

**Comparing the midgut regenerative responses in *Bacillus thuringiensis*-
susceptible and resistant *Heliothis virescens* larvae**

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Anaïs Severiana Castagnola

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Abstract

The crystal (Cry) toxins from *Bacillus thuringiensis* (Bt) display high specificity and toxicity against relevant insect pests and the use of Bt-based products continues to contribute to insect pest management. To protect this investment, further its potential, and investigate possible unintended effects, various research questions have been proposed. One issue related to Bt usage is the evolution of pest resistance to Bt toxins. The midgut epithelium is targeted by Cry toxins killing enterocytes, facilitating invasion of the hemocoel, leading to septicemia and mortality. While resistance may emerge from alterations to these steps, most research efforts have been focused on reduced toxin binding to midgut receptors as resistance mechanism. Lepidopteran crop pest *Heliothis virescens* strains have been hypothesized to have enhanced midgut proliferation and differentiation of stem cell populations allowing for regeneration and resistance to diverse Cry toxins. However, the molecular mechanisms involved are not known. We developed a flow cytometry method to monitor stem cell proliferation and differentiation to compare midgut regenerative responses to Cry intoxication in larvae from susceptible and Bt-resistant strains of *H. virescens*. The structure of the epithelial healing response was studied *in vivo* using hematoxylin-eosin stained midguts derived from larvae fed Cry1Ac toxin. We detected less regenerative cells in midguts from a Bt-susceptible strain (YDK) compared to midguts from resistant (KCB and CXC) strains, and an overall increase in the total number of cells per unit surface area in KCB midguts. Using primary midgut cell cultures, the midgut regeneration response to Cry1Ac in CXC was an increase in available differentiated cells compared to YDK. In contrast, KCB exhibited an increased abundance of stem cells compared to both YDK and CXC. Using a differential proteomics approach we

characterized the proteins secreted by *H. virescens* midgut cells in response to Cry1Ac and identified a relevant role for arylphorin in promoting midgut regeneration in response to Cry1Ac and DiPel intoxication in both susceptible and resistant *H. virescens* larvae. The potential fitness costs associated with altered hexamerin transcript expression were monitored using larval bioassays.

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1 Bt crops: past and future

1.1 Abstract

The development and commercialization of transgenic plants expressing insecticidal toxin genes from the bacterium *Bacillus thuringiensis* (Bt) has revolutionized agriculture in the past two decades. Development of this revolutionary insect pest control technology was facilitated by the identification and characterization of insecticidal Bt proteins and advancements in plant transformation and genetic engineering. While commercialization of this technology is currently limited to a number of countries, these transgenic “Bt crops” are replacing in most cases conventional crop varieties due to their insect resistance, lower spraying requirements, and higher yields. However, concerns related to the increasing adoption of this technology include gene flow to wild relatives, evolution of resistance in target pests, and unintended effects on the environment. In this chapter, we discuss key events in the history of Bt crop development and summarize current regulations aimed at reducing the risks associated with increased adoption of this technology. By analyzing the history of Bt transgenic crops and the current marketplace trends and issues, we aim to examine the outlook of current and impending Bt crops as well as potential issues that may emerge during their future use.

1.2 Introduction

Insecticidal products based on the bacterium *Bacillus thuringiensis* (Bt) have been used for decades to control lepidopteran (caterpillars), dipteran (mosquitoes and black flies), and coleopteran (beetle larvae) pests ([Sanchis 2010](#)). It was estimated that Bt products represent about 80% of all biopesticides, pesticides derived from natural materials, sold worldwide

([Whalon and Wingerd 2003](#)). A major driver in this adoption of Bt products has been the global proliferation of certified organic production, which relies heavily on Bt for insect control. The specificity and high relative toxicity of the insecticidal proteins produced by Bt results in high efficacy and environmental safety when compared to available synthetic pesticides. However, several factors have limited higher adoption of Bt products, including short persistence and low residual activity due to environmental degradation, and poor control of tunneling or root-feeding pests ([Sanahuja, Banakar et al. 2011](#)). These limitations directed the interest in developing alternative systems for more persistent and direct delivery of Bt toxins to control agricultural pests. For example, encapsulation of Bt toxins in non-pathogenic *Pseudomonas fluorescens* cells that were killed before release resulted in increased resistance to environmental degradation and toxicity ([Gaertner 1993](#)). Undoubtedly, the most efficient delivery system developed to control lepidopteran and coleopteran pests is the transformation of plants with insecticidal Bt genes. These transgenic “Bt crops” are protected from insect attack by expression of the transformed Bt toxin genes and accumulation of Bt toxins in the plant tissues. Direct delivery to insects feeding on the plant minimizes exposure to non-target fauna and allows management of otherwise difficult to control tunneling and root-feeding pests.

1.3 Developments conducive to Bt crops

1.3.1 Research on Bt toxin genes

Early research on the identification of Bt proteins responsible for insecticidal activity and progress on methods to transform plants were vital to the development of Bt crops. Discovery, characterization, and classification of Bt isolates using flagellar H antigen typing of vegetative

cells ([de Barjac and Bonnefoi 1968](#)) greatly facilitated the identification of strains producing toxins with potential to control specific pests. More recently, determination of insecticidal activity has been mostly focused on bioassays with purified Bt toxins ([van Frankenhuyzen 2009](#)), because strains within a single Bt serotype can produce multiple and diverse insecticidal components.

Among several virulence factors, Bt cells produce toxins that are responsible for insecticidal activity, and include vegetative insecticidal proteins (Vip), crystal (Cry), and cytolytic (Cyt) toxins. While Vip toxins are produced during the vegetative growth phase ([Estruch, Warren et al. 1996](#)), Cry and Cyt toxins are synthesized during sporulation ([Hannay and Fitz-James 1955](#)) and late exponential growth phase ([Salamitou, Agaisse et al. 1996](#)). Both Cry and Cyt toxins are stored as parasporal crystalline bodies ([Bulla, Kramer et al. 1977](#)), which may be composed of single or multiple toxins contributing to insecticidal activity ([Crickmore, Bone et al. 1995](#)). Determination and compilation of the activity range for individual Bt toxins ([van Frankenhuyzen 2009](#)) allows for the identification of high potency toxins as optimal candidates for expression in transgenic crops to control key insect pests. Due to their earlier discovery and characterization, *cry* toxin genes have been predominantly used for plant transformation, although finding and characterization of *vip* genes has allowed their use to produce transgenic Bt crops (Table 13.1).

The genes encoding Cry ([Kronstad, Schnepf et al. 1983](#)) and Vip ([Franco-Rivera, Benintende et al. 2004](#); [Wu, Guo et al. 2004](#)) toxins are located in plasmids or in the bacterial chromosome. Cloning of the first Cry toxin gene and its expression as an active insecticidal toxin in *Escherichia coli* ([Schnepf and Whiteley 1981](#)) suggested the potential transformation of

Cry toxin genes in diverse organisms to enhance efficacy or increase activity range. Currently, more than 400 Bt toxin genes have been cloned and sequenced, including 218 Cry and 28 Vip toxin holotypes ([Crickmore 2011](#)). Thorough characterization of the mechanisms directing Bt toxin gene expression ([Agaisse and Lereclus 1995](#)), and the small number of genetic loci implicated in controlling this process, greatly facilitated genetic manipulations for efficient expression in heterologous systems ([Andrews, Faust et al. 1987](#)). A diverse range of microorganisms were initially transformed with Bt toxin genes, including alternative *Bacillus* spp. ([Shivakumar, Vanags et al. 1989](#)), *P. fluorescens* ([Obukowicz, Perlak et al. 1986](#); [Gaertner 1993](#)), *Rhizobium* spp. ([Skøt, Harrison et al. 1990](#)), and *Clavibacter xyli* ([Lampel, Canter et al. 1994](#)). Expression of Bt toxin genes in plants required the development of efficient plant transformation and selection methods and the identification of efficient promoter sequences to direct Bt toxin gene expression.

1.3.2 Plant expression systems

The identification of the tandem duplication 35S promoter from the Cauliflower Mosaic Virus (CaMV35S) ([Kay, Chan et al. 1987](#)) and the ubiquitin (ubi) maize (*Zea mays*) promoter ([Christensen, Sharrock et al. 1992](#)), were crucial to achieve enhanced expression of non-plant genes *in planta*, including Bt toxin genes. Although promoters driving expression systemically were initially used for Bt crops, there have also been later examples of the use of tissue-preferred promoters for targeted expression. This strategy is especially desirable when the target pest specializes on feeding on a particular plant tissue. For example, expression of the *cry34Ab1/cry35Ab1* toxin genes to control the root-feeding larvae of the Western corn rootworm

(*Diabrotica virgifera*) has been recently achieved using the *Triticum aestivum* (wheat) peroxidase gene promoter driving root transgene expression ([Gao, Schafer et al. 2004](#)). Expression of the transgene can also be targeted to a particular organelle by using a transit peptide gene, a proposed strategy to help prevent escape of the Bt transgene to wild relatives. For example, the chloroplast transit peptide gene (*cab22L*) from *Petunia hybrida* was used to drive expression of the *cry9c* gene to maize chloroplast in StarLink corn ([Jansens, vanVliet et al. 1997](#)). After this product was withdrawn from the market, alternative promoters such as the rice *rbcS* and its transit peptide sequence (*tp*) have been used to target and enhance expression of *cry* genes in chloroplasts ([Kim, Suh et al. 2009](#)). Alternatively, a chloroplast expression vector containing sites for homologous recombination and the chloroplast promoter *Prrn* was used for directing chloroplast expression of *cry1Ac* ([McBride, Svab et al. 1995](#)) or *cry2Aa* in tobacco ([Kota, Daniell et al. 1999](#)), and *cry1Ab* in cabbage ([Liu, Lin et al. 2008](#)).

1.3.3 Bt toxin gene transformation and selection of transformants

The discovery of effective promoters and the design of *in planta* expression cassettes also advanced the development of effective plant transformation methods. The development of various *Agrobacterium tumefaciens* protocols ([Zambryski, Joos et al. 1983](#)) allowed for stable transformation of Bt genes into dicotyledoneous plants, such as tomato or tobacco. In this system, the *Agrobacterium* Ti plasmid vector is modified by removing tumor-generating portions and inserting the Bt toxin gene of interest and selectable markers so that plant infection resulted in systemic gene integration and subsequent regeneration of transgenic plants. Alternative transformation methods such as electroporation and biolistic bombardment ([Gordon-Kamm,](#)

[Spencer et al. 1990](#)), which could also be used to transform monocotyledoneous crops (maize, rice, or wheat), were also used for development of Bt crops ([Koziel, Beland et al. 1993](#)). In these methods, the plasmid DNA containing a Bt toxin gene of interest and selectable markers are delivered into plant cells using pores induced by electric shock (electroporation) or by coating the plasmid with a heavy metal particle and then using it to bombard plant embryos with a gene gun.

After transformation, successful transformants are usually selected using diverse antibiotic resistance genes. The most commonly used selection gene for Bt crops has been -the neomycin phosphotransferase II (NPTII) gene (*neo*) from *E. coli* ([Betz, Hammond et al. 2000](#)), which inactivates antibiotics like kanamycin and neomycin. An alternative selection approach that has been used for Bt crops is the growth of transformants containing the mannose-6-phosphate isomerase (*Pmi*) gene from *E. coli* on media containing mannose as the only carbon source ([Long 2007](#)). Other examples of selection markers used for selection of plants transformed with Bt genes include the aminoglycoside 39-adenyltransferase (*aadA*) gene that confers resistance to spectinomycin-streptomycin ([Kota, Daniell et al. 1999](#)), and the hygromycin-B phosphotransferase (*aph4*) gene, which allows for transformed cell selection on culture medium containing hygromycin ([Llewellyn, Mares et al. 2007](#)).

1.3.4 Early development and challenges for Bt crops

Once Bt toxin genes of interest were identified and transformation and selection methods were optimized, public and private research groups quickly began to experiment with the goal to develop transgenic Bt plants. Initial attempts transforming full-length crystal toxin genes

resulted in plant toxicity ([Barton, Whiteley et al. 1987](#)), shifting interest to the expression of truncated crystal toxin genes encoding the insecticidal N-terminal toxin half, which were found not to be toxic to the plant. Truncated *cryIA* toxin genes were used to develop transgenic tomato ([Fischhoff, Bowdish et al. 1987](#)), tobacco ([Barton, Whiteley et al. 1987](#); [Vaeck, Reynaerts et al. 1987](#)), and cotton ([Perlak, Deaton et al. 1990](#)). Although some of these initial transgenic Bt plants presented resistance to feeding by selected lepidopteran larvae, it was recognized that the levels of toxin gene expression were still low and performance considered insufficient for commercialization. Low levels of toxin mRNA in the transformed plants suggested that the toxin transcripts were unstable, possibly due to inefficient posttranscriptional processing or rapid turnover ([Barton, Whiteley et al. 1987](#); [Murray, Stock et al. 1991](#)). This phenomenon was originally attributed to the AT-rich nature of Bt toxin genes, which resulted in recognition by the plant cell regulatory mechanisms as foreign, triggering polyadenylation and mRNA instability. Translational efficiency was greatly improved by modification of the Bt toxin gene to match plant-preferred coding sequences, resulting in up to 100-fold higher levels of Cry1Ab toxin in transgenic tobacco and tomato ([Perlak, Fuchs et al. 1991](#)). Laboratory and field tests using transgenic tobacco lines containing this truncated *cryIAb* toxin gene under the control of the CaMV 35S promoter ([Carozzi, Warren et al. 1992](#)) provided evidence of effective protection against tobacco hornworm (*Manduca sexta*) and tobacco budworm (*Heliothis virescens*) ([Warren, Carozzi et al. 1992](#)). Maize plants transformed with an optimized synthetic *cryIAb* gene were reported to be resistant to European corn borer (*Ostrinia nubilalis*) under field conditions ([Koziel, Beland et al. 1993](#)). Further increase in the efficiency of Cry1Ab toxin production in tobacco was achieved using point mutations to avoid inefficient cryptic splice sites

that inhibited nuclear transcript processing and transport to the cytoplasm ([Aarssen, Soetaert et al. 1995](#)). Even higher levels of Cry toxin accumulation were reported for expression of the *cryIAc* toxin gene in tobacco chloroplasts, with accumulation of Cry1Ac protoxin to 5% of the total soluble protein in tobacco leaves ([McBride, Svab et al. 1995](#)).

Prior to the 1960's, intellectual property rights were not readily enforced and the value of scientifically formulated technologies was not realized. A group of biotech companies acknowledged the significance of the above biotechnological developments and shifted the majority of its funding initiatives from chemical manufacturing to basic biotechnology. Companies like Monsanto or Mycogen began experimenting with a variety of promoters, antibiotic resistance genes, transformation-tissue culture systems, and investigating novel Bt insecticidal proteins. These important investments in new technologies compelled the protection of intellectual property rights to secure economic returns. By implementing measures for agricultural companies to protect their investments, the commercial viability of insecticidal transgenic technology was further explored ([Horsch 1993](#)). The patentable portions of the transgenic Bt technologies included promoters, expression strategies, selection markers and techniques, as well as transgenic plants and traits expressed. The strengthening of intellectual property rights clearly indicated the commercial success and marketability of Bt transgenic crops as pest control products, as exemplified in a fourteen-fold increase in expenditure on research and development of plant breeding-related science and technology derived from private funding sources. Public expenditure patterns, which also contribute to investment trends, changed little during this time, further solidifying the causal agent of progress to intellectual property law enforcement ([Fernandez-Cornejo 2004](#)). The actions of early agriculture biotechnology

companies permanently restructured their commercial identity and led the field of agricultural biotechnology from predominantly academic to the most competitive pest management marketplace in history.

Transgenic crops fell under the jurisdiction of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and were therefore considered by regulatory agencies as pesticides for registration purposes ([Earl 1983](#)). Concerns related to changes in Bt toxin behavior after expression in a plant host prevented the use of established data advocating safety of Bt sprays to support safety of Bt crops. Thus, companies soliciting registration were requested to present data detailing toxicological tests with a wide range of organisms (including vertebrates, nematodes, and non-target insects) to support the safety of Bt crops at the toxin amounts produced by the plants. These tests had to be presented for each of the individual insecticidal transgenic traits contained in a plant, so that registration of Bt crops expressing multiple Bt toxin genes would require toxicological assays for each of the toxins produced by the plant. Concerns over the development of resistance to Bt crops, which would also affect alternative Bt-based products, resulted in an additional requisite during registration for a resistance management strategy. These resistance management programs included recommendations for growers, development of resistance monitoring protocols, and resistance control procedures ([Matten, Lewis et al. 1996](#)).

1.4 Commercialization and performance of “first generation” Bt crops

The first commercially available Bt crop was potato expressing the *cry3A* toxin gene (NewLeaf), a product manufactured by an affiliate of Monsanto (NatureMark) that was commercialized in 1995. The expressed *cry3A* gene derived from Bt subsp. *tenebrionis* was

selected due to its high activity against larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*), one of the most economically relevant pest of potato. The amount of Cry3Aa protein expressed within the foliage tissue of the NewLeaf Russet Burbank potatoes was 0.1-0.2% of total leaf protein, representing about a 100-fold higher concentration than the dose needed to kill 95% of neonate *L. decemlineata* larvae, thus fulfilling a high-dose requirement ([Perlak, Stone et al. 1993](#)). Unlike any pest management tool beforehand, NewLeaf potatoes were protected from *L. decemlineata* during all of its life stages and throughout the entire growing season, resulting in significant reductions in insecticide use. Subsequent NewLeaf varieties commercialized in the late 1990's also provided resistance to aphid-transmitted potato viruses ([Lawson, Weiss et al. 2001](#)). Despite remarkable product performance ([Stark 1997](#)), sales and marketing of NewLeaf potato were suspended in 2001 due to issues related to public concerns over transgenic potatoes being used for human consumption.

Alternative transgenic Bt crops were commercialized shortly after NewLeaf potatoes, including maize and cotton, which have since remained the most relevant markets for transgenic seed. Transgenic cotton varieties expressing the *cry1Ac* gene (Bollgard I in the U.S. and Ingard in Australia) were commercialized to control the cotton budworm (*H. virescens*) and the pink bollworm (*Pectinophora gossypiella*) in the U.S. and *Helicoverpa* spp. in Australia. In the first year after its introduction, Bollgard I technology was adopted by more than 5,700 growers in the United States, and contributed to reduction of more than a quarter of a million gallons in chemical insecticide use ([Fraley 1996](#)). Similarly, the average number of sprays to control *Helicoverpa* spp. in Australia was reduced by 56% in Ingard cotton during the first six years after commercialization ([Pitt 2003](#)). Transgenic Bt cotton 'stacked' varieties containing genes

for herbicide resistance were introduced a year later. This technology was rapidly accepted by growers due to its cost-effectiveness under pest pressure ([Martin and Hyde 2001](#)), with an estimated 37% adoption rate in the U.S. by 2001 ([Fernandez-Cornejo and McBride 2002](#)). Concerns with development of resistance led to capping of commercial deployment of Ingard cotton until 2004 at 30% of the cotton area in Australia, until two-toxin gene varieties became available ([Pitt 2003](#)). The growing popularity of transgenic Bt cotton resulted in further reductions in the number of required insecticidal applications in the U.S. by 1999 ([Carriere, Dennehy et al. 2001](#)). Similarly, adoption of Bt cotton in China and India has been reported to result in increased production yields and reduced insecticidal applications ([Pray, Huang et al. 2002](#); [Qaim and Zilberman 2003](#); [Wu, Lu et al. 2008](#)). However, efficient control of targeted pests has resulted in increased populations of secondary pests not controlled by Bt toxins ([Lu, Wu et al. 2010](#)).

Another early success story of the commercialization of transgenic Bt crops was the effective control of the European corn borer (*O. nubilalis*) by Bt maize ([Koziel, Carozzi et al. 1996](#)). Prior to the advent of Bt maize, *O. nubilalis* pest populations were especially devastating due to the burrowing feeding behavior of the larvae, which feed on the whorl, leaf axils, and sheath before boring into the stalk and becoming protected to chemical or foliar insecticidal sprays. Larvae of *O. nubilalis* are highly susceptible to Cry1Ab, which was the toxin gene selected for production of the first round of registered Bt maize varieties, which were based on events 176 (KnockOut from Syngenta and NatureGard from Mycogen), Bt11 (Agrisure from Northup King), or MON810 (Yieldgard from Monsanto) ([Sanahuja, Banakar et al. 2011](#)). Ensuing Bt maize products expressing the *cry1Fa* (event TC1507 in Herculex from Dow

AgroSciences) or *cry9C* (event CBH-351 in StarLink from Aventis CropScience) toxin genes (Table 13.1) were commercialized to target *O. nubilalis* and additional selected species of armyworm (*Spodoptera* spp.). By the year 2000, transgenic Bt maize represented over 85% of all corn grown worldwide ([Shelton, Zhao et al. 2002](#)), and it currently represents 65% of the corn grown in the U.S. ([Service-USDA 2011](#)). These high levels of adoption have resulted in area-wide elimination of *O. nubilalis* along the U.S. corn belt, benefiting both farmers growing transgenic and non-Bt maize ([Hutchison, Burkness et al. 2010](#)). The use of transgenic Bt maize has also been reported to significantly reduce accumulation of ear molds and associated mycotoxins ([Dowd 2000](#); [Hammond, Campbell et al. 2004](#); [Wu 2006](#)), contributing to food safety ([Kershen 2006](#)). On the other hand, Cry9C from StarLink maize represented the first case of unintended entry of transgenic grain in the human food supply. This maize event was approved for domestic animal feed but not for human consumption, yet it was detected in taco shells. This detection resulted in Aventis requesting cancellation of the StarLink registration and the U.S. Food and Drug Administration recommending testing of more than 4 million bushels of corn for the presence of Cry9C until 2007, when monitoring efforts ceased due to lack of detection of significant levels of toxin residue. While there were no documented human allergy cases related to Cry9C, media exposure of the unintended introduction of transgenic Bt maize in the human food supply resulted in public objections to commercialization of transgenic crops.

