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## Functional Characterization of Vesicular Trafficking Genes in the Midgut of *Tetranychus Urticae* via RNA Interference

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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## Abstract

The two-spotted spider mite *Tetranychus urticae* is a polyphagous agricultural pest of economic importance. Previous studies have established that reduced gene expression of *COPB2*, *SNAP- $\alpha$* , and *V-ATPase* genes with RNAi and lowers both the survivorship and fecundity of *T. urticae*. A visible phenotype was also associated with changes to the digestive cells of the midgut after treatment. Serial sections of paraffin embedded RNAi treated mites to determine the changes caused by the transcriptional silencing of the three focal genes. *COPB2* silencing leads to a significant increase in the number of juvenile digestive cells, while *SNAP- $\alpha$*  and *V-ATPase* silencing caused dysfunctional mature digestive cells. The formation and disruption of these digestive cells may provide a potential tool in integrated pest management.

## Keywords

*Tetranychus urticae*, spider mite, *COPB2*, *SNAP- $\alpha$* , *V-ATPase*, RNAi, vesicular trafficking, midgut physiology, histology, integrated pest management.

## Summary for Lay Audience

The spider mite *Tetranychus urticae* is a global pest. Previous studies found that the downregulation of the vesicular trafficking genes *COPB2*, *SNAP-a* and *V-ATPase* resulted in a morphological change, that negatively affected the longevity and reproductive output of *T. urticae*. This study tries to identify changes in terms of what is changing inside the spider mite after genetically silencing these genes with RNA interference. The data suggest RNA interference of these vesicular trafficking genes will cause abnormalities in the life cycle of digestive cells that are found within the midgut of *T. urticae*. RNA interference of *COPB2* causes an increase in the number of digestive cells and increases the proportion of younger digestive cells compared to older digestive cells. The silencing of other similar vesicular trafficking genes produces similar results. The changes caused by RNA interference may not be localized to one gene but to the system they are part of.

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## Abbreviations

<b>ARF</b>	Adenosine diphosphate ribosylation factor
<b>COPB2</b>	Coatomer protein complex subunit beta 2
<b>COPI</b>	Coatomer protein complex 1
<b>COPII</b>	Coatomer protein complex 2
<b>ER</b>	Endoplasmic reticulum
<b>F3R3</b>	Primers of an intergenic region of noncoding DNA
<b>FAA</b>	Formaldehyde, acetic acid and alcohol solution
<b>mRNA</b>	Messenger ribonucleic acid
<b>NSF</b>	N-ethylmaleimide-sensitive factor
<b>PBS</b>	Phosphate buffered saline
<b>PCR</b>	Polymerase chain reaction
<b>SNAP-<math>\alpha</math></b>	N-ethylmaleimide-sensitive factor attachment protein alpha
<b>SNARE</b>	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
<b>RNAi</b>	Ribonucleic acid interference
<b><i>T. urticae</i></b>	<i>Tetranychus urticae</i> (Koch)
<b>TEM</b>	Transmission electron microscope
<b>V-ATPase</b>	Vacuolar-type H <sup>+</sup> adenylyl pyrophosphatase protein

## Chapter One - Introduction

### 1.1 Economic Impact of *Tetranychus urticae* (Koch)

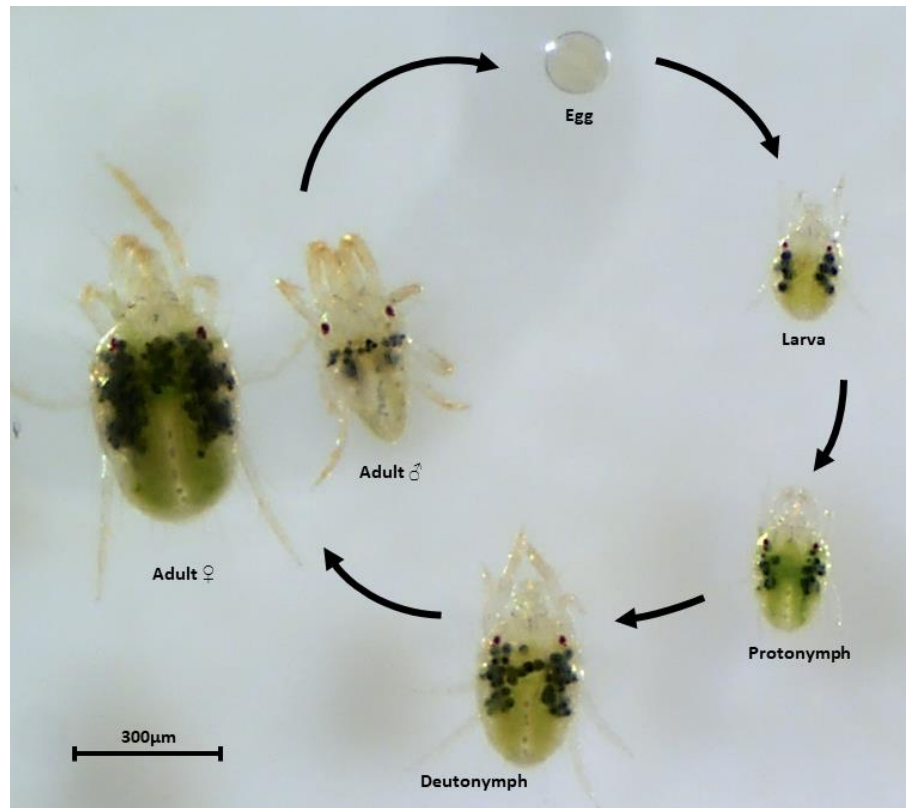
While the advent of civilization was marked by the implementation of agriculture 100,000 years ago, food security and ensuring the physical availability and financial means for an individual to access food is still a paramount concern for countries around the world. According to the United Nations Department of Economic and Social Affairs, the world population is expected to reach 9.7 billion people in 2050 (United Nations, 2019) and experts predict global food production must increase by 50% in order to meet projected demands (Chakraborty, 2011). While pest and disease management practices for crops have advanced in the past century, agricultural crop loss to herbivorous pests is one of the most significant threats to food security in the world, and it is estimated that 10-16% of the global harvest is still lost to plant pathogens and pests (Oerke, 2006). Global food supplies will also be further strained by a warming global climate that will cause increasing extreme weather events such as droughts to occur and introduce invasive species to new regions (Riegler, 2018). These costs in agricultural production are inevitably passed down to the consumer and the lack of food security manifests itself as malnutrition in vulnerable populations and even political instability in countries (Deaton and Lipka, 2015). Therefore, it is ethically important for researchers to recognize emerging agricultural pests and identify the methods to mitigate their potential damage.

The spider mite *Tetranychus urticae* (Koch) stands out from other pests due to their polyphagous nature and their life cycle which give them the ability to adapt to new plant

hosts as well as commercial acaricides (Dermauw et al., 2013). As a generalist herbivore, *T. urticae* is found throughout the world, but is known to thrive in hot and dry conditions and is also known to feed on over 1,100 different species of plants, with 150 of these plants being economically important to farmers (Piraneo et al., 2015). Expenditures towards treatment of spider mite infestations by farmers globally and the damages attributed to crop loss was reported to exceed 1 billion US dollars a year (Godinho, 2011). Despite these costs, the physiology of *T. urticae* is relatively unknown.

## 1.2 Background on Spider Mites

*Tetranychus urticae* is a chelicerate arthropod first identified in 1836 (Koch). The term spider mite alludes to the silk webs that species in the family Tetranychidae produce in order to protect their eggs while on plants. These silk webs also allow for spider mite dispersion to adjacent plants. *Tetranychus urticae* has a short life (Figure 1-1) going from egg to adult in approximately one week at 25°C. Sex is determined by a process known as arrhenotokous parthenogenesis (Krantz et al., 1987), where haploid males emerge from unfertilized eggs and larger diploid females come from fertilized eggs. Female adult mites can lay up to approximately 40 eggs in their lifetime (Godinho, 2011). Spider mites feed on plants with the use of a stylet to puncture through the surface of the leaf cuticle and are known to adapt and become resistant to systemic plant defenses over time (Bensoussan et al., 2016).



**Figure 1-1.** Life cycle of *T. urticae*. Larvae emerge from eggs within 3 days and will molt into the two instars stages of protonymph and deutonymph before becoming an adult in approximately a week. A size bar is added for reference. Picture taken by Zoran Culo and used with permission.

One important element to the success of *T. urticae* lies in its capacity to respond to xenobiotic stresses, including the ability to detoxify plant compounds through the expansion of gene families encoding for detoxification enzymes (Grbić et al., 2011). The high fecundity and short life cycle of the spider mite allow for adaption to occur within a

few generations, and these adaptations are retained even when they move to a new plant host (Dermauw et al., 2013). Detoxification of plant defensive compounds in *T. urticae* is presumably taking place in the midgut. Considering the high fecundity of the spider mite, the required amount of food consumed to keep up with the metabolic demand of egg and silk production is likely substantial.

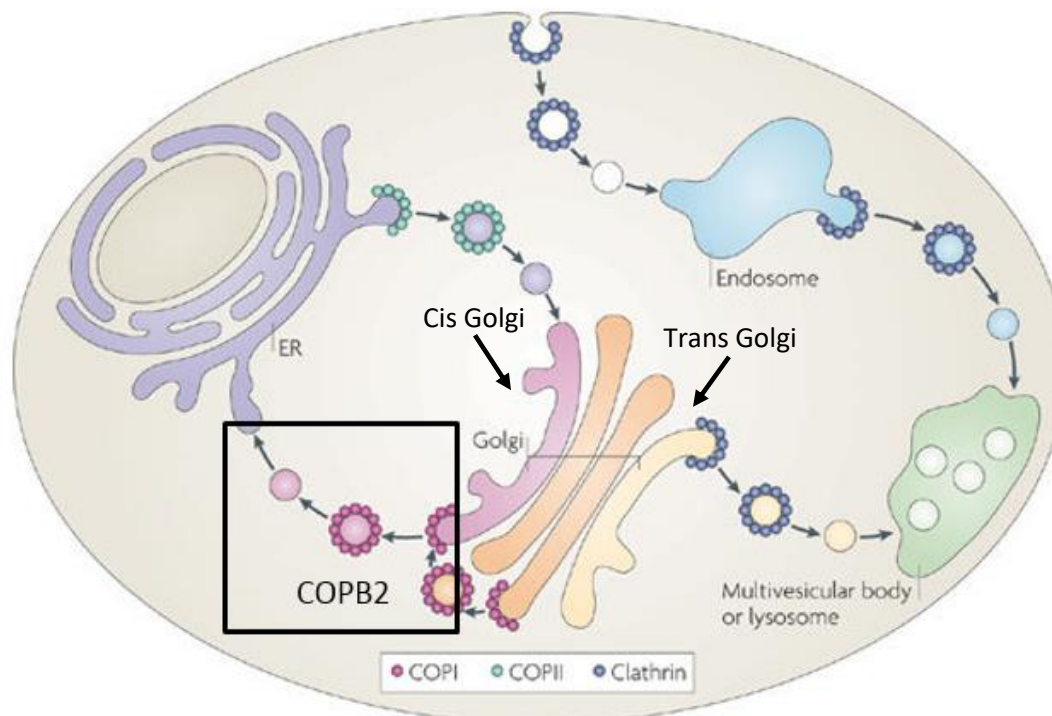
The genome of *T. urticae* was fully sequenced in a previous study (Grbić et al., 2011) and the annotation of genes was achieved using comparative genomics. Arthropods including the red flour beetle (*Tribolium castaneum*) were used to identify gene orthologues in *T. urticae* (Richards et al., 2008). Some of these candidate genes were later found to be sensitive to RNAi as they produced whole-body phenotypes in *T. urticae* after silencing. Initial genomic screens for candidate targets in *T. urticae* identified genes associated with vesicular trafficking and potential gut function, which when silenced significantly reduced spider mite survivorship by 65.4% after 120 h (Kwon et al., 2012; Kwon et al., 2015). The role of vesicular trafficking in the midgut and understanding how digestion occurs in spider mites can reveal their detoxification strategy. By investigating the silencing of these candidate genes from these genomic screens, they may become novel targets for pest management.

## 1.3 Vesicular Trafficking

Vesicular trafficking is the process of the movement of materials and proteins between membrane bound organelles. Mechanistically, this process encompasses the synthesis, movement and the docking of vesicles. Vesicular trafficking allows for the secretion of products that may be used for the purpose of cell to cell communication, and for processes outside or within the cell itself (Rothman, 1994).

There are three different classes of coatomer systems needed to recruit proteins that produce vesicles for the proper functioning of a cell: COPII (coatomer protein complex 2), COPI (coatomer protein complex 1) and clathrin-coated vesicles. After proteins are synthesized in the endoplasmic reticulum (ER), they are further transported towards and throughout the Golgi apparatus in COPII vesicles. At the trans-Golgi, clathrin vesicles are used to export material around or out of the cell through exocytosis and are used to import material into the cell. COPI vesicles are responsible for the reverse movement of material from the trans-Golgi to cis-Golgi and cis-Golgi to ER, in order to ensure the materials necessary for anterograde vesicle movement are not depleted at the ER (Figure 1-2). These three coatomer systems are associated with the normal function, formation, and disassembly of these vesicles in cells for all eukaryotes (Duden, 2003).



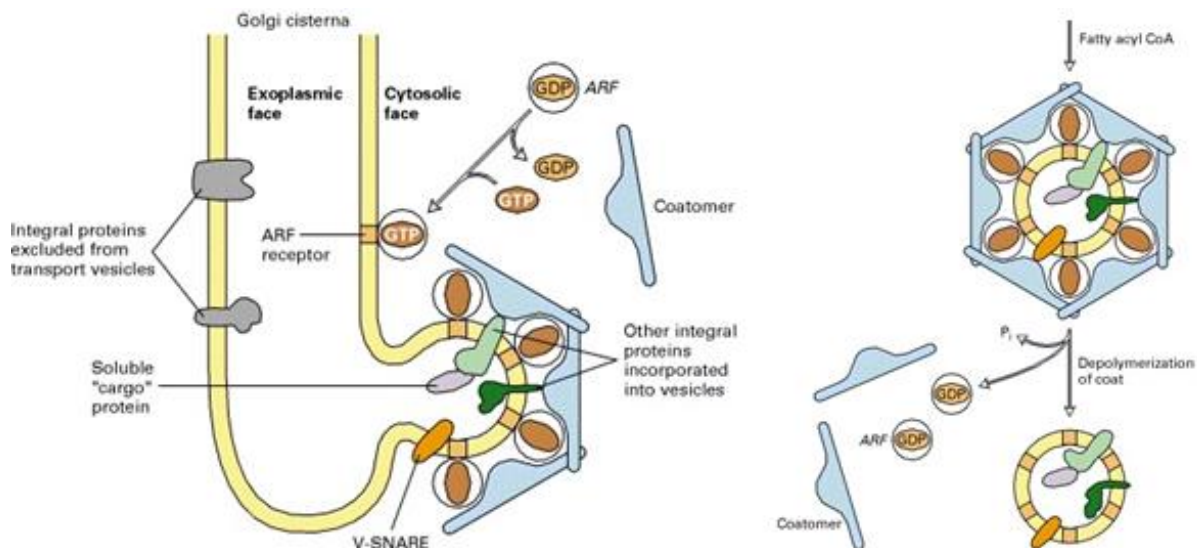


**Figure 1-2.** Overview of the three vesicular trafficking systems in the cell ©. The COPI system is involved in retrograde/reverse trafficking of material and the COPII system is associated with the anterograde/forward trafficking of material (Hsu et al., 2009). Refer to Appendix A for figure permissions and copywrite agreement from Springer Nature.

## 1.4 Coatomer Protein Complex Subunit Beta 2

COPB2 is one of the seven subunits of the COPI, which is responsible for the formation of vesicles used for retrograde protein trafficking (Figure 1-2; Figure 1-3) (Duden, 2003). Retrograde protein trafficking is used to return chaperones, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) receptors and other protein machinery lost from anterograde protein trafficking back to the ER (Bethune

et al., 2006). Cells with a loss of function for the COPI complex gradually lose phospholipids and resident membrane proteins from the rough ER. This eventually prevents further vesicle formation as secretory proteins continue to be synthesized but ultimately, they accumulate in the ER as they cannot be exported. This can lead to problems with organ development, morphological changes or even death for some organisms (Duden, 2003).



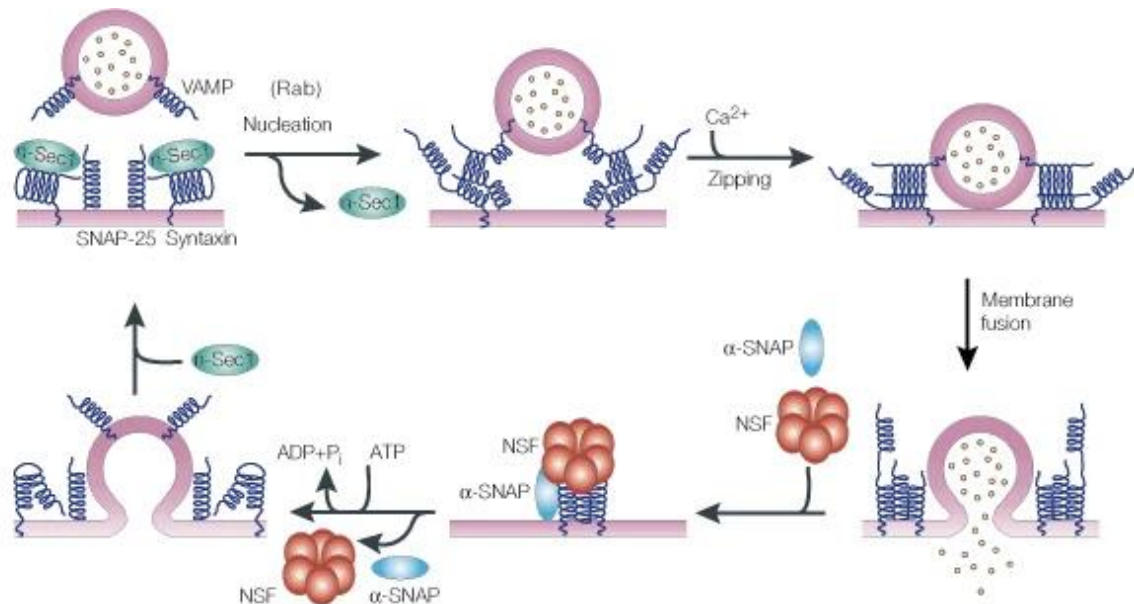
**Figure 1-3.** Diagram of the COPI complex ©. Budding is initiated by ARF (ADP-ribosylation factors) receptors which sequester protein subunits that include COPB2 that allow coatomers to attach. This complex will assist in budding and forming the vesicle. Upon successful formation of a vesicle, the coatomers will detach and become available to form new vesicles (Rothman, 1994). Refer to Appendix A for figure permissions. and copywrite agreement from AAAS.

