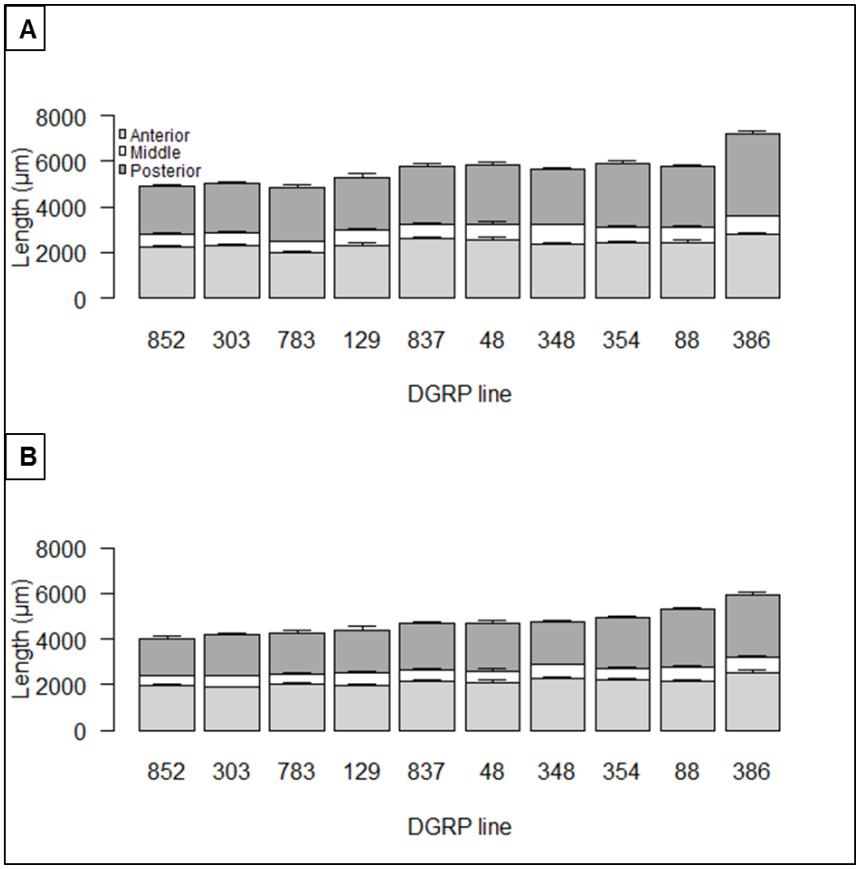

Figure 10. Diet-by-genotype interactions in *Drosophila* Genetic Reference Panel lines. A) Gut lengths of 10 DGRP lines on HY food. B) Gut lengths of 10 DGRP lines on HS food. Bars represent means of gut lengths separated into anterior, middle, and posterior midgut regions on HS and HY diets. Error bars represent standard deviation.

dilp7, but not dilp3, dilp5, upd3, or upd2 and 3, are possible regulators of the gut growth response to diet

With evidence that supported diet-by-genotype interactions were important in the gut growth response, I sought to identify specific genes that mediated this response through evaluation of mutant lines of the *Drosophila* insulin-like peptides (Dilp) and Unpaired (Upd) cytokine families. Ratios of gut lengths of *upd3* KO and *upd2/3* double mutants were not significantly different to the ratio of gut lengths of wild-type flies on HY diet and HS diet (Student's t-test, Figure 11 and Table 7).

