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PATTERNS OF MEMBRANE POTENTIAL OF CELLS ISOLATED FROM THE MIDGUT OF *Heliothis virescens*

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

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by
Richard Melton
May 2020

Accepted by:
Dr. Matthew Turnbull, Committee Chair
Dr. Francis Reay-Jones
Dr. Andrew Mount

ABSTRACT

The midgut is the main point of interaction between a lepidopteran larva and its environment, so understanding how the gut functions is important not just for understanding gut physiology but also the ecology and evolution of these organism. The midgut of these larvae is a fascinating system in which to study developmental, regenerative, and immune physiology. The midgut can exhibit up to a 200-fold increase in size through ontogeny, primarily through addition of new cells at molt, while damaged cells are replaced throughout intermolt periods. In the midgut of lepidopteran larvae, mature cells are produced from stem cells, localized in pockets underneath the mature cells.

Several regulators of stem cell activity are known, but no integrative model has been established. In numerous animal taxa, bioelectric phenomena regulate stem cell activity, including duplication and differentiation. Here, we are using the tobacco budworm, *Heliothis virescens*, to characterize bioelectric patterns in the lepidopteran larval gut. We adapted a method to isolate stem and mature gut cells from physiologically staged fourth instar larvae and assay their membrane potential. As bioelectric phenomena are highly important in gut physiology, our results may be useful in regulating lepidopteran pests. Finally, our results could help further our understanding of how physiology and an organism's environment interact.

DEDICATION

To my Mom and Dad

for their unending love and support

ACKNOWLEDGMENTS

This thesis work would not have been possible without the support and guidance of my advisor Dr. Matthew Turnbull. I would also like to thank my committee Dr. Francis Reay-Jones and Dr. Andrew Mount for their helpful comments on the research and writing. Thanks to Peng Zhang, Daniel Howard, Jessie Parker, and the rest of the Turnbull lab both past and present. Their assistance was critical to the completion of this work.

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CHAPTER ONE

INTRODUCTION

Lepidopteran gut physiology is a fascinating system to study. The midgut is the site of a large amount of stem cell growth of new cells and regeneration/replacement of dead or dying cells (Hakim et al., 2001). The gut epithelium is primarily composed of two cell types: the columnar cells are responsible for nutrient uptake and secretion of digestive enzymes, and the goblet cells which maintain the internal environment of the gut lumen (Hakim et al., 2001). Other cell types such as endocrine and stem cells are present but in much smaller numbers than goblet and columnar cells (Levy et al. 2004). Representative images of midgut epithelium cells can be seen in **Figure 1**. In *Manduca sexta* (Lepidoptera: Sphingidae), the gut can see a 200-fold increase in size between first and last larval instar (Rowland et al. 2016). Most of this growth happens during the molting period. This growth has been shown to be from a large increase in new cell numbers, rather than an increase in cell size (Hakim et al. 2010). Thus, new cells need to be produced due to natural developmental processes and following damage to replace lost or damaged cells.

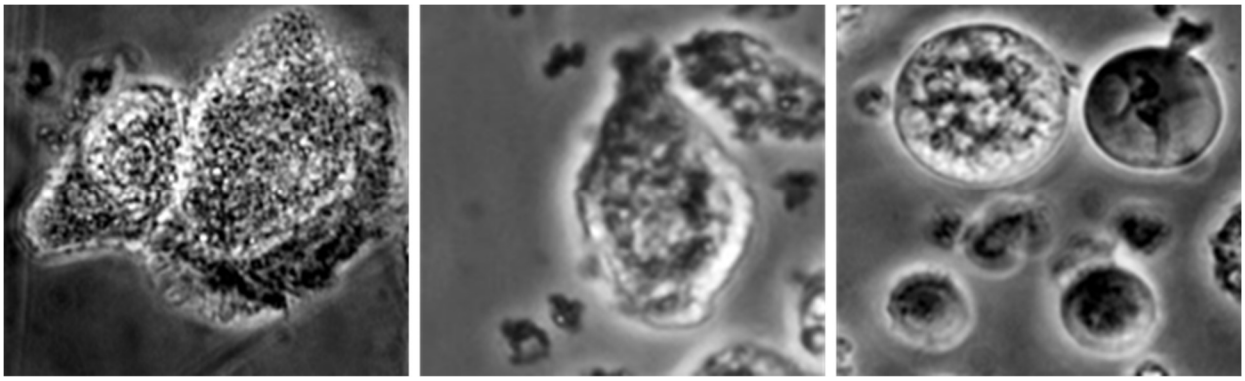

Figure 1. Distinguishing between cell types by morphological characteristics. For the purpose of this research, cells from the lepidopteran larval midgut are grouped into two categories. The first is “mature cells”. These cells are large and generally irregular in shape.

Most of these mature cells are columnar (Left) and goblet cells (Center), although it also includes endocrine cells (not pictured) which generally are smaller and rounder than either of the other two mature cell types. The second category is stem cells, which are generally much smaller than the majority of mature cells and are almost perfectly circular in shape (Right).

New cells in the larval lepidopteran midgut are exclusively stem cell derived (Hakim et al. 2010). This stem cell-based cell replacement allows for extremely rapid growth and regeneration. Several different processes are known to trigger stem cells to begin proliferation; these signals can be modulated through a variety of mechanisms such as paracrine, endocrine, and kinetic modalities. Some proportion of these stem cells will differentiate into mature cells. Following damage (whether by diet or pathogen), holes left by cells that are damaged or dead are filled by these newly generated mature cells (Hakim et al., 2010) (**Figure 2**). Stem cells are also responsible for expanding the gut during growth periods through ontogeny.

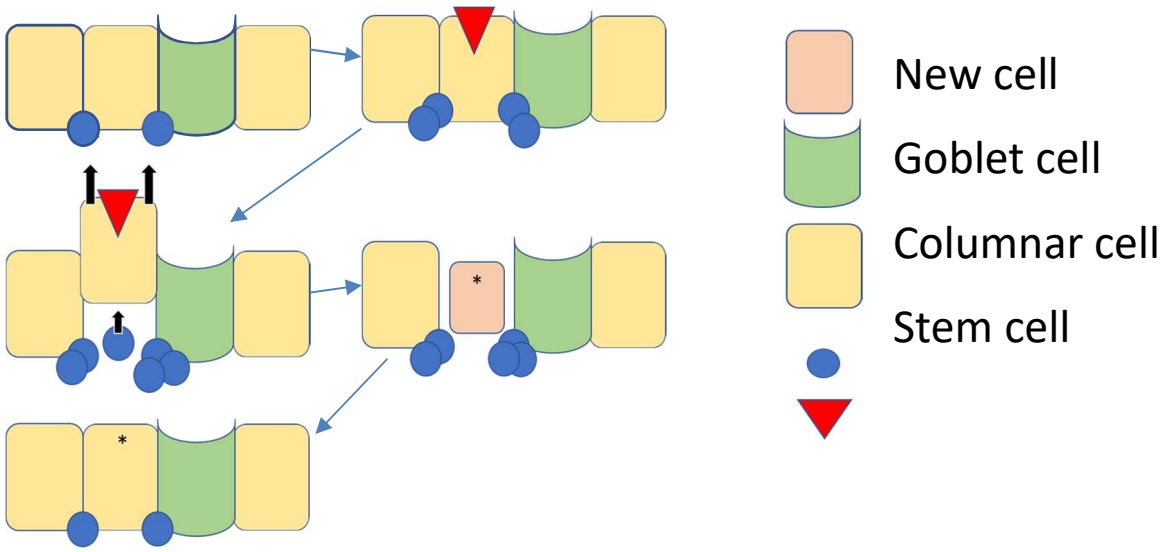

Figure 2. Cell replacement in the gut of lepidopteran larvae. Stem cells are basal to mature cells during homeostatic tissue function. Following damage, stem cells will begin to divide. Once the dead/dying cells are removed stem cells begin to differentiate and fill the place of the removed cell/cells.