A hypomorphic mutation or a partial loss of function of *COPB2* in mice produces phenotypically normal individuals, but they suffer from an increase in autophagic cell death in the brain. A full gene knockout in mice causes low birth weight and death within the first week of life (DiStasio, et al., 2017). The COPI complex is short-lived and dissociates from the vesicle after budding in order to expose SNARE receptors used for docking with a target membrane. SNARE receptors can be found in COPI, COPII and clathrin systems and are responsible for the proper targeting and fusion of vesicles to the membrane of their destination. This communication is associated with the next gene of interest, *SNAP- $\alpha$*  (Duman and Forte, 2003).

## 1.5 N-ethylmaleimide-sensitive Factor Attachment Protein Alpha

*SNAP- $\alpha$*  is a subunit that works with *SNAP- $\beta$* , *SNAP- $\gamma$* , and NSF (N-ethylmaleimide-sensitive factor) to interact with the SNARE protein that is formed between vesicles and target membrane SNARE receptors used in vesicle recognition (Duman and Forte, 2003). Both complexes combine to form the 20S complex that will stabilize vesicle docking and fusion (Figure 1-4). After the successful integration of the vesicle, *SNAP- $\alpha$*  is also responsible for the breakdown of the 20S complex by stimulating NSF ATPase (Barnard et al., 1997). SNARE proteins have regulatory effects on vesicular trafficking, including exocytosis within the cell and a role in the release of neurotransmitters in nerve cells.

Yeast cells with a loss of function from a mutation in *SNAP- $\alpha$*  amass proteins in vesicles released from the ER that accumulate in the cytoplasm as they are unable to dock with the Golgi apparatus (Duden, 2003). *SNAP- $\alpha$*  also has functions relating to cell-matrix adhesion of cells and loss of function causes cell detachment in human intestinal epithelial cells (Naydenov et al., 2014) as well as arrest in cell division for human colon cancer cells (Wang et al., 2017).

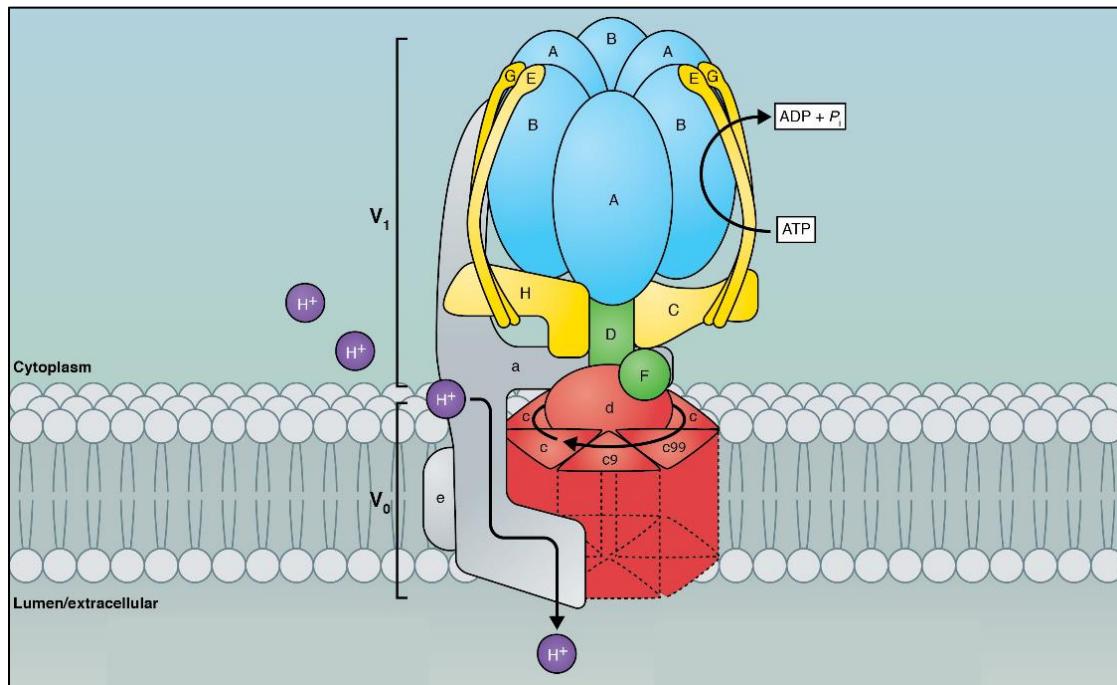


**Figure 1-4.** Formation of the 20S complex ©. Starting from the top-left, after the vesicle and target membrane SNARE receptors bind together, protein subunits including SNAP- $\alpha$  are recruited in order to stabilize successful vesicle fusion. After proper docking, the same protein subunits are used to promote the dissociation of SNARE receptors upon success so that they can be recruited elsewhere in the cell (Chen and Scheller, 2001). Refer to Appendix A for figure permissions and copyright agreement from Springer Nature.

## 1.6 Vacuolar-type H<sup>+</sup> Adenyl Pyrophosphatase Protein

V-ATPase is a conserved enzyme in eukaryotes that is responsible for maintaining the pH gradient between the cytosol and the lumen of the cell and other membrane bound organelles, which allows proper functioning of most cellular machinery (Maxson and Grinstein, 2014). The protein pump is composed of a catalytic unit (V<sub>1</sub>) and a plasma membrane bound unit (V<sub>o</sub>) which uses the energy from ATP hydrolysis to pump hydrogen ions and acidify the lumen of the cell (Figure 1-5). The pH gradient produced by V-ATPase also affects vesicular trafficking, as the protein is responsible for acidifying the interior of vesicles during transit to ensure the proper dissociation and targeting of receptors. This in turn allows for the normal functioning of processes critical to the cell such as protein degradation and neurotransmitter signaling (Maxfield and McGraw, 2004).

V-ATPase is also associated with the upregulation and downregulation of signaling pathways in the cell and overexpression of this protein is seen to be positively associated with the invasive potential of certain cancers (Sennoune et al., 2004). In arthropods, the loss of function in the *V-ATPase* gene is known to cause decreased survivorship and fecundity in the bed bug *Cimex lectularius* (Basnet and Kamble, 2018) and the beetle *Diabrotica virgifera zea* (Velez et al., 2016).

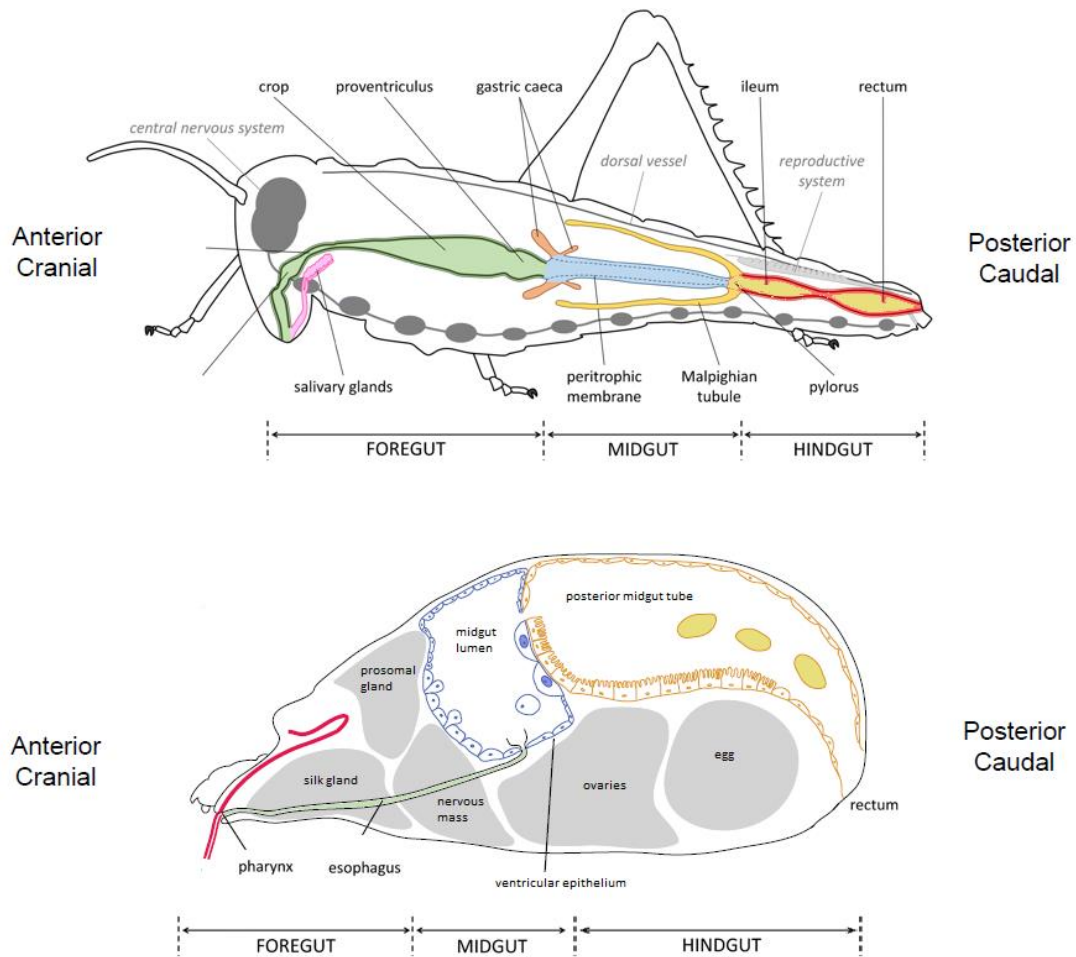


**Figure 1-5.** Illustration of the protein pump V-ATPase and its two components ©. The upper subunit (V<sub>1</sub>) is composed of eight smaller subunits and is responsible for ATP binding and hydrolysis. The force created allows for the lower subunit (V<sub>0</sub>) to rotate and translocate protons through the plasma membrane in order to acidify the lumen. The pH gradient provides the chemical potential that drives certain processes such as protein degradation which are essential to the cell (Maxson and Grinstein, 2014). Refer to Appendix A for figure permissions and copywrite agreement from The Company of Biologists.

## 1.7 Digestion in *T. urticae*

In the digestive system of Acari, the alimentary tract is separated into three components like insects: there is a foregut, midgut and hindgut (Bensoussan et al., 2018). However, unlike insects *T. urticae* lacks certain components in digestion and the movement of nutrients such as a peritrophic matrix, Malpighian tubules, or hemolymph (Mothes and Seitz, 1981). These missing components suggest digestion in *T. urticae* is being performed at the intracellular level via the use of free-floating digestive cells that populate the midgut lumen, as opposed to insects that use extracellular digestion (Bensoussan et al., 2018).

Insect digestion will typically involve the formation of a bolus of food at the foregut that proceeds through the digestive tract (Holtof et al., 2019). After feeding, the bolus is surrounded by the peritrophic matrix which separates the bolus from the digestive tract while allowing substrates and enzymes to be efficiently used. Movement of this bolus is facilitated by muscles contractions along the tract through the midgut until it reaches the hindgut where water is reabsorbed and the bolus is expelled as waste. Nutrient absorption at the midgut is facilitated through passive diffusion through midgut epithelial cells that move nutrient molecules into the hemolymph or Malpighian tubules which transport amino acids from digested protein (Figure 1-6).

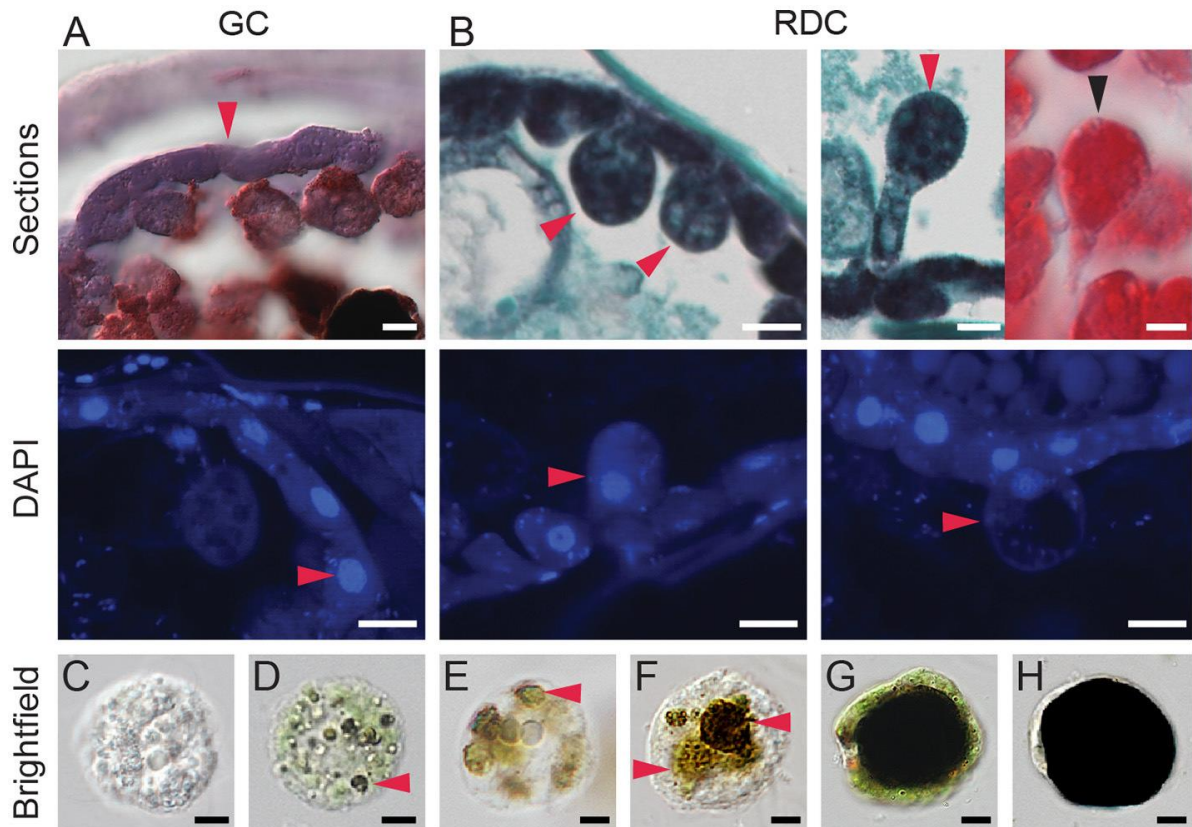


**Figure 1-6.** Comparison of insect and mite digestive systems ©. Both insect and mite digestive systems are structured similarly in terms of having a foregut (green), midgut (blue) and hindgut (yellow). Insects however digest nutrients extracellularly as the bolus is moved through their digestive system. Mites store food material in their midgut where it is taken up by digestive cells which mature, allow for intracellular digestion and are later removed as waste. Refer to Appendix A for figure permissions and copywrite agreement from Springer Nature and the Creative Commons Attribution 4.0 License respectively.



While the foregut and hindgut of *T. urticae* operate similarly to insects, the midgut appears to differ significantly in the mechanics of food digestion. Instead of a tube, the midgut of *T. urticae* appears to be an open area where food enters and is taken up by globular digestive cells for further digestion (Figure 1-7). These digestive cells have nuclei, are visible in large numbers in the midgut, can be seen to accumulate material and are deposited as waste by the mite. Bowman (2019) suggested that the predatory soil mite *Pergamasus longicornis* has a dynamic gut environment and that epithelial cells are seen to interconvert between squamous, columnar or pseudo-stratified cells in order to maximize the amount of food it can ingest as well as portioning it away for long-term storage. The author explains differentiation at the cell-level as opposed to organ-level is ideal for the smaller physiology of mites and this clarifies how *T. urticae* and other mites can thrive without all the aforementioned digestive elements present in insects.

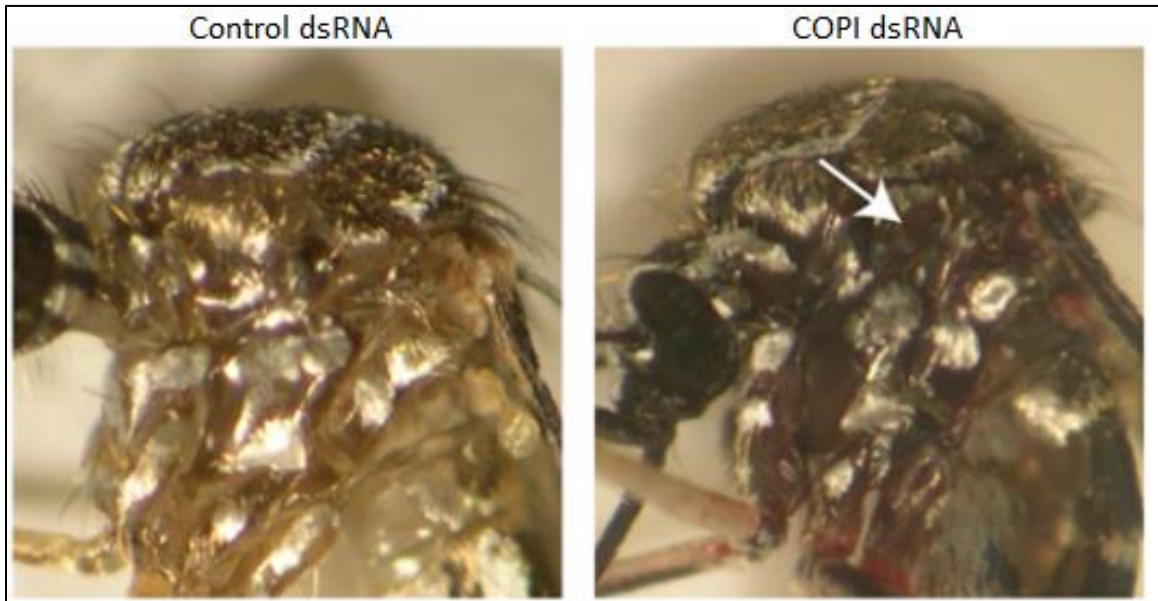
Currently the role these digestive cells perform in *T. urticae* is still unclear, however they can be seen to have a defined life cycle within the midgut (Bensoussan et al., 2018). These digestive cells bud off from midgut epithelial cells into the midgut lumen where they freely float around and progressively accumulate content into the vacuole presumably from secondary plant metabolites and digested material. Over time, these digestive cells grow and darken and after a certain point they are moved from the midgut lumen into the posterior midgut and expelled through the rectum as waste (Figure 1-7).