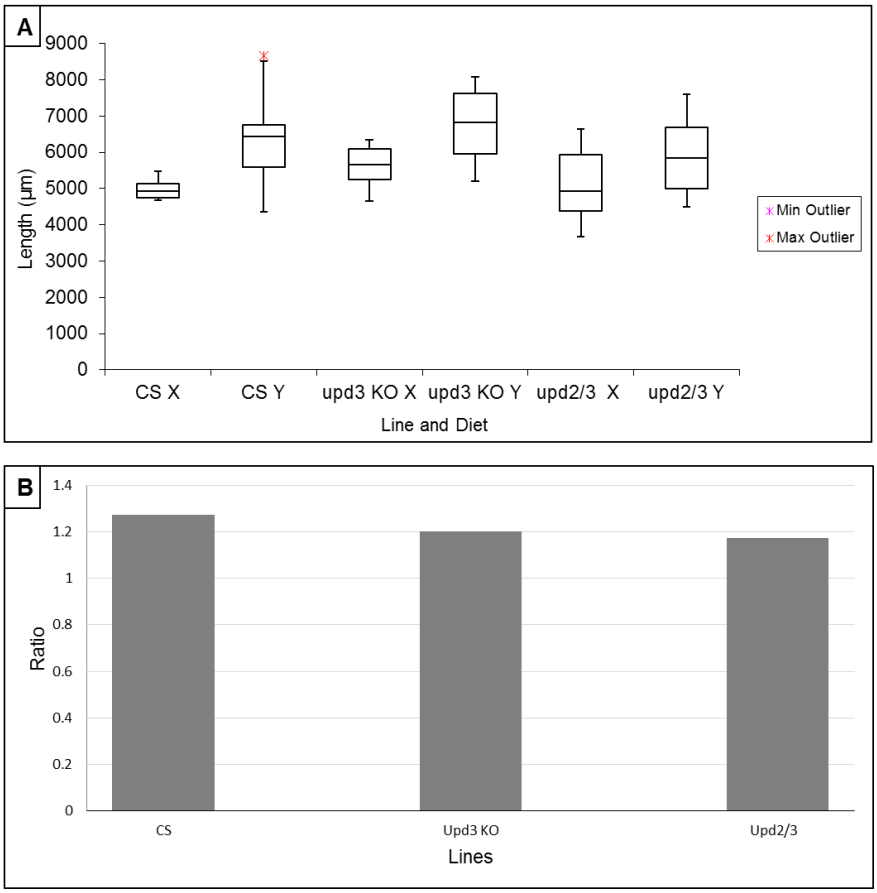

Figure 11. Unpaired knockout mutants on two different diets. A) Box plot displays median and quartile values of gut lengths of wild-type CS, Upd3 KO, and Upd2/3 double mutant flies on HS and HY diet. Error bars represent ±1.5*IQR. One value was excluded as an outlier in the CS on Y food group. B) Ratios of mean gut lengths on HY diet: HS diet. A Student's t-test was performed on ratio values to determine significance.

Of the three *dilp* KO lines included in the screen, only the *dilp*7 KO line appeared to have had a significantly different ratio of HY diet: HS diet gut lengths as compared to the wild-type CS line (Student's t-test, p=0.0042, Figure 12 and Table 9). *Dilp3* KO, *dilp5* KO, and the vehicle control mbGFP were not significantly different from CS (Student's t-test, Table 9). For the moment, only the *dilp7* KO line showed a variable response, suggesting that *dilp7* may be involved in the gut growth pathway.