1.5 Risks associated with the use of Bt crops

Regulation of transgenic Bt crops in the U.S. is currently directed by the Coordinated Framework for the Regulation of Biotechnology, including branches of the Department of

Agriculture Animal and Plant Health Inspection Service (USDA-APHIS), the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). While USDA-APHIS regulates issuance of permits for field release, the EPA assesses the human, environmental and non-target safety of the transgene itself, while FDA assesses the food quality of transgenic crops. Although the commercialization of Bt crops is recognized as one of the most relevant events in the history of agricultural pest control, a number of potential risks associated to the environmental safety and future utility of this technology have been considered. Most relevant identified risks include potential toxicity to non-targets, escape of transgenes in the environment, and development of resistance in targeted insect populations. Although these issues are still a matter of extensive research, strategies to minimize these risks have been proposed and implemented in some cases.

1.5.1 Effects of Bt crops on non-target organisms

The effect of Bt crops on non-target organisms is one of the mandatory components of product registration, although there are also available studies testing safety of Bt crops to non-targets under field conditions. Using NewLeaf potatoes as an initial case study, Stark ([Stark 1997](#)) reported no detrimental effects on non-target, beneficial and predatory insect populations, probably due to reductions in insecticidal applications. Specifically, lady beetles (Coccinellidae) and their important feeding behavior on aphids were unaffected by transgenic potatoes producing Cry3Aa toxin which are insecticidal to closely related *L. decemlineata* larvae ([Dogan, Berry et al. 1996](#)). Results from meta-analysis studies suggest that Bt crops in general support lower numbers of beneficial insects compared to conventional crops when no insecticides are used

([Marvier, McCreedy et al. 2007](#)), which may be due to prey number reduction in Bt plants. In contrast, these studies also found that when insecticidal sprays were used, Bt crops supported higher levels of beneficial insects compared to non-Bt crops, due to more intensive applications needed for non-Bt crops. A compilation of laboratory studies on the influence of Bt crops on 48 species of beneficial predatory insects supports a generally negative impact of Bt crops on predaceous and parasitoid insects ([Lovei, Andow et al. 2009](#)), but whether this is due to the transgene presence or reduction in insect prey populations is unclear. Green lacewings (*Chrysoperla carnea*) have been extensively used as a non-target model organism. Adult lacewings feeding on Bt maize pollen expressing Cry1Ab or Cry3Bb1 did not show significant impact on survival rate, pre-oviposition period, fecundity, fertility or dry weight ([Li, Meissle et al. 2008](#)). Lacewing larvae are not directly affected by Bt toxins ([Rodrigo-Simon, de Maagd et al. 2006](#)), although detrimental effects resulting from low quality or reduced availability of prey in Bt crop fields have been reported ([Romeis, Dutton et al. 2004](#)). Aphids, which are another non-target insect model, do not accumulate Bt toxin after feeding on transgenic Bt plants, which was shown to prevent unintended exposure of predators ([Lawo, Wackers et al. 2009](#)). A meta-analysis of independent studies supported lack of negative effects for Bt crops on honey bees (*Apis mellifera*). Research on potential effects on non-target Lepidopteran populations by Bt crops has been mostly limited to the Monarch butterfly (*Danaus plexippus*) ([Lang and Otto 2010](#)). This focus is probably attributed to the wide attention ([Shelton and Sears 2001](#)) surrounding a single publication ([Losey, Rayor et al. 1999](#)) presenting conclusions that were later demonstrated to be unfounded ([Sears, Hellmich et al. 2001](#); [Gatehouse, Ferry et al. 2002](#)).

The possible leaching of Bt toxins from Bt crop residues into nearby water bodies and the potential effect on aquatic fauna has also been met with controversy ([Waltz 2009](#)). While deposition of Bt plant tissue in the proximity of streams by wind and surface runoff can result in leaching of Bt toxins in the water ([Tank, Rosi-Marshall et al. 2010](#); [Viktorov 2011](#)), their insecticidal properties on aquatic insects have not been demonstrated. The effect of transgenic Bt crop detritus on aquatic ecosystems should be comprehensively explored. In soil ecosystems, Bt crops have generally been found not to adversely affect symbiotic arbuscular mycorrhizal fungi (Liu 2010). While short-term activity shifts in bacterial communities were reported in the presence of residue from Bt corn expressing Cry1Ab toxin (Mulder et al. 2006), no changes in soil microbial population composition or activity were detected in a two-year field study with the same Bt corn varieties (Oliveira et al. 2008). Overall, it is considered that the ecological benefits of reduced synthetic pesticide usage greatly outweigh the minimal consequences to beneficial insect populations ([Gatehouse, Ferry et al. 2011](#)).

1.5.2 Bt transgene escape

The potential escape of Bt genes from transgenic crops into the environment has also been an issue of concern. Some reports suggested introgression into wild maize relatives ([Quist and Chapela 2001](#)), although this conclusion was quickly refuted with issues of contamination and methodology ([Christou 2002](#)). Two main categories of strategies, biological and non-biological, have been considered to prevent transgene escape from transgenic plants. Non-biological methods revolve around mechanical control where pollinating flowers are removed or transgenic crops are secluded from non-transgenic varieties ([Rong, Lu et al. 2007](#); [Kausch,](#)

[Hague et al. 2010](#)). Various molecular strategies for biotechnology-based gene containment have been proposed, including complete transgene excision from seed and pollen ([Luo, Duan et al. 2007](#)), and seed ([Daniell 2002](#)) and male sterility ([He, Abad et al. 1996](#)).

1.5.3 Insect resistance to Bt crops

Resistance to Bt crops has arguably been the main concern related to increased adoption of this technology, mostly because resistance to a specific Bt crop may result in cross-resistance to multiple Bt-based products, including microbial pesticides. Resistance to Bt toxins has been described in a number of laboratory-selected insect strains, evidencing the genetic potential for evolution of resistance to Bt toxins in the field. In the majority of laboratory cases, resistance is associated with alterations in toxin binding to midgut receptors, which is generally transmitted as a single autosomal recessive gene ([Ferré and Van Rie 2002](#)). In agreement with these laboratory reports, resistance to commercial Bt sprays was reported to result from reduced Bt toxin binding in strains of *Plutella xylostella* ([Ferré, Real et al. 1991](#)) and *Plodia interpunctella* ([Van Rie, McGaughey et al. 1990](#)). Based on these data and results from predictive models, three main strategies to delay resistance to Bt crops were outlined ([Gould 1988](#); [Tabashnik 1989](#)): use of refugia in conjunction with expression of high levels of the Bt toxin gene in the plant, rotations between plants expressing Bt toxins with diverse mode of action, and pyramiding of multiple toxin genes within a plant. The initial high dose/refugia strategy used to delay evolution of resistance to single-toxin Bt crops combined the use of mandated 20% refugia and the expression of high Bt toxin doses (25-fold the dose killing 99% of the pest population) in the plant. Exceptions and differences in this regulation occur when there are pests for which Bt crops do

not fulfill the high dose requirement, in which case manufacturer recommended refuge amounts can be raised to as much as 50% of the crop. Refugia are typically areas contiguous to Bt crop fields, and this proximity greatly increases the probability of mating between resistant insects emerging from Bt crops and non-selected insects growing in the refuge. Mating events generate heterozygotes, which combined with a lethal toxin dose decreases the frequency of resistant individuals, assuming that resistance is transmitted as a recessive trait ([Gould 1998](#)). The success of this high dose/refuge strategy is greatly dependent on constant expression of Bt toxin in the plant throughout the season and grower compliance with refuge planting regulations. Both tenets are difficult to achieve, as expression of Bt toxin genes varies depending on diverse factors ([Adamczyk, Adams et al. 2001](#); [Adamczyk and Sumerford 2001](#); [Adamczyk, Perera et al. 2009](#)), and refuge compliance is known to vary among growers and years ([Bourguet, Desquilbet et al. 2005](#); [Gray 2010](#)). In addition, a crucial condition for the effectiveness of refugia is the pest movement throughout a particular crop. In cases of pests with limited movement, such as Western corn rootworm larvae, modeling studies suggest that as grower compliance to refugia decreases resistance emergence sharply increases ([Pan, Onstad et al. 2011](#)). In Bt cotton, resistance models predict faster evolution of resistance populations when larvae move between plants and do not discriminate based on plant genotype for host selection ([Heuberger, Crowder et al. 2011](#)). As an alternative to mandated refugia, non-transgenic crops and wild plant relatives have been used as viable refugia for Bt crops in China ([Qiao, Huang et al. 2010](#)). However, recent reports suggest emergence of resistance to Bt cotton in *Helicoverpa armigera* populations in a region of Northern China with a history of high rate of Bt cotton adoption ([Zhang, Yin et al. 2011](#)). In India, difficulties in monitoring compliance and the lack of control over illegal

transgenic varieties have been suggested as important issues facing resistance management programs for Bt crops ([Jayaraman 2001](#); [Jayaraman 2002](#)). Despite these issues and after more than a decade of use, field evolved resistance to Bt crops can be considered a rare event keeping in mind the level of adoption. Recent reports of field evolved resistance to Bt crops ([van Rensburg 2007](#); [Tabashnik, Van Rensburg et al. 2009](#); [Storer, Babcock et al. 2010](#); [Dhurua and Gujar 2011](#); [Zhang, Yin et al. 2011](#)) are usually correlated with sub-optimal crop growth conditions and most have not been reported to result in crop losses. However, these reports of field-evolved resistance to crops expressing a single Bt toxin have further incentivized the development of alternative approaches to delay resistance in second and third generation Bt crops.

1.6 “Second” and “third” generation Bt crops

High adoption of single transgene Bt crop technology and concerns on potential evolution of insect resistance to these crops promoted the development of “second generation” Bt crops (cotton and maize), which we define in this chapter as those varieties expressing multiple Bt genes with diverse (pyramided) mode of action. Based on the importance of toxin binding to specific receptors in the insect midgut for toxicity and resistance, Bt toxins recognizing alternative midgut receptors are considered as having diverse mode of action by regulatory agencies and therefore are optimum candidates for pyramiding. The use of these pyramided Bt toxin genes greatly reduces the probability of resistance evolution, as target insects would need to develop simultaneous mutations in diverse toxin receptors to acquire resistance ([Roush 1998](#); [Zhao, Cao et al. 2003](#)). Pyramiding of *cryIAc* and *cry2Ab* ([Chitkowski, Turnipseed et al. 2003](#))

or *cry* and *vip* ([Estruch, Carozzi et al. 1997](#)) toxin genes are examples of this strategy to reduce the rate of resistance evolution in second generation Bt crops. However, cross-resistance observed in laboratory-selected insect strains suggests that mechanisms such as altered toxin proteolysis ([Oppert, Kramer et al. 1997](#)) or enhanced midgut healing ([Martinez-Ramirez, Gould et al. 1999](#)) could potentially result in resistance to multiple Bt toxins. In addition, coexistence of single and two-toxin Bt crops may result in faster evolution of resistance to pyramided traits ([Zhao, Cao et al. 2005](#)).

Expression of multiple toxins in second generation Bt crops (Table 13.1) achieves increased control of target pests with low susceptibility to single-toxin Bt crops. For instance, pyramiding of the *cry2Ab2* gene with a chimeric toxin gene (*cry1A.105*) composed of portions of the *cry1Ab*, *cry1Ac*, and *cry1Fa* genes, into maize plants (event MON89034, Yieldgard line of products) expanded the range of control to armyworms (*Spodoptera* spp.) and the black cutworm (*Agrotis ipsilon*). Similarly, Agrisure Viptera maize (events MIR162 and Bt11) producing Vip3Aa20 and Cry1Ab toxins can control corn earworm (*Helicoverpa zea*) and the fall armyworm (*Spodoptera frugiperda*), which display low susceptibility to Cry1A toxins. Control of these pest larvae is also accomplished in cotton varieties producing Cry1Ac and Cry1Fa (events DAS 21023-5 and DAS 24236-5, respectively, WideStrike line of products) or Cry1Ac and Cry2Ab (event 15985, Bollgard II) toxin pyramids in cotton ([Stewart, Adamczyk et al. 2001](#); [Jackson, Bradley et al. 2004](#)). In addition to expanding range of activity to pest species within the same taxonomic order, second generation Bt maize varieties have also been developed to control both lepidopteran and coleopteran pests. For example, the Genuity Vt triple line of maize products produces Cry1Ab and Cry2Ab toxins targeting lepidopteran larvae (event

MON89034-3), and Cry3Bb toxin to control root-feeding Western corn rootworm (*D. virgifera*) larvae (event MON88017-3).

Reductions in mandated refuge size from 20% to 5% have been proposed for pyramided Bt crops based on the increased toxicity against targeted pests and expression of multiple Bt toxins with diverse mode of action. However, it is important to consider that despite the predicted slower evolution of resistance, the evolutionary processes involved in resistance to pyramided Bt crops will be the same as those driving resistance to single toxin varieties ([Ives, Glaum et al. 2011](#)). An alternative to the inherent compliance problems observed with the use of spatial refugia is the use of seed mixtures (refuge in a bag), which is being currently adopted by industry in Bt crops expressing multiple toxins. While this strategy would guarantee the existence of non-Bt refuge plants in fields planted with Bt crops, some models suggest that insect movement between plants may accelerate evolution of resistance ([Heuberger, Crowder et al. 2011](#)). Pests continuously moving and feeding on Bt and non-Bt plants would undergo increased selection pressure in mixtures versus spatial refugia ([Ives, Glaum et al. 2011](#)), which may allow rare broad-spectrum resistance mechanisms to emerge.

The “third generation” of Bt crops, which we define in this chapter as transgenic plants currently in the pipeline for commercialization, are expected to address activity and resistance management issues related to previous Bt crops. These crops are transformed to express pyramids of Bt toxins targeting lepidopteran and coleopteran pests, or combinations of Bt toxins with alternative insecticidal technologies, such as RNA interference (RNAi). Apart from the effects of toxin pyramiding on delaying resistance evolution, expression of multiple toxins also addresses cases of previous Bt crop varieties not fulfilling the high dose tenet for a particular

pest. For example, SmartStax maize from Monsanto and Dow AgroSciences (events MON89034, TC1507, MON88017, and DAS-59122-7) will produce Cry1A.105, Cry2Ab2, and Cry1Fa2 toxins to effectively control a wide range of lepidopteran larvae in addition to producing Cry3Bb1 and Cry34Ab1/Cry35Ab1 toxins to achieve increased efficacy against *Diabrotica* spp.larvae. Combining Bt toxins and insecticidal components with alternative mode of action, such as RNA interference (RNAi) ([Baum, Bogaert et al. 2007](#)) or protease inhibitors ([Cui, Luo et al. 2011](#)), in third generation Bt crops, should address current issues regarding the definition of diverse mode of action used for Bt toxins. Potential challenges with these new Bt crops would include the design and implementation of effective resistance management practices, demonstration that the effects of combining multiple Bt toxins can be predicted from integration of individual toxin assessments, and that novel insecticidal components do not have unforeseen negative impacts on plant innate immune responses to herbivory.

1.7 Conclusions and future prospects

So far, introduction of transgenic Bt crops has had major positive ecological and agricultural consequences ([Betz, Hammond et al. 2000](#)). Despite the highly publicized interchange between non-peer reviewed scientific opinions over yield comparisons ([Sheridan 2009](#)), overall the use of Bt cotton or Bt maize has been reported to result in increased farm income benefits ([Brookes and Barfoot 2009](#)). While assessment of the environmental impact of Bt crops is highly dependent on variables considered, unintended effects pale in comparison to those of alternative pest suppression methods ([Naranjo 2009](#)). In the U.S. alone, the use of Bt maize and cotton has resulted in consistent reductions in chemical insecticide use from 1996-

2009, totaling 64.2 million pounds ([Benbrook 2009](#)). In addition to these economic and environmental benefits, Bt crops can also enhance food safety, as in the case of mycotoxin level reductions reported for Bt maize compared to non-Bt varieties ([Hammond, Campbell et al. 2004](#)).

Current trends in the advancement of Bt crops include the integration of transgenes into other essential crops worldwide, enhancing the quality and efficacy of the transgenes through genetic engineering, discovering new insecticidal Bt toxins ([Kaur 2006](#)), and coupling Bt toxins with non-Bt insecticidal components such as protease inhibitors ([Vaughan 2003](#)) or RNAi ([Baum, Bogaert et al. 2007](#)). These initiatives can broaden activity range, increase insecticidal potency, and further delay the evolution of insect resistance. For example, the development of Bt rice ([Yang, Chen et al. 2011](#)) has extraordinary commercial appeal for agricultural biotechnology ([Xia, Lu et al. 2011](#)), and has recently been suggested to be effective in preventing non-targeted pest outbreaks ([Chen, Tian et al. 2011](#)). The approval of transgenic Bt brinjal as the first genetically modified crop for human consumption in India is expected to result in benefits observed for other Bt crops, although approval has been initially met with controversy ([Seetharam 2010](#)). Other non-essential crops, like sunflower, are being also explored for commercial viability ([Cantamutto and Poverene 2007](#)).

Advances on the characterization of the Bt toxin mode of action have allowed for design of improved Bt toxins amenable to expression in Bt crops. Genetically engineered Cry3A toxins (mCry3A and eCry3.1Ab) displaying increased activity against *D. virgifera* larvae ([Walters, Stacy et al. 2008](#); [Walters, deFontes et al. 2010](#)) are currently used in some Bt maize varieties (Table 13.1). Modified (Mod) Cry1A toxins targeting lepidopteran larvae and effective against

larvae lacking the primary Cry1A toxin receptor ([Soberón, Pardo-López et al. 2007](#)) are a clear alternative to currently used lepidopteran-specific traits. The use of enhancers of Bt toxicity has also been proposed as a strategy to attain high dose mortality levels in pests with low susceptibility to Bt toxins ([Chen, Hua et al. 2007](#); [Gao, Jurat-Fuentes et al. 2011](#)), although their efficacy in transgenic plants has not been reported.

We are at an important moment in the Bt crop marketplace, with many issues simultaneously impacting the future of this technology. Past controversy surrounding some of the publications addressing these issues has hindered advancement of Bt crop research to secure long-term utility of Bt-based biotechnology. New regulatory principles that deviate from the longstanding idea of how selection pressure is established within a Bt crop environment are being developed to address risks related to introduction of second and third generation Bt crops, especially in environments dominated by single Bt gene crop varieties. Commercialization of Bt crops in alternative developing and industrialized countries will result in regulatory challenges due to agricultural infrastructure distinctions, including enforcement, reporting and policing. These issues will have to be addressed using scientifically sound and peer-reviewed discourse exchange to advance dependable knowledge regarding environmental safety of Bt crops and to maintain utility of Bt-based pesticidal products for future generations.

Table 1: Commercially available and projected Bt crops. Data were obtained from ([Center for environmental risk assessment 2009](#))

<i>Event(s)</i>	<i>Trade name(s)</i>	<i>Toxin(s)</i>	<i>M¹</i>	<i>Targeted pest(s)²</i>
<u>Syngenta Maize</u>				
Bt11	Agrisure CB/LL, Agrisure GT/CB/LL	Cry1Ab	D	ECB
MIR604	Agrisure RW, Agrisure RW/GT	Cry3Aa	A	WCR
Bt11, MIR604	Agrisure 3000GT, Agrisure CB/LL/RW	Cry1Ab, Cry3Aa	D, A	ECB, WCR
Bt 11, MIR162	Agrisure Viptera 3110	Cry1Ab, Vip3Aa20	D, A	ECB, FAW, CEW, BCW, WBC
Bt11, MIR604, MIR162	Agrisure Viptera 3111	Cry1Ab, Cry3Aa, Vip3Aa20	D, A, A	ECB, WCR, FAW, CEW, BCW, WBC,
<u>Syngenta Cotton</u>				
COT102, COT67B	VipCot	Vip3Aa19, Cry1Ab	A	CBW, TBW, PBW, FAW, BAW, SBL, CL, CLP
<u>Monsanto Maize</u>				
MON810	YieldGard Corn Borer	Cry1Ab	B	ECB
MON863	YieldGard RW	Cry3Bb1	B	CRW
MON810, MON863	YieldGard VT Triple, YieldGard Plus	Cry1Ab, Cry3Bb1	B, B	CRW, ECB
MON890 34	Genuity VT Double PRO	Cry1A.105/ Cry2Ab2	A	CEW, ECB, FAW
MON890 34, MON880 17	Genuity VT Triple PRO	Cry1A.105/ Cry2Ab2, Cry3Bb1	A, A	CEW, CRW, ECB, FAW

<i>Event(s)</i>	<i>Trade name(s)</i>	<i>Toxin(s)</i>	<i>M¹</i>	<i>Targeted pest(s)²</i>
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Table 1. Continued

MON890 34, TC1507, MON880 17, DAS- 59122-7	Genuity SmartStax	Cry1A.105/ Cry2Ab2, Cry1Fa2, Cry3Bb1, Cry34/35Ab 1	A,B, B, A	BCW, CEW, CRW, ECB, FAW, WBC, SCB, SWCB, SCSB, CEW, SCB, WBC, WCR
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Monsanto Cotton

MON531	Genuity Bollgard, Ingard	Cry1Ac	A	PBW, TBW
MON159 85	Genuity Bollgard II	Cry1Ac/Cry 2Ab2	A/B	CBW, PBW, TBW

Pioneer (DuPont) and Dow Agrosiences Maize

DAS- 06275-8		Cry1F	A	BCW, ECB, FAW, WBC, SWCB, CEW
TC1507	Herculex I	Cry1F	B	BCW, ECB, FAW, WBC, SWCB, CEW
MON810 ,		Cry1Ab	B	ECB
TC1507, MON810	Optimum Intrasect	Cry1F, Cry1Ab	B, B	ECB, WBC, BCW, FAW
DAS- 59122-7	Herculex RW, Optimum AcreMax RW	Cry34/35Ab 1	A	WCR
DAS- 59122-7, TC1507	Optimum AcreMax 1, Herculex Xtra	Cry34/35Ab 1, Cry1F	A, B	WCR, BEC, ECB, FAW, WBC

<i>Event(s)</i>	<i>Trade name(s)</i>	<i>Toxin(s)</i>	<i>M¹</i>	<i>Targeted pest(s)²</i>
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Table 1. Continued

	<u>Pioneer (DuPont) and Dow Agrosiences Cotton</u>			
3006-210-24, 281-24-236	WideStrike	Cry1Ac, Cry1F	A, A	CBW, PBW, TBW, ECB, SBL, BAW, FAW

¹ Method of transgene insertion. Abbreviations are D- Direct DNA transfer, B- Biolistics, A- Agro-mediated transformation.