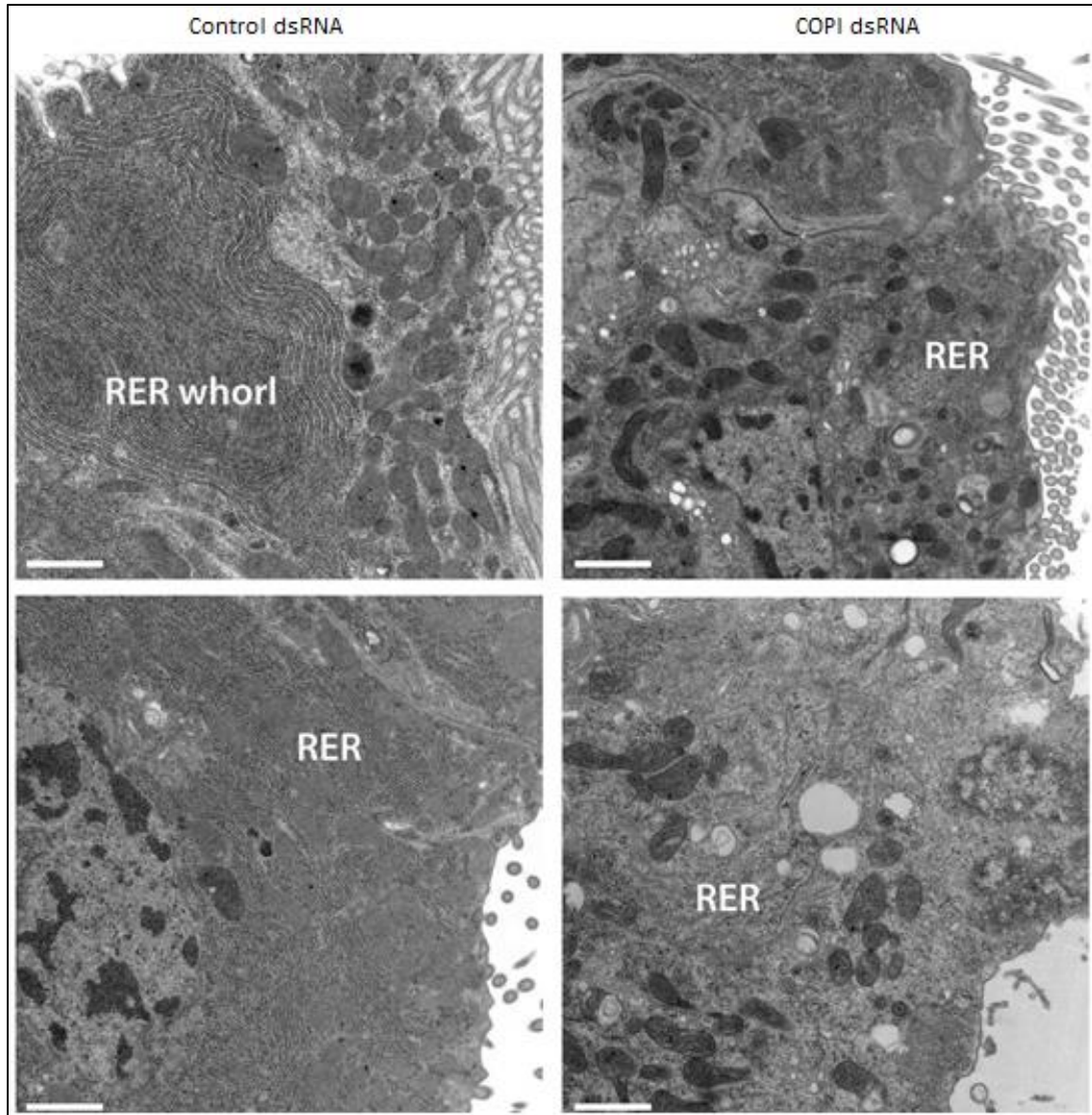
**Figure 1-7.** Life cycle of digestive cells in the midgut of *T. urticae* ©. Top row depicts sections with the budding process in focus stained in Oil Red-O (cryosection), Fast Green and Safranin-O (paraffin section) and Hematoxylin and Eosin (paraffin section) from left to right respectively. Middle row depicts DAPI staining for nuclei of digestive cells as they progressively bud and detach from the midgut epithelium from left to right respectively. Bottom row depicts the six distinct stages of the digestive cell life cycle from left to right respectively which involves the consolidation of smaller vesicles into a single large vacuole that darkens and expands with digested plant material (Bensoussan et al., 2018). Refer to Appendix A for figure permissions and copywrite agreement from the Creative Commons Attribution 4.0 License.

The most objective way to observe normal midgut function for *T. urticae* is to look at the ratio of juvenile to mature digestive cells, as well as the total number of digestive cells within the midgut. Since mature digestive cells are expelled as waste, a constant level of production of digestive cells in the midgut, reflected in a certain ratio between juvenile and mature digestive cells is required to keep up with the normal nutritional and energy demands of *T. urticae*.

Isoe et al., (2011) observed that silencing the COPI system in the mosquito *Aedes aegypti* produced a visible phenotype after feeding on bloodmeal and caused mortality soon after (Figure 1-8). Plastic TEM sections revealed that the phenotype was caused by a breakage in the midgut epithelial layer upon its expansion after feeding. This breakage allowed bloodmeal to seep throughout the body producing the visible phenotype. Silencing of the *COPI* system was observed to cause swelling of the rough endoplasmic reticulum in midgut epithelial cells and presumably made the cells susceptible to breakage during expansion (Figure 1-9). For *T. urticae*, a similar dysfunctional cellular mechanism within midgut epithelial cells could be responsible for the visible phenotypes of silencing. This would explain the changes in the organization of the midgut epithelium and clarify if these changes would also affect the budding behavior and maturation of the midgut digestive cells.



**Figure 1-8.** Phenotype of COPI RNAi-silenced *Aedes aegypti* ©. Gene-specific dsRNA is microinjected into the midgut, which was for rest 48-72 h prior to blood feeding. After feeding on blood mosquitoes die soon after. The blood meal can be seen to seep throughout the entire body (white arrow), as compared to control silenced mosquitoes which suffered no negative effects after feeding. The leakage of blood suggests a breakage somewhere in the midgut environment after expanding to accommodate digestion (Isoe et al., 2011). Refer to Appendix A for figure permissions and copywrite agreement from PNAS.

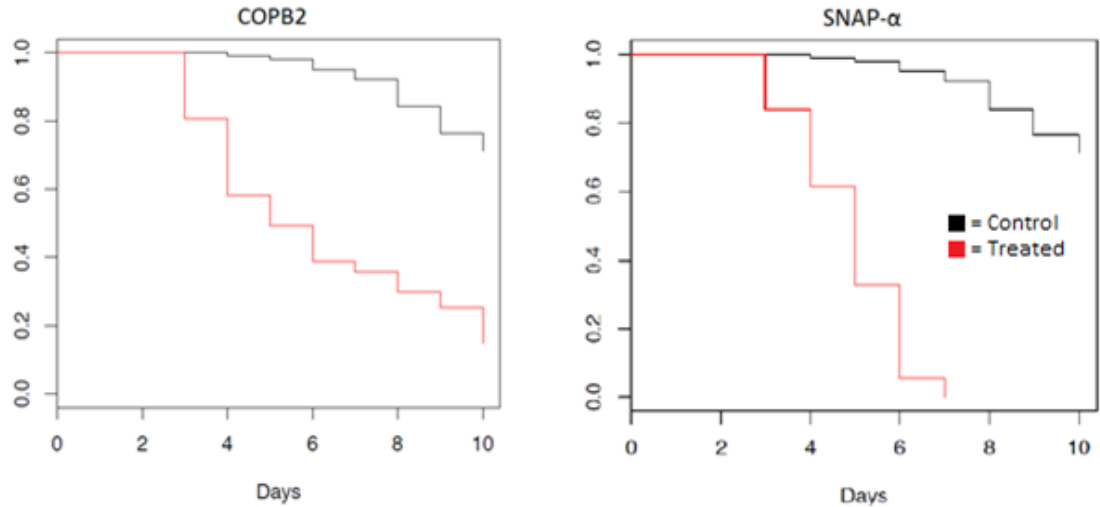


**Figure 1-9.** TEM image of COPI RNAi-silenced *Aedes aegypti* ©. Both the left and right sides depict midgut epithelial cells feeding after RNAi silencing for a control sequence and COPI respectively. In COPI silenced mosquitoes, the rough endoplasmic reticulum (RER) becomes swollen and is responsible for the breakage of the midgut epithelium and leakage of blood meal (Isoe et al., 2011). Refer to Appendix A for figure permissions and copywrite agreement from PNAS.

## 1.8 Delivery of RNAi to Suppress Gene Targets in *T. urticae*

The transcriptional silencing of *COPB2* and *SNAP- $\alpha$*  using RNAi (RNA interference) resulted in significantly affected spider mite fitness due to decreased fecundity and survivorship (Kwon et al., 2012). RNAi inhibits the expression of a specific gene by targeting mRNA (messenger ribonucleic acid) and preventing its translation. In *COPB2* transcriptionally silenced mites, there is a visible reduction in the black spotted globular digestive cells in the midgut, whereas *SNAP- $\alpha$*  and *V-ATPase* silenced mites display an extremely dark phenotype all throughout the body without the median segmentation seen in control mites. (Suzuki et al., 2017).

Both RNAi treatments for *COPB2* and *SNAP- $\alpha$*  were used on newly molted adult female mites and all died approximately a week later (Figure 1-10). Quantitative PCR of the mite samples showed that the transcript levels for the genes were still being expressed at 50% (Bensoussan et al., 2020). This indicates that the partial silencing of these gene transcripts is enough to cause reduced survivorship and produce a visible phenotype in *T. urticae*.



**Figure 1-10.** Survival ship curves of *T. urticae* after RNAi of *COPB2* and *SNAP-α* ©. Experiment was performed over the span of ten days after being treated with dsRNA and compared to control samples which had an intergenic region on the genome (*F3R3*) targeted as a control ( $p < 0.001$  for both experiments [ $n = 50/\text{treatment} \ \& \ 2$  replicates/treatment]) (Bensoussan et al., 2020). Refer to Appendix A for figure permissions and copywrite agreement from the Attribution-NonCommercial-NoDerivatives 4.0 International License.

## 1.9 Objectives

The primary focus of my work was to characterize the role of vesicular trafficking genes in the midgut of *T. urticae* by analyzing the effects of the transcriptional silencing of *COPB2*, *SNAP- $\alpha$* , and *V-ATPase*. The overall goal of my study was to link the physical phenotype arising from RNAi silencing to a biological mechanism using histological analysis and compare to what is already established in the literature. The three objectives are:

1. Examination of the morphological and qualitative changes of cells in the midgut following transcriptional silencing of *COPB2*, *SNAP- $\alpha$* , and *V-ATPase* compared to that of a control.
2. Identification and quantification of differences in digestive cell numbers between transcriptionally silenced mites of *COPB2*, *SNAP- $\alpha$* , and *V-ATPase* versus that of a control.
3. Comparison of the three aforementioned gene silencing phenotypes to that of comparable gene orthologues in another arthropod (*Tribolium castaneum*) in order to identify similarities.



## Chapter Two - Materials and Methods

### 2.1 Spider Mite Rearing

Egg-laying adult females were used to create developmentally synchronized populations in order to ensure the age of the sample population is uniform during treatment. For specimen collection, a colony of London reference strain of *T. urticae* kept on California red kidney bean (*Phaseolus vulgaris*) plants and maintained for more than 100 generation were used. Bean plants were grown in peat-vermiculite mixture at 24°C with 16:8 L:D photoperiod before they were used to replace the older infested bean plants in the spider mite colony. Only newly molted female adult mites gathered from synchronized populations were used for experiments as they are the most active in terms of metabolism. The following protocol for spider mite rearing was adapted from Suzuki et al. 2017.

To separate and treat specifically for a single life cycle stage, 50 female adult mites were taken from the colony and were taken to infest and lay eggs on a single bean leaf inside a vented container for 24 hours at 26°C. These female mites were then removed leaving eggs that will emerge uniformly as deutonymph teleiochrysalis females after 7 days at 27°C. These teleiochrysalis females were moved to a cotton wafer soaked in water placed inside of a Petri dish within a non-vented container for 2 days at 18°C at 100% relative humidity. The lid was then removed, and the container was stored at 26°C at 50% relative humidity to allow for the female mites to molt successfully and be collected for sample processing.

## 2.2 dsRNA Synthesis

The following dsRNA synthesis protocol was adapted from a lab member (Bensoussan et al., 2020). Total RNA was extracted from the frozen female mites with the RNeasy Mini Kit (Qiagen, Valencia, CA) and 3 $\mu$ g was used to make cDNA using the Superscript II cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Template preparation for dsRNAs targeting *F3R3* control, *COPB2*, *SNAP- $\alpha$* , and *V-ATPase* was performed by polymerase chain reaction amplification using specific forward and reverse primers with a minimal T7 promoter sequence at their 5' ends and either cDNAs or genomic DNA as a template (Table 2-1). The length of dsRNA fragments was ~ 600 bp per fragment, as smaller fragments are less effective in transcriptional silencing and larger fragments do not increase effectiveness (Bensoussan et al., 2020). Two separate non-overlapping fragments of dsRNA were used to ensure that the phenotype caused by transcriptional silencing cannot be attributed to off-target effects of silencing. Amplified DNA fragments were purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, New Taipei, Taiwan). Purified fragments were sequenced to confirm their identity. dsRNA fragments were synthesized using 1  $\mu$ g of DNA template with the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific) in 1.5-mL centrifuge tubes. dsRNAs were treated with DNase I for 30 min (Thermo Fisher Scientific), were desaturated at 95°C for 5 min, and were allowed to reach room temperature to facilitate the formation of dsRNA. dsRNA was purified by phenol/chloroform extraction followed by ethanol precipitation. dsRNA was dissolved in nuclease-free water and quantified using the NanoDrop (Thermo Fisher Scientific, Waltham, MA).

**Table 2-1.** List of *T. urticae* orthologues of *Tribolium castaneum* genes identified from previous studies as highly efficient RNAi targets ©. Tc-gene refers to the gene database listing identifier for *Tribolium castaneum*. Tu-ortholog and gene symbol refers to the gene database listing identifier for *T. urticae*. Refer to Appendix A for figure permissions and copywrite agreement from the Attribution-NonCommercial-NoDerivatives 4.0 International License.

Name	Tc-gene	Tu-ortholog	Gene symbol	Cellular function
Ras opposite	TC011120	tetur13g00570	TuRop	SNARE binding
Alpha soluble NSF attachment protein	TC013571	tetur06g05400	TuSnap $\alpha$	Vesicular transport
Shirbire	TC011058	tetur07g07190	TuShi	Actin and microtubule binding activity
Regulatory particle triple-A ATPase 3	TC007999	tetur32g01800	TuRpt3	Subunit of the 26S proteasome
Regulatory particle non-ATPase 7	TC006375	tetur33g01390	TuRpn7	Subunit of the 26S proteasome
Heat shock 70-kDa protein cognate 3	TC004425	tetur08g01320	TuHsc70-3	Protein folding
Signal recognition particle protein 54k	TC002574	tetur17g03110	TuSrp54	Protein secretion
Cactus	TC002003	tetur14g01540	TuCac	Regulation of gene expression
Inverse regulator a	TC008263	tetur04g01220	TuInr-a	RNA cleavage and polyadenylation factor
Gawky	TC006679	tetur05g07970	TuGw	miRNAs mediated gene silencing
Protein phosphatase 1alpha at 96a	TC015321	tetur15g03360 tetur04g05120	TuPp1 $\alpha$ -96a-A TuPp1 $\alpha$ -96a-B	Phosphatase; Regulation of gene expression
Coatomer subunit beta 2	-	tetur24g00150	TuCOPB2	Vesicular trafficking
Vacuolar-type H <sup>+</sup> -ATPase	-	tetur09g04140	TuVATPase	Proton pump, regulation of pH in organelles

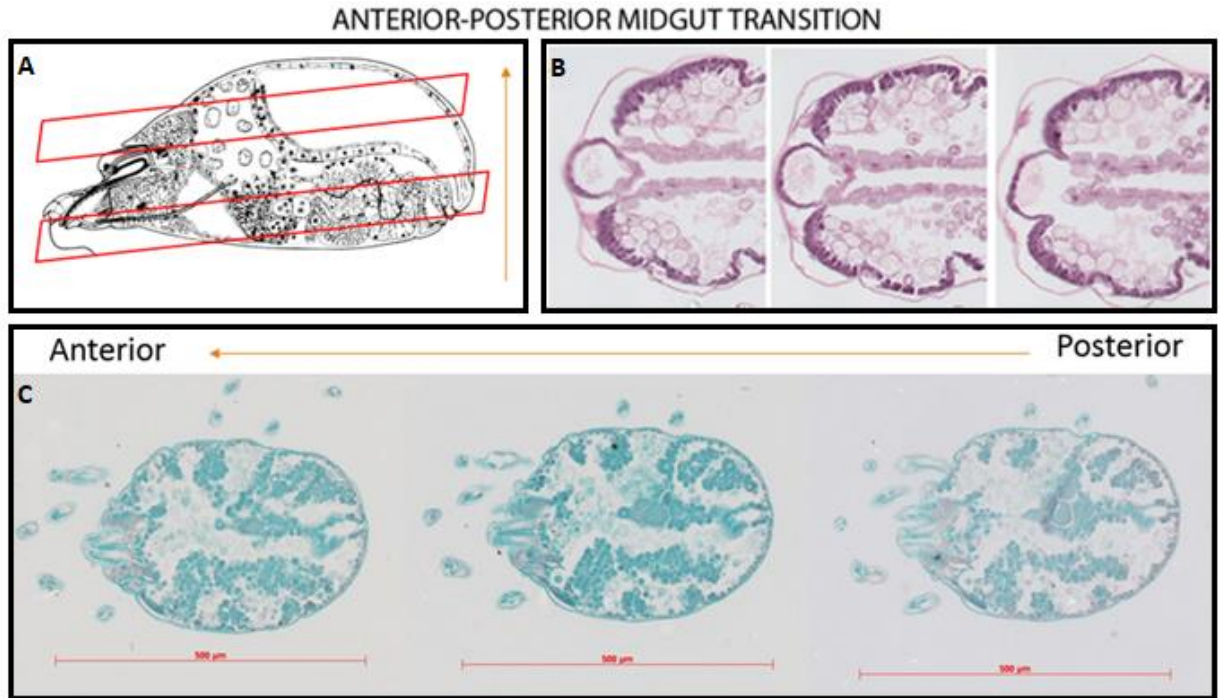
## 2.3 Application of RNAi

Soaking mites in dsRNA with blue dye provides a way to visually track whether the solution was taken up by the mite. This method adapted from Suzuki et al., 2017, using a Kimwipe cloth to absorb this solution and then placing mites overnight on this cloth to allow them to take up the solution. A 15  $\mu$ L solution of dsRNA (160 ng/ $\mu$ L; 0.1% v/v Tween 20) was given to 50 female adult mites of a respective treatment (*F3R3* control, *COPB2*, *SNAP- $\alpha$*  & *V-ATPase*) for 24 hours at 20°C via the Kimwipe method. After treatment, mites were washed in PBS and placed onto bean plants to recover. Spider mites phenotypically changed based on the gene silenced 48 h after treatment and died 3-10 days

after treatment (Figure 1-8). Therefore, my experiment focused on treated adult female mites 48 h after treatment to identify changes caused by the disruption of physiological processes and to prevent the attribution of morphological changes to the compounded effects of stress. To test for the effect of the presence of RNAi in cells, mites treated with RNAi for an intergenic region on the genome as a control (dsRNA *F3R3*) are compared histologically against mites treated for *COPB2*, *SNAP- $\alpha$* , and *V-ATPase*. The control treatment does not trigger RNAi but exposes mites to dsRNA treatment.