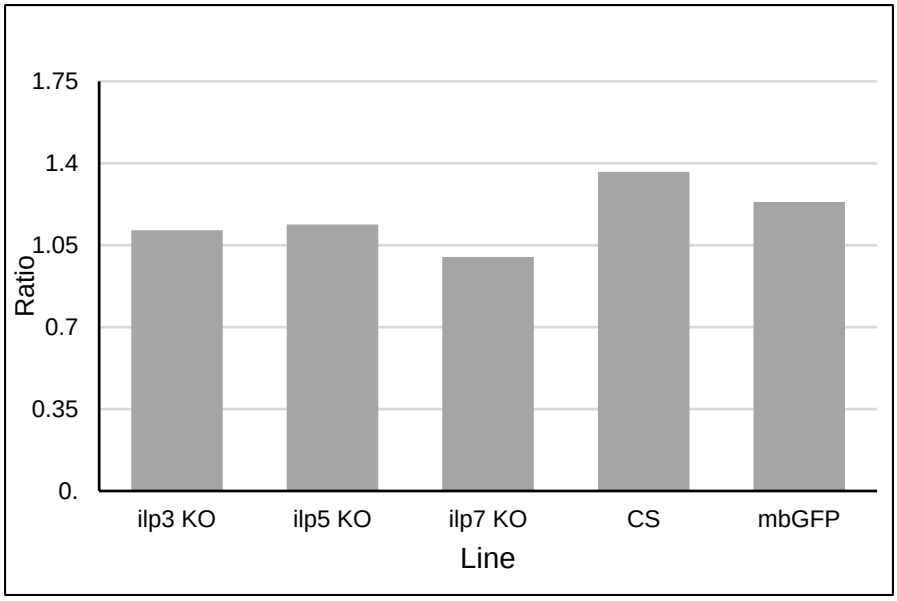

Figure 12. ***Drosophila* insulin-like peptide mutants on two different diets.** Ratio of HY diet: HS diet gut lengths of three *dilp* KO lines and two control lines.

Hr38, sd, and Abd-B are candidates for genes involved in the gut resizing and stem cell proliferation pathways

Previous studies have identified *dilp3,* when secreted from the visceral muscle, as a regulator of stem cell proliferation and important in gut resizing in response to fasting and feeding (O'Brien, et al., 2011). I therefore screened for genes that were predicted to regulate *dilp3*. Preliminary data identified a set of 24 visceral muscle transcription factors that bind to the promoter of *dilp3* and I evaluated each through RNAi and gut length analysis on two diets. As there was only one repeat, no statistical analysis could be performed at this time. Instead, candidate transcription factors for further study were isolated through identification of clear outliers with a strong loss of adaptive gut growth using the range between the HY diet: HS diet gut length ratios of the two control lines, CS and mbGFP (Figure 13B). RNAi lines *Hr38*, *sd*, and *Abd-B* appeared to exhibit less adaptive gut growth, suggesting that these three genes are involved in the stem cell proliferation pathway involved in gut resizing.