The genus Heliothis has several species that are known as major pests (Fitt 1989), including *H. virescens* Noctuidae:Heliothis. *Heliothis virescens* are typically an agricultural problem throughout North and South America, primarily feeding on tobacco, cotton, tomato, sunflower and soybeans. Heliothines lead to a large amount of crop damage each year (Bottrell, 1977). These pests can be particularly troublesome for crops for several reasons, one of which is the tendency of these larvae to feed on plant structures that are high on nitrogen such as reproductive structures and growing points (buds). These feeding habits can incur major impacts on crop yield (Hardwick, 1965). The major pest species within the heliothines are polyphagous causing major damage to several different plant species.

One of the more common control techniques used to combat lepidopteran pests is the use of plants genetically engineered with Bt-cry toxins, so common that almost 80% of cotton and corn in the U.S.A. is Bt-recombinant (Wechsler, 2018). This method is so widely used that resistance has developed in many insect species to several Bt toxins (Reisig et al., 2018; Tabashnik et al., 2003). The toxins work by binding to mature cells causing pores within the gut leading to the loss of cells and eventually death of the organism (Carriere et al., 2010; Coates, 2016; de Bortoli and Jurat-Fuentes, 2019). Several resistance modalities have been demonstrated in lab colonies, including activation and receptor binding (Coates, 2016). Another mechanism of resistance or survival may be related to gut stem cell replenishment rate: increased rates of

midgut stem cell division and/or differentiation may replace mature cells sufficiently to overcome sublethal crytoxin damage (Spies et al.,1985; Loeb et al., 2000).

Numerous soluble factors regulating stem cell division and differentiation have been identified in lepidopteran midguts, such as ecdysone and 20-hydroxyecdysone which are known to regulate proliferation and differentiation, respectively, in *Spodoptera littoralis* larval gut stem cells (Smagghe et al., 2005). Bioelectric mechanisms (Levin et al. 2013) may also play a role. Bioelectricity, which in the broad sense is the generation, maintenance, and dynamism of charge gradients across living cell membranes, is linked to nearly all aspects of cellular and tissue physiology, including developmental phenomena. In planaria, an animal known for its regenerative properties, stem cell behavior can be manipulated by treating wound sites with Ivermectin, a Cl^- channel agonist (Ferenc and Levin, 2019). It was demonstrated in the frog *Xenopus laevis* that hyperpolarized the membrane potential of cells at wound sites via incubation in Na^+ ion rich media increases regeneration rate by altering stem cell division and differentiation rates (Tseng & Levin 2012). These examples suggest that bioelectricity, specifically membrane potential (V_{mem}), could be playing an important role in regulating stem cell activity. Across animal phyla, stems cells are depolarized relative to mature cells, and embryonic stem cells are even more depolarized (Levin 2017). Further, there are differences in cell membrane potential of different types of terminal or mature cells reflecting the different task these cells have. The question at hand remains: in lepidopterans, specifically the midgut, is membrane potential just a readout of cell physiology or is it a determining factor in controlling cell behavior?

The main goal of this project was to characterize membrane potential patterns within the gut mature and stem cells of *H. virescens* larvae. I hypothesized that stem cells would be depolarized relative to mature cells. I further predicted that following dietary challenge, I would observe changes in membrane potential of stem cells suggesting a change in the behavior of these cells in response to the damage caused by the dietary assay. The results of this work will not only allow us to better understand the role of membrane potential in *H. virescens* stem cells, but potentially stem cell systems in general. Thus, *H. virescens* midguts could serve as an alternative model for understanding stem cell behavior.

CHAPTER TWO

DYNAMICS OF LEPIDOTERAN LARVAE MIDGUT CELL MEBRANE POTENTIAL

1. Abstract

In the midgut of lepidopteran larvae, mature cells are produced from stem cells, localized in pockets underneath the mature cells. Several modalities, including small soluble molecules such as endocrine and paracrine factors as, are known to regulate lepidopteran gut stem cell activity. In numerous animal taxa, bioelectric phenomena regulate stem cell activity, including duplication and differentiation. Here, we used the tobacco budworm, *Heliothis virescens*, to characterize bioelectric patterns in the lepidopteran larval gut. We adapted a method to isolate stem and mature gut cells from physiologically staged fourth instar larvae and characterized cell membrane potential using both cationic and anionic membrane potential sensitive dyes DiBac4(3) and Rhodamine 6G. We found that gut stem cells are depolarized compared to mature cells, a general trend seen in other stem cell systems. As bioelectric phenomena are highly important in gut physiology, our results, including ability to manipulate gut bioelectricity, may be useful in regulating lepidopteran pests. Finally, our results could help further our understanding of how the physiology of an organism interacts with the environment in which they live.

2. Introduction

The midgut of larval Lepidoptera is the site of food digestion, nutrient uptake and transport, and consequently encounters numerous stresses from growth to toxins. Given this, the midgut is also the site of substantial stem cell proliferation and differentiation that facilitate the regeneration and replacement of dead or dying cells (Billingsley and Lehane, 1996). The luminal face of the gut epithelium is primarily composed of two cell types: columnar cells which are responsible for nutrient uptake and secretion of digestive enzymes and goblet cells which maintain the internal environment of the gut lumen. In the vasal face of the epithelium there are pockets of stem cells intermixed with endocrine cells (Loeb et al., 2000). New columnar and goblet cells are thought to be exclusively stem-cell derived, with little stem cell activity (division or differentiation) except immediately prior to or during intramolt periods and following periods of damage (Hakim et al. 2010; Hakim et al., 2001). During intramolt periods, midgut stem cells are highly proliferative, facilitating incredible increases in gut size and cell number: for example, the gut of the larval noctuid *Trichoplusia ni* exhibits a 2.6-fold increase in total cell populations from 3rd to 5th instar (Engelhard et al., 1991).