## 2.4 Fixation and Embedding of Samples for Microscopy

Spider mites were collected and immersed in 2.5% FAA (formalin, acetic acid, alcohol) overnight at 4°C to fix the samples. Fixed samples were washed in PBS (phosphate buffered saline) buffer with a detergent (1% v/v Triton X-100), then dehydrated via graded changes of ethanol (30%, 50%, 70%, 100%, 100% EtOH; 45 mins each). Specimens were then cleared with xylene, embedded in paraffin and placed into 1 × 1 cm molds in batches of 25 mites. Paraffin-embedded specimens were sectioned at a thickness of 10  $\mu$ m on the Leica RM2255 microtome and produced 3 slides worth of serial sections per block. Sections were then arranged into ribbons on microscope slides for clarity. Slides were kept at 4°C until they are deparaffinized for the staining of tissue (Figure 2-1).



**Figure 2-1.** Examples of the arrangement of paraffin samples into serial sections and histological dyes used ©. Individual sections are grouped and ordered based on the presence of anatomical structures and their relative sizes compared to adjacent samples. Refer to Appendix A for figure permissions and copywrite agreement from the Attribution-NonCommercial-NoDerivatives 4.0 International License.

(A) Sagittal cross-section of a mite depicting how the longitudinal sections (red boxes) were cut for the experiment; structures such as the posterior midgut tube, ovaries, and midgut epithelium will determine the approximate location of the section in the body.

(B) Longitudinal image set of a mite stained with Hematoxylin and Eosin. Hematoxylin stains DNA/RNA blue and Eosin stains the extracellular matrix and cytoplasm pink.

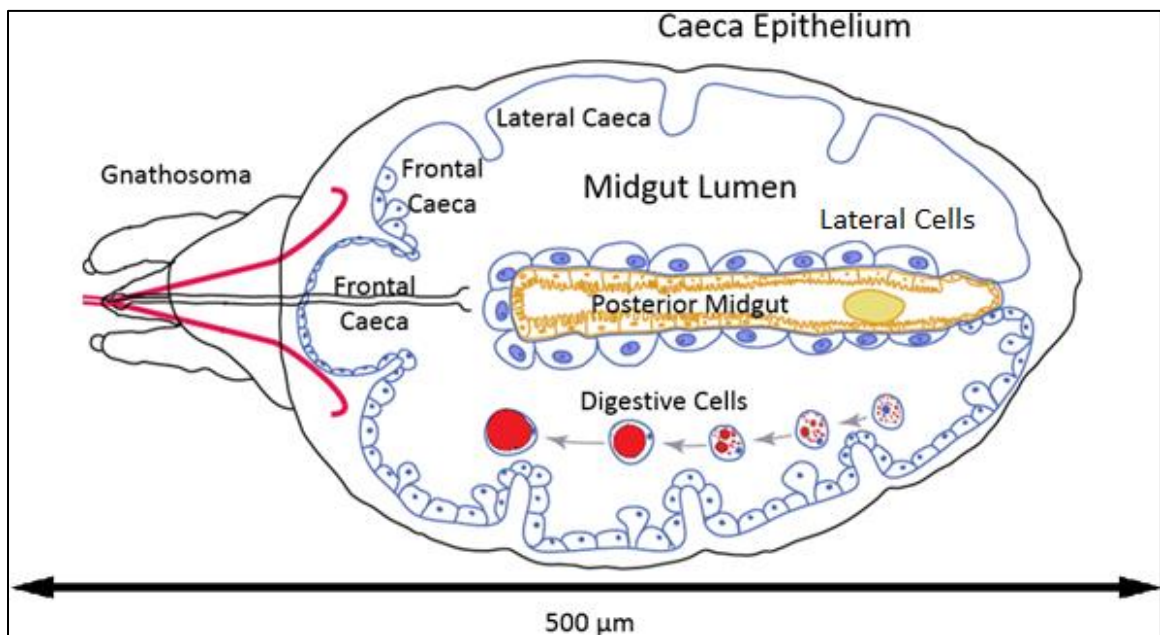
(C) Longitudinal image set of a mite stained with Fast Green and Safranin-O. Fast Green stains cellulose and cytoplasm green and Safranin-O stains DNA/RNA red.

Comparison of serial sections between RNAi induced two spotted spider mites and negative control RNAi silenced spider mites in terms of the type of midgut cells affected should reveal the role that the vesicular trafficking genes *COPB2*, *SNAP- $\alpha$*  and *V-ATPase* have in the establishment of normal gut tissues. Mounted slides were imaged with a microscope mounted camera so that a digital image can be kept for analysis and archiving. Hematoxylin and Eosin were used for staining paraffin sections as Hematoxylin stains DNA/RNA in cells and Eosin stains proteins, which assists in identifying qualitative changes in cells between the control and experimental group. Fast Green and Safranin-O are also dyes that were used on sections, with Safranin-O staining for DNA/RNA in cells and Fast Green staining for proteins.

## 2.5 Computational Analysis of Samples

Images were taken with a Zeiss LSM 510 at 40x magnification with Köhler illumination between imaging sessions to ensure consistency. Serial sections were composed based on the spatial position and relative proportion of prominent structures in the mite gut and the head region. Most notably, the size and position of the midgut caeca and the posterior midgut tube, as well as the presence of silk glands and ovaries indicate if the sectioned material is closer to the anterior or posterior end of the spider mite (Figure 2-2). For computational analysis, the program ImageJ was used with the FIJI add-on suite in order to allow for the quantification of data. Digital segmentation of gut tissue aided in highlighting possible structures resembling digestive cells and the consistent measurement of structures with regards to the micrometer scale.

For quantitative analysis, 20 histologically comparable mites (biological replicates) were selected from the serial sections of each of the 4 treatments (*F3R3* control, *SNAP-α*, *V-ATPase* & *COPB2*). Additionally, 2 adjacent serial images of the same mite (technical replicates) were used in calculating the average of digestive cells and the proportion of juvenile to mature digestive cells per section for statistical analysis (One-way ANOVA analysis followed by Tukey post-hoc test).



**Figure 2-2.** Morphological features of the midgut in *T. urticae* ©. Presence and size of certain structures allow for the spatial determination of where a specific section is located. Samples collected for quantitative use must have these labeled structures present in the image in order to allow for a comparative basis for analysis. A summary of the life cycle of the digestive cell is also included for reference, with the vacuole development shown in red from right to left. Image adapted with labels for structures (Bensoussan et al., 2018). Refer to Appendix A for figure permissions and copyright agreement from the Creative Commons Attribution 4.0 License.

## Chapter Three – Results

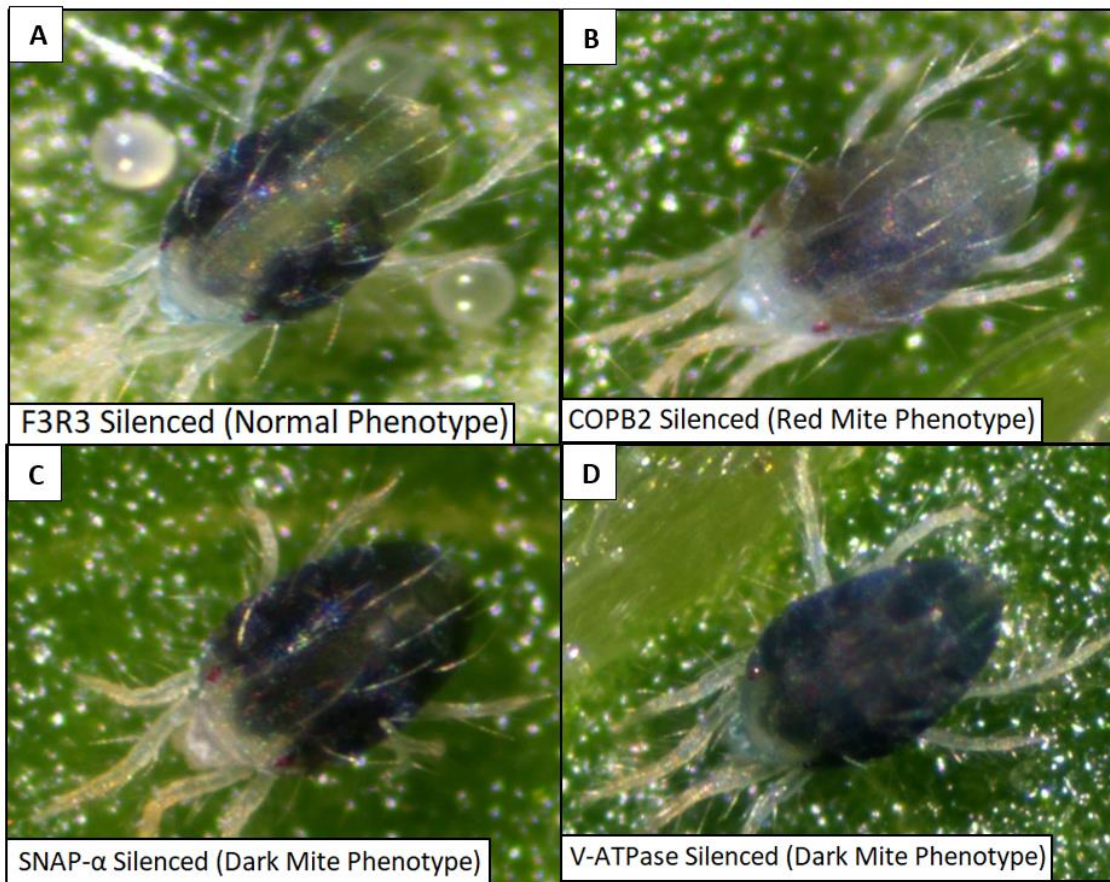
### 3.1 Morphological Differences Observed Between Phenotypes

#### 3.1.1 Changes in the Life cycle of Digestive Cells

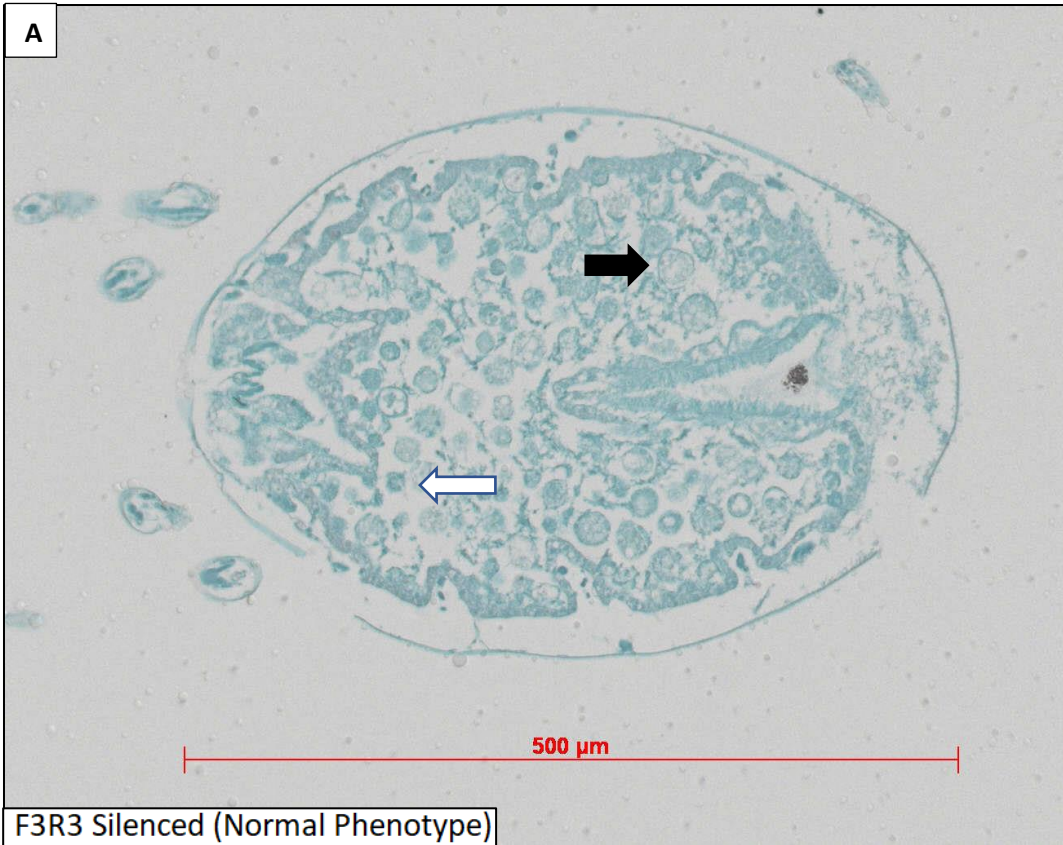
There was an increased number of mature digestive cells in the *V-ATPase* & *SNAP- $\alpha$*  RNAi silenced mites and the increased number of juvenile digestive cells in the *COPB2* RNAi silenced mites (Figure 3-2). Furthermore, as *SNAP- $\alpha$*  RNAi silenced mites had lighter and more consistent pigmentation of their digestive cells than *V-ATPase* RNAi silenced mites (Figure 3-3).

The distribution of the digestive cells in *COPB2* RNAi silenced mites were different from other treatments, as the juvenile digestive cells accumulated in large groups close to the caeca near the midgut epithelium rather than being evenly spread out (Figure 3-2; Figure 3-3).



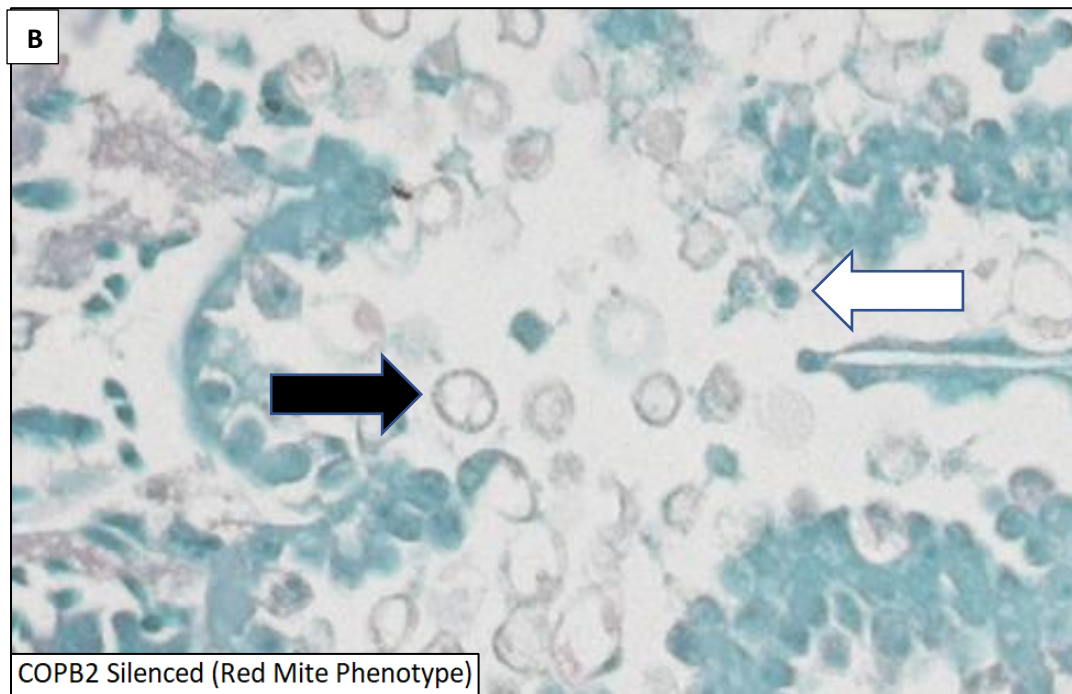
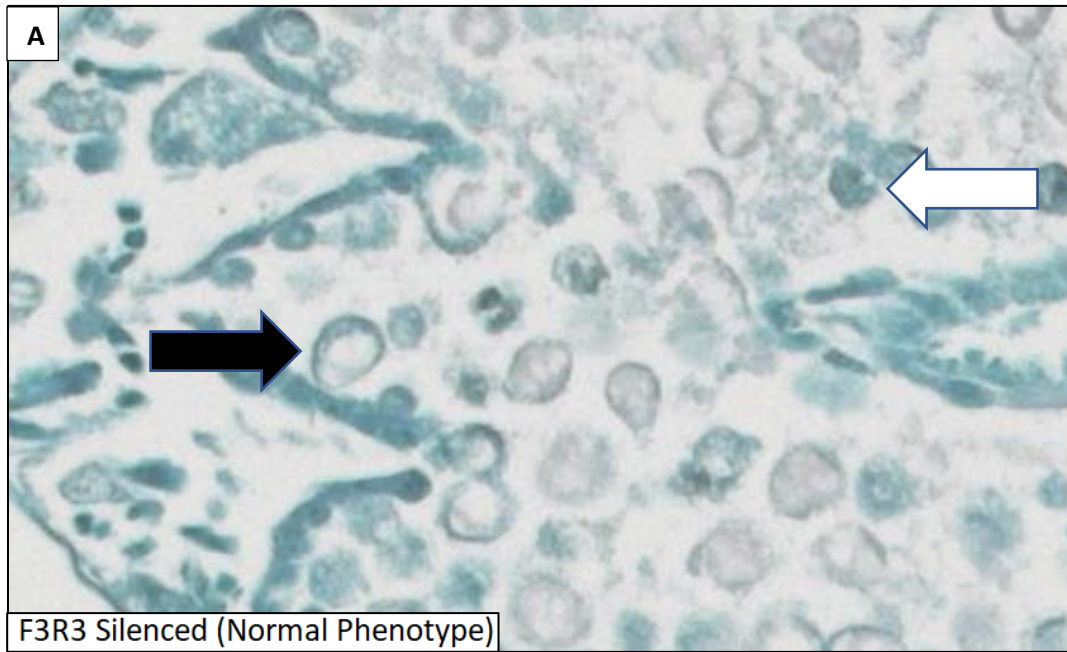