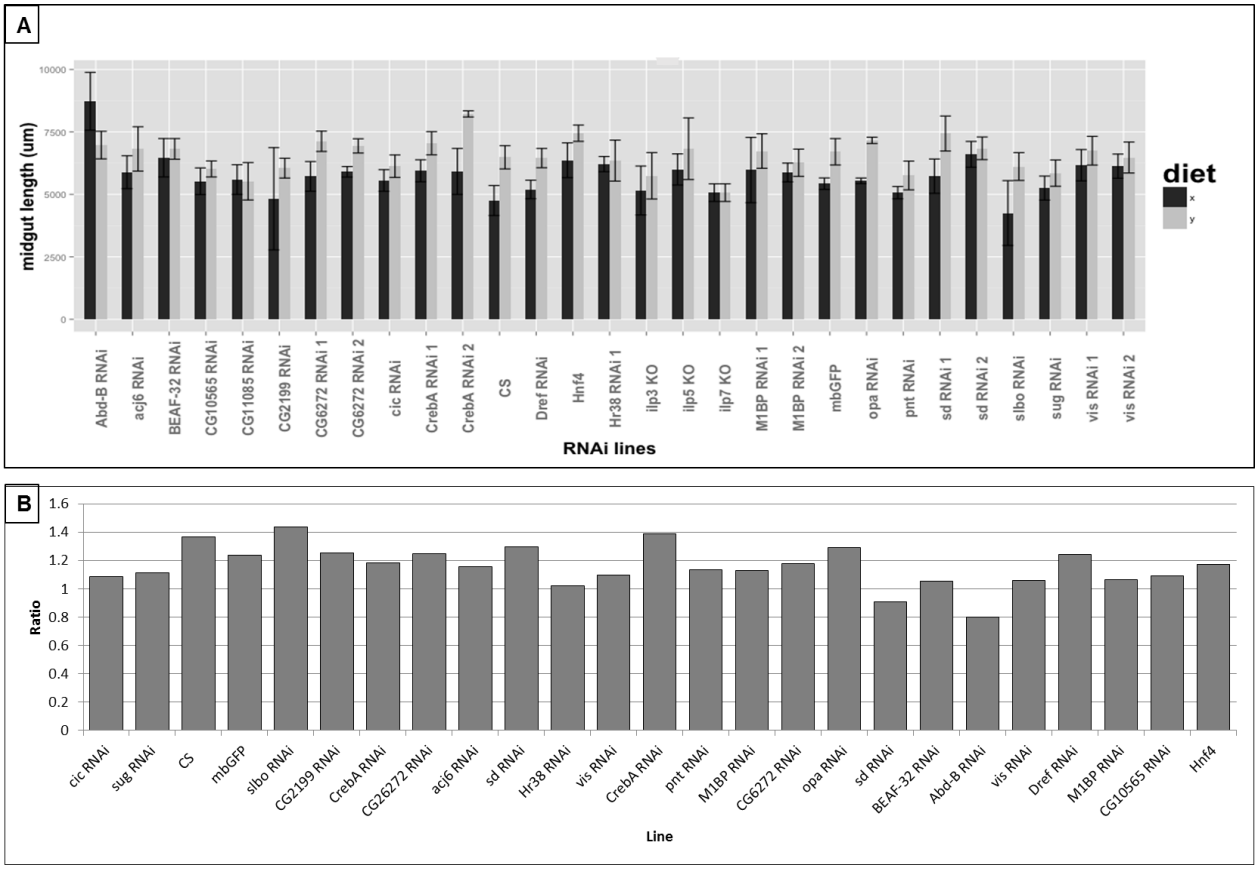

Figure 13. TF RNAi screen for genes involved in midgut response to diet. Error bars represent standard error. A) Gut lengths of 24 RNAi, 3 *dilp* KO, and 2 control lines on two diets. Some lines could not be analyzed due to incomplete data collection for one or more diets. B) Ratio of HY diet: HS diet gut lengths of 24 RNAi lines and 2 control lines with data for both diets. The blue box represents a range based on the ratios of the two control lines. Genes of interest are lines that fall clearly outside of that range and are marked with a red square.

Discussion:

This study has demonstrated that gut resizing due to different ratios of macronutrients is reversible, different diets induce significant changes at a cellular level, and diet-by-genotype interactions are important in determining different responses to the two diets. In addition, it has identified candidate genes for future study of pathways involved in gut resizing and stem cell proliferation.

Drosophila guts remain plastic days into their adult life and gut lengths can dramatically increase or decrease when switched between diets. This suggests that gut resizing responds to not only availability of food, as found by O'Brien et al. (2011), but also ratios of macronutrients in the food. The occurrence of this phenotype was not consistent across all experimental groups, however, as gut lengths of flies that were switched from the normal sucrose food (S food) to the high sugar food (HS food) did not exhibit a significant change in gut length. A possible explanation for this lack of response is that flies that were reared on the normal sucrose food experienced a less dramatic change in diet than flies that were reared on either HS or HY food and shifted to the opposite diet, as there is approximately a ten-fold difference in yeast and approximately two-fold difference in sugar per unit volume of food between the two experimental diets. It is also likely that dietary protein plays a large role in the gut resizing response, as the change in dietary protein is more dramatic than the change in dietary sugar between diets. There is a much smaller difference in yeast per unit volume between the standard sucrose diet and the HY and HS diets, two-fold and five-fold difference respectively, while the difference in sugar per unit volume was increased. This implies that the ratio of dietary protein is likely to have a greater impact, as a smaller difference in the amount of protein can effect a greater change in response. Dietary protein dilution has also been implicated as important in overconsumption of food, so flies may have consumed more food in order to compensate for the lack of dietary yeast resulting in a less apparent change in gut length, although one would expect gut lengths to also decrease on HY food if that were the case (Gosby, et al., 2014). It is also possible that gut resizing requires a longer time when switched from S food to HS food and that a greater change would be apparent if the flies were kept on the HS food for additional days after being shifted from the normal food. Alternatively, these data could support

gut resizing as a matter of growth regulation rather than true resizing, as there is little loss of cells when nutrients become unavailable and growth when nutrients do become available.