A common control technique for lepidopteran larvae in cotton and corn is the use of toxins from the bacterium *Bacillus thuringiensis* (Bt); approximately 80% of cotton and corn in the US is Bt-recombinant (Wechsler, 2018). Although there is uncertainty regarding the specific mode of action, toxin ingestion, activation and binding induces gut cell death, resulting in loss of mature cell numbers and eventually death of the organism (Carriere et al., 2010; de Bortoli and

Jurat-Fuentes, 2019). Selection stemming from the pervasive use of this method has led to the development of resistance to several Bt-cry toxins in lepidopteran lineages (Reisig et al., 2018; Tabashnik et al., 2003). While several resistance modalities have been demonstrated in laboratory colonies, including activation and receptor binding (Coates, 2016), an intriguing mechanism may be related to gut stem cell replenishment rate: increased rates of midgut stem cell division and/or differentiation may replace mature cells sufficiently to overcome sublethal Bt toxin damage (Spies et al.,1985; Loeb et al., 2000). These studies were done *in vitro* and not *in vivo* but the results imply that in vivo results would be similar, that stem cells *in vivo* might help overcome a lower dosage by mitigating the damage. Given this, better understanding of the mechanisms regulating lepidopteran midgut stem cell division and duplication may enhance control opportunities.

Numerous soluble factors regulating lepidopteran midgut stem cells have been identified. Ecdysone and 20-hydroxyecdysone regulate proliferation and differentiation, respectively, in *Spodoptera littoralis* gut stem cells (Smagghe et al., 2005), and arylphorin and 20-hydroxyecdysone induce differentiation in *Bombyx mori* (Cermenati et al., 2007). Additionally, several midgut derived paracrine factors (termed MGDF 1-4) have mitogenic activity in *Heliothis virescens* (Loeb et al., 1999; Loeb, 2010). Significantly greater knowledge exists regarding regulation of *Drosophila melanogaster* adult intestinal stem cells, as the Wnt, Notch, Hippo/Yorkie, JNK, JAK/STAT and EGFR pathways are all implicated in stem cell behavior, as has also been observed in mice (Lin et al., 2008). Importantly, signaling patterns during stem cell replacement of gut cells lost through damage appear to involve many of the pathways engaged during ontogeny (Jiang et al 2016).

An additional modality observed to regulate cell decisions regarding quiescence, duplication and differentiation are bioelectric signals. All cells generate, maintain and modulate voltage differences across their plasma membrane (membrane potential, V_{mem}). These gradients are used to power many different forms of work including cross-membrane transport, but they also have been linked to cell-cycle regulation (Blackison et al., 2009). Of relevance here, it has been demonstrated across a range of animals that stem and other highly dividing cells are depolarized (more positive internal to outside) relative to terminal cells (Binggeli and Weinstein, 1986), and that V_{mem} is not only a readout of cell state, but also can affect cell differentiation patterns (Levin et al., 2017). This then suggests that modification of stem cell V_{mem} might lead to targeted manipulation of duplication and differentiation patterns in the lepidopteran midgut, enhancing control opportunities.

Given the above and lack of knowledge of the relationship between cell type and V_{mem} patterning in insects, the main goal of this study was to characterize the patterns of V_{mem} in different cell types collected from the gut tissue of lepidopteran larvae, through ontogeny (development) and after dietary damage. Understanding these patterns will allow us to better understand the system and serve as a starting point for future manipulations of cellular and tissue physiology. In this study we found that, as observed in other animals, stem cells in the lepidopteran gut are depolarized relative to mature cells. We found that this pattern is consistent through ontogeny as well as following dietary challenge. Additionally, following dietary challenge-induced loss of mature cells, we observed significant alterations in midgut stem cell

V_{mem}. Together, our data suggest that V_{mem} may serve as an indicator of functional changes in lepidopteran midgut stem cell populations and is worthy of future attempts to manipulate for control purposes.

3. Materials and Methods

Insect husbandry

Heliothis virescens were obtained from Benzon Research (Carlisle, PA) as 2nd and 3rd instar larvae. Insects were maintained on artificial diet at 22 C° or 26 C° until use, and in some instances, cohorts were temporarily incubated at 4 C° for 12 hours to delay development. In the latter instances, cold storage was performed >48 hours prior to experimental use. Larvae were staged according to head capsule width and slippage (Strand et al., 1988) and interval since molt.

Gut cell isolation

After being anesthetized on ice for 20-30 minutes, larvae were surface sterilized in a washing solution (30% sterile distilled-deionized water, 3% commercial dish soap, 67% commercial bleach). Larval midguts were removed through a dorsal cut in sterile Ringers solution (137mM NaCl, 1.8mM $CaCl_2$2.7mM KCl and 2.4mM $NaHCO_3$) (Barbosa 1974). Excised midguts were incubated in Ringers until dissections were completed. Midguts were then transferred to Incubation Media [3:1 Graces Insect Media:Ringers; Castagnola et al. (2011)], cut into 2-3 pieces each, and incubated for 90 minutes. Following incubation, guts were triturated until no distinct tissue remained visible. The homogenate was pipetted through a 70 µm cell

strainer (Fisher Scientific). The flow-through containing the cells was transferred into a 15 ml conical tube and centrifuged (400 g for 5 minutes at 4 C°). The resulting cell pellet was resuspended in 3 ml of incubation media, and an aliquot of resuspended cells counted by hemocytometer. The remaining cells were plated in a multiwell (6-well) plate and allowed to attach for 10-15 minutes before imaging.

Bioassays

For analysis of developmental patterns, larvae were staged and then dissected as described above. For dietary challenges, 24 hours prior to dissection, ten 4th instar larvae were moved to a 30 mm petri dish with a thin layer of 2% agar and either artificial diet supplied by Benzon Inc. or washed organic Iceberg lettuce bought from local stores. For each replicate, five larvae were placed on each diet.

Fluorescence staining and imaging

To allow V_{mem} quantification, cells were incubated with V_{mem}-sensitive fluorescent dyes. One millimolar stocks of the anionic dye DiBac4(3) and the cationic Rhodamine 6G, which increasingly fluoresces with depolarization and hyperpolarization, respectively, were generated in ddH_2O and added to a final concentration of 1 μM to the incubation media containing cells. The dyed media containing cells was transferred from the conical tube to a 6-well plate and incubated at room temperature in the dark for 20 minutes. For each well, three fields of view were haphazardly selected, and Bright Field (BF) and fluorescence [FITC for DiBac4(3) or TRITC for Rhodamine 6G] images captured. All imaging was done on a Nikon TE2000 inverted

microscope with standard FITC and TRITC fluorescent optics as well as phase contrasts for bright field using a Nikon Di2 monochrome camera. The exposure time was constant per dye for each replicate. These produces were done following the methods outlined in Zhang and Turnbull 2018.

Data analysis

Corresponding stacks of BF and fluorescence images for each field of view were generated in FIJI (ImageJ 1.52h). For each set of stacked images, 25 mature and 25 stem cells (based on cell morphology) were haphazardly selected on the Bright Field layer using the free-hand selection tool, and area, mean pixel intensity, standard deviation, and perimeter of each ROI (region of interest) were measured on the fluorescent layer. Data were exported to Excel (Microsoft, 2016) and normalized mean intensity of each cell was calculated following the protocol in Zhang and Turnbull (2017).

Statistical Analysis

Comparisons of cell area between mature and stem cells were analyzed using a T-test. All other multiple comparisons mainly the comparisons of membrane potential between cell type, ontogenic stage, and diet type were completed using an analysis of variance (ANOVA) and Tukey's HSD post-hoc test. All statistical analyses were performed in R (R studio, 2020), and all figures generated in DataGraph 4.5.1.