²Common insect name abbreviations are: ECB- European corn borer; WCR- Western corn rootworm; FAW- Fall armyworm; CEW- Corn earworm; BCW- Black cutworm; WBC- Western bean cutworm; BEC- Bean cutworm; CBW- Cotton bollworm; TBW- Tobacco budworm; PBW- Pink bollworm; BAW- Beet armyworm; SBL- Soybean looper; CL- Cabbage looper; Cotton leaf perforator; SCB- Sugar cane borer; SWCB- Southwestern corn borer; SCSB- Southern cornstalk borer; SPB- Spotted bollworm

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2 Monitoring stem cell proliferation and differentiation in primary midgut cell cultures from *H. virescens* larvae using flow cytometry

2.1 Abstract

In the midgut of *Heliothis virescens* larvae, proliferation and differentiation of stem cell populations allow for midgut growth and regeneration. Basic epithelial regenerative function can be assessed *in vitro* by purifying these two cell type populations, yet efficient high throughput methods to monitor midgut stem cell proliferation and differentiation are not available. We describe a flow cytometry method to differentiate stem from mature midgut cells and use it to monitor proliferation, differentiation and death in primary midgut stem cell cultures from *H. virescens* larvae. Our method is based on differential light scattering and vital stain fluorescence properties to distinguish between stem and mature midgut cells. Using this method, we monitored proliferation and differentiation of *H. virescens* midgut cells cultured in the presence of fetal bovine serum (FBS) or AlbuMAX II. Supplementation with FBS resulted in increased stem cell differentiation after 5 days of culture, while AlbuMAX II-supplemented medium promoted stem cell proliferation. These data demonstrate utility of our flow cytometry method for studying stem cell-based epithelial regeneration, and indicate that AlbuMAX II-supplemented medium may be used to maintain pluripotency in primary midgut stem cell cultures.

2.2 Introduction

In lepidopteran larvae growth and regeneration of digestive epithelia are dependent on stem cell proliferation and differentiation ([Loeb and Hakim](#) ; [Hakim, Baldwin et al. 2001](#)). Gut

stem cells have unlimited self-renewal capacity and through asymmetrical cell division they can generate two types of cells, one functional replicate and another that differentiates to a mature form ([Sadrud-Din, Loeb et al. 1996](#)). This process allows the population of stem cells to remain constant while simultaneously providing new mature cells to maintain gut homeostasis ([Watt and Hogan 2000](#); [Hakim, Baldwin et al. 2010](#)).

Gut regeneration in lepidopteran larvae has been mostly studied in reference to its relevance to susceptibility to entomopathogens ([Kirkpatrick, Washburn et al. 1998](#); [Forcada, Alcacer et al. 1999](#); [Martinez-Ramirez, Gould et al. 1999](#); [Hoover, Washburn et al. 2000](#)), or parasite infection and colonization ([Kotsyfakis, Ehret-Sabatier et al. 2005](#); [Vlachou, Schlegelmilch et al. 2005](#)). Most of the information on this regenerative process is derived from *in vitro* studies using primary midgut cell cultures ([Hakim, Caccia et al. 2009](#); [Hakim, Caccia et al. 2009](#); [Hakim, Baldwin et al. 2010](#); [Hakim, Baldwin et al. 2010](#)). This primary cell culture system allows intrinsic studies on midgut epithelial regeneration ([Hakim, Baldwin et al. 2001](#); [Goto, Loeb et al. 2005](#); [Loeb 2005](#); [Loeb 2006](#)) or pathogenesis ([Loeb, Hakim et al. 2000](#); [Loeb, Martin et al. 2001](#); [Loeb, Martin et al. 2001](#)), circumventing potential background from other tissues. In vertebrate systems, discrimination of mature and stem cells from *in vitro* cultures by flow cytometry is typically accomplished using proliferation markers or cell immunophenotyping ([Keeney and Sutherland 2000](#); [Kang and Alvarado 2009](#); [Preffer and Dombkowski 2009](#)). In lepidopteran models, there has been a lack of information on stem cell-specific antigens, which has prevented the use of immunophenotyping and multiparameter flow cytometry. Alternative methods to discriminate mature cells from stem cells using flow cytometry have been reported ([Goodell, Brose et al. 1996](#)), although they have not been

attempted on insect cell cultures. Due to lack of stem cell-specific biomarkers for primary midgut cell cultures in lepidopteran systems, there are no available *in vitro* methods to allow quantitative high throughput detection of lepidopteran midgut stem cells themselves, their proliferation or their differentiation. In primary cultures, mature and stem cells have been discriminated based on gross morphological attributes alone ([Hakim, Caccia et al. 2009](#)). The goal was to develop a method that would allow quantitative discrimination of mature and stem cells using primary midgut cell cultures from *H. virescens* larvae as model, and to use this method in monitoring midgut stem cell proliferation and differentiation. Using differential light scattering and vital staining properties for each cell type, we developed a flow cytometry method able to discriminate mature cells from stem cells in primary cultures from *H. virescens* midgut larvae. Furthermore, we report successful use of this method to monitor stem cell proliferation and differentiation after incubation with supplemented media, which allowed identification of media additives that maintain pluripotency and viability of the stem cells.

2.2 Materials and methods

2.2.1 Insects

H. virescens eggs were obtained from laboratory colonies of Benzon Research (Carlisle, PA), or were kindly supplied by Dr. Fred Gould (North Carolina State University). No differences between larvae hatched from eggs of either source were observed in our experiments. Upon hatching, larvae were reared on artificial diet (BioServ, NJ) at 28°C on an 18:6 LD photoperiod. Early fourth instar larvae, as determined by time since hatching and size, were

anesthetized on ice for 10 min and midguts carefully dissected under sterile conditions and used for preparation of primary cell cultures as described below.

2.2.2 Establishment of primary mature and stem midgut cell cultures

All dissections and transfers were done in the sterile environment of a biosafety cabinet. Fourth instar larvae were anesthetized on ice for 15 min, and then surface sterilized for 30s in a cleaning solution (10% Palmolive detergent plus 0.1% Clorox) before dissecting the midgut. After dissection, midguts were cleaned for food, peritrophic matrix, and malphigian tubules using forceps, and then briefly washed in sterile Ringer's ([Barbosa 1974](#)) containing 0.5% (v/v) gentamicin (Invitrogen, CA), 0.1% Clorox, and 1x antibiotic/antimycotic (Invitrogen, CA). Incubation media was prepared by mixing in a 3:1 ratio supplemented Grace's Insect Medium (containing lactalbumin hydrolysate and yeastolate; Invitrogen, CA) containing 1x antibiotic/antimycotic and 0.1% gentamicin with sterile Ringer's. Five to six clean midguts were cut in sections with micro-scissors and incubated in 2 ml of incubation media for 90 min at room temperature. After this incubation, midgut tissue was homogenized carefully by pipetting and sieved through 70 mm cell strainers (BD Biosciences, NJ) into a sterile 50 ml conical tube. Tubes were centrifuged (400 g for 5 min at 4°C) and the supernatant discarded. The pellet containing midgut mature and stem cells was suspended in 1 ml of incubation media. Stem cells were separated from mature cells using a density gradient as described elsewhere ([Loeb, Jaffe et al. 1999](#)). Briefly, samples were overlaid on 3 ml of Ficoll-Paque (GE Life Sciences, NJ) in a 15ml conical tube and centrifuged (600 g for 15 min at 4°C). After centrifugation, stem cells were collected from the top 0.99 ml, the immediate 2.75 ml containing debris were discarded,

and the bottom 0.25ml containing the pellet was collected for mature cells. Ficoll-Paque was eliminated from stem and mature cell samples by washing twice with incubation media (600 g for 5 min at 4°C). Final stem and mature cell pellets were suspended in 0.35 or 1 ml, respectively, of incubation media. Stem and mature cell samples that were prepared simultaneously were pooled and the number of cells counted using a hemocytometer. Using this procedure, we repeatedly obtained approximately 8×10^5 stem cells and 1×10^7 mature cells from 30 larvae. Stem cells were diluted to 4×10^5 cells/mL and mature cells were diluted to 4×10^5 cells/mL with incubation media (as described above) and kept in a sterile incubator at 26°C.

2.2.3 Vital staining of primary cell cultures

We used calcein acetoxymethylester (calcein AM) to fluorescently stain viable cells in mature and stem cell cultures, as described in the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, CA). Using this procedure, activation of calcein AM by intracellular esterases fluorescently labels viable cells in a culture. Calcein AM was briefly acclimated to room temperature, and then diluted to a 20 mM working solution with DMSO. Viable cell staining was done by incubating calcein AM solution with cell cultures (2 µl of working solution per ml of cell culture) for 20 min at room temperature protected from light

2.2.4 Flow cytometry

Primary cell cultures were collected in culture tubes and used for flow cytometry analysis using a LSRII flow cytometer (BD Bioscience, CA). Photomultiplier tube voltages were set to 620 eV for forward scatter channel (FSC), 228 eV for side scatter channel (SSC), and 368 eV for

the FL1 channel. The threshold was set at 60,200 on the FSC channel to exclude debris. During analysis, distinct cell populations emerged based upon SSC in the y-axis and green fluorescence FL1 (calcein fluorescence) in the x-axis. Data from gating 5,000 cells for each sample were analyzed using the DiVa software (BD Bioscience, CA). Using freshly prepared primary midgut stem and mature cell cultures, two gates to differentiate stem from mature cells were created based on the data from SSC- FL1 dot plots. Primary cell cultures treated with 50% ethanol (v/v) were used to define a third gate containing dead cells. Data were analyzed and graphed using Cyflogic TM software ([http://www. cyflogic.com](http://www.cyflogic.com); CyFlo Ltd, Finland). Statistical significance was tested using the SigmaPlot v11.0 software (Systat Software Inc., IL). When testing for differences in cell numbers between two samples we used the Student's t-test method ($P=0.05$), while comparisons between multiple samples were tested for significance with analysis of variance (ANOVA) using the Holm–Sidak and Dunn's pair wise multiple comparison procedures ($P=0.05$).

2.2.5 Primary cell culture treatments

To determine the effect of media additives on midgut cell cultures, stem or mature cell cultures (4×10^5 cells/mL) were diluted to 2×10^5 cells/mL in (1ml final volume) incubation media alone, or containing either 40 g/L of AlbuMAX II (Invitrogen, CA) or 10% FBS (Biowhittaker, MD). Cells were incubated in wells of a 24-well cell culture-treated plate at 26°C for five days, as this was the tested time frame resulting in more clear differences in cell number and type between control and experimental treatments, and with lower overall cell mortality

(data not shown). Differentiation and proliferation in each sample were measured after the five-day incubation using staining and flow cytometry as detailed.

2.3 Results

2.3.1 Morphology of primary midgut stem and mature cell cultures

Cells in the three main developmental stages of stem, differentiating, and mature are in the midgut of *H. virescens* larvae. These cells can be easily identified in primary cultures based on the gross morphology under light microscopy ([Loeb, Jaffe et al. 1999](#); [Hakim, Caccia et al. 2009](#)). After purifying mature cells from stem cells in primary culture, we observed that most cells in the mature cultures displayed brush border-type lining (Fig. 1a), typical of midgut columnar cells ([Hakim, Caccia et al. 2009](#)). Also detected albeit in lower numbers were mature cells with alternative morphologies, putative goblet, and neuroendocrine cells. In stem cell cultures, almost all cells presented spherical shape and clear surface, and they were much smaller than mature cells (Fig. 1b). This morphology is typical of lepidopteran midgut stem cells ([Baldwin and Hakim 1991](#); [Loeb and Hakim 1996](#); [Hakim, Caccia et al. 2009](#)). Putative differentiating cells with amorphous morphology and heterogeneous surface (Fig. 1c) were more abundant in mature cell cultures, but were also detected in stem cell cultures.

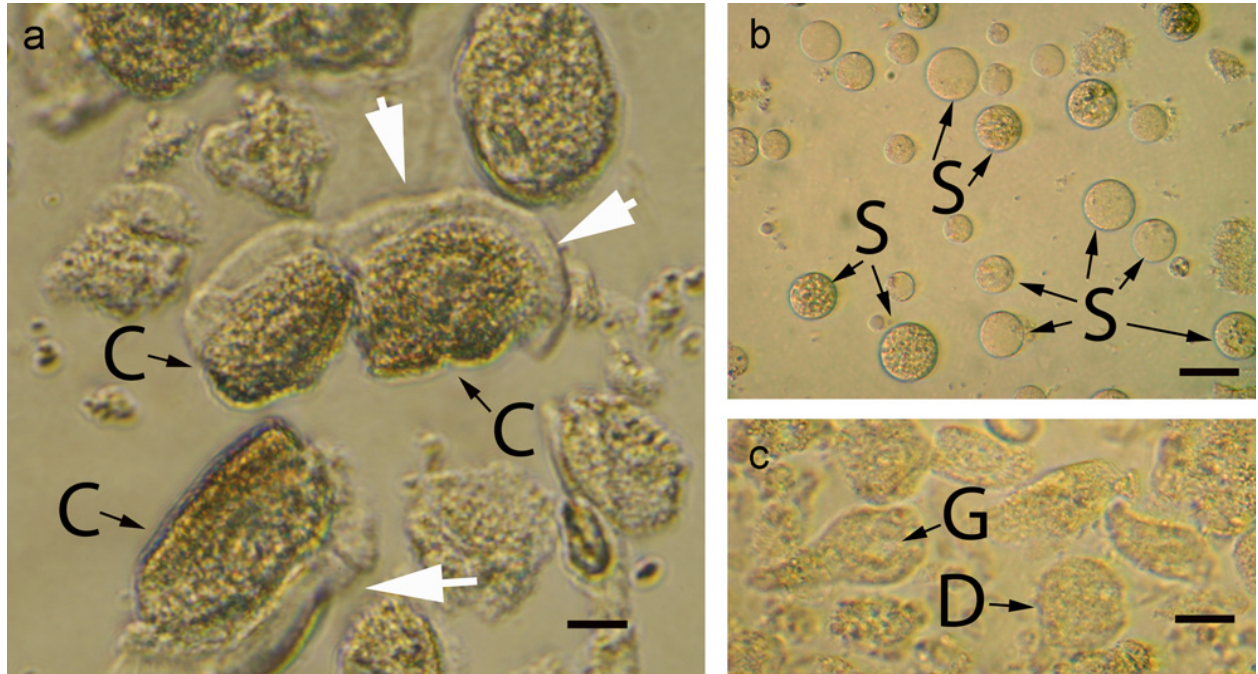


Figure 1: Morphological identification of *H. virescens* midgut cell types in primary cell culture using light microscopy. Primary mature (a and c) and stem (b) midgut cell cultures are shown. White arrows indicate the presence of brush border membrane on the surface of columnar midgut cells. Black arrows point to specific cell types: C=columnar cells, S=stem cells, G=goblet cell, and D=differentiating cell. Bars are 10 μm in all pictures.

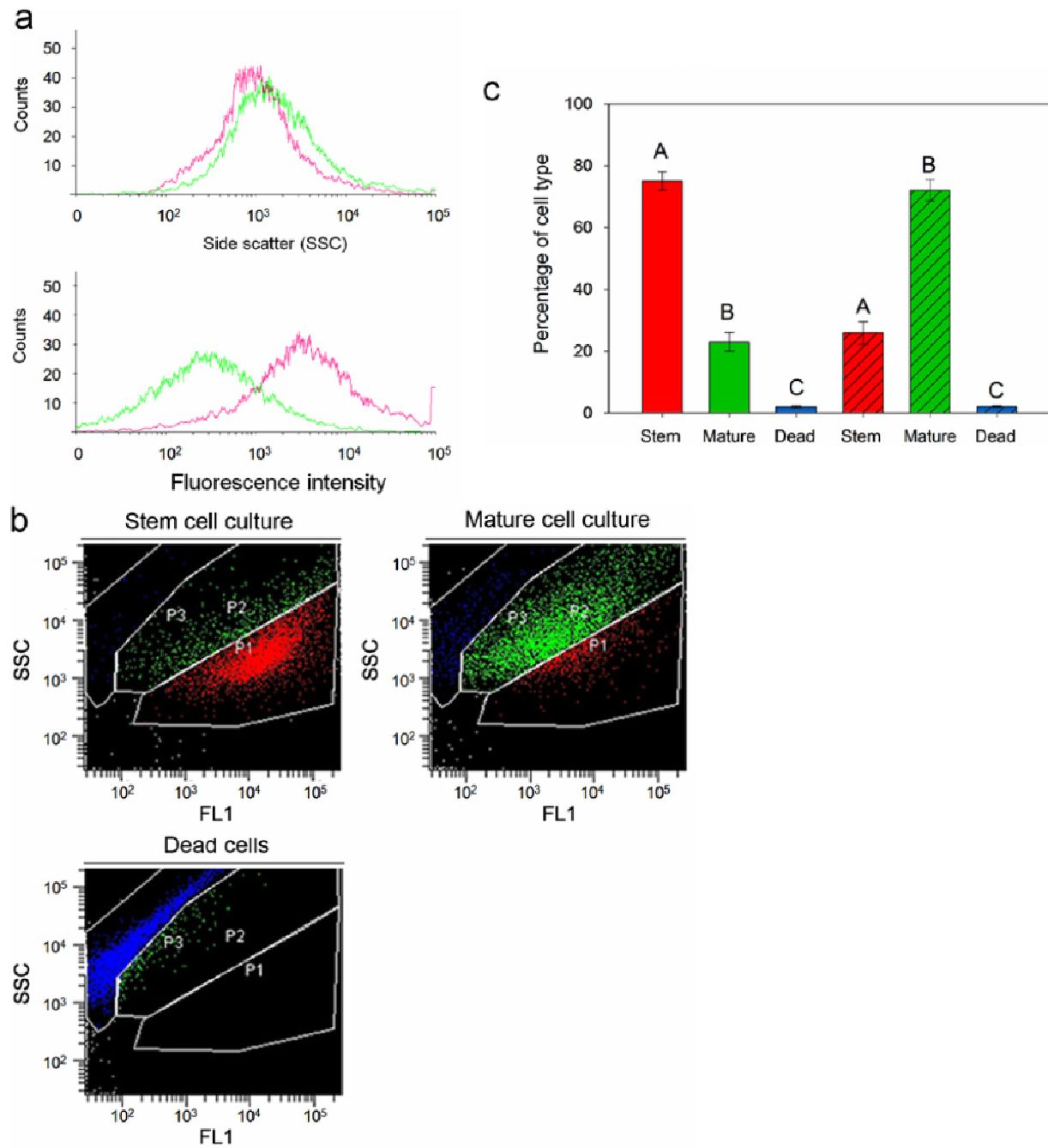
2.3.2 Separation of mature, stem and dead cells using flow cytometry

Forward and side light scattering (FSC and SSC, respectively), are parameters used to detect and characterize cell populations in flow cytometry. While FSC reflects the size of the cell, SSC is related to the cell morphological complexity. Even though we attempted to use FSC to differentiate between midgut mature and stem cells, high heterogeneity in the mature cell population limited efficacy of this parameter (data not shown). Based on the observed lower membrane complexity in stem cells, we hypothesized that they would have much lower SSC

compared to mature and differentiating cells. Measurements of SSC of purified stem cell populations in a flow cytometer showed a shift to lower SSC signal intensity when compared to mature cell samples (Fig. 2a). In order to more efficiently gate the two cell types, an additional parameter was needed. During assays to monitor cell viability, we found that stem cells displayed higher vital fluorescence than mature cells (Fig. 2a). This fluorescence is dependent on removal of the acetomethoxy group from calcein AM by intracellular esterases and binding of calcium to the entrapped calcein ([Decherchi, Cochard et al. 1997](#)). Even though mature cells displayed lower vital staining than stem cells, their fluorescence was much higher than dead cells (Fig. 2b), indicating their viability. Based on the differential SSC and calcein AM fluorescence properties displayed by mature and stem cells, we were able to establish dual parameter gating for these two cell types. Dead cells, displaying variable SSC and low vital fluorescence, were confined to a third gate. Primary midgut stem cell cultures were gated as displaying low SSC and high fluorescence, while mature cell cultures were gated at high SSC but lower fluorescence (Fig. 2b). Even though most of the cells were successfully detected in their respective gate, some cells in stem and mature cell cultures (about 20%) were detected outside their respective gates, suggesting that our purified cultures contained a small percentage of contaminating cell types (Fig. 2c).

Figure 2: Establishment of flow cytometry gates for *H. virescens* midgut stem and mature

cells. (a) Side light scatter (SSC) monitored for midgut mature (green) and stem (red) cells using flow cytometry. (b) Calcein AM fluorescence in primary midgut mature (green) and stem cell (red) cultures as detected using a Q3 fluorescein filter in the flow cytometer. (c) Distribution of primary midgut stem (solid bars) or mature (hatched bars) cells in gates established using SSC and calcein AM (FL1) fluorescence as gating parameters. Data shown are representative flow cytometry plots and mean cell percentage numbers in mature and stem cell cultures from three experiments with 5,000 events considered for each experiment. Bars denote standard error of the mean for each sample; statistically significant differences are denoted by different letters for each column (Student's t-test, $P=0.05$). Stem cells (red) displayed higher FL1 fluorescence and lower SSC than mature cells (green). Dead cells (after ethanol treatment) were detected as displaying the lowest vital staining.



2.3.3. Functional analysis of stem and mature cell gates

To test whether stem cells were effectively gated to the stem cell gate in our method, we measured and compared proliferation in mature and stem cell gates after incubation of stem cell cultures for five days in incubation media alone (control) or supplemented with 40 g/L AlbuMAX II. Since only stem cells are capable of proliferation in lepidopteran midgut ([Hakim, Baldwin et al. 2010](#)), we expected to observe increased cell numbers in the stem cell gate in the presence of media containing AlbuMAX II compared to control media. In agreement with our prediction, and as shown in Fig. 3, the number of cells in the stem cell gate significantly increased between control and AlbuMAX II-treated cultures (Student's t-test, $P=0.05$). In contrast, no significant differences (Student's t-test, $P=0.05$) were found between cell numbers in the mature cell gate in control and AlbuMAX II-treated cultures. These results confirmed the presence of stem cells (i.e. capable of proliferation) in our stem cell gate, while mature cells (in capable of proliferation) were present in the mature cell gate.