Corresponding to large-scale morphological changes induced by diet, I also showed changes at the cellular level. The total number of enterocytes was significantly higher in flies reared on HY food than HS food, but not the cell counts for either intestinal stem cells or enteroendocrine cells. A higher number of enterocytes is expected as the gut lengths of flies on HY food do increase, but the lack of difference in ISC counts between the two experimental groups is surprising as O'Brien et al. (2011) found that stem cell populations as well as total cell populations increased in adaptive gut growth induced by feeding. It is possible that the mechanisms for proliferation of ISCs differ in response to availability of food versus availability of specific macronutrients. Although total numbers of ISCs and enteroendocrine cells did not significantly differ between the two diets, the density of these cell types did, with flies on the HS diet exhibiting a higher density of ISCs and enteroendocrine cells. The difference in diet may have stimulated increased development of stem cells into daughter enteroblasts, which in turn could have differentiated into enterocytes in a preferential manner. Future experiments, therefore, would examine the effect of diet on pathways involved in determining differentiation of enteroblasts. Although the density of enterocytes did not significantly differ between the two experimental groups, there was still a lower density of enterocytes on the HY food which suggests larger enterocytes. To confirm this comparison, additional experiments to directly compare the size of enterocytes in three dimensions between the two experimental groups would be necessary.

Surface area, through imaging of the brush border, was measured and found to be significantly different between the two experimental groups, with more surface area of brush border per enterocyte on the HY diet. Length of brush border per cell as well as the surface area

per fold of the brush border were found to be significantly larger in guts of flies that were reared on HY food rather than HS food. This increase in surface area is likely functionally significant and would allow for increased absorption of nutrients in anticipation of greater availability of dietary protein, which is in line with past studies that have shown changes in height and density of crypts and villi in mammals (Dunel-Erb, et al., 2001). In addition, this supports the conclusion that enterocytes in the R4 region of the midgut of flies fed the HY diet are both more numerous and larger, as there is more surface area associated with each EC. Taken together, all of these changes converge to increase absorption surface through greater numbers of ECs, larger ECs, and more surface area per EC.

The role of diet-by-genotype interactions in the gut growth response was supported by three sets of experiments. In addition to experiments with *D. melanogaster*, analysis of 12 different species of *Drosophila* has further supported the importance of the gene-by-diet interaction. Different species of *Drosophila* showed variable gut resizing in response to the two diets, with two lines even exhibiting a reversed phenotype. One explanation for these differences is that these species are adapted to different environments and the availability of nutrients in their natural habitats may have selected for different responses to increase fitness and survival in their specific niche.

Diet-by-genotype interactions within a species were found to strongly affect variations in response to diet across multiple experiments. An initial analysis of 30 Global Diversity Lines showed that naturally variant genotypes could result in vastly different responses to diet. Further quantification of this interaction through analysis of ten lines from the DGRP revealed differences in the adaptive response of different regions as posterior midgut resizing was less dependent on genetic variation, although all regions exhibited some degree of dependence on genetic variation,

diet, and the interaction between the two. Interestingly, gain of mass did not affect gut resizing when compared to the effect of diet (data not shown), although the small sample size of ten could be a factor in this result. The strength of DGRP experiments lies in the large number of fully genotyped lines, allowing for analysis of genetic variation on phenotypes, analyzing only a small subset of the DGRP does weaken the significance of the results. In addition, ten randomly chosen lines out of more than 200 are unlikely to fully represent the naturally occurring genetic variation of the DGRP and its effect on gut resizing responses. The DGRP data included in this thesis will be part of a GWAS and the analysis of the full set of lines should yield more compelling results. This quantitative genetic variation within a subset of a population (DGRP), at a global scale (Global Diversity Lines), and across 12 *Drosophila* species provides the substrate for selection and evolution. Further work is required to understand how quantitative variation in gut function corresponds to parallel variation in whole-organism physiology, function and health.

The importance of diet-by-genotype interactions in the adaptive gut response has been well supported, and screening for and testing specific genes that mediate this response was the next step. Although previous studies have implicated *dilp3* in the adaptive gut growth pathway, this study found that the *dilp7* knockdown line to be the only line with significant differences from the control wild-type line (Table 9). This difference could be attributed to different pathways for gut resizing due to fasting and feeding as opposed to differences in ratios of macronutrients, although further experiments directly comparing the two conditions would be necessary to confirm. I also found differences in stem cell proliferation response to the two diets, contrasting with previous studies (O'Brien, et al., 2011). While *upd2* and *upd3* have been found to regulate gut renewal through ISC proliferation after bacterial infection, the lack of effect on adaptive gut growth indicates that there are separate pathways for gut resizing due to diet versus recovery from

challenge (Osman, et al., 2012). A possible future experiment would be to test the effect of *upd1* on gut resizing through knockdown of expression, as *upd1* is involved in upkeep of ISC populations (Osman, et al., 2012).