4. Results

General Morphology

As has been reported in other publications (Hakim et al, 2001; Loeb et al., 2000), we observed in preliminary observations that cells isolated from the midgut of *H. virescens* larvae fall into two populations based on visual morphology: large, irregularly-shaped cells are likely columnar and goblet cells, and smaller, rounder cells a likely of a mix of stem and endocrine cells (Levy et al. 2004) (Figure 3A). Size distribution appears largely continuous (Figure 3B), although area significantly differs between cell type ($t(111.62)=11.42$, $p < 0.0001$) (Figure 3C).

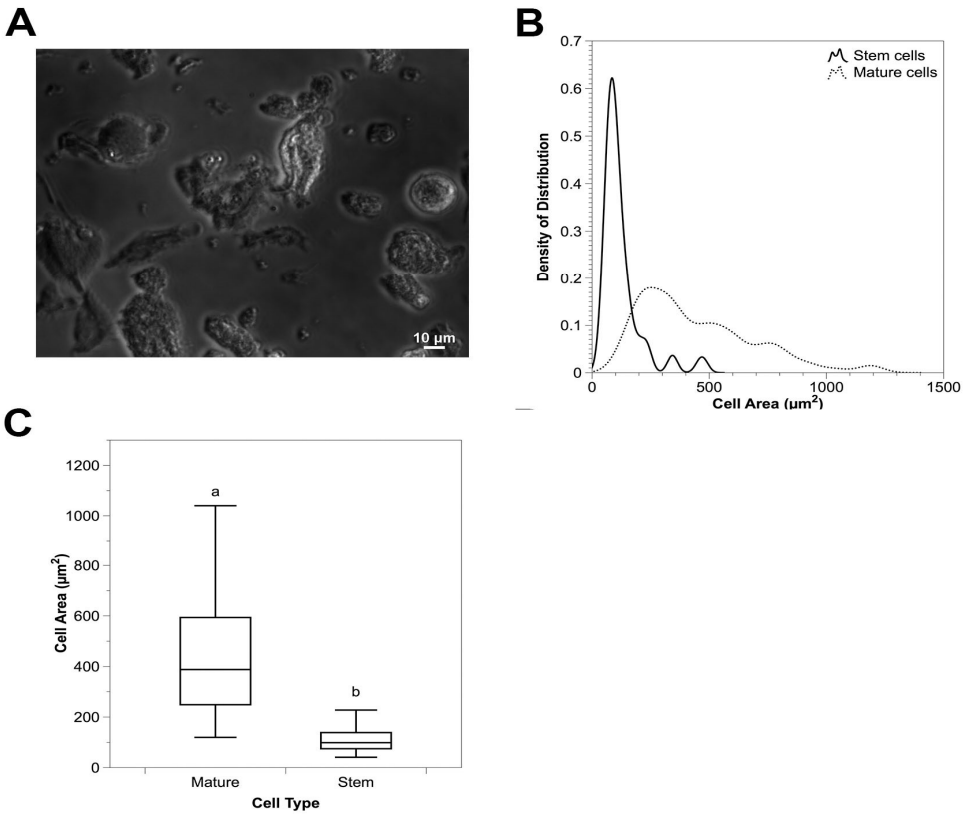

Figure 3. Gut cell isolation from *H. virescens* and morphological distinction. A) Representative image of midgut cells isolated from 4th instar *H. virescens*. B) Distribution of cell

size. C) Isolated midgut mature cells are significantly larger than stem cells [$t(111.62)=11.42$, $p < 0.0001$].

Ontogeny

We next examined cellular patterns through ontogeny. We observed that the above described size and shape dimorphism of the cells was consistent through ontogeny from early 4th instar to early 5th instar when looking at the area of mature vs stem cells (Figure 4) [$F(7,2363)=279.1$, $p <0.001$].

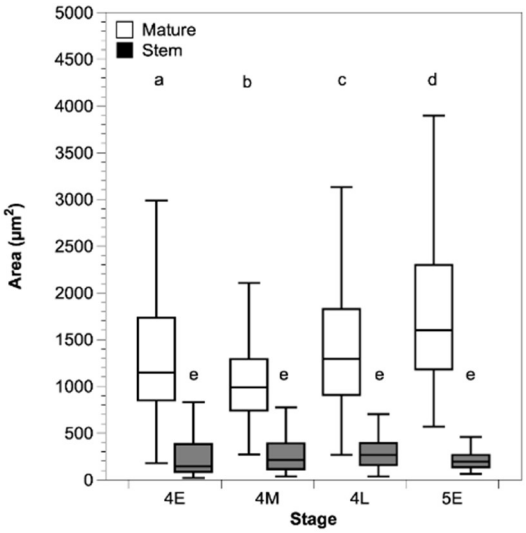

Figure 4. Ontogenic patterns in *H. virescens* gut cell morphology. Ontogenically staged larvae were dissected and cells isolated from the midguts. The area of a representative number of cells was collected. This shows that throughout ontogeny mature cells are larger than stem cells.

To characterize patterns of membrane potential associated with development of the gut, we stained cells isolated from 4th through early 5th instar larvae with the V_{mem}-sensitive dye Rhodamine 6G, for which intensity increases with hyperpolarization, and observed that stem cells consistently fluoresce less intensely than mature cells (Figure 5) ($F(2,2363)=179.53$), $p <$

0.001); this indicates that stem cells are depolarized relative to mature cells. The Rhodamine 6G intensity data suggest there may be some difference within stem cells late in 4th instar, as small cells exhibit higher intensity (are more hyperpolarized) at this time than at other periods, while early 5th instar mature cells similarly are significantly more hyperpolarized than mature cells isolated from other instars. Regardless of developmental period, as found in other animal systems, stem cell populations are depolarized relative to terminal populations.