2.3.4. Monitoring stem cell proliferation and differentiation using flow cytometry.

To test the use of our flow cytometry method in monitoring stem cell proliferation and differentiation, we incubated midgut stem cell cultures prepared from *H. virescens* larvae for 5 days in incubation media alone (control), or supplemented with 40g/L AlbuMAX II or 10% FBS. In agreement with data in Fig. 3, stem cell cultures incubated with AlbuMAX II presented a significantly higher cell number (Holm–Sidak method, $P=0.05$) compared to controls (Fig. 4a). In contrast, the number of cells after five days in FBS-treated cultures was not significantly

different from either controls or AlbuMAX II-treated cultures (Holm–Sidak method, $P=0.05$).

These data suggested that stem cell cultures responded differently to AlbuMAX II and FBS.

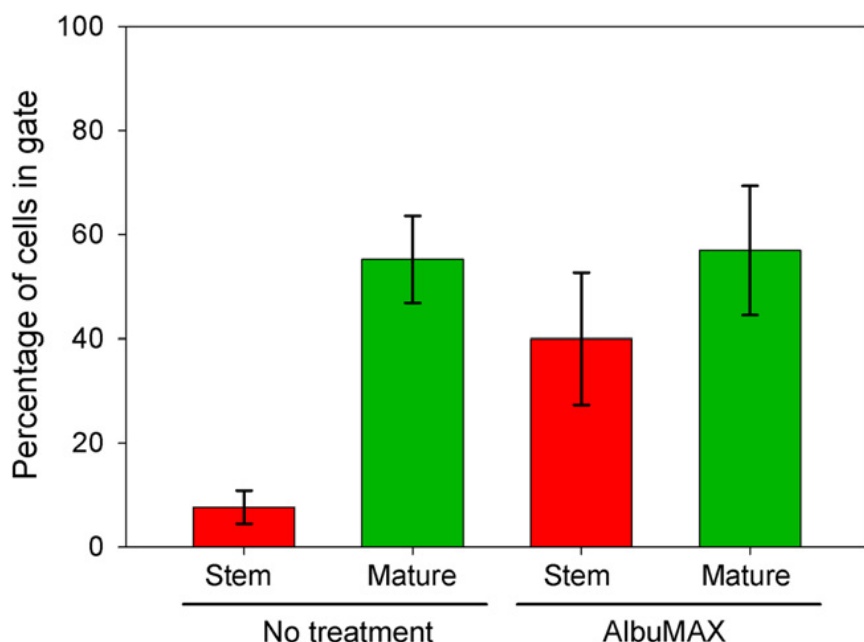
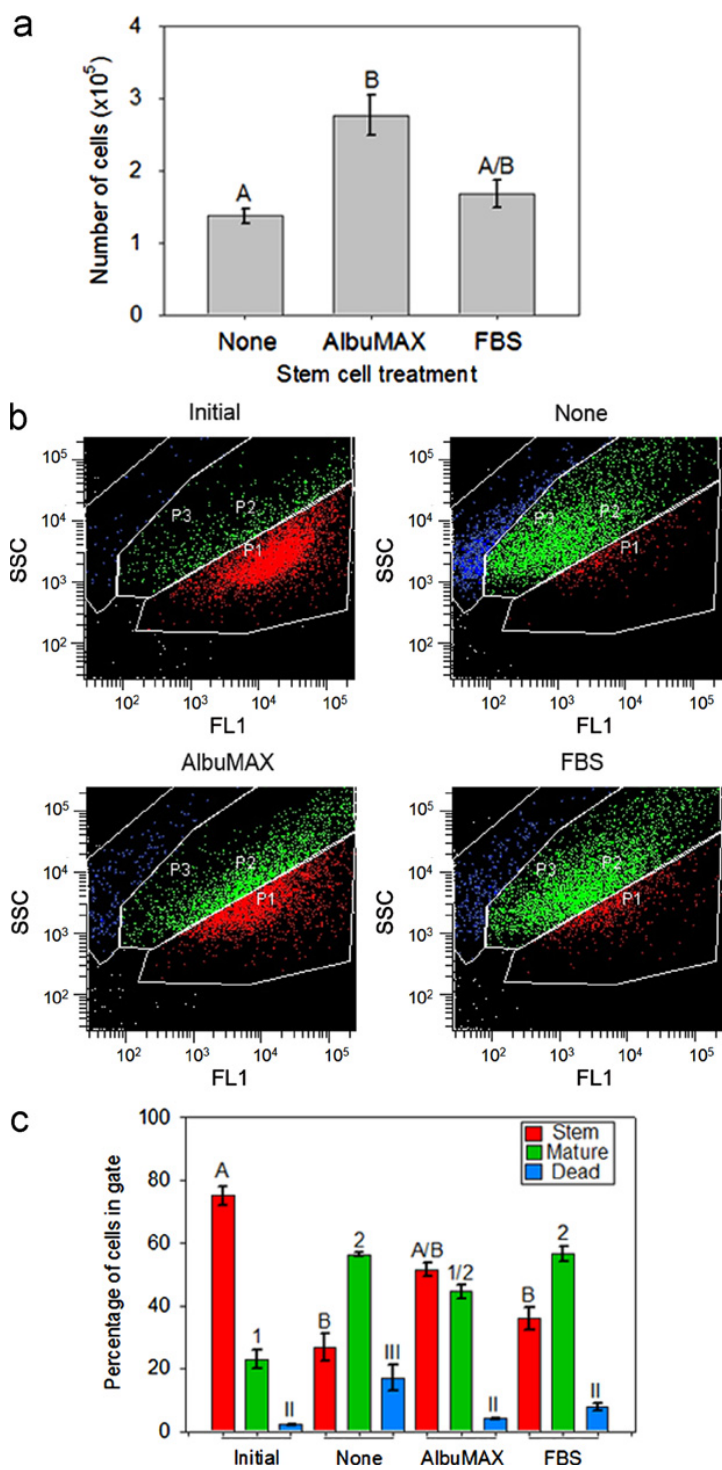


Figure 3: Proliferation of *H. virescens* primary midgut stem cell cultures in the presence of 40g/L AlbuMAX II in incubation media. Purified primary midgut stem cell cultures (2×10^5 cells) were incubated for 5 days in incubation media alone or containing 40g/L AlbuMAX II. Bars denote the percentage of cells in each of the flow cytometry gates established using SSC and FL1 (calcein AM) fluorescence. Data shown are the means and standard errors from five independent experiments and cell preparations performed at least in triplicate. Mean (7 standard error) for the final total stem cell numbers in each treatment were: $0.67 (70.27) \times 10^4$ cells for no treatment and $10.05 (75.85) \times 10^4$ cells for AlbuMAX II-treated cells. Mean (7 standard error) for the final total mature cell numbers in each treatment were $5.63 (71.98) \times 10^4$ for no treatment and $4.32 (70.52) \times 10^4$ for AlbuMAX II-treated cultures.

Using our flow cytometry method, we compared the number of cells in each gate (stem, mature, and dead) in freshly prepared and the corresponding treated cell cultures (Fig. 4b). In agreement with Fig. 2c, we observed a small percentage (about 20%) of differentiating mature cells in our freshly prepared stem cell cultures (Fig. 4b and c). After incubation for five days in incubation media alone (control), we observed a significant (Holm–Sidak method, $P < 0.05$) increase in the number of mature and dead cells, together with a drastic decrease in the number of stem cells (Fig. 4b and c). In contrast, incubation in incubation media containing either AlbuMAX II or FBS resulted in higher proportion of stem cells (Fig. 4b and c), although only in the AlbuMAX II-treated cultures the number of stem cells was not significantly different from the freshly prepared (initial) untreated cultures (Dunn’s method, $P < 0.05$). Interestingly, there were no differences in the proportion of mature cells for all three treatments (between 52% and 56%), while the number of dead cells was only significantly increased (Dunn’s method, $P < 0.05$) in cultures incubated in incubation media alone (17% with media alone versus 3.9% and 7.7% in AlbuMAX II and FBS treatments, respectively). Changes in cell morphology and number, as suggested by the flow cytometry results, were confirmed using microscopy observations (data not shown).

Figure 4: Increased proliferation and/or differentiation in primary stem cell cultures in the presence of FBS or AlbuMAX II as media additives. Primary midgut stem cell cultures from *H. virescens* larvae (2×10^5 cells) were incubated for 5 days in incubation media alone (none), or containing 40g/L of AlbuMAX II (AlbuMAX II) or 10% FBS (FBS). (a) Proliferation was quantified by counting cells in a flow cytometer. Bars represent the mean and standard error calculated from at least 6 experiments performed with 3 independent stem cell cultures. Different letters on top of bars denote significant differences (ANOVA Dunn's method, $P=0.05$). (b) Representative plots (SSC versus FL1 fluorescence) of stem cell cultures stained with calcein AM. Freshly prepared stem cell cultures before incubation (initial) are shown for comparison in the same culture after five day incubation with incubation media alone (none), or supplemented with either AlbuMAX II at a final concentration of 40g/L (AlbuMAX II) or 10% FBS (FBS). Events in each gate are colored based on SSC and FL1 fluorescence parameters as blue (dead cells), green (mature cells), or red (stem cells). (c) Percentage of cells in each of the three gates determined in (b). Bars are colored corresponding to the specific gate in (b). Data in each bar are the mean and the corresponding standard error calculated from a minimum of three independent experiments performed in triplicate. Different letters, numbers, or symbols denote significant differences (ANOVA Dunn's and Holm–Sidak methods, $P=0.05$). Within each stem cell treatment, mean (7 standard error) for the final total cell number in the stem, mature, and dead cell cytometry gates, respectively, were: $6.00 (71.14) \times 10^4$, $2.11 (70.78) \times 10^4$, and $0.14 (70.02) \times 10^4$ for initial samples; $1.50 (70.17) \times 10^4$, $3.77 (70.66) \times 10^4$, and $1.38 (70.45) \times 10^4$ for no treatment; $7.46 (71.03) \times 10^4$, $7.76 (71.28) \times 10^4$, and $0.48 (70.06) \times 10^4$ for AlbuMAX II-treated;

and $3.85 (70.28) \times 10^4$, $4.92 (70.05) \times 10^4$, and $0.53 (70.07) \times 10^4$ for FBS-treated stem cell cultures, respectively.



2.4 Discussion

Primary midgut cell cultures from lepidopteran larvae are a relevant model to study gut epithelial development, physiology, and response to intoxication ([Hakim, Caccia et al. 2009](#); [Hakim, Baldwin et al. 2010](#)). Previous studies using these primary midgut cultures relied on gross morphology to differentiate mature cells from stem cells and to quantify stem cell differentiation ([Loeb and Hakim 1996](#); [Sadrud-Din, Loeb et al. 1996](#)). Using differential morphological and vital staining attributes, we developed a flow cytometry method that allows quantitative discrimination between mature and stem cells in primary midgut cell cultures from *H. virescens* larvae. Furthermore, we demonstrate the utility of this method to monitor stem cell proliferation and differentiation. Further work will be necessary to test utility of our method in monitoring stem cell proliferation and differentiation in alternative in vitro systems. We initially attempted immunophenotyping of stem cells by targeting Delta, a stem cell marker in adult *Drosophila* midgut ([Ohlstein and Spradling 2007](#)), and proliferating cell nuclear antigen (PCNA), reported as marker for proliferative cells in the midgut of *Locusta migratoria* ([Zudaire, Simpson et al. 2004](#)). However, no Delta orthologues have been reported for Lepidoptera, and antisera to PCNA did not efficiently differentiate mature and stem cells in primary midgut cell cultures from *H. virescens* larvae (data not shown). In developing our technique, we took advantage of the distinct morphology and calcein AM fluorescence displayed by midgut stem when compared to mature cells in primary culture. As previously reported ([Loeb and Hakim 1996](#)), *H. virescens* midgut mature cells had a much more complex morphology than stem cells, resulting in higher scatter laser-filter readings in the flow cytometer. Our second parameter, calcein AM staining, has been previously used to discriminate between cell subpopulations. For

example, higher calcein fluorescence was reported as a useful parameter to differentiate and detect cancer stem cells ([Allen, Hart et al. 2009](#); [Chu and Lee 2009](#)) or human fibroblasts under stress ([Uggeri, Gatti et al. 2004](#)). Since higher fluorescence is dependent on calceinAM cleavage, our data suggest that increased staining of *H. virescens* midgut stem cells is probably due to higher esterase activity compared to mature cells. Although we did not test this possibility, there are examples in the literature of differential staining of cells due to differential intracellular esterase activity ([Afrimzon, Deutsch et al. 2008](#)). Alternatively, it is also possible that mature cells maybe more efficient than stem cells in calcein AM efflux, as found for subpopulations of cancer stem cells ([Allen, Hart et al. 2009](#)). Further work would be necessary to determine the specific mechanism yielding higher calcein AM fluorescence in *H. virescens* midgut stem compared to mature cells. Using our flow cytometry method, we monitored changes in the proportions of mature and stem cells to identify media additives that would maintain pluripotency of primary midgut stem cell cultures. We selected FBS as a common media supplement that promotes growth in insect cell cultures ([Ferkovich and Oberlander 1991](#)), and AlbuMAX II as a serum-free culturing alternative that promotes human embryonic stem cell renewal *in vitro* ([Garcia-Gonzalo and Belmonte 2008](#)). Both supplements contain bovine serum albumin and associated lipids, but FBS also contains additional growth actors. Based on this difference, we expected to observe higher levels of mature cells when stem cell cultures were incubated with FBS compared to treatment with AlbuMAX II. Although we did not detect significant differences in the proportion of mature cells among our three treatments, we found that AlbuMAX II was the only treatment that maintained a similar proportion of stem cells compared to freshly prepared cultures. The increased proportion of mature cells with no

significant change in the proportion of stem cells detected for AlbuMAX II correlates with the higher number of total cells detected for this treatment. These data suggest that as previously reported for human embryonic stem cells ([Garcia-Gonzalo and Belmonte 2008](#)), treatment with AlbuMAX II mostly promotes stem cell proliferation, thus maintaining pluripotency in the culture. Even though there are previous reports on peptides inducing lepidopteran midgut stem cell proliferation *in vitro* ([Takeda, Sakai et al. 2001](#); [Blackburn, Loeb et al. 2004](#); [Goto, Loeb et al. 2005](#)), previous attempts did not identify alternatives to FBS in promoting growth in insect cell cultures ([Ferkovich and Oberlander 1991](#)). In this regard, this is the first report on the use of a commercially available media supplement alternative to FBS to induce lepidopteran midgut stem cell proliferation.

The similar levels of differentiation observed for stem cell cultures treated with incubation media alone or in combination with AlbuMAX II or FBS suggest that differentiation factors are produced endogenously in stem cell cultures. These factors may be produced by the low percentage of mature cells present in the stem cell cultures, as detected by our flow cytometry method. In support of this hypothesis, previous reports demonstrate the existence of growth factors in conditioned media from primary midgut cell cultures ([Sadrud-Din, Loeb et al. 1996](#)).

Treatment with either FBS or AlbuMAX II significantly reduced cell mortality when compared to control cultures, suggesting that these supplements are also effective in improving mature cell viability. Current models of epithelial regeneration based on stem cell proliferation and differentiation suggest that daughter cells from dividing stem cells follow diverse cellular lineages to maintain homeostasis ([Blanpain, Horsley et al. 2007](#); [Ohlstein and Spradling 2007](#)).

Our data suggest that AlbuMAX II induces midgut stem cells to divide asymmetrically, whereas while one daughter cell remains as undifferentiated, the other daughter cell enters into differentiation to become a mature cell. Through this process the number of stem cells is maintained, while the number of mature cells in the culture is increased, as observed in our AlbuMAX II treatment. Even though further research is needed to confirm this hypothesis, it would suggest the possibility of maintaining continuous cultures of lepidopteran pluripotent stem cells that may be induced to differentiate to mature cells using conditioned media and/or hormones ([Loeb and Hakim 1996](#); [Sadrud-Din, Loeb et al. 1996](#)), or specific differentiation factors ([Loeb, Jaffe et al. 1999](#); [Loeb and Jaffe 2002](#)). Generation of inducible primary midgut stem cell cultures and tools to monitor their proliferation and differentiation would open new avenues of research to characterize midgut epithelium physiology, development and growth, or response to injury and pathogenic attack

3 Identification of the Cry1Ac-induced secretome in primary *Heliothis virescens* midgut cell cultures

3.1 Abstract

Insecticidal crystal (Cry) toxins produced by the bacterium *Bacillus thuringiensis* (Bt) target cells in the midgut epithelium of susceptible insect larvae. While the mode of action of Cry toxins has been extensively investigated, the midgut response to Cry intoxication is not well characterized. In this work, we investigate the response of primary midgut cell cultures from *Heliothis virescens* larvae to intoxication by Cry1Ac, the most active Cry toxin against this lepidopteran insect. Incubation of these midgut cell cultures with Cry1Ac resulted in differential proliferative response when compared to control treatments. The molecular factors inducing this proliferative response were localized to the proteins secreted by the cultured cells, or secretome. Using differential proteomic analysis of secretomes from control and Cry1Ac-treated midgut cell cultures, we identified proteins with increased abundance correlating to Cry1Ac treatment. In these analyses, we identified *H. virescens* arylphorin precursor as the protein with the largest quantitative difference in Cry1Ac-induced secretome compared to control treated cells. To confirm differential increased expression of arylphorin upon exposure of *H. virescens* larvae to Cry1Ac treatment, we used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) targeting members of the hexamerin family of proteins in an available *H. virescens* transcriptome. These experiments identified multiple arylphorin and hexamerin-like transcripts that are up-regulated during larval recovery from Cry1Ac intoxication. Our data suggest a role for arylphorin in midgut regeneration after intoxication by Cry1Ac, and identify proteins with differential levels in response to Cry1Ac intoxication in *H. virescens* primary midgut cell

cultures. This first characterization of the insect midgut cell secretome contributes to the functional characterization of the insect midgut epithelium and identifies proteins that may be involved in midgut homeostasis.

3.2 Introduction

Insecticidal Cry toxins synthesized by the bacterium *Bacillus thuringiensis* (Bt) are a powerful agricultural tool used to control destructive lepidopteran and coleopteran crop pests ([Bravo, Likitvatanavong et al. 2011](#)). During Cry intoxication of susceptible larvae, the gut epithelial barrier becomes disrupted allowing invasion of the hemocoel by gut bacteria and ultimately leading to death by septicemia ([Broderick, Robinson et al. 2009](#); [Raymond, Johnston et al. 2009](#)). Lepidopteran larvae can recover from exposure to sub-lethal Cry toxin doses ([Dulmage and Martinez 1973](#)) by regenerating their midgut epithelium ([Spies and Spence 1985](#)). Enhanced midgut epithelial recovery relates to resistance against Cry toxins ([Forcada, Alcacer et al. 1999](#); [Martinez-Ramirez, Gould et al. 1999](#)), which further highlights the importance of the gut epithelial response in determining susceptibility to Cry toxins. However, while the Cry toxin mode of action has been extensively characterized and reviewed ([Bravo, Gill et al. 2007](#)), there is a lack of detailed information on the molecular regulation of the midgut epithelium response to Cry intoxication.

Several studies have given insight into the cues associated with the lepidopteran midgut response to Bt intoxication. The midgut response to treatment with Cry1Ab protoxin in *Choristoneura fumiferana* and *Manduca sexta* larvae was characterized using subtraction hybridization libraries ([Meunier, Prefontaine et al. 2006](#)) and custom microarrays ([van Munster,](#)

[Prefontaine et al. 2007](#)). In these assays, general down-regulation of metabolic enzymes and up-regulation of genes involved in detoxification, stress, or immune response were detected. Similar studies of the response to Cry1C intoxication or baculovirus infection in *Spodopera exigua* larvae identified a family of proteins responding to pathogens (REPAT), involved in mitigation of pathological effects ([Herrero, Ansems et al. 2007](#)). Specific REPAT and arylphorin genes were found to be up-regulated in response to intoxication with a Bt pesticide (Xentari) in larvae of *S. exigua*, and this activation was found to be constitutive in larvae from a Xentari-resistant strain of *S. exigua* ([Hernández-Martínez, Navarro-Cerrillo et al. 2010](#)). However, the specific functional roles of REPAT and arylphorin proteins in midgut regeneration after pathologic attack have not been elucidated. The mitogen-activated protein kinase p38 pathway has been reported to be involved in defense against Cry intoxication in both *Aedes aegypti* and *Manduca sexta* larvae ([Cancino-Rodezno, Alexander et al. 2010](#); [Cancino-Rodezno, Alexander et al. 2010](#)). Activation of this pathway results in diverse yet specific responses to intoxication ([Porta, Cancino-Rodezno et al. 2011](#)), including the observed shedding of aminopeptidase-N from *Lymantria dispar* midgut cells upon intoxication with Cry1Ab ([Valaitis 2008](#)).

Primary midgut cell cultures from lepidopteran larvae have been used as an *in vitro* model to study the molecular cues, such as growth factors and cytokines, directing midgut regeneration ([Hakim, Baldwin et al. 2010](#)). A number of peptidic midgut differentiation factors (MDFs) from mature cell conditioned media and hemolymph have been identified to induce differentiation ([Loeb, Jaffe et al. 1999](#)) and/or proliferation ([Hakim, Blackburn et al. 2007](#)). In some cases, production of MDFs by midgut cells was demonstrated through immunodetection in

luminal secretions and midgut cell microvilli ([Loeb, Coronel et al. 2004](#)). Primary midgut cell cultures from *Heliothis virescens* larvae are capable of regeneration after intoxication with Bt toxin ([Loeb, Martin et al. 2001](#)), and the MDF1 growth factor was detected in mature *H. virescens* midgut cells upon Cry intoxication ([Goto, Takeda et al. 2001](#)). While these cultured midgut cells control the activation of the regenerative response to Cry intoxication ([Loeb, Martin et al. 2001](#)), there is no data available on the specific factors involved in regulation of this process. Given that these growth factors are secreted by midgut cells, proteomic analysis of the proteins secreted by midgut cells (midgut secretome) should allow identification of proteins involved in epithelial response to intoxication. While the midgut lumen ([Pauchet, Muck et al. 2008](#)) and peritrophic matrix ([Campbell, Cao et al. 2008](#)) proteomes have been characterized in *Helicoverpa armigera* larvae, the proteins secreted by lepidopteran midgut cells into the lumen in response to Cry intoxication (Cry-induced secretome) have not been described to date.