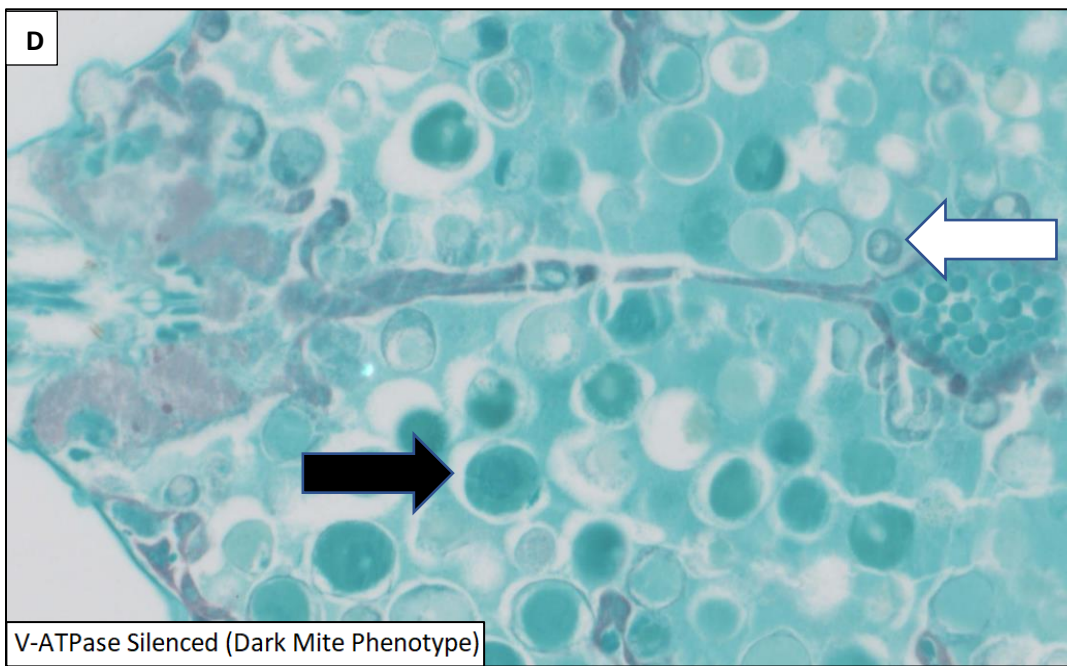
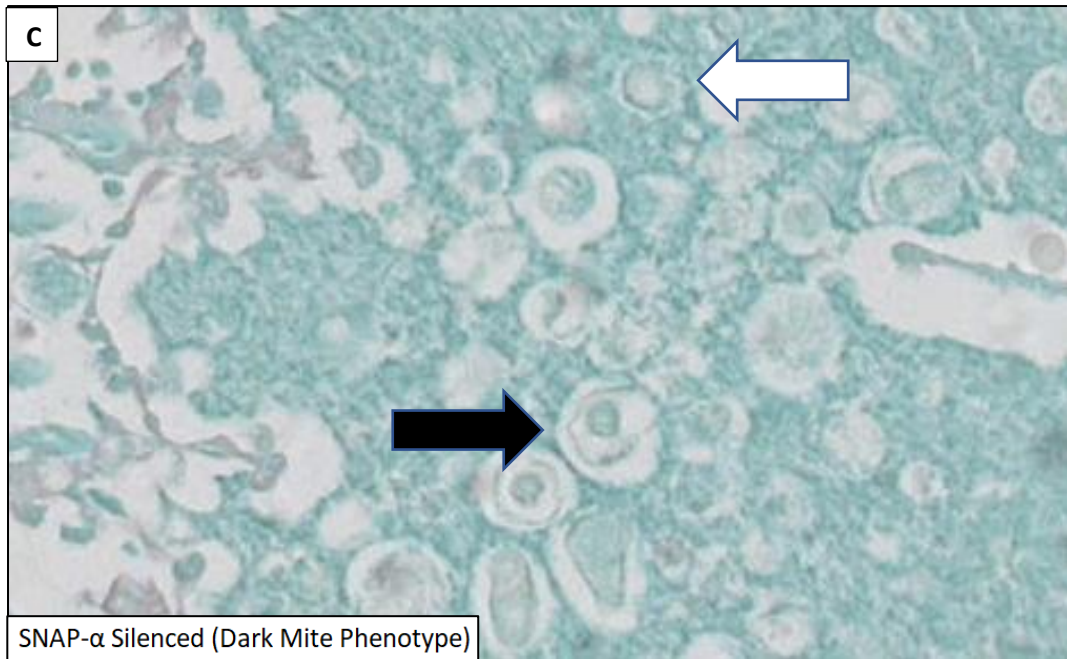
**Figure 3-1.** Summary of whole-body RNAi phenotypes in *T. urticae*. *F3R3* control silenced (**A**), *COPB2* silenced (**B**), *SNAP-α* silenced (**C**), and *V-ATPase* silenced (**D**). *F3R3* silenced mites appear similar to untreated mites (Figure 1-1). *COPB2* has a “colorless” appearance and *SNAP-α* & *V-ATPase* has a “dark” mite appearance 48 h after resting from RNAi silencing treatment.





**Figure 3-2.** Comparison of paraffin embedded RNAi silenced phenotypes. *F3R3* control silenced (**A**), *COPB2* silenced (**B**), *SNAP- $\alpha$*  silenced (**C**), and *V-ATPase* silenced (**D**). Images depict a distinction between mature (black arrows) and juvenile (white arrows) digestive cells. The main morphological landmarks used for quantitative analysis include the size and position of the posterior midgut tube, the lack of ovary structures and the presence of three frontal caeca. Mites are positioned with the anterior end on the left side and the posterior end on the right side for all images.





**Figure 3-3.** Comparison of digestive cells in RNAi silenced phenotypes in *T. urticae*. Images depict a distinction between mature (black arrows) and juvenile (white arrows) digestive cells. In *F3R3* control RNAi silenced mites (**A**), juvenile digestive cells appear as small defined nucleated bodies with strong pigmentation and mature digestive cells appear as larger bodies that lack pigmentation with their nuclei offset towards the outer membrane. Both types of digestive cells are seen to be evenly spaced throughout the midgut lumen. In *COPB2* RNAi silenced mites (**B**), both types of digestive cells are like that of the control; however, juvenile digestive cells are seen to aggregate and stick in large masses. In *SNAP- $\alpha$*  RNAi silenced mites (**C**), juvenile digestive cells appear like the control and mature digestive cells are found to have stronger pigmentation centered around a darker body within the digestive cell. The midgut lumen is also seen to be strongly stained which is a feature missing from the control RNAi silenced mites. In *V-ATPase* RNAi silenced mites (**D**), both types of digestive cells are structurally like *SNAP- $\alpha$*  silenced mites, but with stronger staining of the cell body. The midgut lumen is also like that of *SNAP- $\alpha$*  RNAi silenced mites as there is considerable staining.

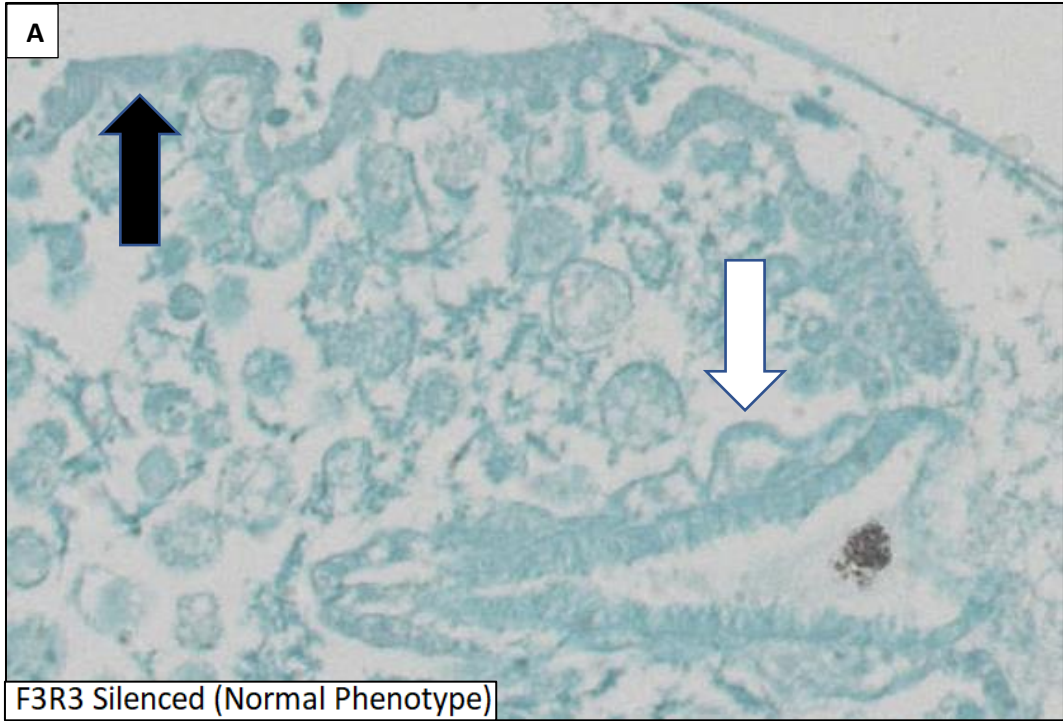
### 3.1.2 Lumen, Caeca Epithelium and Lateral Cells

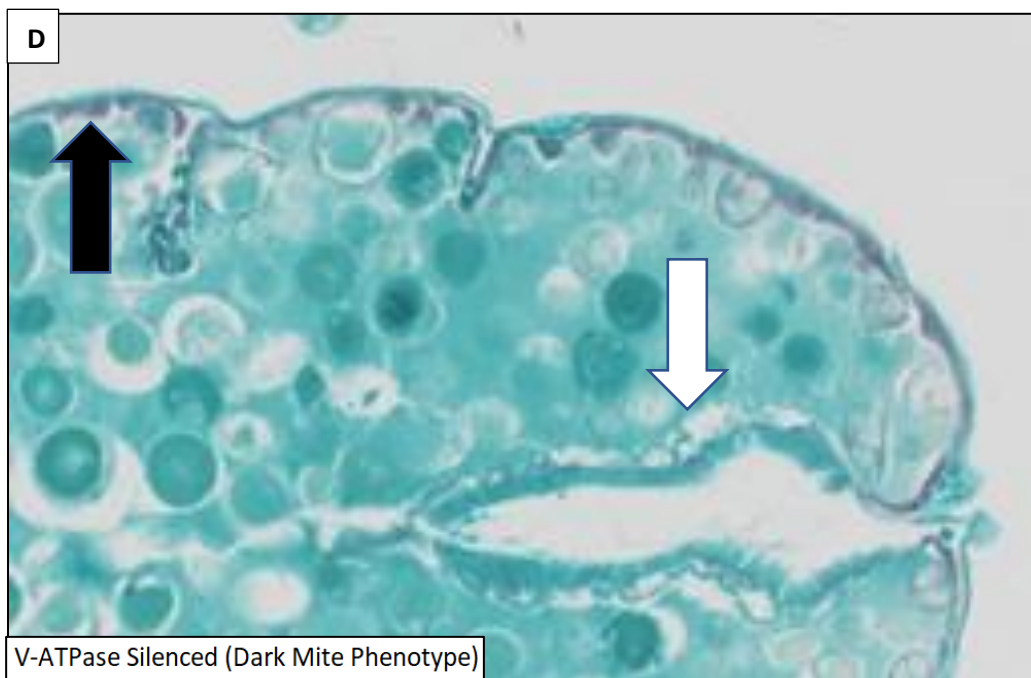
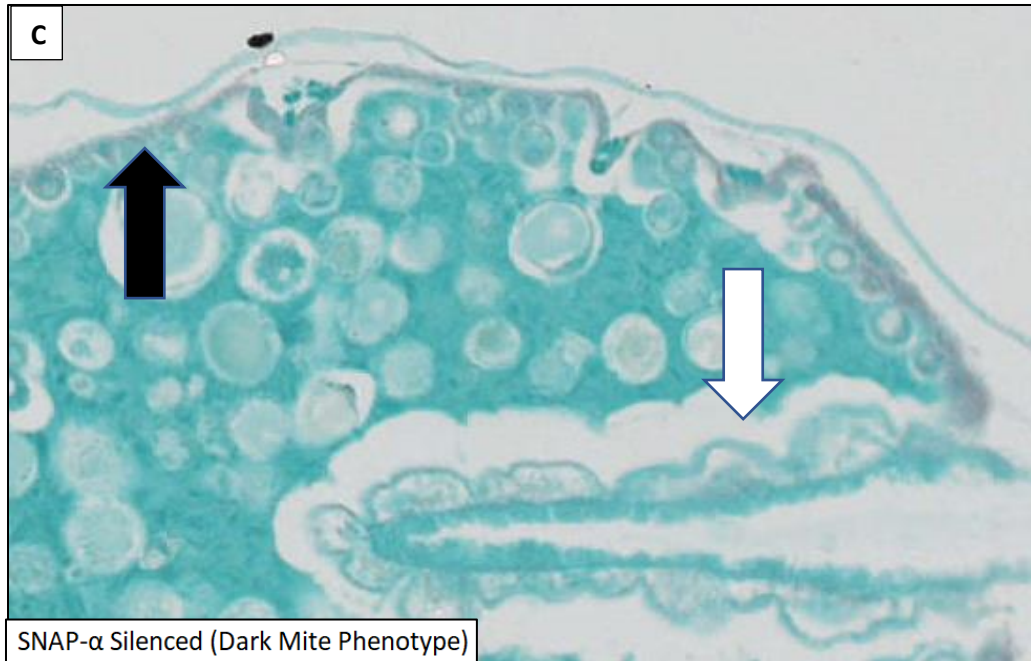
The midgut lumen of *SNAP- $\alpha$*  and *V-ATPase* silenced mites were seen to be extensively pigmented in all samples. This pigmentation of the midgut lumen is not seen in either *COPB2* or the *F3R3* control RNAi silenced mites (Figure 3-2).

In *F3R3* control RNAi silenced mites the caeca epithelium is composed of a simple thin squamous layer of cells that is consistent all around the midgut and are slightly thinner towards the anterior end of the mite (Figure 3-4 A). In mites with the silenced *SNAP- $\alpha$*  and *COPB2* genes, the caeca epithelium cells became distinctly rounded cells that invaginate towards the midgut lumen (Figure 3-4 B & C). In mites with the silenced *V-ATPase* gene, the caeca epithelium becomes flattened and thinner in comparison to the *F3R3* control silenced mites (Figure 3-4 A & D).

The lateral cells of the posterior midgut, which face away from the posterior midgut tube into the midgut lumen (Figure 2-2), were thicker after RNAi silencing for *SNAP- $\alpha$*  and *COPB2* and thinner with *V-ATPase* (Figure 3-4).







**Figure 3-4.** Comparison of caeca epithelium and lateral cells in RNAi silenced phenotypes in *T. urticae*. Images depict cells of the caeca epithelium (black arrows) and the lateral cells that face away from the posterior midgut tube (white arrows). In *F3R3* control RNAi silenced mites (**A**), the caeca epithelium of the midgut is composed of a simple squamous layer of cells which appear to bud off to form the juvenile digestive cells of the midgut. For the experimental RNAi silenced mites (**B**, **C**, & **D**), this caeca epithelium was affected in terms of its morphology. The lateral cells were also affected by RNAi silencing.

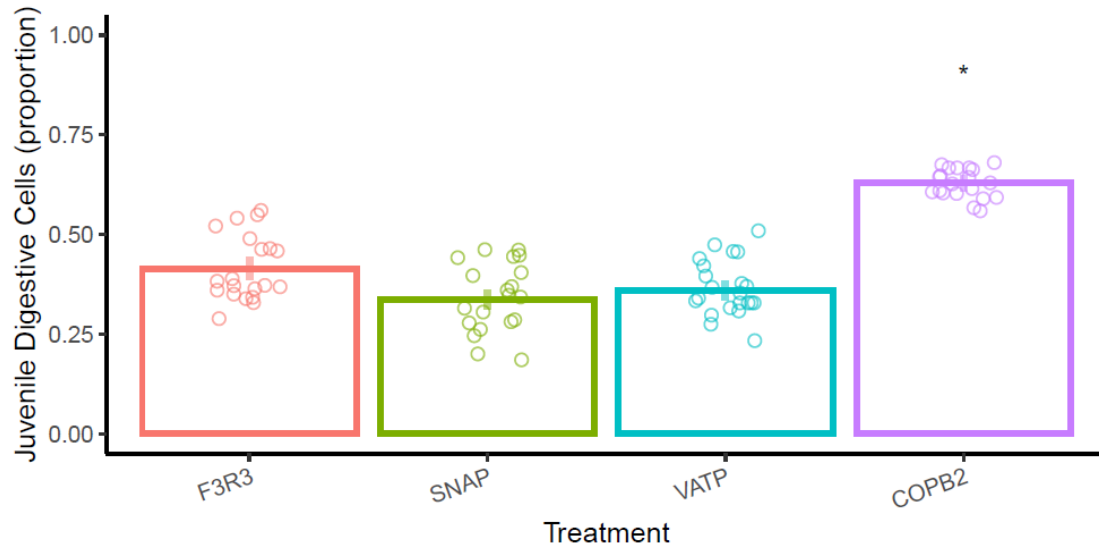
## 3.2 Quantitative Differences Observed Between Phenotypes

### 3.2.1 Ratio of Juvenile to Mature Digestive Cells

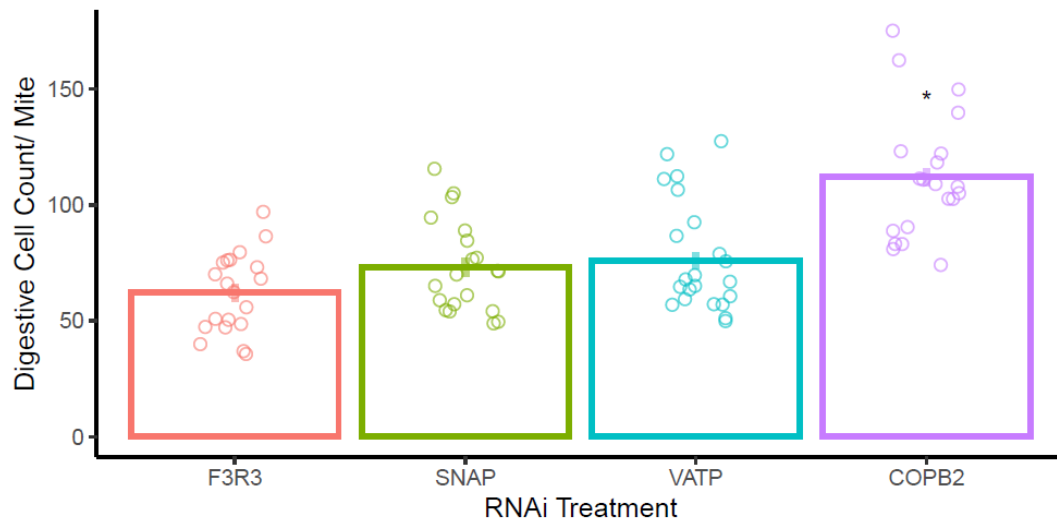
Only in *COPB2* RNAi silenced mites, the proportion of smaller juvenile to larger mature digestive cells in the midgut was 1.6-fold higher compared to *F3R3* control silenced mites (Figure 3-5,  $p < 0.05$ ). In the "dark" mite phenotypes of *SNAP- $\alpha$*  and *V-ATPase* RNAi silenced mites, the ratio of smaller juvenile to larger digestive cells was not detected to differ significantly compared to *F3R3* control silenced mites. (Figure 3-5).

### 3.2.2 Overall Number of Digestive Cells

In *COPB2* RNAi silenced mites, the overall number of digestive cells in the midgut was 1.5-fold higher compared to *F3R3* control silenced mites (Figure 3-6,  $p < 0.05$ ). In *SNAP- $\alpha$*  and *V-ATPase* RNAi silenced mites, there was no statistically significant difference detected between the experimental results and the *F3R3* control silenced mites (Figure 3-6).