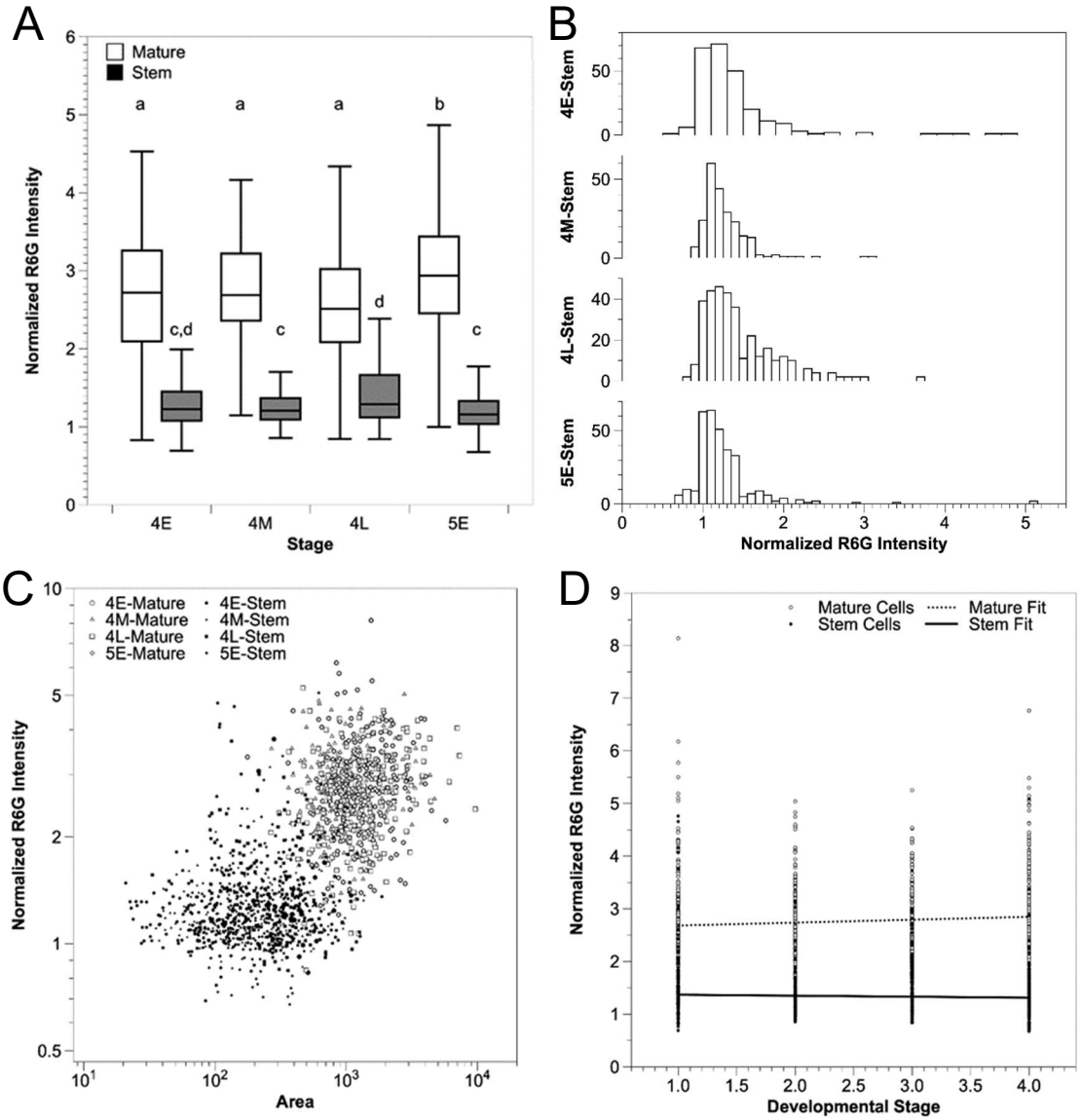

Figure 5. Membrane potential of gut stem and mature cells during 4th and early 5th larval instars. A) Rhodamine 6G(R6G) intensity of cells based on morphological type through ontogeny; populations with different letters significantly differ at $p < 0.05$. B) Distribution of stem cell population intensities through ontogeny. C) Scatter plot of the normalized intensity, demonstrating mature and stem cell populations largely are distinct based on both area and normalized Rhodamine 6G intensity. D) Rhodamine 6G intensity is consistent within population throughout ontogeny.

Dietary Assay

When looking at the effect of diet on cell number and membrane potential, we observed a significant reduction in total cell number when larvae were fed lettuce prior to gut cell isolation (F (3,8)=16.88, $p < 0.014$) (Figure 6A). While stem cell numbers decreased relative to artificial diet control, the reduction was not significant (F (3,12)=1.793, p=0.202) (Figure 6B). There was a significant reduction in mature cell numbers following lettuce feeding as compared to control (Figure 6B) (F93,8)=16.88), $p < 0.001$).

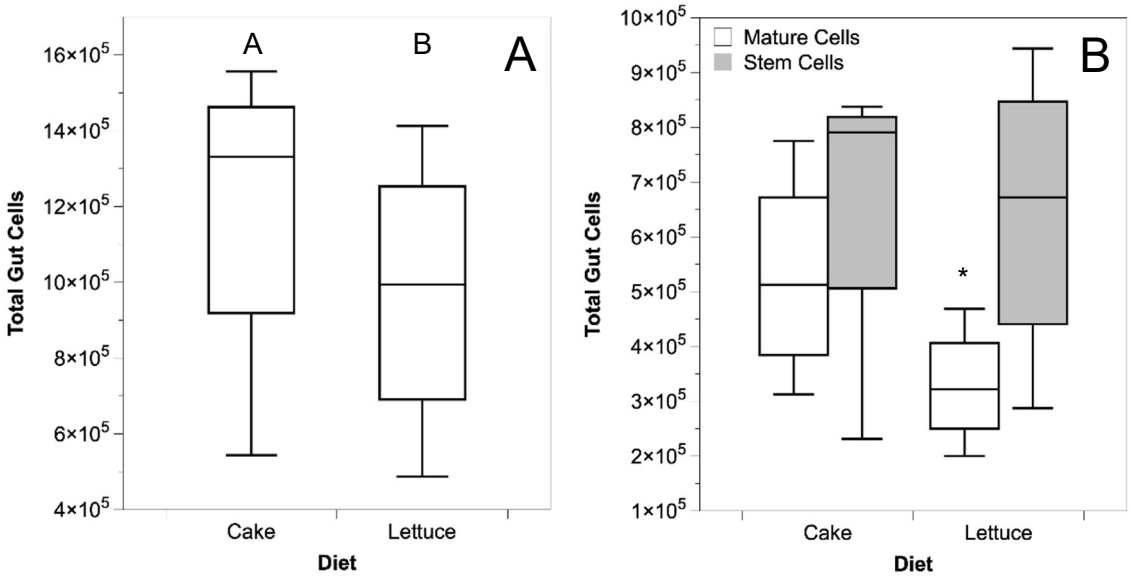

Figure 6. *Heliothis virescens* larvae exhibit cell population shifts when fed on lettuce relative to artificial cake diet. A) Total number of cells on both diets, showing a reduction following the dietary challenge. B) The total number of cells separated out by classification, showing an increase in stem cells following dietary challenge.

Given these findings, we utilized organic lettuce as a mild (sublethal) dietary challenge to test whether alterations in cell number via cell death resulted in alterations in membrane potential. To quantify midgut cell V_{mem} following dietary challenge, we utilized both DiBac4(3) and Rhodamine 6G, which fluoresce more intensely when cells are depolarized and hyperpolarized, respectively. We again recovered two size dimorphic cell populations in the dietary assay (Figure 7A-B), and broadly observed that stem cells are relatively depolarized in comparison to mature cells (Figures 7C-E). V_{mem} results differed between the two dyes for both populations of cells. Stem cells stained with Rhodamine 6G were statistically indistinguishable (F (3,896) = 660, p=0.48) between diet treatments; however, they significantly differed in the DiBac4(3) staining (F (3,896) = 279.7, p<0.001). Conversely, mature cells isolated from lettuce-fed individuals exhibited significantly different V_{mem} as compared to artificial diet controls with Rhodamine 6G (F (3,896) = 660, p<0.001), but not with DiBac4(3) (F (3,896) = 279.7, p=0.70). In both statistically different instances - stem cells isolated from lettuce-fed individuals stained with DiBac4(3), and mature cells isolated from lettuce-fed individuals stained with Rhodamine 6G - the cells exhibited significant depolarization relative to controls. Examination of the distribution of cell intensities shows that the stem cells are uniformly depressed in intensity in lettuce-fed pools stained with Rhodamine 6G (Figure 7C). The distribution of stem cell intensities in DiBac4(3) more clearly shows what may be a dimorphic pool (Figure 7E). This

implies that DiBac4(3) may be a better tool to observe stem cell depolarization, due to how close stem cell intensities were to the background fluorescence of Rhodamine 6G.