In this work, we characterize the secretomes from primary mature midgut cell cultures prepared from larvae of *H. virescens* after treatment with buffer, Cry1Ac, or Cry3Aa toxins. Our goal was to characterize the *H. virescens* midgut cell secretome and identify the changes induced by exposure to Cry1Ac toxin, the most active Bt toxin against *H. virescens*. Using midgut stem cell bioactivity assays ([Castagnola, Eda et al. 2011](#)), we detected differential effects on midgut stem cell proliferation between secretomes from control and Cry1Ac-treated mature midgut cell cultures. We identified proteins and compared secretomes from control and Cry1Ac-treated mature midgut cell cultures using 1 dimensional gel electrophoresis coupled to liquid chromatography and mass spectrometry (1D Gel LC/MS/MS). Relative quantification of proteins in each secretome by spectral counting allowed us to detect proteins with increased

levels correlating with Cry1Ac intoxication. In these analyses, we identified arylphorin as the secretome protein with the highest differential quantitative variance in Cry1Ac compared to control treatments. We confirmed up-regulation of arylphorin gene expression after Cry1Ac intoxication of *H. virescens* larvae using qRT-PCR and Western blotting. Our work represents the first description of the Cry1Ac-induced midgut secretome, and contributes to the characterization of the midgut response to Cry intoxication in Lepidoptera.

3.3 Materials and methods

3.3.1 Insects

Eggs from the Bt-susceptible laboratory strain (YDK) of *H. virescens* ([Gould 1995](#)) were kindly supplied by Dr. Fred Gould (North Carolina State University). Upon hatching, larvae were reared on artificial diet (BioServ, Frenchtown, NJ) at 28°C on an 18:6 L,D photoperiod. Fourth instar larvae were anesthetized on ice for 20 min and midguts dissected under sterile conditions and flash frozen for subsequent RNA and protein isolation, fixed with formalin for histopathological staining, or used for preparation of primary midgut cell cultures.

3.3.2 Bacterial toxins

Bacillus thuringiensis var. *kurstaki* strain HD73 producing Cry1Ac toxin and var. *tenebrionis* producing Cry3Aa were obtained from the *Bacillus* Genetic Stock Center (BGSC, Columbus, OH). Bacterial culturing, toxin purification and activation were as described elsewhere ([Perera, Willis et al. 2009](#)). Purified toxins were quantified by the method of Bradford ([Bradford 1976](#)) using the Coomassie Plus Protein Assay (Pierce) with BSA as the standard.

3.3.3 Preparation of histological midgut sections

Fourth instar *H. virescens* larvae were fed an LC₁₀ dose (0.2 µg/mL) of Cry1Ac toxin or a corresponding volume of control buffer (20 mM TRIS/HCl pH 8.0 0.3 M NaCl) by droplet feeding. A 0.04% trypan blue solution (Invitrogen, Carlsbad, CA) was incorporated into all solutions to monitor the larvae swallowing the dose. Midguts were dissected at 1.5 hours post-feeding and their median region sliced in and immediately fixed in 10% neutral buffered formalin solution (3.7% formaldehyde, 30 mM NaH₂PO₄, 46 mM Na₂HPO₄). Sliced midgut tissues were processed using the Tissue-Tek VIP processor (Sakura, Torrance, CA) and embedded in Paraplast embedding medium (Sigma-Aldrich, St. Louis, MO). Block sections were obtained by cutting 5 µm slices using a Microm HM355s microtome (Thermo Scientific, Kalamazoo, MI), which were mounted on Fisherbrand Superfrost Plus slides (Fisher Scientific, Waltham, MA). Slides were stained with hematoxylin and eosin (H&E) according to a standard procedure ([Slaoui and Fiette 2011](#)). Stained midgut sections were visualized using an Olympus DP72 microscope (Olympus, Tokyo, Japan) at 600x magnification.

3.3.4 Establishment of primary midgut cell cultures

All dissections and transfers were done in the sterile environment of a biosafety cabinet. Fourth instar larvae were anesthetized on ice for 15 min, and then surface sterilized for 30s in a cleaning solution (10% Palmolive detergent plus 0.1% Clorox) before dissecting the midgut. After dissection, midguts were cleaned for food, peritrophic matrix, and Malpighian tubules using forceps, and then briefly washed in sterile Ringer's ([Barbosa 1974](#)) containing 0.5% (v/v)

gentamicin (Invitrogen, Carlsbad, CA), 0.1% Clorox, and 1x antibiotic/antimycotic (Invitrogen). Incubation media was prepared by mixing in a 3:1 ratio of working Grace's (supplemented Grace's Insect Medium [Invitrogen] containing 1x antibiotic/antimycotic and 0.1% gentamicin) in sterile Ringer's. Five to six clean midguts were cut in sections with micro-scissors and incubated in 2 ml of incubation media for 90 min at room temperature. After this incubation, midgut tissue was homogenized carefully by pipetting and sieved through 70 mm cell strainers (BD Biosciences, NJ) into a sterile 50 ml conical tube. Tubes were centrifuged ($400 \times g$ for 5 min at 4°C) and the supernatant discarded. The pellet containing midgut mature and stem cells was suspended in 1 ml of working Grace's media. When needed, stem cells were separated from mature cells using a density gradient as described elsewhere (Loeb and Hakim, 1999). Briefly, samples were overlaid on 3 ml of Ficoll-Paque (GE LifeSciences, NJ) in a 15 ml conical tube and centrifuged ($600 \times g$ for 15 min at 4°C). After centrifugation, stem cells were collected from the top 0.99 ml, the immediate 2.75 ml containing debris were discarded, and the bottom 0.25 ml containing the pellet was collected for mature cells. Ficoll-Paque was eliminated from stem and mature cell samples by washing twice with incubation media ($600 \times g$ for 5 min at 4°C). Final stem and mature cell pellets were suspended in 0.35 or 1 ml, respectively, of working Grace's. Stem and mature cell samples that were prepared simultaneously were pooled and the number of cells counted using a hemacytometer (Bright-Line, Horsham, PA). Using this procedure, we repeatedly obtained approximately 8×10^5 stem cells and 1×10^7 mature cells from 30 larvae. Stem cells were diluted to 4×10^5 cells/mL and mature cells to 3.5×10^6 cells/mL with working Grace's (as described above) and kept in a sterile incubator at 26°C .

3.3.5 Monitoring of primary cell culture regeneration

Mixed primary midgut cell cultures containing stem and mature cells were diluted to 3.5×10^6 cells/ml in working Grace's media and then 1 mL seeded in each well of a 12-well plate. Each mixed cell culture was treated with 2 μ g of Cry1Ac toxin for a series of time intervals (see Figures for details) at 26°C. After treatment, cultures were collected into sterile microfuge tubes and centrifuged at 400 x g for 5 min at room temperature. Supernatants were removed except for 50 μ l, which was used to re-suspend the cell pellets. Trypan blue (Invitrogen) was added to a final 0.04% concentration and cell mortality was monitored by light microscopy in a hemacytometer (Bright-Line, Horsham, PA), with dead cells visualized as having a nucleus stained blue or enucleated but stained blue.

3.3.6 Preparation of midgut cell secretomes

Purified mature midgut cells were diluted in Grace's insect medium (Invitrogen) to a concentration of 3.5×10^6 cells/mL, then 1 mL per well used to seed a 12 well plate (Corning, Corning, PA). Purified Cry1Ac toxin (2 μ g/ml), Cry3Aa protoxin (2 μ g/ml) or the corresponding volume of control buffer (20 mM TRIS/HCl, 0.3 M NaCl pH 8.0) were added to the cultures and incubated for 18 hours at 26°C. Then, media supernatant containing the proteins secreted by the midgut cells was collected by centrifugation (2,000 x g for 20 min at 4°C). Collected samples (2 ml) were concentrated to 50-100 μ l and media exchanged to 20 mM TRIS/HCl pH 8.0 buffer using centrifugal filter devices (3-kDa MWCO, Millipore, MA) following manufacturer's instructions. Proteins in concentrated samples were quantified using

the Qubit fluorometer (Invitrogen) and then diluted to 2 mg/ml in 20 mM TRIS/HCl pH 8.0 buffer. These samples were either submitted for 1DGel LC/MS/MS analysis at NextGen Science (Ann Arbor, MI), or stored at -80° C until used for bioactivity assays as described below.

3.3.7 Stem cell bioactivity assays

Purified stem cell cultures (4×10^5 cells/ml) were incubated for 24 hours with Cry1Ac-induced secretome from primary midgut mature cell cultures. As controls we used secretomes from primary midgut mature cell cultures treated with control buffer, and incubation with purified Cry1Ac toxin (1 µg). After treatment, stem cells were stained with calcein AM (Invitrogen) following manufacturer's instructions. Stem cell differentiation and proliferation were analyzed using differential fluorescence in an LSRII flow cytometer (BD Bioscience, CA) as described previously ([Castagnola, Eda et al. 2010](#)).

3.3.8 Proteomic analysis of primary midgut cell culture secretome

Proteins in secretome samples (10 µg) were separated by 1D SDS-10%PAGE and each of the sample lanes sliced in five cross-sections that were subjected to in-gel digestion in a ProGest workstation (Genomic Solutions, Ann Arbor, MI). Briefly, samples were reduced with DTT at 60°C, then allowed to cool to room temperature before being alkylated with iodoacetamide. Tryptic digestion was done at 37°C for 4 hours, then reactions stopped by addition of formic acid. Analysis of peptides generated by the tryptic digestion through liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) was performed at NextGen Sciences (Ann Arbor, MI) using a ThermoFisher LTQ Orbitrap XL mass spectrometer. Tandem mass spectra were analyzed using Mascot (Matrix Science, London, UK) and queried against a custom

database containing a complete *H. virescens* transcriptome (Perera et al. in preparation, 203,652 entries). Search parameters included a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Iodoacetamide derivative of cysteine was specified as a fixed modification, while S-carbamoylmethylcysteine cyclization (N-terminus), deamidation of asparagine and glutamine, and oxidation of methionine and acetylation of the N-terminus were specified in as variable modifications.

Scaffold (version Scaffold_3_00_07, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50% probability as specified by the Peptide Prophet algorithm ([Keller, Nesvizhskii et al. 2002](#)). Protein identifications were accepted if they could be established at greater than 90% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm ([Nesvizhskii, Keller et al. 2003](#)). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Determination of high quantitative variance between treatments was performed in Scaffold using spectral counts as relative abundance parameter.

3.3.9 Electrophoresis and western blotting

Mature midgut cell cultures (3.5×10^6 cells) were pelleted ($21,135 \times g$ for 10 min at room temperature) and resuspended in deionized water and quantified using Qubit fluorometer (Invitrogen). Samples were solubilized in loading buffer ([Laemmli 1970](#)) and heat denatured for 5 min, then 7.5 μ g were loaded per lane on a SDS-10%PAGE gel. After electrophoresis, gels

were stained for total protein using the ProtoBlue Safe (National Diagnostics, Atlanta, GA), or electrotransferred overnight at 4°C to PVDF Immobilon-P filters (Millipore) using 20 mV constant voltage.

Filters were blocked by incubation for one hour at room temperature in blocking buffer (PBS pH 7.4 plus 0.1% Tween-20 and 3% BSA). Primary rabbit antisera against *H. virescens* p76 ([Leclerc and Miller 1990](#)) was generously provided by Dr. Kent Shelby (USDA-ARS Biological Control of Insects Research Laboratory, Columbia, MO). A dilution of the p76 antisera (1:10,000) was added to the blocking buffer and incubated for one hour at room temperature. After washing 6 times (10 min each) with washing buffer (PBS pH 7.5 plus 0.1% Tween-20 and 0.1% BSA), filters were probed with a 1:10,000 dilution of anti-rabbit antisera conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. After incubation, filters were washed as above and developed using the Super Signal West Pico chemiluminescence kit (Pierce) and photographic film.

3.3.10 Real-time PCR

Third instar *H. virescens* larvae were exposed to artificial diet containing Cry1Ac toxin at a concentration of 1 µg of Cry1Ac toxin per mL of diet. Midguts were dissected from larvae after 0, 2, 4, 6, or 18 hours of incubation. A group of larvae that had been exposed to contaminated diet for 2 hours was removed and placed on regular diet, and their midguts were dissected after 4, 6, or 18 hours after this transfer. Total RNA was purified from a pool of three dissected midguts using TriReagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. Integrity of the purified total RNA was checked by 2% agarose gel

electrophoresis. Total RNA was quantified using a nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and a Qubit fluorometer (Invitrogen, Carlsbad, CA). Contaminating DNA was eliminated using digestion with DNase I (GE Healthcare, Piscataway, NJ) for one hour at 37°C, then the samples were further purified using the RNeasy (Qiagen, Hilden, Germany.) cleaning protocol. Purified total RNA was used to synthesize cDNA with the Superscript VILO cDNA synthesis kit (Invitrogen), following to manufacturer's instructions. Samples were quantified using a nanodrop spectrophotometer prior to real time PCR.

Relative quantification of gene expression was measured in a 7900HT Fast Real-Time system (Applied Biosystems, Carlsbad, CA) on standard mode using the SDS 2.3 and SDS RQ manager software to collect cycle threshold (Ct) values. Primers targeting transcripts in the hexamerin gene family in a *H. virescens* transcriptome (Perera *et al.*, in preparation) were designed using the D-Lux designer software (<http://escience.invitrogen.com/lux/index.jsp>). As endogenous control for relative quantification, we used primers targeting a *H. virescens* tubulin transcript from the transcriptome with high similarity to alpha-tubulin from *Xestia cnigrum* (accession number EU100015). Primers and cDNA concentrations were validated using absolute quantification efficiency curve and dissociation curve analyses. Fluorescence-based quantification of PCR products generated in real time was measured using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Amplification reactions contained 5 µM forward and reverse primers, 10 ng of cDNA template, 10 µl of SYBR Green Master Mix, and DEPC-treated water to a final volume of 20 µl. The amplification program included initial steps of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 55°C for 20 sec. For relative quantification of gene expression we used the $2^{-\Delta\Delta C_t}$ method ([Livak and](#)

[Schmittgen 2001](#)) comparing transcript levels of target and endogenous control genes between treatments.

3.4 Results

3.4.1 Histopathology of Cry1Ac intoxication in *H. virescens* larvae

After individual larvae were droplet-fed a sublethal dose of Cry1Ac toxin, the histopathological structural changes of the dissected midguts were observed by H&E staining (Fig. 5). In larvae treated with buffer control, we observed an ordered epithelial structure with a population of regenerative cells associated with the basal lamina and mature cells occupying the apical portion of the epithelium (Fig. 5A). Under insecticidal conditions, the mature cells appeared damaged, with typical acidophilic (pink) stain observed along the apical surface (Fig. 5B). Regenerative cells in Cry1Ac-treated midguts appeared elongated towards the apical epithelial surface, indicative of differentiation (Fig. 5B). These differentiating cells appeared in proximity to damaged columnar cells. Some differentiating regenerative cells appeared to contain vacuoles characteristic of goblet cells (asterisks). In general, a lower number of undifferentiated regenerative cells were observed in the midguts of Cry1Ac-treated larvae.

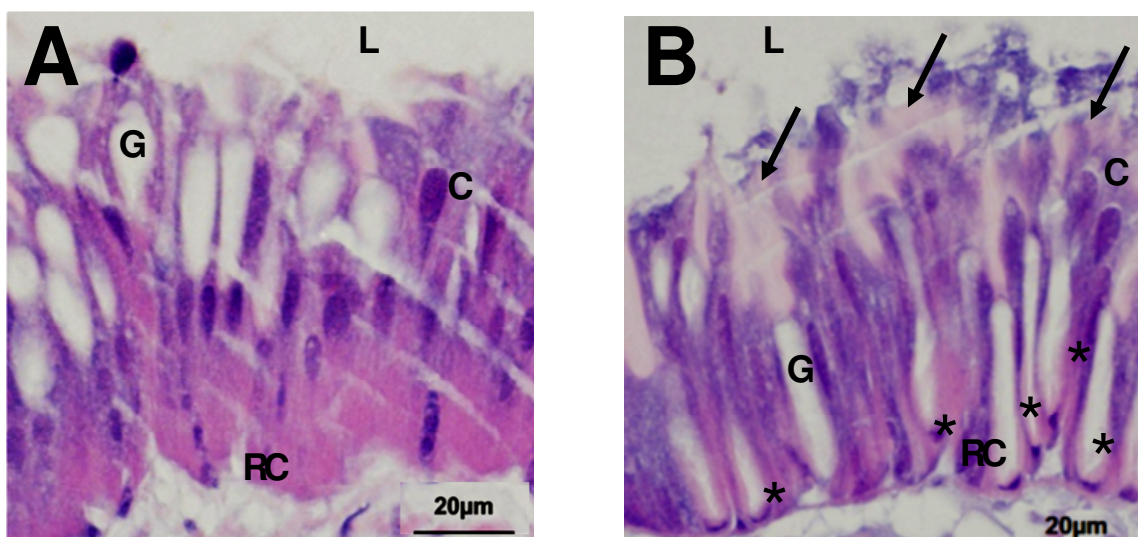


Figure 5: Structural changes in the midgut epithelium of *H. virescens* larvae after Cry1Ac intoxication. Midgut epithelium tissue sections from *H. virescens* larvae were stained with H&E after treatment with buffer control (A) or a sublethal dose of Cry1Ac toxin (B). Control cross sections had the typical midgut epithelial organization with regenerative cells (RC) localized in proximity to the basal lamina and goblet (G) and columnar (C) cells found toward the apical side just below the lumen (L). After sublethal intoxication with Cry1Ac, stress features in the epithelium included reduced organization and elongated (asterisks) or damaged (black arrows) cells. Reference bar = 20µm.

3.4.2 Primary *H. virescens* midgut cell culture regeneration after exposure to Cry1Ac toxin

We established primary mixed midgut cell cultures from *H. virescens* larvae to assess their regenerative potential under insecticidal conditions. Two hours after exposure to Cry1Ac toxin, we detected an increase in total cell numbers (Fig 6). This change consisted of a greater than 2-fold increase in the number of dead cells, with a smaller increase in the number of living

cells. One hour later, there was resurgence in the number of alive cells and a decrease in the number of dead cells, suggestive of culture regeneration. After 18 hours, the total number of cells doubled, indicating that the cells were actively dividing in culture under continual insecticidal conditions. While the number of vital cells did not change significantly over the 18 hour period (ANOVA, $P = 0.608$), there was a significant increase in the number of dead cells (ANOVA, $P < 0.05$) when comparing the 30 min and 6 or 18 hour time points, implicating asymmetrical cell division.

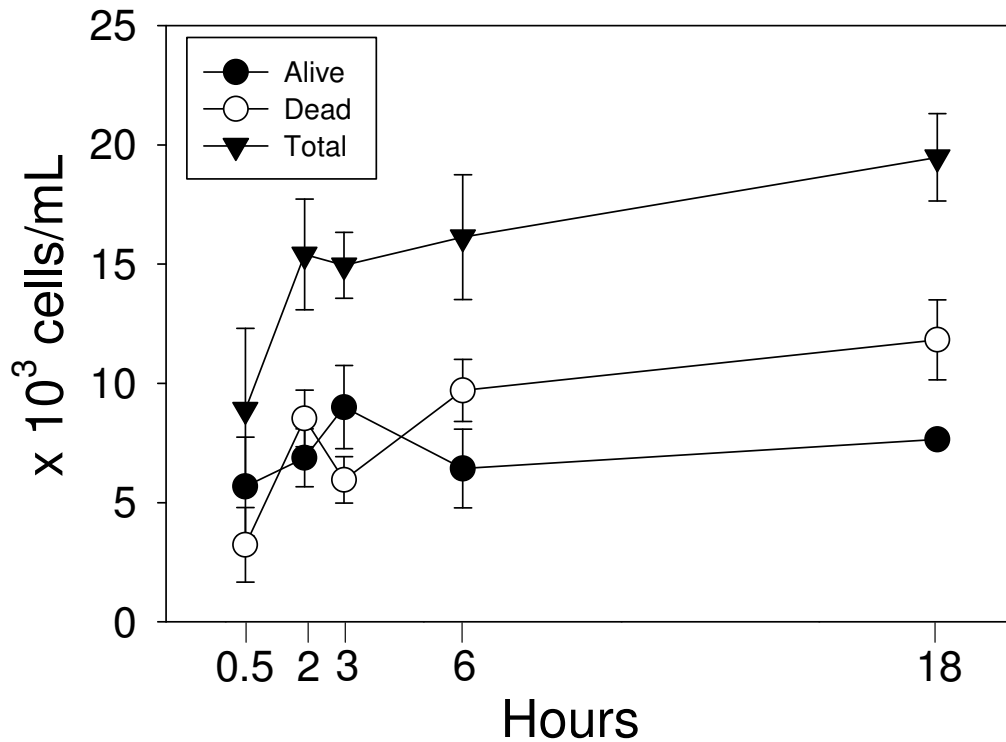


Figure 6: Renewal of primary *H. virescens* midgut cell cultures during incubation with Cry1Ac toxin. Primary cultures were incubated with 2 μ g of Cry1Ac toxin, and the number of alive (closed circles) and dead (open circles) cells at different intervals were estimated using trypan blue exclusion. Total cell numbers (inverted triangles) were obtained by adding the number of alive and dead cells at each time point analyzed. Data shown are the mean and standard error from duplicate counts of two independent cultures derived from 30 larvae each.

3.4.3 Secretome bioactivity on primary midgut stem cell cultures

The regenerative properties of secretomes, collected from midgut mature cell cultures treated with control buffer or Cry1Ac toxin, were tested on fresh midgut stem cell preparations using calcein differential staining properties ([Castagnola, Eda et al. 2011](#)). Freshly prepared

midgut stem cell cultures contained predominantly stem cells (75%), with minimal proportion (25%) of contaminating mature cells (Fig. 7, green bars). The effects of all treatments on stem cells were observed at a prolonged time point (24 hours) to maximize the probability of observing a differential effect.