**Figure 3-5.** Proportion of juvenile digestive cells compared to mature digestive cells in *T. urticae* RNAi silencing treatment. Error bars are +/- 1 standard error, (One-way ANOVA analysis followed by Tukey post-hoc test, \* =  $p < 0.05$  as compared to *F3R3* silenced control [n = 20 biological replicates/treatment & 2 technical replicates/biological replicate]). Only *COPB2* silencing was detected to have an increased proportion of juvenile digestive cells compared to *F3R3* control silencing.



**Figure 3-6.** Averages digestive cell count per *T. urticae* RNAi silencing treatment. Error bars are +/- 1 standard error, (One-way ANOVA analysis followed by Tukey post-hoc test, \* =  $p < 0.05$  as compared to *F3R3* control [n = 20 biological replicates/treatment & 2 technical replicates/biological replicate]). Only *COPB2* silencing was detected to have an increased average of digestive cells compared to *F3R3* control silencing.

### 3.3 Qualitative Differences Observed Between Orthologues

Between the dark mite silencing phenotypes of *SNAP- $\alpha$* , *V-ATPase*, *ROP*, *RPN7* and *SRP54* (Figure 3-7 B - F) and the colorless mite silencing phenotypes *COPB2* and *HSC70* (Figure 3-7 G & H), certain distinguishing features are shared in terms of effects caused by RNAi silencing. Some of these properties include the pigmentation of the midgut lumen and the whole-body phenotype (Table 3-1). It is noted that RNAi silencing changes were not observed in tissue such as the ovaries or eggs (Figure 3-7).

In the dark mite silencing phenotypes, *SNAP- $\alpha$*  and *SRP54* (Figure 3-8 B & F) appear very similar in terms of the strong pigmentation defining the nuclei of juvenile digestive cells as well as the weak pigmentation present in the mature digestive cells. For *V-ATPase*, *ROP* and *RPN7* (Figure 3-8 C, D, & E), the mature digestive cells are lighter in pigmentation compared to the surrounding midgut lumen and more numerous compared to the juvenile digestive cells, which appear darkly pigmented and consistent throughout. The pigmentation of the midgut lumen is lighter in *SNAP- $\alpha$*  and *SRP54* (Figure 3-8 B & F) compared to *V-ATPase*, *ROP* and *RPN7* (Figure 3-8 C, D, & E). For the colorless mite silencing phenotypes of *COPB2* and *HSC70* are similar (Figure 3-8 G & H), as there is a significant increase in the darkly pigmented juvenile digestive cells compared to the mature digestive cells, which have little pigmentation. However, *HSC70* (Figure 3-8 H) has pigmentation of the midgut lumen, which is not present in *COPB2* (Figure 3-8 G). For the silencing phenotype of *RPT3* (Figure 3-8 I), the juvenile and mature digestive cells appear to be hollowed out and are unlike any other silencing phenotype. The midgut lumen

is also pigmented similar to the dark mite phenotype, but the juvenile digestive cells aggregate similar to those of the colorless mite phenotype.

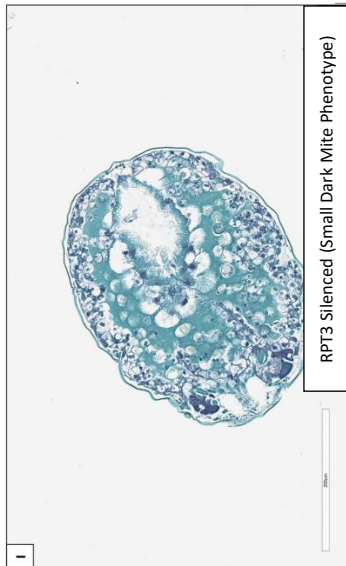
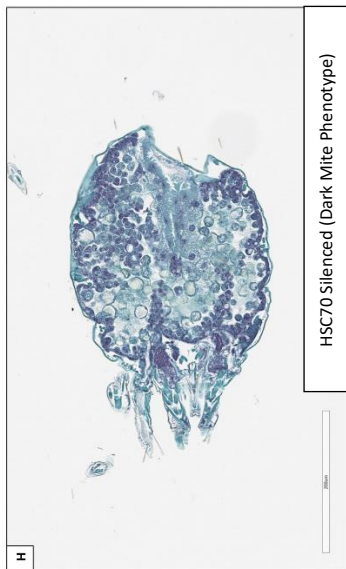
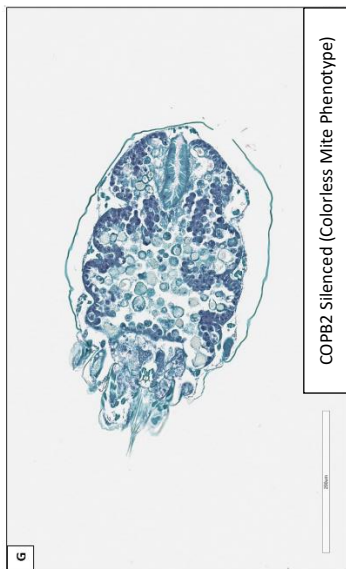
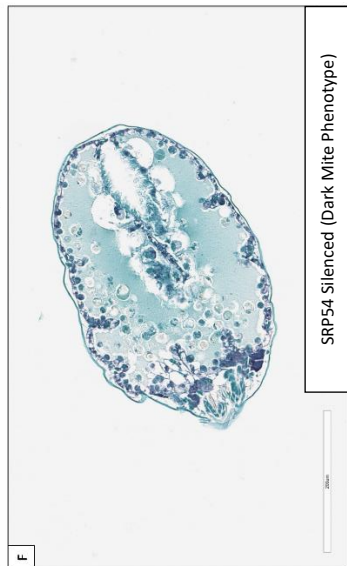
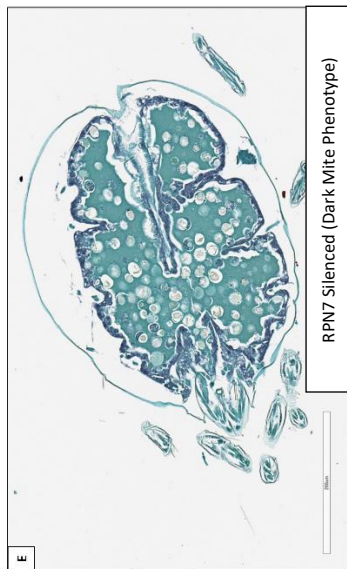
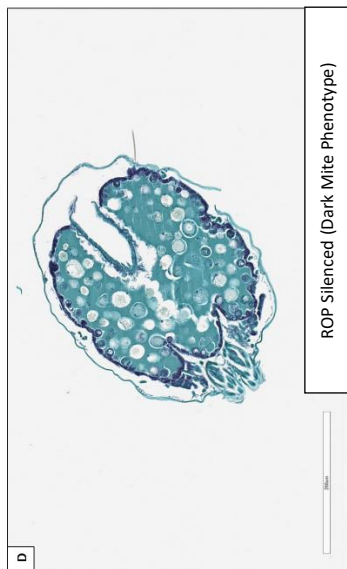
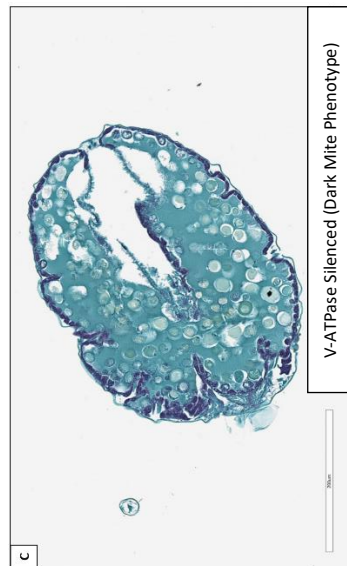
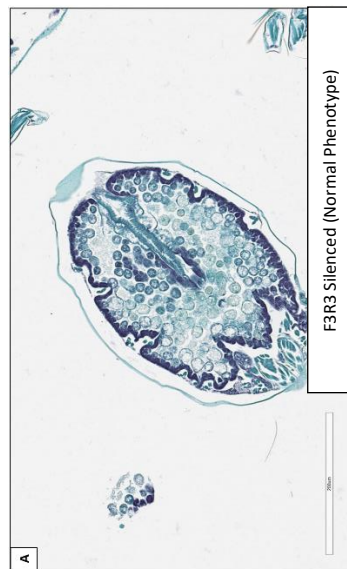
For the silencing phenotypes of *SNAP- $\alpha$* , *RPN7*, *COPB2* and *HSC70* (Figure 3-9 B, E, G & H), these phenotypes displayed a midgut epithelium that is simple and squamous. However, the digestive cells which are budding appear to be arrested in the early stages for *SNAP- $\alpha$* , *RPN7* and *COPB2* and in the late stage for *HSC70*. For the silencing phenotypes of *V-ATPase* and *ROP* (Figure 3-9 C & D), the midgut epithelium appears to adopt a thinner and flattened organization. For the silencing phenotype of *SRP54* and *RPT3* (Figure 3-9 F & I), the midgut epithelium appears similar to the digestive cells and appear hollowed out with the exception of the nucleus. The midgut epithelium is also seen to flatten with *SRP54* and adopt a pseudostratified appearance for *RPT3*.

For the silencing phenotypes of *SNAP- $\alpha$* , *RPN7*, *COPB2* and *HSC70* (Figure 3-10 B, E, G & H), the lateral cells appear to become distorted in shape and thicker. For the silencing phenotypes of *V-ATPase* and *ROP* (Figure 3-10 C & D), the lateral cells appear to be thinner. For the silencing phenotype of *SRP54* and *RPT3* (Figure 3-10 F & I), the lateral cells appear similar to the digestive cells and appear empty inside apart from the nucleus and are extremely enlarged.

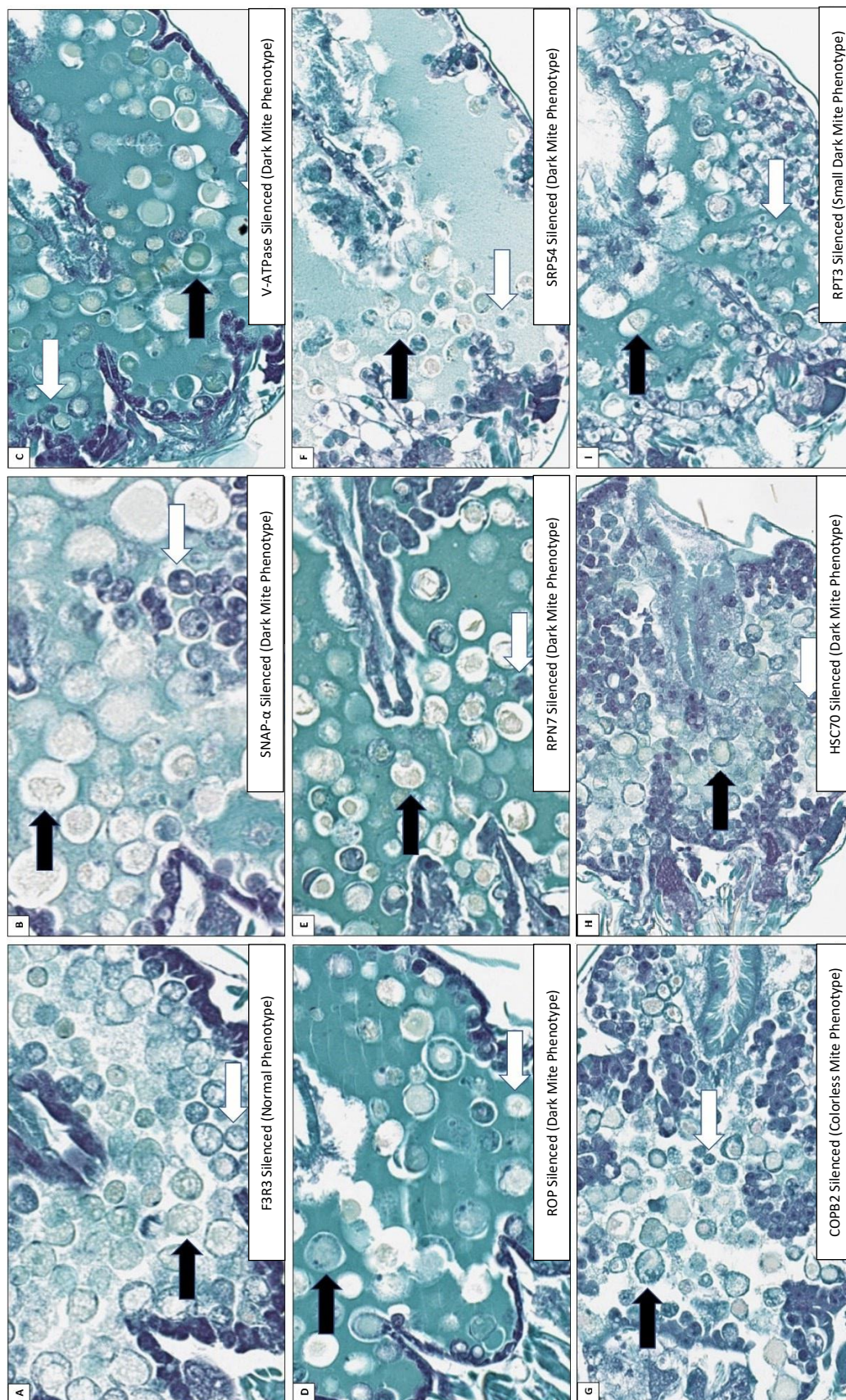


**Table 3-1.** Summary of phenotypes upon RNAi of *T. urticae* orthologues derived from *Tribolium castaneum* RNAi sensitive genes. Genes denoted by green are those which were the focus of this paper. Genes denoted by yellow are orthologue genes in *T. urticae* identified from the red flour beetle (*Tribolium castaneum*). The chart organizes various qualitative changes from digestive cell behavior and structures in the midgut as compared to *F3R3* control silenced mites. The chart and subsequent figures were organized based on the whole-body phenotype (dark mite, colorless mite, & small black mite).

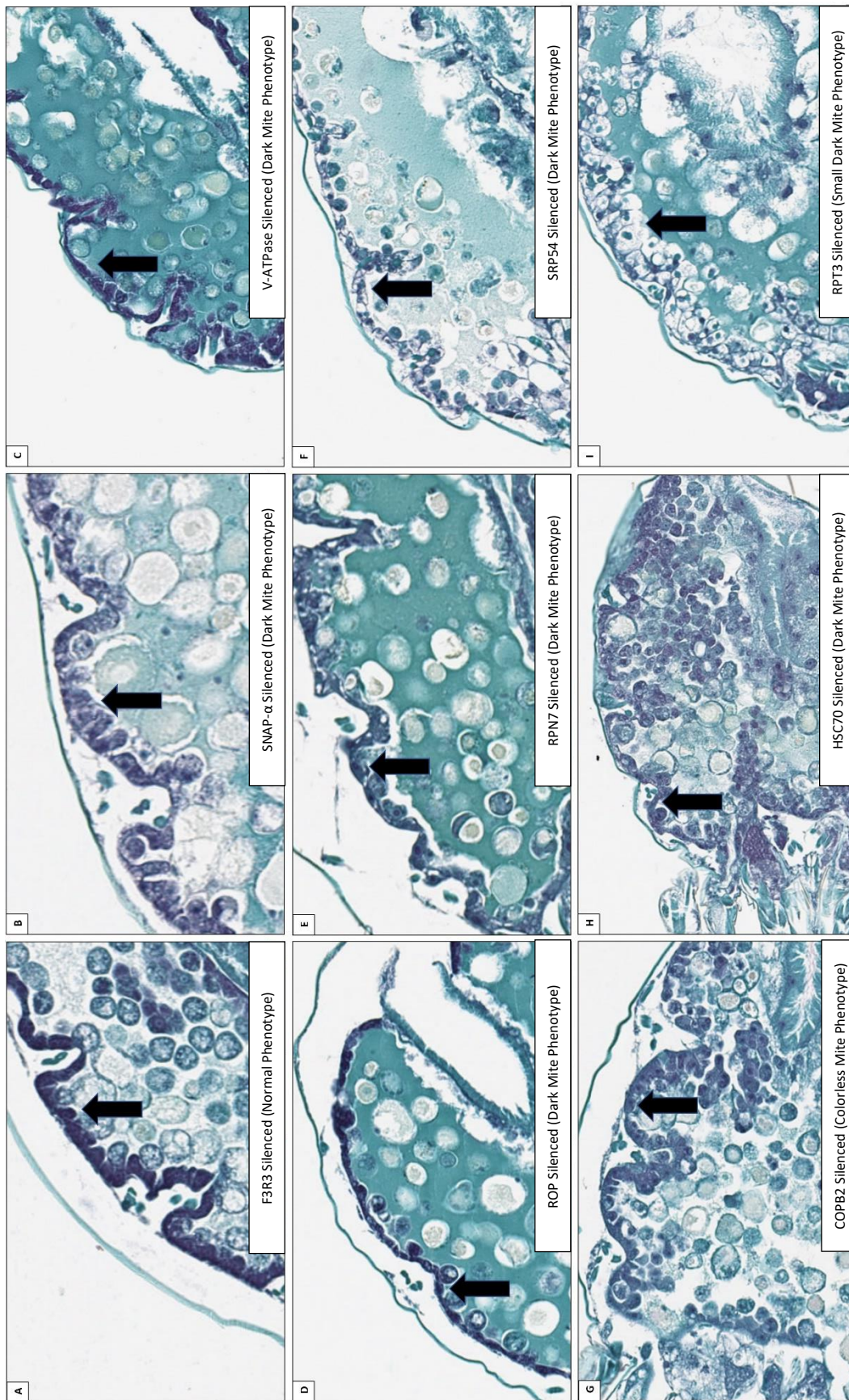
RNAi target	Gene Function	RNAi Whole Body Phenotype	Midgut Epithelium	Midgut Lumen Pigmentation	Ratio of Mature/Juvenile Digestive Cells	Number of Digestive Cells	Digestive Cell Behavior	Lateral Cells of the Posterior Midgut
<b>SNAP-<math>\alpha</math></b>	Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus. Belongs to the SNAP family.	Dark Mite	Budding cells are arrested in early stage	Light lumen staining	Appears to have more mature digestive cells to juvenile digestive cells	Unclear increase	Mature cells become heavily pigmented	Lateral cells are thicker
<b>V-ATPase</b>	Acidify a wide array of intracellular organelles and pump protons across the plasma membranes of numerous cell types.	Dark Mite	Thin & flattened epithelium	Dark lumen staining	Appears to have more mature digestive cells to juvenile digestive cells	Unclear increase	Mature cells become heavily pigmented	Lateral cells are thinner
<b>ROP</b>	Associated with the docking of vesicles involved in exocytosis, and predicted to have SNARE binding activity.	Dark Mite	Thin & flattened epithelium	Dark lumen staining	Appears to have more mature digestive cells to juvenile digestive cells	Unclear	Mature cells become heavily pigmented	Lateral cells are thinner
<b>RPN7</b>	A regulatory subunit of the 26S proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins.	Dark Mite	Budding cells are arrested in early stage	Dark lumen staining	Appears normal	Unclear	Mature cells become heavily pigmented	Lateral cells are thicker
<b>SRP54</b>	Cotranslational targeting of proteins destined for secretion or membrane insertion as part of the SRP cycle.	Dark Mite	Expanded empty epithelial cells	Light lumen staining	Appears normal	Unclear	Mature cells become slightly pigmented	Lateral cells are larger and extremely perturbed
<b>COPB2</b>	The Golgi coatamer complex that constitutes the coat of nonclathrin-coated vesicles and is essential for Golgi budding and vesicular trafficking.	Small Red Mite	Budding cells are arrested in early stage in large groups	Lumen staining not present	Significantly more juvenile digestive cells to mature digestive cells	Overall Significant Increase	Juvenile cells appear to clump in large groups near the epithelium	Lateral cells are thicker
<b>HSC70</b>	Facilitates the proper folding of newly translated and misfolded proteins, as well as stabilize or degrade mutant proteins.	Small Red Mite	Budding cells are arrested in late stage	Light lumen staining	Appears to be more juvenile digestive cells to mature digestive cells	Appears to increase	Mature cells contents lack pigmentation	Lateral cells appear to thicken and clump together
<b>RPT3</b>	Part of the 26S proteasome and is involved in the ATP-dependent degradation of ubiquitinated proteins.	Small Dark Mite	Expanded epithelial cells that are pseudostratified	Dark lumen staining	Unclear	Appears to increase	Juvenile cells appear heavily pigmented compared to matured cells	Lateral cells are larger and extremely perturbed



**Figure 3-7.** Comparison of phenotypes upon RNAi of *T. urticae* orthologues derived from the following *Tribolium castaneum* RNAi sensitive genes for comparison: *F3R3* control silenced (**A**), *SNAP- $\alpha$*  silenced (**B**), *V-ATPase* silenced (**C**), *ROP* silenced (**D**), *RPN7* silenced (**E**), *SRP54* silenced (**F**), *COPB2* silenced (**G**), *HSC70* silenced (**H**) and *RPT3* silenced (**I**). The following images in this paper are taken at 40x magnification on a Zeiss LSM 510 microscope, stained with Fast Green and Safranin-O, organized on ImageJ and are taken with consideration to the previously established morphological landmarks in order to establish a comparable basis for study (Figure 2-2).