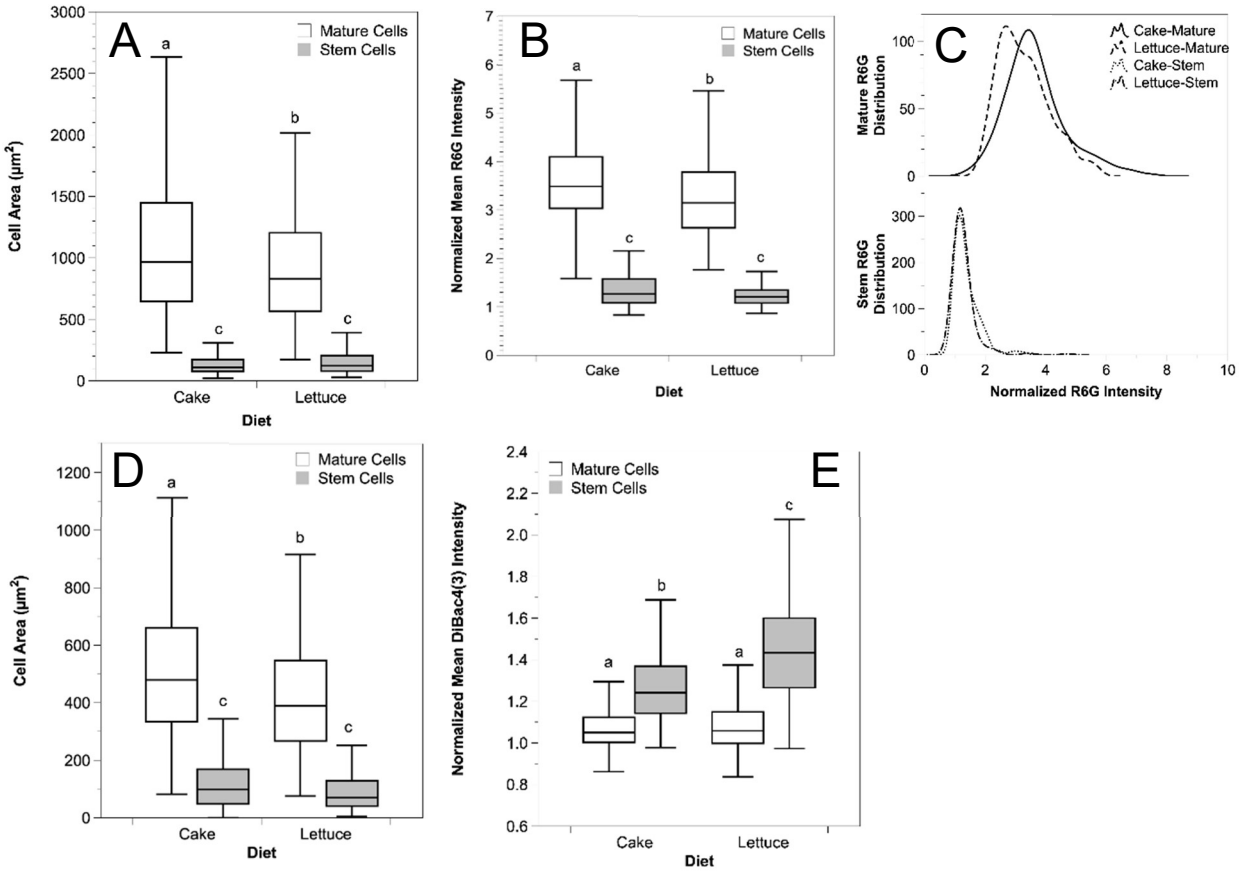

Figure 7. Membrane potential shifts in stem cells in lettuce but not artificial diet-fed individuals. A) Area and B) normalized mean intensity of midgut cells incubated with Rhodamine 6G following feeding with artificial (cake) diet or iceberg lettuce. C) Distribution of normalized mean intensity for mature and stem cell populations stained with Rhodamine 6G. D) Area, E) normalized mean intensity of DiBac4(3)-stained cells isolated from artificial diet or lettuce fed larvae.

5. Discussion

Bioelectric patterns are known to drive important processes in many different animal systems. The role of bioelectricity in the development and regeneration in the lepidopteran system is not well studied. The first step was to reliably distinguish between different populations of cells *ex vivo*. Traditionally, the morphology of the cells is used to distinguish between the different types of cells (Loeb et al.,2000). We observed that various cell types generally found in the gut epithelium of caterpillars can be placed in one of two populations (Figure 3A): mature cells which were typically non-uniform in shape and small round cells which are a combination of endocrine and stem cells. Additionally, we found that different cell populations could be distinguished by size (cell area). When area and shape were combined, we found that columnar and goblet cells were large and irregular and stem and endocrine cells were small and round. These patterns of size and shape are consistent with other studies that have looked at isolated populations of gut epithelial cells (Loeb and Hakim, 1996; Hakim et al., 2001). We note, however, that these differences in size were continuous and there could be some ambiguity relating to the presence of both endocrine cells as well as differentiating stem cells, which are known to be different at least in morphology from that of non-differentiating stem cells, in that stem cells are small and round and mature cells are typically larger and irregular in shape (Sadrud-din et al., 1996).

To characterize the baseline membrane potential patterns between our different cell populations, we used both DiBac4(3) and Rhodamine 6G. With both dyes we observed that stem cells (small round) are depolarized relative to mature cells and these patterns were constant

throughout ontogeny (from late 3rd to early 5th instar). Similar patterns have been seen in other stem cell systems (Levin et al. 2012).

Next, we characterized V_{mem} patterns during repair and regeneration of the epithelium following a dietary antagonist. We found that guts dissected from larvae fed on lettuce had a significantly lower mature cell count than guts from larvae fed on artificial diet, suggesting the diet treatment caused some damage and loss of cells. We then used the dyes mentioned above to record the membrane potential. We observed that the cells from the lettuce treatment were more depolarized than the cells from the artificial diet treatment. It has been suggested that new stem cells are predicted to be more depolarized than older cells (Levin et al. 2012). Our data thus imply that the stem cells in the lettuce treatment are newer, suggesting proliferation and possibly differentiation into mature cells, could be happening to fill the holes that would have been left by dying or dead mature cells (Loeb et al., 2000). These trends align with the idea that the gut stem cells act to rapidly repair the gut following damage, which has been suggested to be a major point of defense within the caterpillar, given that stem cell-based repair of the gut has been implicated in both the survival of larvae on sublethal doses of Bt toxins as well as viral infection (Spies, 1985 Hoover et al., 1998).