When looking at percentage of each cell type (stem, mature or dead), treatment with buffer secretome resulted in reduced numbers of stem and increased mature and dead cells (Fig. 7, red bars) when compared to fresh stem cell cultures, supporting that this secretome contained factors inducing differentiation. In contrast, treatment with Cry1Ac-induced secretome (Fig. 7, orange bars) resulted in the highest levels of dead cells concomitant with disappearance of stem cells, while the number of mature cells was not different from the buffer secretome treatment. However, incubation of stem cells with purified Cry1Ac toxin did not result in alteration of the stem cell or dead populations when compared to fresh cultures, while the number of mature cells was similar to the buffer and Cry1Ac secretome treatments (Fig. 7, gray bars). This observation suggests that the Cry1Ac-induced secretome induced cell death, an observation that was not evident in treatments with Cry1Ac alone. Whether the cell types accounting for the increased dead cell numbers were stem, mature or both was not determined.

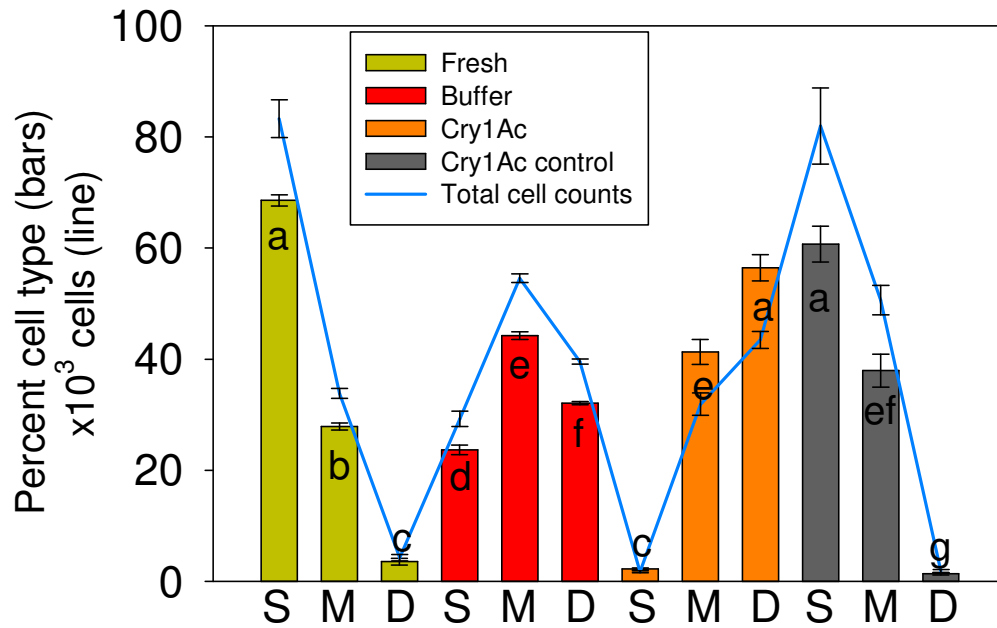


Figure 7: The Cry1Ac-induced secretome causes increased cell death in *H. virescens* primary midgut stem cell cultures. Cell type composition of primary stem cell cultures was determined using a fluorescence-based method. Stem cells (S) displayed lower side scatter light due to their less complex morphology and higher vital fluorescence than mature cells (M). Dead cells (D) were identified based on their absence of vital staining, which does not allow determination of specific cell types in this group. Using flow cytometer gates total events were counted and gated into individual cell type populations. The total number of cell types (blue line) in each treatment is also shown, and was used to calculate the percent cell type (bars). Both buffer (red bars) and Cry1Ac (orange bars) secretomes induced stem cell differentiation, although a higher proportion of dead cells was detected with the Cry1Ac secretome. The abundant dead cell population is absent from treatment with purified Cry1Ac toxin (gray bars). Different letters in the bars denote statistically significant differences (Kruskal-Wallis ANOVA on Ranks, $P = <0.001$).

3.4.4 Identification of proteins from mature midgut cell secretomes

The secretomes produced after incubation of *H. virescens* primary mature midgut cell cultures with buffer, Cry1Ac, or Cry3Aa were collected and proteins identified and quantified by mass spectrometry using a custom *H. virescens* transcriptome database (Perera et al. in preparation). A total of 269 proteins were identified from the secretomes (False Discovery Ratio [FDR] 0.4 %), with 128 proteins being common to all three secretomes. Up to 214 and 215 proteins were identified in the Cry1Ac and Cry3Aa secretomes, respectively, while 222 were identified in the buffer-induced secretome (Fig. 8). Between 17 to 19 proteins appeared to be specific to each secretome, with a similar number (11 to 18) being common between secretome paired comparisons.

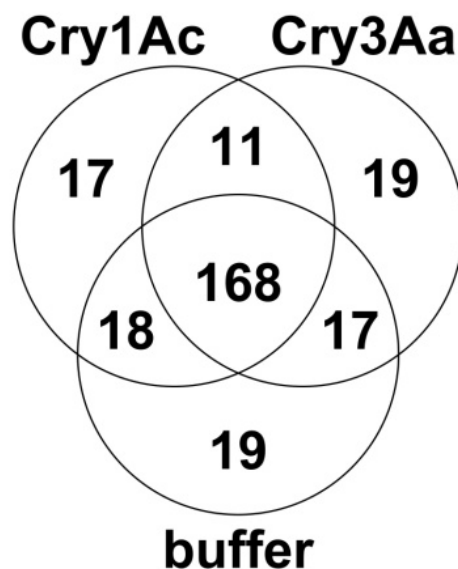


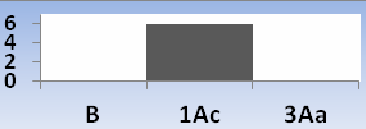
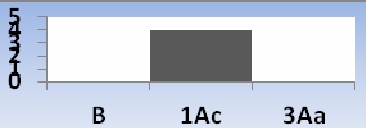
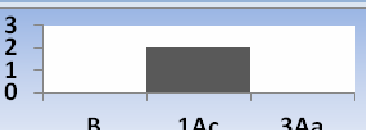
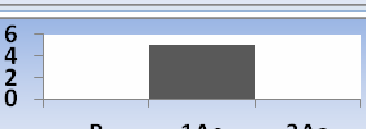
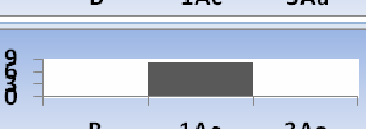
Figure 8: Protein Venn diagram presenting the number of identified proteins in each of the analyzed secretomes (Cry1Ac, buffer and Cry3Aa). A total of 269 proteins (0.4% protein FDR) were identified from 8,965 spectra (7.1% peptide FDR).

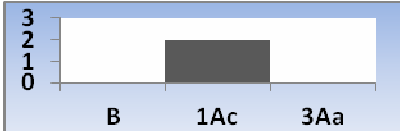
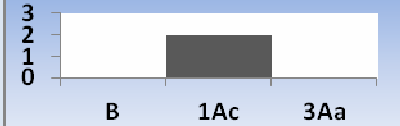
Using the secretomes induced by buffer or Cry3Aa, an inactive toxin against *H. virescens* larvae ([van Frankenhuyzen 2009](#)), we identified proteins unique to the Cry1Ac secretome for further analysis. To focus our analysis on secreted and membrane proteins that could interact with midgut stem cells, we used predicted Gene Ontology (GO) terms to categorize proteins and assign a putative subcellular localization. Using this analysis, we eliminated 10 of the 17 proteins that were only found in the Cry1Ac secretome from further analysis based on being ribosomal or predicted to reside in mitochondria. Two of the other seven proteins (Table I) were predicted to reside in the cytoplasm and matched with proteins with functions related to cytoskeletal rearrangements: a predicted protein from *Tribolium castaneum* with high homology

to the dedicator of cytokinesis (DOCK) protein 1 (accession number XP_972351.1), and myosin from the jumping ant *Harpegnathos saltator* (accession number EFN88334.1). A homolog to the cytosolic/microsomal glutathione S-transferase (GST) from *Choristoneura fumiferana* (accession number AAF23078.1) was also identified as unique to the Cry1Ac secretome, while other GST isoforms were detected in the buffer and Cry3Aa secretomes. Three proteins unique to the Cry1Ac secretome matched to proteins that were predicted to be secreted: the macrophage migration inhibitory factor from *Bombyx mori* (accession number NP_001040199.1), the chymotrypsin inhibitor CI-8A from *B. mori* (accession number AAK52495.1), and the arylphorin subunit precursor from *Spodoptera litura* (accession number CAB55605.1).

When performing quantitative variance analyses comparing all the proteins identified in the three secretomes, arylphorin was the only protein with significant relative amount differences among secretomes. Two different contigs (899 and 448) identified as homologs of *S. litura* arylphorin were detected. While contig 899 had the highest quantitative significance among the secretomes, peptides matching to this contig were found in all three secretomes, albeit they were found at higher (approx. 5-fold) relative levels in Cry1Ac secretome (Table 2). In contrast, peptides matching to contig 448 were only detected in the Cry1Ac secretome. This may indicate that the peptides identified in contig 448 may represent part of a midgut-specific arylphorin that is expressed in response to Cry1Ac intoxication.

Table 2: Cytosolic, membrane, and secreted proteins identified as unique to the Cry1Ac secretome. Spectral counts, the number of tandem mass spectra obtained for each protein are shown as a surrogate for protein abundance in each secretome.

Contig number	Database match (accession number)	Organism	Description	Predicted Molecular weight (kDa)	E- value	Predicted location	Spectral counts								
5748	NP001040199	<i>Bombyx mori</i>	Macrophage migratory inhibitory factor	48	4.21187e-15	S	 <table><thead><tr><th>Secretome</th><th>Spectral counts</th></tr></thead><tbody><tr><td>B</td><td>0</td></tr><tr><td>1Ac</td><td>5.5</td></tr><tr><td>3Aa</td><td>0</td></tr></tbody></table>	Secretome	Spectral counts	B	0	1Ac	5.5	3Aa	0
Secretome	Spectral counts														
B	0														
1Ac	5.5														
3Aa	0														
1991	XP972351	<i>Tribolium castaneum</i>	Dedicator of cytokinesis (DOCK) 1	227	0.0	M	 <table><thead><tr><th>Secretome</th><th>Spectral counts</th></tr></thead><tbody><tr><td>B</td><td>0</td></tr><tr><td>1Ac</td><td>4.5</td></tr><tr><td>3Aa</td><td>0</td></tr></tbody></table>	Secretome	Spectral counts	B	0	1Ac	4.5	3Aa	0
Secretome	Spectral counts														
B	0														
1Ac	4.5														
3Aa	0														
106	AAD09820	<i>Heliothis virescens</i>	Calcium-transporting atpase sarcoplasmic endoplasmic reticulum type (calcium pump)	331	0.0	I/M	 <table><thead><tr><th>Secretome</th><th>Spectral counts</th></tr></thead><tbody><tr><td>B</td><td>0</td></tr><tr><td>1Ac</td><td>2.5</td></tr><tr><td>3Aa</td><td>0</td></tr></tbody></table>	Secretome	Spectral counts	B	0	1Ac	2.5	3Aa	0
Secretome	Spectral counts														
B	0														
1Ac	2.5														
3Aa	0														
1567	AAK52495	<i>Bombyx mori</i>	Chymotrypsin inhibitor	122	2e-130	S	 <table><thead><tr><th>Secretome</th><th>Spectral counts</th></tr></thead><tbody><tr><td>B</td><td>0</td></tr><tr><td>1Ac</td><td>5.5</td></tr><tr><td>3Aa</td><td>0</td></tr></tbody></table>	Secretome	Spectral counts	B	0	1Ac	5.5	3Aa	0
Secretome	Spectral counts														
B	0														
1Ac	5.5														
3Aa	0														
448	CAB55605	<i>Spodoptera litura</i>	Arylphorin precursor	175	0.0	S	 <table><thead><tr><th>Secretome</th><th>Spectral counts</th></tr></thead><tbody><tr><td>B</td><td>0</td></tr><tr><td>1Ac</td><td>7.5</td></tr><tr><td>3Aa</td><td>0</td></tr></tbody></table>	Secretome	Spectral counts	B	0	1Ac	7.5	3Aa	0
Secretome	Spectral counts														
B	0														
1Ac	7.5														
3Aa	0														

Contig number	Database match (accession number)	Organism	Description	Predicted Molecular weight (kDa)	E- value	Predicted location	Spectral counts
Table 2. Continued							
1527	AAF23078	<i>Choristoneura fumiferana</i>	Glutathione s-transferase	147	1.70127e-57	I	
181	EFN88334	<i>Harpegnathos saltator</i>	Myosin	276	0.0	I	

3.4.6 Arylphorin expression in midguts from Cry1Ac-fed *H. virescens* larvae

To confirm the proteomic identification of arylphorin as differentially secreted after exposure to Cry1Ac toxin, we used Western blotting with antisera against the p76 arylphorin subunit of *H. virescens* (Fig. 9). Under non-insecticidal conditions, arylphorin expression increased after larvae fed on artificial diet for two hours. After this time, p76 arylphorin levels decreased and did not increase again until 18 hours, when larvae were approximating pupation. At this time point we detected a high molecular weight band, which may represent the arylphorin hexamer.

In contrast, when larvae were continuously exposed to Cry1Ac toxin, p76 arylphorin expression steadily increased over the 2 to 6 hour time period, and then sharply decreased by the 18 hour time point. This sharp decrease was probably due to larvae susceptibility to toxin and diminished larval health. When larvae were fed Cry1Ac toxin for two hours and then allowed to recover in toxin-free diet, p76 arylphorin levels were found to be increased compared to initial levels two hours after feeding on toxin-free diet. However, and as observed in larvae exposed to Cry1Ac toxin, p76 arylphorin levels decreased to reach undetectable levels at the 18 hour time point.

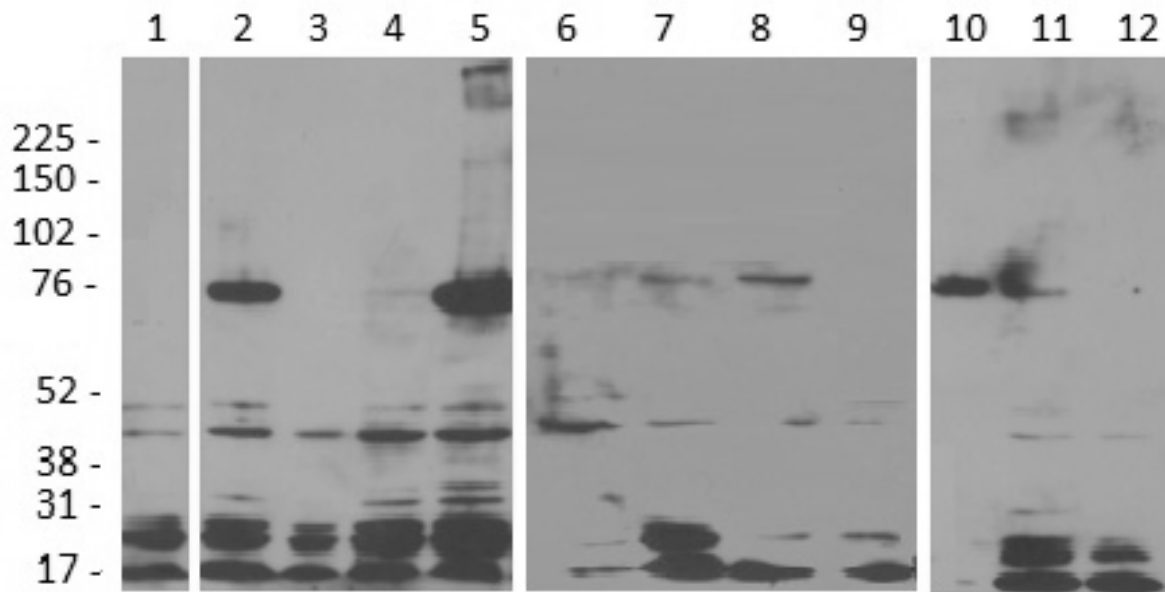


Figure 9: Western blotting detection of p76 arylphorin in *H. virescens* larvae feeding on artificial diet with buffer control (lanes 2-5), diet containing Cry1Ac toxin (lanes 6-9), or with Cry1Ac-contaminated diet for two hours followed by feeding on uncontaminated diet (lanes 10-12). Levels of arylphorin in untreated larvae are shown in lane 1 for comparison. Molecular weight markers (in kDa) are shown on the left; the expected size for the p76 arylphorin subunit was approximately 76 kDa. Larval incubation times were two hours (lanes 2, and 6), four hours (lanes 3, 7, and 10), six hours (lanes 4, 8 and 11), and 18 hours (lanes 5, 9, and 12).

3.4.7 Transcriptional changes in arylphorin and hexamerin after exposure to Cry1Ac

To characterize expression patterns of arylphorin and hexamerin genes during Cry1Ac intoxication and recovery, we used quantitative real time PCR (qRT-PCR) with primers targeting arylphorin and hexamerin family contigs in a custom *H. virescens* transcriptome. The levels of

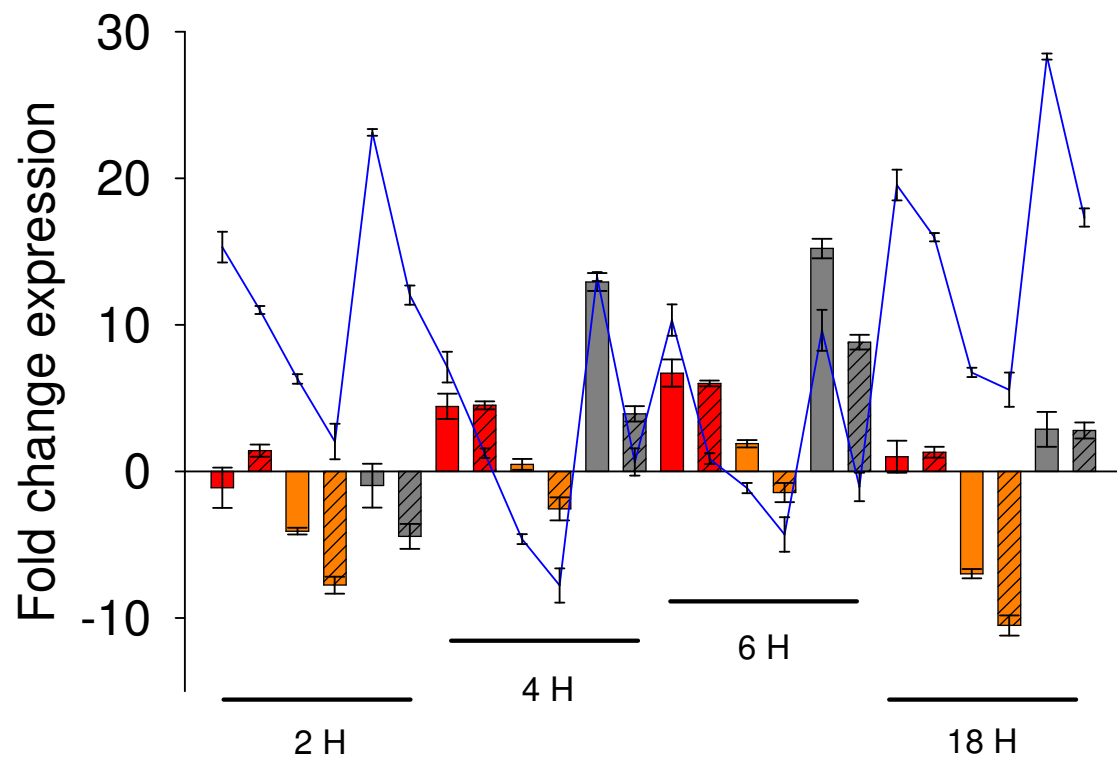
arylphorin transcripts analyzed corresponded with our Western blot results (Fig. 9). There was an initial decrease after two hours of exposure to diet, followed by a sharp increase after 18 hours, and this general trend was also observed for all the hexamerin contigs analyzed (Fig. 10). The initial decrease of these aerylphorin and hexamerin transcripts corresponds with a known function of this family of proteins; to provide an amino acid source when one is not available from feeding. The increased transcript levels after 18 hours incubation on diet with buffer incorporated was most likely due to the larvae approaching the pupation stage.

Under continuous exposure to Cry1Ac, all the hexamerin transcripts were found to be initially in relative low abundance compared to the buffer controls (Fig. 10). In contrast to the response observed in buffer treatments, levels of aerylphorin and hexamerin transcripts increased after 4 and 6 hours to drastically decrease after 18 hours. These differences between buffer and Cry1Ac treatments suggested that aerylphorin and hexamerin-like transcripts may be specifically implicated in the midgut response to Cry1Ac intoxication.