The transcription factor RNAi screen identified several candidate genes for study: *hormone receptor-like in 38* (*Hr38*), *scalloped* (*sd*), and *abdominal B* (*Abd-B*). These transcription factors are of interest as they bind to the *dilp3* promoter and future experiments would seek to trace the upstream pathways of these transcription factors and *dilp3*, which has been found to be important in stem cell proliferation when it is released from the visceral muscle (O'Brien, et al., 2011).

While the importance of macronutrient ratios at both the organ level and the cellular is clear, further studies are needed to identify the genes and individual components of the pathways involved in this complex response. In addition, results suggest that different genes may mediate adaptive gut growth in response to lack of food versus changes in dietary macronutrients. Further studies to examine this possibility and compare the diet-by-genotype interactions of these responses would be necessary.

In conclusion, this thesis demonstrates that the gut can plastically and reversibly resize in response to variations in availability of dietary nutrients, dependent on a panel of transcription factors and *dilp7*, but not Upd ligands. This morphological change is accompanied by cellular changes with possible functional significance for nutrient absorption. There is quantitative genetic variation in the gut's response to diet, within species, across geographical scales, and across species; this variation may be an important causal factor in animal health and how it responds to diet. This thesis, therefore, is foundational in understanding how the effects of diet on gut function integrate into animal health systems. Most importantly, these underlying mechanisms may be conserved in vertebrates, including humans.

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Supplementary Data:

Table 1. Genotypes of flies used in cellular characterization studies		
Line	Genotype	
esgTS	w	esgGal4/CyOlacZ UAS-GFP tubGal80ts
prosv1TS a142-GFP	w	UAS-GFP tubGal80ts prosv1-Gal4 / TM6B

Table 2. Adaptive growth after switching between diets. A student t-test was performed between the final diet and the starting diet.

Diet	Switched	Switched From	Mean Gut Length (µm)	StDev	p value
HS	No	None	4967.589947	893.167552	
HY	No	None	7176.614183	1050.45653	
HS	Yes	HY	5183.469432	1107.6292	4.6867-06***
HY	Yes	HS	6860.610726	1088.26003	6.0421E-06***
S	No	None	5821.078085	780.070974	
HS	Yes	S	5525.672267	1282.08079	0.5027
HS	Yes	S	7467.745425	1051.5449	0.00032**

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 3. Cellular consequences of different diets. A student t-test was performed between the values of the HS and HY diet for each group.

	Mean Total Cells	StDev Total Cells	p value
ISCHS	735.8219321	349.0396364	0.8826
ISCHY	758.8479516	372.5122626	
ECHS	2085.852996	906.9065041	0.009771**
ECHY	3225.757688	1112.899395	
EECHS	169.9760845	70.46413176	0.7289
EECHY	179.9318332	72.08450691	

	Mean Cells/µm2	StDev Cells/µm2	p value
ISCHS	0.003349745	0.001355502	0.01880*
ISCHY	0.002072632	0.000907284	
ECHS	0.006904469	0.00315123	0.3135
ECHY	0.005843824	0.001728686	
EECHS	0.000755298	0.000336316	0.001466**
EECHY	0.000393604	0.000156769	

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 4. Absorption surface changes on different diets. A t-test was performed between values on HS and HY diets

Diet	Height (nm)	StDev	p value
HS	5270.58795	2381.761682	0.00000014581***
HY	12033.799	3201.576106	

Diet	Surface Area of Fold (nm^2)	StDev	p value
HS	246609293.9	138745902.2	0.00000103792***
HY	710547209.8	314047584.4	

Diet	Length Per Cell (nm)	StDev	p value
HS	17112.88129	5561.158847	0.004705**
HY	30482.0145	4731.271685	

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 5. MANCOVA analysis of 10 DGRP lines.