As has been reported previously (Loeb et al., 2000; Loeb et al, 2003; Hakim et al., 2009; Castagnola et al, 2010), stem cells of the larval lepidopteran *H. virescens* have the characteristic morphology of being small and round relative to the larger, more irregularly shaped columnar cells and the goblet cells. It should be noted that endocrine cells, which co-localize on the basal face of the pseudostratified gut epithelium (Levy et al. 2004) also are small and round.

Therefore, we likely have conflated their values (morphology, membrane potential, and numbers) with those of the stem cells. This could be an issue given endocrine cells are mature cells and likely have v_{mem} readouts similar to other mature cells. But given the consistency of the data presented here, the patterns observed are likely real. Future work would benefit from a method to remove the ambiguity caused by the inclusion of endocrine cells.

There are many examples of V_{mem} changes being linked to cell processes. One such example is the proliferation of vertebrate cells, where hyperpolarization is a necessary step in S-phase induction. G2/M induction has also been linked to depolarization activity in the cell (Blackiston et al., 2009). Outside the cell cycle, V_{mem} pattern changes have also been implicated in certain developmental pathways. In the fly *Drosophila melanogaster*, for example, depolarization during wing development increased potassium uptake via inward rectifying potassium channels leads to fluxes of Ca^{2+}, which in turn induces the release of the BMP ligand Decapentaplegic (Dpp) (Dahal et al., 2017; George et al., 2019). Dpp is required for the formation of proper wing patterns. Intriguingly, Dpp release may also be induced by exogenous depolarization, supporting that bioelectric signals are enough to regulate (at least some) ligand signaling pathways.

Bioelectric signaling has also been shown to be important in regenerative processes. In the frog *Xenopus laevis,* limb regeneration is limited to early stages of development. Removal of a limb post-regenerative state followed by incubation of the wound in a media with increased concentrations of Na^{+} was shown to induce Notch and BMP signaling. These signals lead to cell proliferation at the wound site (Tseng and Levin, 2013). Similarly, the well-known regenerative abilities of planarians can be manipulated by treating wound sites with Ivermectin, a Cl^{-} channel

agonist. Here, modification of wound site bioelectric patterns results in changes in regenerative abilities (Ferenc and Levin, 2019). Thus, across hundreds of millions of years of evolution and divergent animal phylogenies, there is a linkage and even dependence between bioelectric phenomena and developmental and regenerative processes.

This linkage highlights the importance of this work as a first step in understanding the role stem cell play in tissue regeneration and development, particularly the role that membrane potential plays in these processes. And given the relative importance of development and regeneration to the survival of insects, this work could serve as a first step in better understanding how insects survive our efforts to limit population size and thus allow us to better defend against these pests.

CHAPTER THREE

CONCLUSION

In this thesis work, I characterized the patterns of membrane potential in cells isolated from the midgut of larval *Heliothis virescens*. Although stem and mature cell membrane potential have been quantified in other organisms, this has not yet been characterized in a lepidopteran species. We found that the mature or large irregular cells were more hyperpolarized than the stem or small round cell. This pattern was consistent through ontogeny and following the treatment of different diets. Additionally, we saw indication that stem cells of the experimental group were more depolarized than the control, following the dietary damage. This is in line with what other studies have reported in other animal systems, that stem cells are depolarized relative to mature cells (Binggeli and Weinstein, 1986). This suggests that these patterns could be conserved across evolutionary time.

Our characterization with membrane potential sensitive dyes provides basic insight into the idea of lepidopteran stem cells behaving in a similar way to stem cells found in other organisms. This work was done by isolating cells from gut tissue and staining and imaging shortly after. Future studies could look at characterizing these patterns over time in a primary culture that can be maintained for weeks. In the past, primary culture has been used to investigate the effects of Bt toxins on gut cultures (Loeb et al. 2001). The latter method could be modulated to track the shifts in membrane potential through time by taking a subset of cells and staining

with a membrane potential sensitive dye. This would allow us to observe shifts in membrane potential through proliferation and differentiation, granting more insight into the role membrane potential plays in stem cell behavior. Additionally, attempts could be made to modify membrane potential to observe if altering membrane potential changes cell behavior. Primary culture of *H. virescens* midgut cells has been done (Loeb et al., 2003; Castagnola et al. 2010; Hakim et al, 2009), but getting a culture technique that is sterile and viable can be difficult. We tried to culture midgut cells by dissecting in sterile conditions in the presence of multiple concentrations of antibiotics, but the longest we could get viable cells was four days post-isolation (data not shown).

Primary culture could also serve as a method to eliminate some of the ambiguity caused by the inclusion of endocrine cells. This ambiguity is problematic when trying to distinguish between stem cells and mature cells (endocrine cells included). Stem cells divide in the midgut tissue (Castagnola et al., 2010), so tracking differentiation and proliferation could grant insight into a better way to distinguish between stem cells and other "small and round cells" found in the gut tissue. Though this will do little to distinguish between endocrine cells and quiescent or undifferentiated stem cells. Other than morphology, there needs to be a better method to distinguish between stem cells and other cell types. This can be done in several different ways. We could use vital dyes to distinguish between dead/dying cells and those that are viable. Based on what is seen in other studies, mature cells should dye after a few days leaving mostly stem cells (Castagnola et al. 2010). Flow cytometry has also been identified as a potential method to distinguish cell types (Castagnola et al. 2010). Additionally, RNAseq could be used on the different populations of cells to identify differences in gene expression between mature and stem

cells. Using combinations of these approaches, stem cell specific markers could be designed to unequivocally distinguish between mature and stem cells.

The main potential application of this work is in population control of insects. Though a specific control technique has not been suggested from this work, this study grants insight in the proper functioning of the lepidopteran gut. Since the gut is the main point of interaction between a lepidoptera larvae and the environment in which it lives, a better understanding of how these larvae survive our control techniques could allow us to better defend against them.

References

1. Barbosa P. Manual of basic techniques in insect histology. Bulletin of the ESA 1975 Mar 17,;21(1):12.

2. BOTTRELL DG, ADKISSON PL. Cotton insect pest management. 1977.

3. Binggeli R, Weinstein RC. Membrane potentials and sodium channels: Hypotheses for growth regulation and cancer formation based on changes in sodium channels and gap junctions. Journal of Theoretical Biology 1986 Dec 21,;123(4):377-401.

4. Blackiston DJ, McLaughlin KA, Levin M. Bioelectric controls of cell proliferation: Ion channels, membrane voltage and the cell cycle. Cell Cycle 2009 Nov 1,;8(21):3527-36.

5. Carrière Y, Crowder DW, Tabashnik BE. Evolutionary ecology of insect adaptation to bt crops. Evolutionary Applications 2010 Sep;3(5-6):561-73.

6. Castagnola A, Eda S, Jurat-Fuentes JL. Monitoring stem cell proliferation and differentiation in primary midgut cell cultures from *Heliothis virescens* larvae using flow cytometry. Differentiation 2011;81(3):192-8.

7. Coates BS. Bacillus thuringiensis toxin resistance mechanisms among lepidoptera: Progress on genomic approaches to uncover causal mutations in the european corn borer, *Ostrinia nubilalis*. Current Opinion in Insect Science 2016 Jun;15:70-7.