To further examine the role of aerylphorin and hexamerins in recovery from Cry1Ac intoxication, we monitored their transcript levels after a 2 hour exposure to Cry1Ac toxin. Two hours after the larvae were allowed to recover in toxin-free diet, we observed high relative levels of all analyzed transcripts (Fig. 11), which was qualitatively reminiscent of the increased transcript levels observed when larvae were continuously exposed to Cry1Ac toxin for four hours (Fig. 10). After this time, the pattern of transcript levels was similar in larvae continuously exposed to Cry1Ac and larvae recovering from intoxication. Hexamerins up-regulated between these two conditions may be important in a midgut response to intoxication. Interestingly, expression profiles, besides three transcripts, look very similar otherwise indicating that there were similar

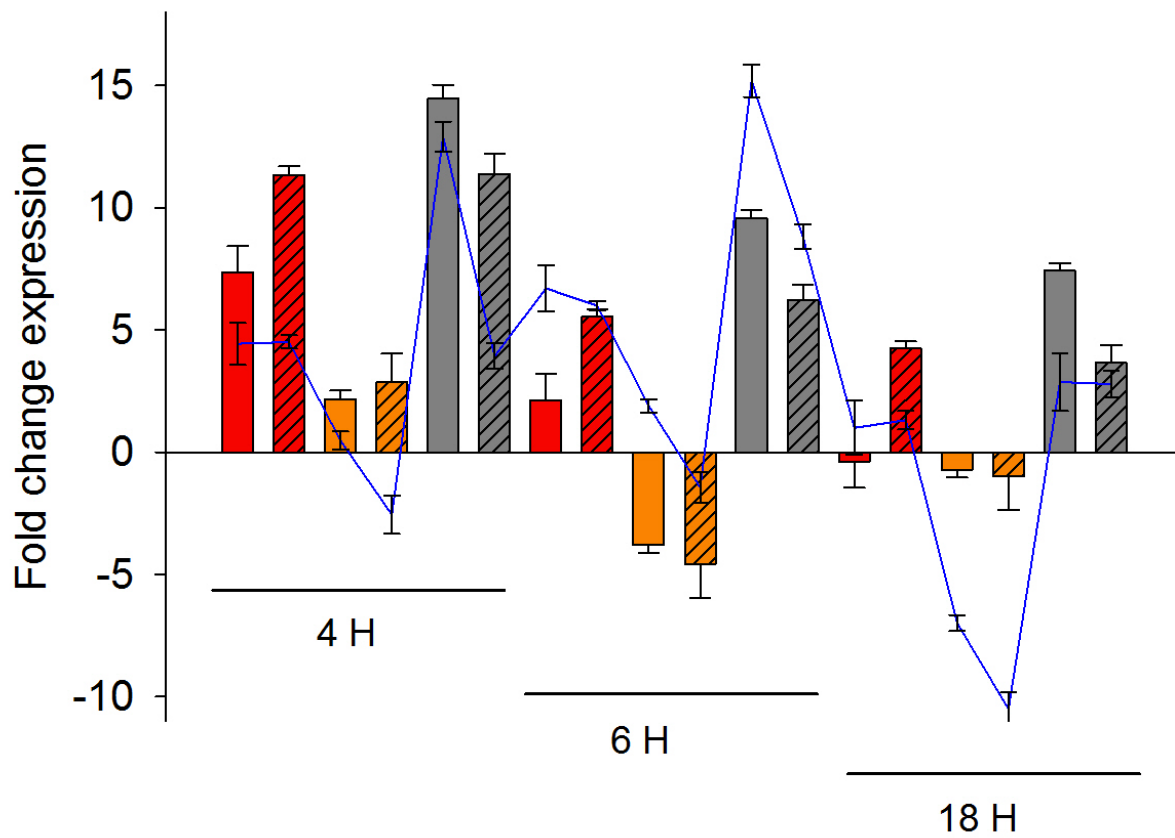
hexamerin-related healing and starvation responses in larvae. There was an increase in fold change of all hexamerin transcripts in the buffer treatment after 2 or 18 hours. Under Cry1Ac treatment conditions, hexamerin-like transcript 623 and arylphorin precursors 899 and 448 were both up-regulated compared to buffer conditions at the 4 and 6 hour time points. All hexamerin transcripts were higher after exposure to Cry1Ac and continually decreased over the next 12 hours. Conversely, after Cry1Ac removal all hexamerin transcript levels returned to initial conditions.

Figure 10: During Cry1Ac intoxication there is increased arylphorin expression compared to other hexamerins. Transcript levels of hexamerin and arylphorin-like transcripts in *H. virescens* larvae fed on diet containing buffer (blue line) or Cry1Ac (bars), as were determined using dissected midguts and qRT-PCR. Time after initial exposure to diet is shown in the X axis. Relative quantification of gene expression calculations were normalized to initial (time zero) levels using tubulin gene expression as an endogenous control. Each time point sample consisted of three pooled midguts, PCR reactions were performed a minimum of three times, and using at least three technical replicates per experiment.



- Hexamerin-like transcript (3478)
- Hexamerin-like transcript (623)
- Arylphorin precursor (899)
- Arylphorin precursor (448)
- Riboflavin binding hexamerin precursor (22964)
- Hexamerin 2 beta (1673)
- Buffer control

Figure 11: Recovery after Cry1Ac intoxication relates to increased hexamerin transcript levels. Hexamerin and arylphorin transcript levels after feeding for two hours on Cry1Ac-containing diet (blue line) are compared to levels in insects exposed to Cry1Ac and allowed to recover (bars) demonstrated distinct differences between arylphorins (orange) and other hexamerins (red). Time after initial exposure to diet is shown in the X axis. Relative quantification of gene expression calculations were done using time zero midguts and tubulin gene expression as an endogenous control. Each time point sample consisted of three pooled midguts, assays were performed a minimum of three times, and using at least three technical replicates per experiment.



- Hexamerin-like transcript (3478)
- Hexamerin-like transcript (623)
- Arylphorin precursor (899)
- Arylphorin precursor (448)
- Riboflavin binding hexamerin precursor (22964)
- Hexamerin 2 beta (1673)
- Cry1Ac

3.5 Discussion

The biological activity of an epithelium during molting ([Tettamanti, Grimaldi et al. 2007](#)), Bt intoxication ([Martinez-Ramirez, Gould et al. 1999](#)), digestion ([Santos, Ribeiro et al. 1984](#)), and degeneration ([Rost-Roszkowska, Jansta et al. 2010](#)) can be visualized by various structural characteristics. The shape and orientation of columnar cells, the reduced number of regenerative cells ([Sousa, Santos et al. 2010](#)) and the abundance of autophagic vacuoles ([de Sousa, Wanderley-Teixeira et al. 2009](#)) are structural characteristics which can be visualized using histological midgut cross sections. The epithelial organization of midguts derived from *H. virescens* larvae was detected using the standard histopathological method of H&E staining. The number of available regenerative cells was compared in midguts derived from larvae which had been fed control diet or diet containing Cry1Ac toxin, and this estimation was further studied using established *in vitro* methodologies ([Castagnola, Eda et al. 2011](#)). In larvae fed Cry1Ac, we found increased vacuolization in the apically-located columnar cells, indicating apoptosis and autophagy. The newly regenerated columnar cells were more basally located. Previous reports suggest that increased granulation in the cytoplasm of columnar cells indicates apocrine secretion and the release of digestive enzymes ([Pinheiro, Quagio-Grassiotto et al. 2008](#)). After ingestion of plants expressing Bt toxins the insect midgut epithelium shows degeneration of the microvilli, aberrant goblet cell localization, and stressed morphology ([Sousa, Santos et al. 2010](#)). These effects were similarly detected in midguts derived from Cry1Ac fed larvae.

In previous reports primary *H. virescens* midgut cell cultures were described to be susceptible to Bt toxin and attempted to regenerate by producing increased differentiating cells ([Loeb, Martin et al. 2001](#)). When toxin treatments were washed away, the proportion of each

midgut cell type returned to stasis as indicated by a decrease in abundance of differentiating cells. In correlation with these findings, our mixed midgut cell cultures increased in the number of total cells after continuous exposure to toxin. This increase in the total number of cells over time indicates that mixed midgut cell cultures actively divide in the presence of toxin. The increase in the number of regenerative cells triggered to differentiate in stained midgut epithelium cross-sections corresponded to the observed stem cell differentiation response to secretome *in vitro*. These results further demonstrate the regenerative capabilities of the *in vitro* *H. virescens* midgut cell culture, mimicking epithelial cross talk *in vivo*. Based on these observations, we expected that the growth factors implicated in a healing response would be present in the secreted proteome of mature midgut cell cultures.

Epithelial stem cell responses have been previously characterized to be indicators of a wide variety of biological active properties ([Pitsouli, Apidianakis et al. 2009](#)) and therefore a stem cell assay was an appropriate choice to measure secretome bioactivity. In order to distinguish the stem cell unique response to the different secretomes, we incubated stem cells with toxin-induced secretomes and compared their bioactivity to control secretomes. Cell regeneration occurs as less abundant stem cells in mixed midgut cell cultures are induced to proliferate and differentiate to mature cells, because stem cells are the only cell type capable of division in these cultures ([Losick, Morris et al. 2011](#)). In primary mixed midgut cell cultures the changes in the cell type ratios can be due to a number of variables. The conditions which are known to impact *in vitro* cell culture changes are 1) the stem cell secretome and putative stem-to-stem interactions ([Lin 2002](#)), 2) the mature cell secretome ([Buhrke, Lengler et al. 2011](#)), and 3) membrane-bound mature cell interaction factors ([Brown, Gregory et al. 2000](#)). We purified the

stem cells from mature cells in order to limit the impact of these variables and maximize detection of effects induced by mature cell secretome on stem cells. Fresh stem cells were incubated with mature cell secretome in order to analyze the potential for a stem cell bio-activity response ([Pitsouli, Apidianakis et al. 2009](#)). The two interactions *in vitro* ([Loeb, Martin et al. 2001](#)) are a demonstration of the cross-talk healing response that happens *in vivo* ([Slack 2000](#)) after toxin ingestion ([Martinez-Ramirez, Gould et al. 1999](#)). We observed high mortality when treating stem cells with buffer-induced secretome, which is probably due to the short lifespan of the contaminating mature midgut cells in stem cell cultures. However, the Cry1Ac-induced secretome induced higher mortality and differentiation, supporting that differentiation factors are present in this secretome.

In order to identify the proteins present in biologically active secretome, we used LC/MS/MS. In these assays the protein with the most significant difference in relative amounts was arylphorin, displaying both high quantitative variance and Cry1Ac specificity. Previous reports have presented evidence for a role of arylphorin in inducing an immune response larvae after infection with bacteria ([Freitak, Wheat et al. 2007](#)). Additionally, recent several studies have reported bioactivity of α -arylphorin as a midgut cell mitogen in Lepidoptera, promoting regeneration of midgut cell cultures ([Blackburn, Loeb et al. 2004](#); [Hakim, Blackburn et al. 2005](#); [Hakim, Blackburn et al. 2007](#)). Although arylphorin has never been directly collected from the midgut in response to intoxication, the hemolymph and the midgut have been shown to work in concert with respect to arylphorin. It was established that the midgut epithelial tissue works as a sensing organ which can trigger arylphorin expression in the hemolymph ([Palli and Locke 1987](#)) and viral infection causes down-regulation of arylphorin which facilitates larval parasitization by

wasps ([Kunkel, Grossniklausbuergin et al. 1990](#)). Our data represents the first indication that arylphorin may be directly involved in the midgut healing response after Cry1Ac intoxication.

Arylphorin belongs to a class of storage proteins called hexamerins, which are exploited by the organism to build new tissues ([Telfer and Kunkel 1991](#)). Hexamerins provide an amino acid source to the insect during developmental phases when they do not normally feed, and are utilized for new tissue formation during molting, when the insect undergoes extreme physiological and morphological change ([Haunerland 1996](#)). In order to investigate the potential for a hexamerin-based mechanism of regeneration in the Bt toxin damaged midgut, we performed larvae bioassays and analyzed hexamerin expression. Larvae were exposed to Cry1Ac and then allowed to recover and the midguts were collected for transcript investigation by qRT-PCR and Western blotting. We found that expression of selected genes in the hexamerin family is induced after larvae were starved and also in the larvae which were allowed to recover from intoxication. Larvae that were continually fed with diet contaminated with Cry1Ac toxin stopped feeding, thus prohibiting proper midgut digestion, metabolism and nutrient uptake. Initially arrested metabolism may explain the observed decreased expression of members in the hexamerin gene family, but after an extended time up-regulation of arylphorin was detected. All of the arylphorin transcripts investigated with buffer-fed larvae had increased expression as the larvae transitioned into the pre-pupae stage, as expected from reports of role of this protein in metabolism and pupation ([Haunerland 1996](#); [Kiran Kumar, Ismail et al. 1998](#); [Nagamanju, Hansen et al. 2003](#)). In larvae treated with control diet we detected a putative hexameric form of midgut arylphorin by Western blotting detected after 18 hours. Although this work provides

support for an alternate function for arylphorin besides fat body storage and ecdysis, these mechanisms may not be necessarily mutually exclusive and may work in concert.

There were a number of non-hexamerin secreted proteins expressed by mature midgut cells in response to intoxication. The macrophage migration inhibitory factor, for which contig 5748 has matching peptides, has been traditionally characterized to signal an inflammatory response ([Cohen 1977](#)). It has an established role in chemotaxis ([Fan, Hall et al. 2011](#)) and has long been considered a secreted cytokine ([Taniguchi 1988](#)). Antitrypsin isoform 1, contig 1567, is a protease inhibitor secreted to inhibit serine proteases. It is in the serpin family ([Belorgey, Irving et al. 2011](#)) of protease inhibitors ([Zou and Jiang 2005](#)) and covalently binds to the protease resulting in an inactive serpin-protease complex. This protein is also a known prophenoloxdase activator ([Soderhall, Cerenius et al. 1994](#)) which in the midgut would activate various immune response pathways that could help recover from toxin presence ([Zhu, Guo et al. 2011](#)). Glutathione-s-transferase, contig 1527, which was only present in the Cry1Ac secretome, is a significant finding for this *in vitro* cell culture system. This protein is often expressed, and at times over-expressed, in response to various xenobiotics ([Yang, Li et al. 2011](#)). This protein detoxifies compounds which are harmful to the organism. Detection of this protein after Cry1Ac intoxication suggests that the primary cell cultures are attempting to heal and regenerate because there is not a whole organism present in this case and must be triggered by midgut cells present alone. Although completely overcoming toxicity is not evident in this susceptible strain, this *in vitro* primary cell culture system could be used to examine the secretome expression when there is evident of Bt-resistance.

There are multiple proteins identified in the secretome that indicated a metabolic response to intoxication instead of the arrested metabolism and paralytic response that one would expect. The increased metabolic response during Cry1Ac intoxication may be due to system differences between *in vivo* larval midgut responses and *in vitro* cell culture systems. For example, adenylate kinase, contig 3061, was identified in the Cry1Ac-specific secretome. Adenylate kinase ([Muller, Schlauderer et al. 1996](#)) is a phosphorylating enzyme associated with the nucleus and mitochondria catalyzing the reversible phosphate group transfer between ATP and ADP. In larval midguts there are multiple immune response factors from other tissues that influence the midgut response. Mature cells in culture are being exposed to a much lower concentration per unit surface area than if the toxin had been ingested, which also may influence immune responses. The presence of intracellular proteins in the Cry1Ac treated secretome is evidence that the cells are being lysed and their intracellular components have become solubilized. Because these cells have undergone Cry interaction and consequently died, these proteins can give us insight into how these cells responded to the onslaught and also to how their neighboring cells react to cells dying around them. It is not surprising that the majority of these intracellular proteins have been previously identified as cell death response proteins or involved in various intracellular cell death pathways ([Loeb, Hakim et al. 2000](#); [Tettamanti, Grimaldi et al. 2007](#)). In our study, abundance of the apoptosis-like response explains the increased abundance of dead cells after 24 hour exposure to the Cry1Ac mature cell secretome. One example is the increased expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), contig 5781, when midgut cells are exposed to Cry1Ac. It was recently characterized that increased GAPDH expression occurs in response to the apoptosis-inducing agent actinomycin D ([Courtiade, Muck et al. 2011](#)).

Also, GAPDH is a well characterized metabolite that is an intermediate in glycolysis and gluconeogenesis as well as other biosynthetic pathways ([Sirover 2011](#)). This metabolite participates in a high number of metabolic pathways, indicating that the cultures are responding to intoxication by mounting and activating an immune healing response *in vitro*, which similar studies have described in response to the insecticide Pyridalyl ([Powell, Ward et al. 2011](#)). The peptides of contig_1390 are closely related to the cytochrome c oxidase assembly protein cox15, which is associated with the mitochondria and various posttranslational modifications, protein turnover, chaperone activation, and protein complex assembly. Proteins within this family have been previously described to induce apoptosis and were up-regulated in the Cry1Ac-specific mature cell secretome. Down-regulation of cytochrome c oxidase has been previously reported as a biomarker for apoptosis inhibition in insecticide resistance in strains of *Drosophila melanogaster* ([Song and Scharf 2009](#)).

Our proteomic analysis of the midgut cell secretome in response to Cry1Ac intoxication has identified arylphorin and proteins in the hexamerin family as potentially involved in the cross-talk between mature and stem cells resulting in regeneration of the damaged midgut. Since midgut regeneration has been previously suggested as a putative resistance mechanism to Cry toxins ([Forcada, Alcacer et al. 1999](#); [Loeb, Martin et al. 2001](#)), our data provide potential target proteins to focus on when comparing susceptible and resistant larvae. Our analysis also identified additional proteins with differential expression in response to Cry1Ac intoxication, which suggests a potential role for these proteins in defense or pathological processes. Further work will concentrate on testing the functional role of these proteins in insect midgut defense and as potential targets for the development of inhibitors with insecticidal effect.

4 Characterization of the Cry1Ac-induced midgut regenerative response in susceptible and resistant strains of *Heliothis virescens*

4.1 Abstract

The bacterium *Bacillus thuringiensis* (Bt) produces crystal (Cry) toxins with potent insecticidal properties which have been extensively studied to elucidate their mechanism of action. The necessity and importance of each step in the mode of action of Cry toxins for effective insecticidal activity is still highly debated, especially in the case of laboratory-selected resistant strains displaying over a 1,000-fold resistance when compared to susceptible insects. Our work was focused on two strains of the lepidopteran crop pest *Heliothis virescens*, KCB and CXC, that have been characterized to tolerate multiple and diverse Cry toxins and the commercial Bt product DiPel. Histopathological analysis of dissected *H. virescens* larval midguts revealed that midguts from resistant strains KCB and CXC contained higher numbers of regenerative cells compared to a reference susceptible strain (YDK). Using a fluorescence-based flow cytometry method we quantified stem cell proliferation and differentiation in primary cultures exposed to proteins secreted (secretomes) by mature midgut cells exposed to Cry1Ac toxin. Stem cell regeneration in the presence of secretome was observed to be distinct for each strain. Thus, while the CXC secretome induced higher differentiation, the treatment of stem cells with the KCB secretome resulted in increased proliferation. We further tested the existence of regenerative factors involved in resistance to diverse toxins in midgut secretomes by feeding secretomes collected from resistant larvae to susceptible insects and monitoring their tolerance to DiPel. In these bioassays we observed that secretomes from CXC or KCB midgut cells significantly increased tolerance of YDK larvae to DiPel. Interestingly, we also observed

increased mortality induced by treatment with secretomes, suggesting the presence of activators of cell death pathways in these secretomes. Identification of proteins in these secretomes by LC/MS/MS allowed the characterization of the proteins differentially secreted by midgut cells in the CXC and KCB strains compared to YDK larvae. In general, proteins identified by tandem mass spectrometry as specific to the CXC secretome included aminopeptidase, citrate synthase, myosin, ribosomal proteins, oligopeptidase, ribophorin and catalase. In contrast, the only protein identified specific to the KCB secretome was histone. The most statistically significant protein identified in all the analyzed secretomes was the hexamerin protein arylphorin. In these assays, arylphorin expression was found to be augmented in resistant compared to susceptible larvae, with higher expression in response to feeding on diet containing DiPel compared to Cry1Ac. Since hexamerins are crucial to larval development and molting, we also measured fitness parameters in susceptible and resistant larvae feeding on artificial diet. Larvae from the resistant strains displayed slower development and longer larval stages than susceptible larvae. Taken together, these data suggest a role for arylphorin and hexamerins in midgut regeneration and tolerance to DiPel. Characterization of the secretomes from midgut cells of the CXC and KCB larvae suggests the importance of some of these midgut proteins in resistance.

4.2 Introduction

Bacillus thuringiensis (Bt) is an ubiquitous bacterium producing crystal (Cry) toxins with insecticidal activity. Recent estimations suggest that Bt-based insecticidal technologies represent approximately 2% of the insecticide market ([Bravo, Likitvivatanavong et al. 2011](#)). Each step in the mode of action of Cry toxins has been extensively investigated and varies with different

insecticidal toxins, the targeted insect pest, and method of admission to the insect. The Cry toxin mode of action includes crystal solubilization and proteolytic toxin processing by midgut proteases ([Choma, Surewicz et al. 1990](#); [Forcada, Alcacer et al. 1996](#); [Kirouac, Vachon et al. 2006](#)), followed by toxin binding to a primary receptor ([Johnson 1994](#); [Gomez, Sanchez et al. 2002](#); [Gómez, Pardo-López et al. 2007](#)). According to the pore formation model, binding to a cadherin receptor leads to further toxin processing and oligomerization ([Rodriguez-Almazan, Zavala et al. 2009](#)), followed by binding to secondary receptors, oligomer insertion in the cell membrane and pore formation, and cell death by osmotic shock ([Pacheco, Gomez et al. 2009](#)). In an alternative model, binding to cadherin activates intracellular cell death pathways resulting in enterocyte death ([Zhang, Candas et al. 2006](#); [Song and Scharf 2009](#); [Cancino-Rodezno, Alexander et al. 2010](#)). Both field-evolved resistance to crops expressing Bt toxins and laboratory-selected Bt resistance occur when any step in the mode of action is surpassed by the insect, resulting in a genetically based decrease in susceptibility ([Tabashnik, Van Rensburg et al. 2009](#)).

The majority of current resistance models are based on the most common resistance mechanism, the alteration of toxin binding to midgut receptors ([Caccia, Hernandez-Rodriguez et al. 2010](#); [Gouffon, Van Vliet et al. 2011](#)). However, this mechanism would only result in resistance against a single toxin or a group of toxins sharing a common receptor. This dogma currently dictates all resistance management strategies for Bt-based insecticidal technologies ([Downes, Mahon et al. 2010](#); [Tamez-Guerra 2010](#); [Jurat-Fuentes, Karumbaiah et al. 2011](#); [Li, Gao et al. 2011](#)). However, there are several examples of laboratory-selected insect strains that are resistant to diverse Cry toxins, in which toxin binding does not differ from binding in

susceptible insects. In this regard, it is recognized that larvae can recover from sub-lethal Cry toxin doses and fast midgut epithelium recovery has been reported to correlate with resistance to Cry toxins not sharing binding sites ([Forcada, Alcacer et al. 1999](#); [Sayyed, Raymond et al. 2004](#)).