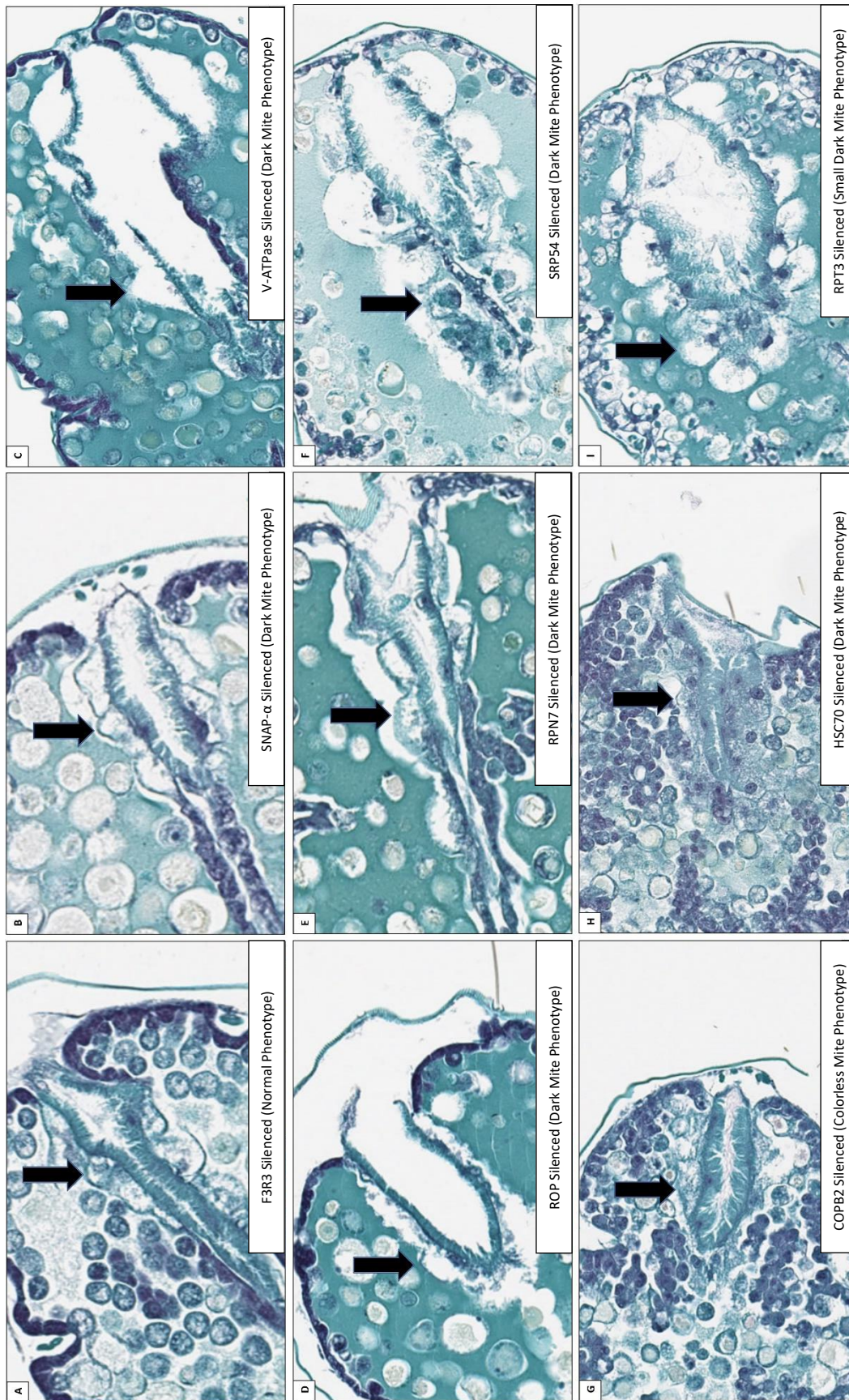


**Figure 3-8.** Comparison of digestive cell phenotypes upon RNAi of *T. urticae* orthologues derived from *Tribolium castaneum* RNAi sensitive genes. Images depict a distinction between mature (black arrows) and juvenile (white arrows) digestive cells. *SNAP- $\alpha$*  and *SRP54* (**B & F**) appear similar in terms of the strong pigmentation defining the nuclei of juvenile digestive cells as well as the weak pigmentation present in mature digestive cells. In *V-ATPase*, *ROP* and *RPN7* (**C, D, & E**), the mature digestive cells are lighter in pigmentation compared to the surrounding midgut lumen and more numerous compared to the juvenile digestive cells which appear darkly pigmented and consistent throughout. Pigmentation of the midgut lumen is lighter in *SNAP- $\alpha$*  and *SRP54* (**B & F**) compared to *V-ATPase*, *ROP* and *RPN7* (**C, D, & E**). *COPB2* and *HSC70* are similar (**G & H**), However, *COPB2* (**G**) appears to not have some pigmentation of the midgut lumen that is present in *HSC70* (**H**). For *RPT3* (**I**), the juvenile and mature digestive cells appear to be hollowed out and are unlike any other silencing phenotype. The midgut lumen is also pigmented similar to the dark mite phenotype, but the juvenile digestive cells are seen to aggregate together similar to that of the colorless mite phenotype.



**Figure 3-9.** Comparison of caeca epithelium phenotypes upon RNAi of *T. urticae* orthologues derived from *Tribolium castaneum* RNAi sensitive genes. Images depict cells of the caeca epithelium (black arrows). For *SNAP- $\alpha$* , *RPN7*, *COPB2* and *HSC70* (**B**, **E**, **G** & **H**), the midgut epithelium is simple and squamous. The digestive cells which are budding appear to be arrested in the early stages for *SNAP- $\alpha$* , *RPN7* and *COPB2* and in the late stage for *HSC70*. For *V-ATPase* and *ROP* (**C** & **D**), the midgut epithelium appears to adopt a thinner and flattened organization. For the silencing phenotype of *SRP54* and *RPT3* (**F** & **I**), the midgut epithelium appears similar to the digestive cells and appear hollowed out with the exception of the nucleus. The midgut epithelium is also seen to flatten with *SRP54* and adopt a pseudostratified appearance for *RPT3*.





**Figure 3-10.** Comparison of lateral cell phenotypes upon RNAi of *T. urticae* orthologues derived from *Tribolium castaneum* RNAi sensitive genes. Images depict cells the lateral cells that face away from the posterior midgut tube (black arrows). For *SNAP- $\alpha$* , *RPN7*, *COPB2* and *HSC70* (**B**, **E**, **G** & **H**), the lateral cells appear to become distorted in shape and thicker. For *V-ATPase* and *ROP* (**C** & **D**), the lateral cells appear to be thinner. For *SRP54* and *RPT3* (**F** & **I**), the lateral cells appear similar to the digestive cells and appear empty inside apart from the nucleus and are extremely enlarged.

## Chapter Four – Discussion

### 4.1 Characterization of Changes Caused by RNAi

Although it is apparent that all the silencing phenotypes produce significant changes in the midgut of *T. urticae*, the role of this study was to understand how these affected mechanisms affected midgut tissues and how this led to their characteristic phenotype. While most of the RNAi silenced genes targeted the vesicular trafficking system, silencing showed changes primarily in the digestive cells and the midgut epithelium from where these cells bud off.

From the silencing of *SNAP- $\alpha$*  and *V-ATPase*, the “dark” mite whole-body phenotype could be seen histologically as the heavy staining present in the midgut lumen of *T. urticae* which was indicative of free-floating plant material that was not seen in *COPB2* or *F3R3* control silencing (Figure 3-1 C & D; Figure 3-2 C & D). Neither *SNAP- $\alpha$*  and *V-ATPase*, differed significantly in terms of their juvenile to mature digestive cell ratio compared to the *F3R3* control silenced samples (Figure 3-5). Data suggests that the “dark” mite phenotype is not specific to the silencing of one gene, but rather a characteristic of dysfunction in vesicular docking proteins. For *COPB2*, the “colorless” mite whole-body phenotype can be attributed to the absence of midgut lumen staining and the lack of mature digestive cells which provide the visible dark spots in control silenced mites (Figure 3-1 B; Figure 3-2 B).

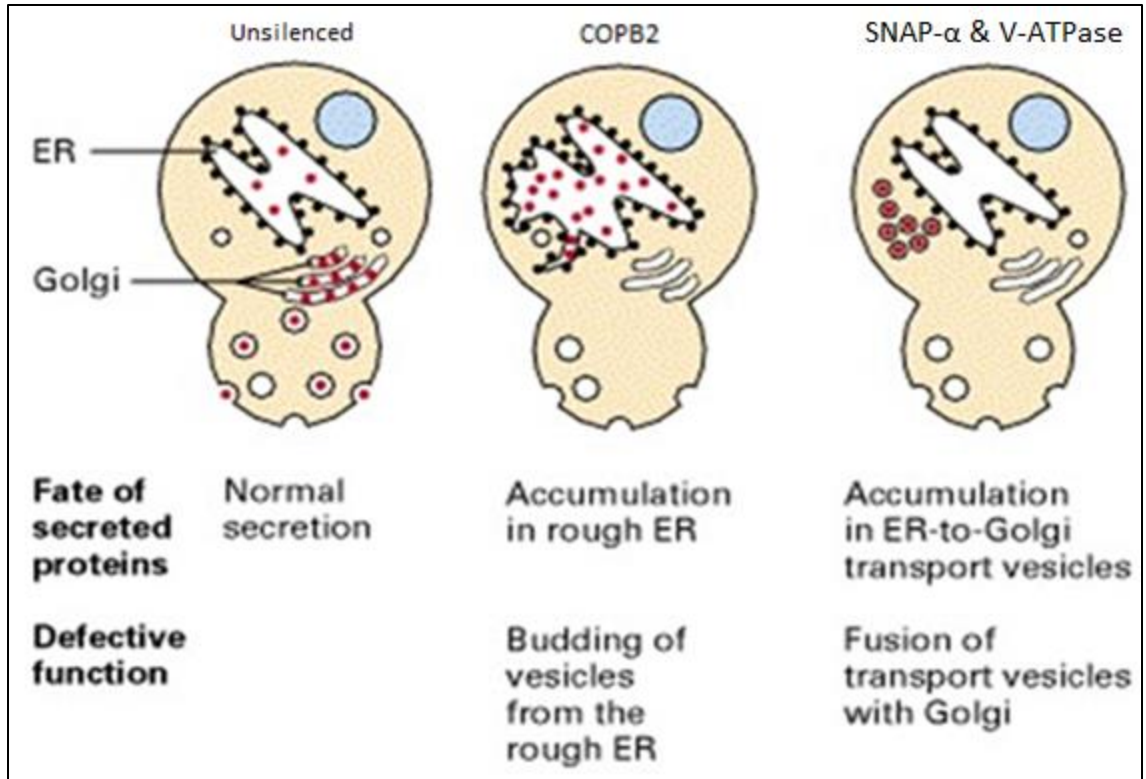
From the image data, *T. urticae* had two-times more midgut digestive cells when the *COPB2* gene was silenced (Figure 3-7,  $p < 0.05$ ). Between the two “dark” mite phenotypes, the mature digestive cells of *SNAP- $\alpha$*  appeared to be pigmented lighter in contrast to the staining of the midgut lumen, whereas *V-ATPase* had mature digestive cells pigmented darker in contrast to the staining of the midgut lumen (Figure 3-3 C & D). This difference in pigmentation in the vesicles of digestive cells could be reflective of changes to the pH from the dysfunctional *V-ATPase* gene. For the phenotype of silenced *COPB2*, this 1.5-fold increase in juvenile digestive cells is apparent in all samples with many of these cells exhibiting dark pigmentation similar to mature digestive cells and adhering to each other in large groups along the midgut epithelium (Figure 3-3 B). The juvenile digestive cells could be unable to bud off the midgut epithelium. This change suggests that *COPB2* is critical to either digestive cell development. RNAi silencing changes were not observed in tissue such as the ovaries or eggs. This suggests that the method for dsRNA uptake does not affect targets outside of the digestive system in *T. urticae*.

## 4.2 Analysis of RNAi Silencing on Digestive Cell Function

The digestive system of *T. urticae* is structurally segmented, like it is in insects, as there is a foregut, midgut and hindgut present. However, *T. urticae* lacks certain features in assisting digestion and the movement of nutrients such as a peritrophic matrix, Malpighian tubules, or hemolymph in comparison to insects (Mothes and Seitz, 1981). While these features are key to extracellular digestion in insects, the free-floating digestive cells in the midgut of *T. urticae* may serve as the facility where plant material is digested

and assist in moving nutrients throughout the body. By silencing genes critical to the function of these midgut digestive cells, *T. urticae* may be unable to absorb nutrients after feeding on plants and render them unable to dedicate energy towards other biological processes such as egg production. This is supported by the fact that RNAi-silenced mites had reduced fecundity (Bensoussan et al., 2020).

From what is known of the genes and the respective phenotypes of *SNAP- $\alpha$* , *V-ATPase* and *COPB2*, the general symptoms appear consistent with yeast models of perturbed vesicular trafficking (Figure 4-1). In yeast strains where vesicle signaling genes are silenced, exported protein accumulated in vesicles and are secreted out of the cell as vesicles are unable to bind properly with the cell membrane due to the lack of interfacing proteins that can interact (Rothman, 1994). For the RNAi silenced phenotype of *SNAP- $\alpha$*  & *V-ATPase*, the midgut lumen of *T. urticae* was full of stained material while the larger mature digestive cells were extensively pigmented in comparison to the *F3R3* control silenced samples. As the midgut digestive cells are key to intracellular digestion in *T. urticae*, their inability to export digested food material and nutrients maybe reflected by the darkened digestive cells, which differ in pigmentation to the undigested food material in the midgut lumen (Figure 3-3 C & D).



**Figure 4-1.** Yeast models of disturbed vesicular trafficking ©. In reference to a functional unsilenced model of vesicular trafficking, *COPB2* RNAi silencing leads to the accumulation of protein in the endoplasmic reticulum, due to the depletion of phospholipids and resident membrane proteins from the rough ER required for the formation of vesicles which are lost from the lack of reverse vesicular trafficking. *SNAP- $\alpha$*  and *V-ATPase* RNAi silencing leads to the accumulation of protein in vesicles, due to the inability for the vesicle to bind to their target destinations (Rothman, 1994). Refer to Appendix A for figure permissions and copyright agreement from Springer Nature.

In yeast strains where the COPI complex is silenced, exported protein accumulated in the endoplasmic reticulum, due to the depletion of phospholipids and resident membrane

proteins from the lack of retrograde vesicular trafficking (Rothman, 1994). For the phenotype of *COPB2*, the midgut of *T. urticae* was seen to be primarily composed of numerous darkened juvenile digestive cells that were unable to progress in size and appear to adhere to other digestive cells in masses along the edges of the midgut caeca. The increase in juvenile midgut digestive cells can be also attributed to either an inability of the mite to recognize and expel these cells as waste, or the mite's midgut attempting to make up for the reduction in the digestive capability of these cells (Figure 3-3 B). As all the *T. urticae* samples in this experiment were taken after feeding and a 24 hour period of RNAi silencing, the changes in the midgut epithelium seen after silencing may reflect a state of digestion or reflect the fact that the midgut itself is at capacity and bloated instead of a result of silencing interfering with budding behavior of midgut digestive cells.

To further validate the results and confirm the function of digestive cells in *T. urticae*, similar vesicular trafficking genes could be silenced in future work. Some of these genes have already been tested with varying degrees of success in reducing survivorship and fecundity, while also producing similar phenotypes to the one tested in this paper (Table 3-1; Grbić et al., 2011). From preliminary testing, silencing the Ras opposite gene (*ROP*) and the 26S proteasome non-ATPase regulatory subunit gene (*RPN7*) produced the same “dark” body phenotype similar to *SNAP- $\alpha$*  and *V-ATPase* silencing (Figure 3-8; B - E). The heat shock cognate 70 gene (*HSC70*) was also silenced in *T. urticae* and produced the same “colorless” body phenotype as *COPB2* while appearing histologically similar in terms of juvenile digestive cell abundance (Figure 3-8; G & H). These results suggest that the whole-body phenotypes seen during silencing are not a result of the dysfunction of a certain

gene, but the dysfunction of the larger vesicular trafficking system they are associated with. The *ROP* gene is associated with neurotransmitter exocytosis and is primarily expressed in the central nervous system of *Drosophila melanogaster* (Salzberg et al., 1993). *HSC70* gene is a necessary component in clathrin-mediated endocytosis and a key system in cellular vesicular trafficking (Eisenberg and Greene, 2007). *RPN7* is a gene associated with the degradation of ubiquitinated proteins in *Saccharomyces cerevisiae* and was the only gene in this study that was not clearly linked to vesicular trafficking (Isono, et al., 2004).