8. Dahal GR, Pradhan SJ, Bates EA. Inwardly rectifying potassium channels influence *Drosophila* wing morphogenesis by regulating dpp release. Development (Cambridge, England) 2017 Aug 1,;144(15):2771-83.

9. de Bortoli CP, Jurat-Fuentes JL. Mechanisms of resistance to commercially relevant entomopathogenic bacteria. Current Opinion in Insect Science 2019 Jun;33:56-62.

10. Engelhard EK, Keddie BA, Volkman LE. Isolation of third, fourth, and fifth instar larval midgut epithelia of the moth, *Trichoplusia ni*. Tissue and Cell 1991;23(6):917-28.

11. Fitt GP. The ecology of *Heliothis* species in relation to agroecosystems. Annual Review of Entomology 1989 Jan;34(1):17-53.

12. Ferenc NN, Levin M. Effects of ivermectin exposure on regeneration of *D. dorotocephala* planaria: Exploiting Human-Approved ion channel drugs as morphoceuticals. Macromolecular Bioscience 2019 Mar;19(3):e1800237,n/a.

13. G. Cermenati, P. Corti, S. Caccia, B. Giordana, M. Casartelli. 2007. A morphological and functional characterization of *Bombyx mori* larval midgut cells in culture. Invertebrate Survival Journal J4 119-126

14. George LF, Pradhan SJ, Mitchell D, Josey M, Casey J, Belus MT, Fedder KN, Dahal GR, Bates EA. Ion channel contributions to wing development in *Drosophila melanogaster*. G3 (Bethesda, Md.) 2019 Apr 9,;9(4):999-1008.

15. Hakim RS, Baldwin K, Smagghe G. Regulation of midgut growth, development, and metamorphosis. Annual Review of Entomology 2010 Jan 7,;55(1):593-608.

16. Hardwick DF. The corn earworm complex. Memoirs of the Entomological Society of Canada 1965 Jan 1,;97(40):1-247.

17. Hoover K, Yee J, Schultz C, Rocke D, Hammock B, Duffey S. Effects of plant identity and chemical constituents on the efficacy of a baculovirus against *Heliothis virescens*. Journal of Chemical Ecology 1998 Feb;24(2):221-52.

18. Jiang H, Tian A, Jiang J. Intestinal stem cell response to injury: Lessons from *Drosophila*. Cellular and Molecular Life Sciences : CMLS 2016 Sep;73(17):3337-49.

19. Lehane MJ, Billingsley PF. Biology of the insect midgut. Dordrecht: Springer Netherlands; 1996.

20. Levin M. Reprogramming cells and tissue patterning via bioelectrical pathways: Molecular mechanisms and biomedical opportunities. Wiley Interdisciplinary Reviews: Systems Biology and Medicine 2013 Nov;5(6):657-76.

21. Levin M, Pezzulo G, Finkelstein JM. Endogenous bioelectric signaling networks: Exploiting voltage gradients for control of growth and form. Annual Review of Biomedical Engineering 2017 Jun 21, 19(1):353-87.

22. Levin M, Stevenson CG. Regulation of cell behavior and tissue patterning by bioelectrical signals: Challenges and opportunities for biomedical engineering. Annual Review of Biomedical Engineering 2012 Aug 15,14(1):295-323.

23. Lin G, Xu N, Xi R. Paracrine wingless signalling controls self-renewal of *Drosophila* intestinal stem cells. Nature 2008 Oct 23, 55(7216):1119-23.

24. Loeb MJ. Factors affecting proliferation and differentiation of lepidopteran midgut stem cells. Archives in Insect Biochemistry and Physiology 2010;74(1):1-16.

25. Loeb MJ, Hakim RS. Insect midgut epithelium in vitro: An insect stem cell system. Journal of Insect Physiology 1996;42(11):1103-11.

26. Loeb MJ, Hakim RS, Martin P, Narang N, Goto S, Takeda M. Apoptosis in cultured midgut cells from *Heliothis virescens* larvae exposed to various conditions. Archives of Insect Biochemistry and Physiology 2000 Sep;45(1):12-23.

27. Loeb MJ, Jaffe H, Gelman DB, Hakim RS. Two polypeptide factors that promote differentiation of insect midgut stem cells in vitro. Archives of Insect Biochemistry and Physiology 1999;40(3):129-40.

25. Raziel S. Hakim, Kate M. Baldwin, Marcia Loeb. The role of stem cells in midgut growth and regeneration. In Vitro Cell Developmental Biology -Animal 2001 Jun 1,;37(6):338-42.

28. Reisig DD, Huseth AS, Bacheler JS, Aghaee M, Braswell L, Burrack HJ, Flanders K, Greene JK, Herbert DA, Jacobson A, et al. Long-term empirical and observational evidence of

practical *Helicoverpa zea* resistance to cotton with pyramided bt toxins. Journal of Economic Entomology 2018 Aug 3,;111(4):1824-33.

29. Rowland IJ, Goodman WG. Magnetic resonance imaging of alimentary tract development in *Manduca sexta*. PloS One 2016;11(6):e0157124.

30. S. M. Levy, A. M. F. Falleiros, E. A. Gregório, N. R. Arrebola, L. A. Toledo. The larval midgut of anticarsia gemmatalis (hübner) (lepidoptera: Noctuidae): Light and electron microscopy studies of the epithelial cells intestino médio de larvas de *Anticarsia gemmatalis* (hübner) (lepidoptera: Noctuidae): Estudo das células epiteliais ao microscópio de luz e eletrônico. Brazilian Journal of Biology 2004 Aug 1,;64(3b):633-8.

31. Sadrud-Din, S. Loeb, M. Hakim, R. In vitro differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae. The Journal of Experimental Biology 1996 Jan 1,;199:319-25.

32. Seth J Wechsler. Trends in the adoption of genetically engineered corn, cotton, and soybeans. Amber Waves 2018 Dec 1,:1-7.

33. Smagghe G, Vanhassel W, Moeremans C, Wilde D, Loeb MJ, Blackburn MB, Hakim RS. Stimulation of midgut stem cell proliferation and differentiation by insect hormones and peptides. Annals of the New York Academy of Sciences 2005 Apr;1040(1):472-5.

34. Spies AG, Spence KD. Effect of sublethal *Bacillus thuringiensis* crystal endotoxin treatment on the larval midgut of a moth, *Manduca*: SEM study. Tissue and Cell 1985;17(3):379-94.

35. Bruce E. Tabashnik, Yves Carrière, Timothy J. Dennehy, Shai Morin, Mark S. Sisterson, Richard T. Roush, Anthony M. Shelton, Jian-Zhou Zhao. Insect resistance to transgenic bt crops: Lessons from the laboratory and field. Journal of Economic Entomology 2003;96(4):1031-8.

36. Tseng A, Levin M. Cracking the bioelectric code. Communicative & Integrative Biology 2013 Jan 1,;6(1).

37. Zhang P, Turnbull MW. Virus innexin expression in insect cells disrupts cell membrane potential and pH. The Journal of General Virology 2018 Oct;99(10):1444-52.