	Df	Pillai	approx F	num Df	den Df	Pr(>F)
	1	0.98952	7330.6	3	233	< 2.2e-16 ***
ral	8	0.78251	10.4	24	705	< 2.2e-16 ***
diet	1	0.40068	51.9	3	233	< 2.2e-16 ***
mg	1	0.00708	0.6	3	233	0.6460105
ral:diet	7	0.21712	2.6	21	705	0.0001102 ***
Residuals	235					

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 6. Proportion variance of MANCOVA test for 10 DGRP lines. Sums of squares for each factor in the MANCOVA was divided by the total sums of squares.

Gut Region	ral	diet	mg	ral:diet	Residuals
anterior	0.2523752	0.1713156	0.00163602	0.04059232	0.5340809
middle	0.2627577	0.1481608	0.00229523	0.03273783	0.5540484
posterior	0.3546817	0.2082599	0.00193409	0.0277464	0.4073779

Table 7. *upd3* KO and *upd2/3* double mutants on HY and HS diets. A t-test was performed on difference in gut length on X and Y food

Line and Diet	Length	StDev	p value
CSHS	4988.66667	303.271935	
CSHY	6347	1450.33238	
upd3 KOHS	5643.92224	508.593892	0.65425246
upd3 KOHS	6781.86514	942.387351	
upd2/3 double mutant HS	5095.34684	942.460294	0.49857305
upd2/3 double mutant HS	5988.84596	1037.83521	

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 8. Genotypes of TF RNAi lines

Number	Genotype	Number	Genotype
25995	cic RNAi	31936	pnt RNAi
26760	CHES-1-like RNAi (checkpoint supp)	32548	GAL4 C578
27026	sug RNAi (sugarbabe)	32858	M1BP RNAi (motif-1 bp)
CS	CS	33652	CG6272 RNAi
mbGFP	mbGFP	34706	opa RNAi (odd paired)
27043	slbo RNAi (slow border cells)	35481	sd RNAi(scalloped)
27082	CG2199 RNAi	35642	BEAF-32 RNAi
27648	CrebA RNAi (cyc. AMP resp bpA)	35647	Abd-B RNAi (abdominal B)
29377	Hr38 RNAi (hormone receptor-like in 38) (JF02541)	35738	vis RNAi
29331	CG6272 RNAi	36760	CG3376 RNAi
29335	acj6 RNAi (abnormal chemosensory jump 6)	36865	Dref RNAi (DNA Replication element)
29352	sd RNAi(scalloped)	41937	M1BP RNAi (motif-1 bp)
29376	Hr38 RNAi (hormone receptor-like in 38) (JF02540)	42516	CG11085 RNAi
30881	ilp2 KO	42548	drm RNAi (drumstick)
29544	vis RNAi	42377	MiMic AstC-R2 (allostatin C receptor)
30882	ilp3 KO	42516	CG11085 RNAi
30884	ilp5 KO	42525	CG11617 RNAi
30887	ilp7 KO	43205	CG10565 RNAi
31900	CrebA RNAi	43516	Hnf4 (Hepatocyte nuclear factor 4)

Table 9. Insulin-like peptide mutants on HY and HS diets. A student t-test was performed between the wild-type group (CS) and each of the three mutant groups and the vehicle control

Line	Relative Growth	p-value
ilp3 KO	1.114110973	0.1248
ilp5 KO	1.138441589	0.4931
ilp7 KO	0.999987572	0.004155**
CS	1.363619536	
mbGFP	1.234997845	0.5755

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 10. Analysis of Variance Table for 12 Species

Response: gutLength

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
species	10	131390554	13139055	17.8779	< 2.2e-16 ***
food	1	16461098	16461098	22.3981	6.635e-06 ***
species:food	10	60733612	6073361	8.2638	7.012e-10 ***
Residuals	110	80842776	734934		

* significant at p<0.05; ** significant at p<0.01, ***significant at p<0.0001

Table 11. Proportion of Variance of 12 Species

terms	proportionVarianceExplained
species	0.453966222
food	0.056874579
species:food	0.209840111
Residuals	0.279319087