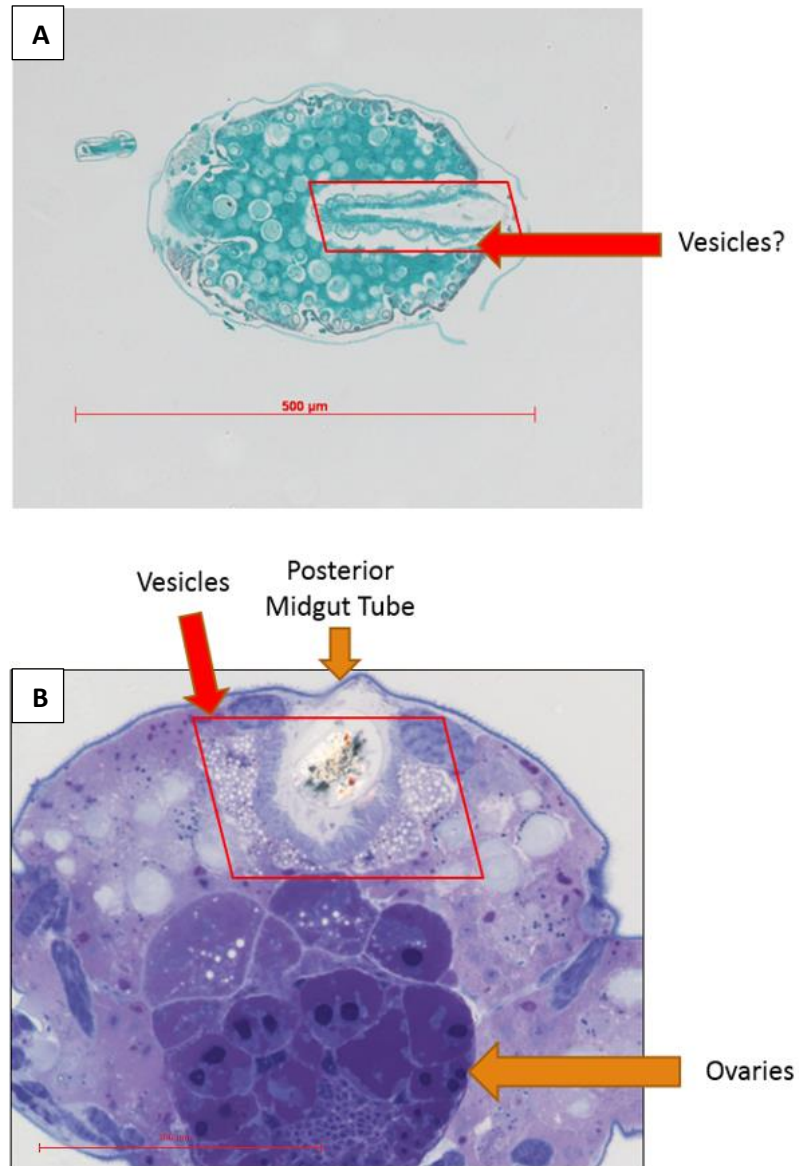
### 4.3 Limitations and Future Studies

The protocol used in this project went through many different iterations before producing useful data. There are three main histological methods that were attempted for this project: plastic embedding, OCT cryosectioning and paraffin embedding. Previous attempts by the Grbić Lab to observe the midgut used plastic sectioning, however this process was expensive and time consuming. There was also no guarantee that a batch would be successful until the end of processing, which meant that it took considerable time to evaluate changes to the protocol. OCT cryosectioning was attempted as it did not require a lengthy tissue fixation protocol and was the most accessible in terms of relative cost. Unfortunately, due to the size and nature of the specimen, it was extremely difficult to quickly freeze the mite in a way that preserved the midgut region. In the resulting images, the sectioned mites varied in size and lacked many of the structures and digestive cells present in the midgut. This lack of consistency made OCT cryosectioning infeasible to use for the purpose of this project.



Paraffin embedding of tissue was eventually chosen as the embedding process worked in preserving the midgut region and was less labor intensive than plastic embedding. However, when comparing plastic sections to paraffin sections of *T. urticae*, processing and imaging artifacts were apparent in paraffin samples. The most notable being an empty halo being formed between the posterior midgut tube and the midgut lumen in paraffin samples (Figure 4-2 A). In plastic embedded samples, this space is occupied by numerous small vesicle-like structures within lateral cells and their disappearance may be a result of the use of xylene to clear samples during the paraffin embedding process (Figure 4-2 B). The location of these vesicles to the posterior midgut tube and the ovaries, this missing structure could be how the mite supply the ovaries with nutrition. Therefore, it would be important to produce plastic sections of RNAi-silenced *T. urticae* to identify this structure and characterize if it is affected by the silencing of these vesicular trafficking genes.

In terms of future directions, there are many improvements that can be made now that protocol is proven to be reliable. With regards to workflow, fully autonomous work was unfeasible at the beginning due to the nature of the image capture device on the microscope. Mites would have to be located on an individual basis and tracked through different slides in order to produce a full serial. This would be painstaking work, as it falls on the researcher to keep track of upwards of 6 different instances of the same mites on a single slide and then reassemble it by manually later. However, with a slide scanner which allowed for the whole imaging of an entire slide with no loss of magnification. It would then be possible in the future to save time by automating the process of identifying individual mites based on their orientation and relative position on the slide.



**Figure 4-2.** Description of paraffin imaging artifacts. Top image depicts a longitudinal paraffin section (A) and the bottom image depicts a transverse plastic section of *T. urticae* (B). Red boxes indicate the position of the midgut posterior tube. The use of xylene as a clearing agent is suspected to have washed away the vesicles seen in the plastic section between the posterior midgut and the midgut lumen producing the white “halo” seen in the paraffin section.

## 4.4 Conclusion

The effects of RNAi silencing are characterized by massive changes occurring in the midgut of *T. urticae* for all treatment associated phenotypes. RNAi silencing of these genes primarily affect the life cycle of digestive cells in the midgut and produce the whole-body phenotype.

*COPB2* RNAi silencing results in dysfunction of the COPI system which manifests itself as an increase in juvenile midgut digestive cells that are unable to expand in size but can mature and fill with food material. This causes the “colorless” mite phenotype due to the visible reduction in the mature digestive cells which appear as spots on the mite. For *SNAP- $\alpha$*  and *V-ATPase* genes, RNAi silencing leads to dysfunction in terms of vesicular docking which manifests itself as changes in midgut digestive cells which intake food content but are unable to export digested content. This leads to content that fills up the midgut lumen as indicated by the extensive dark pigmentation that leads to the “dark” mite phenotype. Both findings are verified as the silencing of similar orthologue genes for these vesicular systems produce a similar visible phenotype.

Further investigation should be conducted in terms of plastic sectioning and TEM imaging of midgut epithelial cells, midgut cells and posterior midgut lateral cells to identify cellular mechanisms that cause the respective phenotypes in *T. urticae*. This knowledge can be of use in the field of agriculture pertaining to integrated pest management strategies for crops through the use of RNAi.

## 4.5 References

- Barnard R. J., Morgan A., & Burgoyne R. D. (1997). Stimulation of NSF ATPase Activity by  $\alpha$ -SNAP is Required for SNARE Complex Disassembly and Exocytosis. *J Cell Biol*, **139**, 875 — 883.
- Basnet S. & Kamble S. T. (2018). RNAi-Mediated Knockdown of vATPase subunits Affects Survival and Reproduction of bed bugs (Hemiptera: Cimicidae). *J Med Entomol*, **55**, 540—546.
- Bensoussan N., Santamaria M. E., Zhurov V., Diaz I., Grbić M. & Grbić V. (2016). Plant-Herbivore Interaction: Dissection of the Cellular Pattern of *Tetranychus urticae* Feeding on the Host Plant. *Front Plant Sci*, **7**, 1105.
- Bensoussan N., Zhurov V., Yamakawa S., O’Neil C. H., Suzuki T., Grbić M. & Grbić V. (2018). The Digestive System of the Two-Spotted Spider Mite, *Tetranychus urticae* Koch, in the Context of the Mite-Plant Interaction. *Front Plant Sci*, **9**, 1206.
- Bensoussan N., Dixit S., Tabara M., Letwin D., Milojevic M., Antonacci M., Jin P., Arai Y., Bruinsma K., Suzuki K., Fukuhara T., Zhurov V., Geibel S., Nauen R., Grbić M. & Grbić V. (2020) Environmental RNA interference in two-spotted spider mite, *Tetranychus urticae*, reveals dsRNA processing requirements for efficient RNAi response. *bioRxiv*. <https://doi.org/10.1101/2020.05.10.087429>
- Bethune J., Wieland F., & Moelleken J. (2006). COPI-mediated transport. *J Membrane Biol*, **211**, 65 — 79.

- Bowman C. E. (2019). The gut epithelium from feeding to fasting in the predatory soil mite *Pergamasus longicornis* (Mesostigmata: Parasitidae): one tissue, two roles. *Exp Appl Acarol*, **77**, 253 — 357.
- Cazaux M., Navarro M., Bruinsma K. A., Zhurov V., Negrave T., Grbić V., & Grbić M. (2014). Application of Two-spotted Spider Mite *Tetranychus urticae* for Plant-pest Interaction Studies. *J Vis Exp*, **89**, 1 — 7.
- Chakraborty S. (2011). Climate change, plant diseases and food security: an overview. *Plant Pathol*, **60**, 2 — 14.
- Chen Y. A. & Scheller R. H. (2001). SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*, **2**, 98 —106.
- Deaton J. & Lipka B. (2015). Political instability and food security. *J Food Secur*, **3**, 29 — 33.
- Dermauw W., Wybouw N., Rombauts S., Menten B., Vontas J., Grbić M., Clark R. M., Feyereisen R., & Leeuwen T. V. (2013). A link between host plant adaption and pesticide resistance in the polyphagous spider mite *Tetranychus urticae*. *PNAS*, **110**, 113 — 112.
- DiStasio A., Driver A., Sund K., Donlin M., Muraleedharan R. M., Pooya S., Kline-Fath B., Kaufman K. M., Prows C. A., Schorry E., Dasgupta B., & Stottmann R. W. (2017). Copb2 is essential for embryogenesis and hypomorphic mutations cause human microcephaly. *Hum Mol Gen*, **26**, 4836 — 4848.

- Duden R. (2003). ER-to-Golgi transport: COP I and COP II function. *Mol Membr Biol*, **20**, 197 — 207.
- Duman J. G. & Forte J. G. (2003). What is the role of SNARE proteins in membrane fusion? *Am J Physiol Cell Physiol*, **285**, 237 — 249.
- Eisenberg E. & Greene L. E. (2007). Multiple roles of auxilin and HSC70 in clathrin-mediated endocytosis. *Traffic*, **8**, 640 — 646.
- Godinho A. (2011, November 23). Spider mite's secrets revealed. *Instituto Gulbenkian de Ciencia*.
- Grbić M., Van Leeuwen T., Clark R., et al. (2011). The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature*, **479**, 487 — 492.
- Holtof M., Lenaerts C., Cullen D & Broeck J. (2019). Extracellular nutrient digestion and absorption in the insect gut. *Nature*, **377**, 397 — 414.
- Hsu V., Lee S., & Yang J. (2009). The evolving understanding of COPI vesicle formation. *Nat Rev Mol Cell Biol*, **10**, 364 — 369.
- Isoe J., Stover W., Miesfeld R. B., & Miesfeld R. L. (2011). Defects in coatamer protein I (COPI) transport cause blood feeding-induced mortality in yellow fever mosquitoes. *Proc Natl Acad Sci*, **108**, 211 — 217.
- Isono E., Saeki Y., Yokosawa H., & Toh-e A. (2004). RPN7 is required for the structural integrity of the 26S proteasome of *Saccharomyces cerevisiae*. *J Biol Chem*, **279**, 27168 — 27176.

- Iwasaki S., Kobayashi M., Yoda M., Sakaguchi Y., Katsuma S., Suzuki T., & Tomari Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell*, **39**, 292 — 299.
- Jonckheere W., Dermauw W., Zhurov V., Wybouw N., Van den Bulcke J., Villarroel C. A., Greenhalgh R., Grbić M., Schuurink R. C., Tirry L., Baggerman G., Clark R. M., Kant M. R., Vanholme B., Menschaert G., & Van Leeuwen T. (2016). The Salivary Protein Repertoire of the Polyphagous Spider Mite *Tetranychus urticae*: A Quest for Effectors. *Mol Cell Proteomics*, **15**, 3594 — 3613.
- Krantz G. W., Helle W., & Sabelis M. W. (1987). Spider mites: Their biology, natural enemies and control (World crop pests Vols. 1A and 1B). Amsterdam: *Elsevier Science Publishers*.
- Kwon D., Park J., & Lee S. (2012). Screening of lethal genes for feeding RNAi by leaf disc-mediated systematic delivery of dsRNA in *Tetranychus urticae*. *Pestic Biochem Phys*, **105**, 69 — 75.
- Kwon D, Park J., Ashok P. A., Lee U., & Lee S. (2015). Screening of target genes for RNAi in *Tetranychus urticae* and RNAi toxicity enhancement by chimeric genes. *Pestic Biochem Phys*, **130**, 1 — 7.
- Maxfield F. R. & McGraw T. E. (2004). Endocytic recycling. *Nat Rev Mol Cell Biol*, **5**, 121 — 132.
- Maxson M. E. & Grinstein S. (2014). The vacuolar-type H<sup>+</sup>-ATPase at a glance – more than a proton pump. *J Cell Sci*, **127**, 4987 — 4993.

- May A., Whiteheart S. & Weis W. (2001). Unraveling the Mechanism of the Vesicle Transport ATPase NSF, the N-Ethylmaleimide-sensitive Factor. *J Biol Chem*, **276**, 21991 — 21994.
- Mothes U. & Seitz K. A. (1981). Functional microscopic anatomy of the digestive system of *Tetranychus urticae* (Acari, Tetranychidae). *Acarologia*, **22**, 257 — 270.
- Naydenov N., Feygin A., Wang L., & Ivanov A. (2014). N-Ethylmaleimide-sensitive Factor Attachment Protein  $\alpha$  ( $\alpha$ SNAP) Regulates Matrix Adhesion and Integrin Processing in Human Epithelial Cells. *J Biol Chem*, **289**, 2424 — 2439.
- Niu J., Jian H., Xu J., Chen C., Guo Q., Liu Q., & Guo Y. (2012). RNAi silencing of the *Meloidogyne incognita* RPN7 gene reduces nematode parasitic success. *Eur J Plant Pathol*, **134**, 131 — 144.
- Oerke E. C. (2006). Crop losses to pests. *J Agric Sci*, **144**, 31 — 43.
- Piraneo T., Bull J., Morales M. A., Lavine L. C., Walsh D. B., & Zhu F. (2015). Molecular mechanisms of *Tetranychus urticae* chemical adaptation in hop fields. *Sci Rep.* **5**, 17090.
- Richards S., Gibbs R.A., et al. (2008). The genome of the model beetle and pest *Tribolium castaneum*. *Nature*, **452**, 949 — 955.
- Riegler M. (2018). Insect threats to food security. *Science*, **361**, 846 — 847.
- Rothman J. (1994). Mechanisms of intracellular protein transport. *Nature*, **372**, 55 — 63.
- Sabelis M. W. (1985). Spider mites: Their biology, natural enemies and control. Helle W. (Eds.). Amsterdam: *Elsevier Science Publishers*.



- Salzberg A., Cohen N., Halachmi N., Kimchie Z., & Lev Z. (1993). The *Drosophila* RAS2 and ROP gene pair: a dual homology with a yeast RAS-like gene and a suppressor of its loss-of-function phenotype. *Dev*, **117**, 1309 — 1319.
- Sennoune S. R., Bakunts K., Martinez G. M., Chua-Tuan J. L., Kebir Y., Attaya M. N., & Martinez-Zaquilan R. (2004). Vacuolar H<sup>+</sup>-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. *Am J Physiol Cell Physiol*, **286**, 1443 — 1452.
- Shih C. T., Poe S. L., & Cromroy H. L. (1976). Biology, life table, and intrinsic rate of increase of *Tetranychus urticae*. *Ann Entomol Soc Am*, **69**, 362 — 364.
- Suzuki T., Nunes M. A., España M. U., Namin H. H., Jin P., Bensoussan N., Zhurov V., Rahman T., De Clercq R., Hilson P., Grbić V., & Grbić M. (2017). RNAi-based reverse genetics in the chelicerate model *Tetranychus urticae*: A comparative analysis of five methods for gene silencing. *PLOS One*, **12**, 1 — 22.
- Ulrich J., Dao V. A., Majumdar U., Schmitt-Engel C., Schwirz J., Schultheis D., Ströhlein N., Troelenberg N., Grossmann D., Richter T., Dönitz J., Gerischer L., Leboulle G., Vilcnskas A., Stanke M., & Bucher G. (2015). Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. *BMC Genomics*, **16**, 674.
- United Nations Department of Economic and Social Affairs. (2019). World population prospects 2019. (Report No. ST/ESA/SER.A/423). New York, NY: United Nations.

- Velez A. M., Khajuria C., Wang H., Narva K. E. & Siegfried B. D. (2016). Knockdown of RNA interference pathway genes in Western Corn Rootworms (*Diabrotica virgifera zae*) Demonstrates a possible mechanism of resistance to lethal dsRNA. *PLOS One*, **11**, 1 — 12.
- Wang Y., Chai Z., Wang M., Jin Y., Yang A., & Li M. (2017). COPB2 suppresses cell proliferation and induces cell cycle arrest in human colon cancer by regulating cell cycle-related proteins. *Exp Ther Med*, **15**, 777 — 784.

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<b>Article Title</b>	Yeast Systems Biology: The Continuing Challenge of Eukaryotic Complexity.	<b>Publication Type</b>	e-Journal
<b>Date</b>	01/01/1984	<b>Start Page</b>	3
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<b>Country</b>	United States of America	<b>Volume</b>	2049

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<b>Title</b>	The Functional Characterization of the Midgut of Tetranychus urticae via RNA	<b>Institution name</b>	University of Western Ontario
<b>Instructor name</b>	Sean Pham	<b>Expected presentation date</b>	2020-11-13

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Title, description or numeric reference of the portion(s)	Overview of the Secretary Pathway	Title of the article/chapter the portion is from	Yeast Systems Biology: The Continuing Challenge of Eukaryotic Complexity.
Editor of portion(s)	Oliver, Stephen G	Author of portion(s)	Oliver, Stephen G
Volume of serial or monograph	2049	Issue, if republishing an article from a serial	N/A
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## Curriculum Vitae

**Name:**

Sean Pham

**Post-Secondary Education and Degrees:**

University of Western Ontario

London, Ontario, Canada

2012 – 2017 B.Sc. (Specialization in Biology)

**Honours and Awards:**

Western Graduate Research Scholarship

2017 – 2019

Western Scholar Athlete

2018 – 2019

**Related Work Experience:**

Graduate Teaching Assistant

University of Western Ontario

2017 – 2019