The tobacco budworm, *Heliothis virescens*, is a common agricultural pest controlled with Bt insecticidal products containing multiple Cry toxins and transgenic crops expressing Bt toxins. Although there are *H. virescens* strains resistant to Cry toxins by diminished receptor expression and toxin binding capacity ([Gahan, Gould et al. 2001](#)), there are recorded *H. virescens* resistant strains in which toxin binding does not appear to be altered. One such *H. virescens* strain, KCB, is resistant to multiple Cry toxins found within the commercially available product DiPel ([Forcada, Alcacer et al. 1999](#)). Another example is resistant strain CXC which was selected for resistance to Cry1Ac and developed cross-resistance to Cry2Aa, which does not share binding sites with Cry1Ac in *H. virescens* ([Gahan, Ma et al. 2005](#)). Understanding the resistance mechanisms in these toxins is highly relevant to delay and manage development of resistance to Bt products and transgenic crops expressing diverse Cry toxins. One explanation for the broad range resistance of CXC and KCB is the phenomena known as enhanced midgut regenerative capabilities, where midgut stem cells are more abundant and/or multiply to mature forms more quickly after Cry intoxication than in susceptible strains. This enhanced midgut regeneration is probably directed by midgut expression of highly biologically active growth factors. However, there has been limited molecular evidence verifying the enhanced regeneration resistance mechanism, mostly due to lack of midgut cell type markers, growth factor pathway activation identification, and dependable *in vitro* models to measure

changes in regenerative responses due to environmental challenges and cues; issues which have only recently been addressed ([Castagnola, Eda et al. 2011](#)).

In this study, we characterize the proteins secreted by primary midgut mature cell cultures (secretomes) from *H. virescens* larvae of resistant (CXC and KCB) or susceptible (YDK) strains to elucidate potential composition differences that may correlate with resistance to diverse Cry toxins. The existence of midgut growth factors in secretomes from resistant insects was supported by results from bioassays in which secretomes from CXC and KCB midgut cells increased tolerance of YDK larvae to DiPel. Using tandem mass spectrometry we identified and quantified proteins unique to the secretomes from KCB and CXC larvae. Arylphorin was identified as displaying a relevant change in expression after Cry1Ac intoxication, which was confirmed by quantitative PCR targeting hexamerin transcripts in larvae exposed to Cry1Ac or DiPel. Determination of potential fitness costs in CXC and KCB larvae suggested slower development and longer larval stages in these larvae when compared to susceptible insects.

4.3 Materials and Methods

4.3.1 Insects and midgut dissection

H. virescens eggs from the laboratory strains YDK, KCB, CXC and YHD2 ([Gould 1995](#)) were kindly supplied by Dr. Fred Gould (North Carolina State University). Upon hatching, larvae were reared on artificial diet (BioServ) at 28°C on an 18:6 LD photoperiod. Fourth instar larvae were anesthetized on ice for 20 min and midguts carefully dissected under sterile conditions and used for preparation of primary cell cultures as described below. For bioassays fourth instar larvae were starved for one hour in the incubator in empty cups and then placed on

diet containing Cry1Ac toxin, DiPel, or secretome for the designated time period. After incubation larvae were then anesthetized on ice for 20 min and midguts were collected with RNase displace conditions and flash frozen using liquid nitrogen.

4.3.2 Bacterial toxins

Bacillus thuringiensis var. *kurstaki* strain HD73 producing Cry1Ac toxin and var. *tenebrionis* producing Cry3Aa were obtained from the *Bacillus* Genetic Stock Center (BGSC, Columbus, OH). Bacterial culturing, toxin purification and activation were as described elsewhere ([Perera, Willis et al. 2009](#)). Purified toxins were quantified by the method of Bradford ([Bradford 1976](#)) using the Coomassie Plus Protein Assay (Pierce) with BSA as the standard.

4.3.3 Preparation of histological midgut sections

Fourth instar *H. virescens* larvae were fed an LC₁₀ dose (0.2 µg/mL) of Cry1Ac toxin or a corresponding volume of control buffer (20 mM TRIS/HCl pH 8.0 0.3 M NaCl) by droplet feeding. A 0.04% trypan blue solution (Invitrogen, Carlsbad, CA) was incorporated into all solutions to monitor the larvae swallowing the dose. Midguts were dissected at 1.5 hours post-feeding and their median region sliced in and immediately fixed in 10% neutral buffered formalin solution (3.7% formaldehyde, 30 mM NaH₂PO₄, 46 mM Na₂HPO₄). Sliced midgut tissues were processed using the Tissue-Tek VIP processor (Sakura, Torrance, CA) and embedded in Paraplast embedding medium (Sigma-Aldrich, St. Louis, MO). Block sections were obtained by cutting 5 µm slices using a Microm HM355s microtome (Thermo Scientific, Kalamazoo, MI), which were mounted on Fisherbrand Superfrost Plus slides (Fisher Scientific,

Waltman, MA). Slides were stained with hematoxylin and eosin (H&E) according to a standard procedure ([Slaoui and Fiette 2011](#)). stained midgut sections were visualized using an Olympus DP72 microscope (Olympus, Tokyo, Japan) at 600x magnification.

4.3.4 Primary midgut cell cultures

All dissections and transfers were done in the sterile environment of a biosafety cabinet. Fourth instar larvae were anesthetized on ice for 15 min, and then surface sterilized for 30s in a cleaning solution (10% Palmolive detergent plus 0.1% Clorox) before dissecting the midgut. After dissection, midguts were cleaned for food, peritrophic matrix, and Malphigian tubules using forceps, and then briefly washed in sterile Ringer's (Barbosa, 1974) containing 0.5% (v/v) gentamicin (Invitrogen, Carlsbad, CA), 0.1% Clorox, and 1x antibiotic/antimycotic (Invitrogen). Incubation media was prepared by mixing in a 3:1 ratio of working Grace's (supplemented Grace's Insect Medium [Invitrogen] containing 1x antibiotic/antimycotic and 0.1% gentamicin) in sterile Ringer's. Five to six clean midguts were cut in sections with micro-scissors and incubated in 2 ml of incubation media for 90 min at room temperature. After this incubation, midgut tissue was homogenized carefully by pipetting and sieved through 70 mm cell strainers (BD Biosciences, NJ) into a sterile 50 ml conical tube. Tubes were centrifuged (400 x g for 5 min at 4°C) and the supernatant discarded. The pellet containing midgut mature and stem cells was suspended in 1 ml of working Grace's media. When needed, stem cells were separated from mature cells using a density gradient as described elsewhere (Loeb and Hakim, 1999). Briefly, samples were overlaid on 3 ml of Ficoll-Paque (GE LifeSciences, NJ) in a 15 ml conical tube and centrifuged (600 x g for 15 min at 4°C). After centrifugation, stem cells were collected from

the top 0.99 ml, the immediate 2.75 ml containing debris were discarded, and the bottom 0.25 ml containing the pellet was collected for mature cells. Ficoll-Paque was eliminated from stem and mature cell samples by washing twice with incubation media ($600 \times g$ for 5 min at 4°C). Final stem and mature cell pellets were suspended in 0.35 or 1 ml, respectively, of working Grace's. Stem and mature cell samples that were prepared simultaneously were pooled and the number of cells counted using a hemacytometer (Bright-Line, Horsham, PA). Using this procedure, we repeatedly obtained approximately 8×10^5 stem cells and 1×10^7 mature cells from 30 larvae. Stem cells were diluted to 4×10^5 cells/mL and mature cells to 3.5×10^6 cells/mL with working Grace's (as described above) and kept in a sterile incubator at 26°C .

4.3.5 Midgut cell secretome collection and identification

Purified mature midgut cells were diluted in Grace's insect medium (Invitrogen) to a concentration of 3.5×10^6 cells/mL, then 1 mL per well used to seed a 12 well plate (Corning, Corning, PA). Purified Cry1Ac toxin ($2 \mu\text{g/ml}$) or the corresponding volume of control buffer (20 mM TRIS/HCl, 0.3 M NaCl pH 8.0) were added to the cultures and incubated for 18 hours at 26°C . Then, media supernatant containing the proteins secreted by the midgut cells was collected by centrifugation ($2,000 \times g$ for 20 min at 4°C). Collected samples (2 ml) were concentrated to 50-100 μl and media exchanged to 20 mM TRIS/HCl pH 8.0 buffer using centrifugal filter devices (3-kDa MWCO, Millipore, MA) following manufacturer's instructions. Proteins in concentrated samples were quantified using the Qubit fluorometer (Invitrogen) and then diluted to 2 mg/ml in 20 mM TRIS/HCl pH 8.0 buffer. These samples were either

submitted for 1DGel LC/MS/MS analysis at NextGen Science (Ann Arbor, MI), or stored at -80° C until used for bioactivity assays as described below.

4.3.6 Proteomic analysis of primary midgut cell culture secretome

Proteins in secretome samples (10 µg) were separated by 1D SDS-10%PAGE and each of the sample lanes sliced in five cross-sections that were subjected to in-gel digestion in a ProGest workstation (Genomic Solutions, Ann Arbor, MI). Briefly, samples were reduced with DTT at 60°C, then allowed to cool to room temperature before being alkylated with iodoacetamide. Tryptic digestion was done at 37°C for 4 hours, then reactions stopped by addition of formic acid. Analysis of peptides generated by the tryptic digestion through liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) was performed at NextGen Sciences (Ann Arbor, MI) using a ThermoFisher LTQ Orbitrap XL mass spectrometer. Tandem mass spectra were analyzed using Mascot (Matrix Science, London, UK) and queried against a custom database containing a complete *H. virescens* transcriptome (Perera et al. in preparation, 203,652 entries). In the instance where no matching sequences were found in the available transcriptome, the most similar protein in searches of the NCBI nr-insecta database using Scaffold and Mascot algorithms. Briefly, the search parameters included a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Iodoacetamide derivative of cysteine was specified as a fixed modification, while S-carbamoylmethylcysteine cyclization (N-terminus), deamidation of asparagine and glutamine, and oxidation of methionine and acetylation of the N-terminus were specified in as variable modifications.

Scaffold (version Scaffold_3_00_07, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50% probability as specified by the Peptide Prophet algorithm ([Keller, Nesvizhskii et al. 2002](#)). Protein identifications were accepted if they could be established at greater than 90% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm ([Nesvizhskii, Keller et al. 2003](#)). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Determination of high quantitative variance between treatments was performed in Scaffold using spectral counts as relative abundance parameter.

4.3.7 Flow cytometry to monitor stem cell bioactivity

Purified stem cell cultures (4×10^5 cells/ml) were incubated for 12 hours with Cry1Ac-induced secretomes from primary midgut mature cell cultures from resistant strains KCB and CXC and susceptible strain YDK. As controls we used equal volumes of working Graces or treated stem cells with purified Cry1Ac toxin (1 μ g). After treatment, stem cells were stained with calcein AM (Invitrogen) following manufacturer's instructions. Stem cell differentiation and proliferation were analyzed using differential fluorescence in an LSRII flow cytometer (BD Bioscience, CA) as described previously ([Castagnola, Eda et al. 2010](#)).

4.3.8 Quantitative PCR

Midguts from third instar *H. virescens* larvae were collected from a bioassay of diet incorporated Cry1Ac toxin (1 µg/mL) or 0.2% DiPel (v/v). Midguts were collected at time points 0 hours, 2 hours, 4 hours, 6 hours, and 24 hours. A portion of the 2 hour incubations were removed from treatment and placed on regular diet and these midguts were collected at the next time points 4 hours, 6 hours and 24 hours.

Total RNA was purified from a pool of three dissected midguts using TriReagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. Integrity of the purified total RNA was checked by 2% agarose gel electrophoresis. Total RNA was quantified using a nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and a Qubit fluorometer (Invitrogen, Carlsbad, CA). Contaminating DNA was eliminated using digestion with DNase I (GE Healthcare, Piscataway, NJ) for one hour at 37°C, then the samples were further purified using the RNeasy (Qiagen, Hilden, Germany.) cleaning protocol. Purified total RNA was used to synthesize cDNA with the Superscript VILO cDNA synthesis kit (Invitrogen), following to manufacturer's instructions. Samples were quantified using a nanodrop spectrophotometer prior to real time PCR.

Relative quantification of gene expression was measured in a 7900HT Fast Real-Time system (Applied Biosystems, Carlsbad, CA) on standard mode using the SDS 2.3 and SDS RQ manager software to collect cycle threshold (Ct) values. All primers used in qRT-PCR are presented in supplementary Table A. Primers targeting transcripts in the hexamerin gene family in a *H. virescens* transcriptome (Perera *et al.*, in preparation) were designed using the D-Lux

designer software (<http://escience.invitrogen.com/lux/index.jsp>). As endogenous control for relative quantification, we used primers targeting a *H. virescens* tubulin transcript from the transcriptome with high similarity to alpha-tubulin from *Xestia cnigrum* (accession number EU100015). Primers and cDNA concentrations were validated using absolute quantification efficiency curve and dissociation curve analyses. Fluorescence-based quantification of PCR products generated in real time was measured using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Amplification reactions contained 5 µM forward and reverse primers, 10 ng of cDNA template, 10 µl of SYBR Green Master Mix, and DEPC-treated water to a final volume of 20 µl. The amplification program included initial steps of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 55°C for 20 sec. For relative quantification of gene expression we used the $2^{-\Delta\Delta C_t}$ method ([Livak and Schmittgen 2001](#)) comparing transcript levels of target and endogenous control genes between treatments.

4.3.9 DiPel and secretome incorporation bioassays

Larval tolerance under 0% and 0.2% (v/v) DiPel intoxication was monitored in the presence of 0.27 mg of secretome protein per ml of diet. First instar larvae (n=32) were fed diet containing DiPel at either 0% or 0.2% and secretome at 28°C on an 18:6 L:D photoperiod. Mortality was monitored everyday for 5 days. Percent mortality was normalized by subtracting the mortality observed with 0% DiPel. When this calculation yielded a negative value 0% mortality was reported.

4.3.10 Determination of fitness costs in resistant larvae

Neonates of the YDK, CXC, KCB, and YHD2-B strains were placed on artificial diet and incubated at 28°C on an 18:6 LD photoperiod. Fitness parameters monitored included larval weight at fourth instar, days from neonate to pupation, pupal weight at first day of pupation, and days until moth emergence. The experiment was performed using 90 larvae per strain, and the experiment duplicated once (n= 180 per strain). Total time until adult emergence gives an estimation of time between generations.

4.4 Results

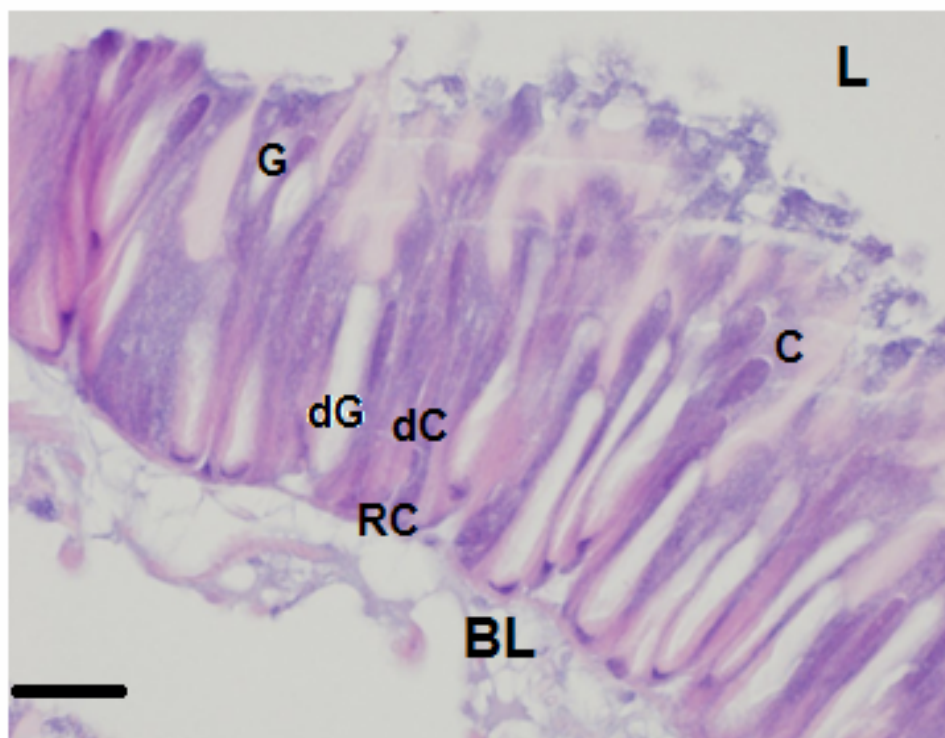
4.4.1 Histopathology of resistant *H. virescens* strains under Cry1Ac intoxication

Individual larvae from DiPel-resistant strains (CXC and KCB) and a susceptible strain (YDK) were fed Cry1Ac toxin by droplet feeding and structural changes of dissected midguts were observed by H&E staining. In Cry1Ac-intoxicated midguts of YDK larvae (Fig. 1A) we observed an abundance of damaged mature cells and differentiating regenerative cells. The columnar and goblet cells still associated with the apical side of the epithelium appeared damaged, and newly differentiated cells with elongated morphology appeared pushing through the epithelium from the basal side. There were a minimal number of regenerative cells remaining associated with the basal lamina. Newly differentiated goblet and columnar cells were seen in proximity to damaged and sloughing mature cells. The midgut histopathology of stained midguts from CXC (Fig. 1B) had distinct differences from midguts derived from KCB (Fig. 1C) larvae. Mature cells in midguts from CXC larvae displayed high levels of granulation at the apical side of the epithelium, corresponding to cellular sloughing and blebbing (arrow). Well-

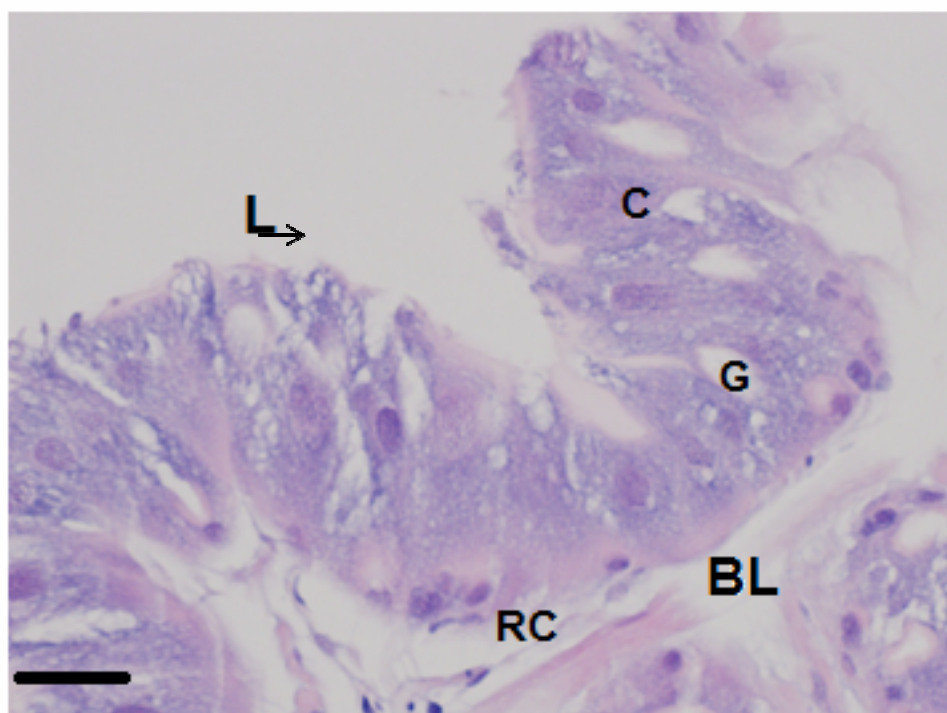
organized stem cell nidi were observed associated with the basal lamina. In contrast, KCB midguts had an increased number of goblet cells distributed throughout the epithelium, granules without apparent organization, and regenerative cell types dispersed along the basal lamina. There appear to be lower numbers of differentiating cells in midguts from resistant larvae compared to midgut from the YDK strain.

Figure 12: Histopathology of Cry1Ac intoxication in susceptible and resistant *H. virescens* larvae. Larvae from the YDK (A), CXC (B) and KCB (C) strains of *H. virescens* were treated with 0.2 µg/mL of Cry1Ac by droplet feeding. After 1.5 hours their midguts were dissected, sectioned and stained with H&E. L, lumen; C, columnar cell; G, goblet cell; RC, regenerative cell; BL, basal lamina; dG, differentiating goblet cell; dC, differentiating columnar cell. Arrows indicate cells being sloughed off into the midgut lumen. Reference bar = 20µm.

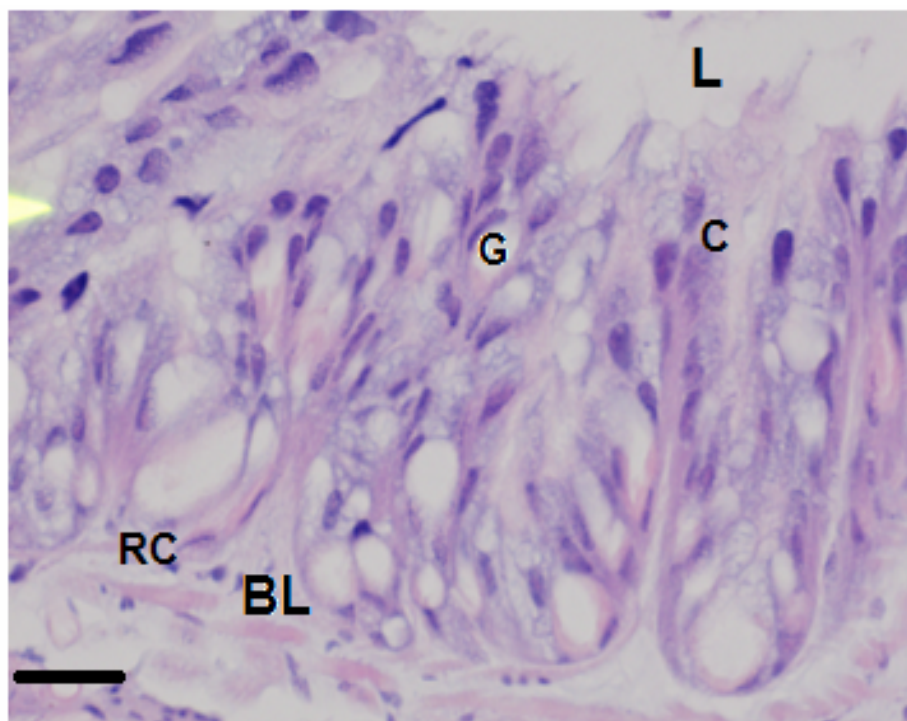
A



B



C



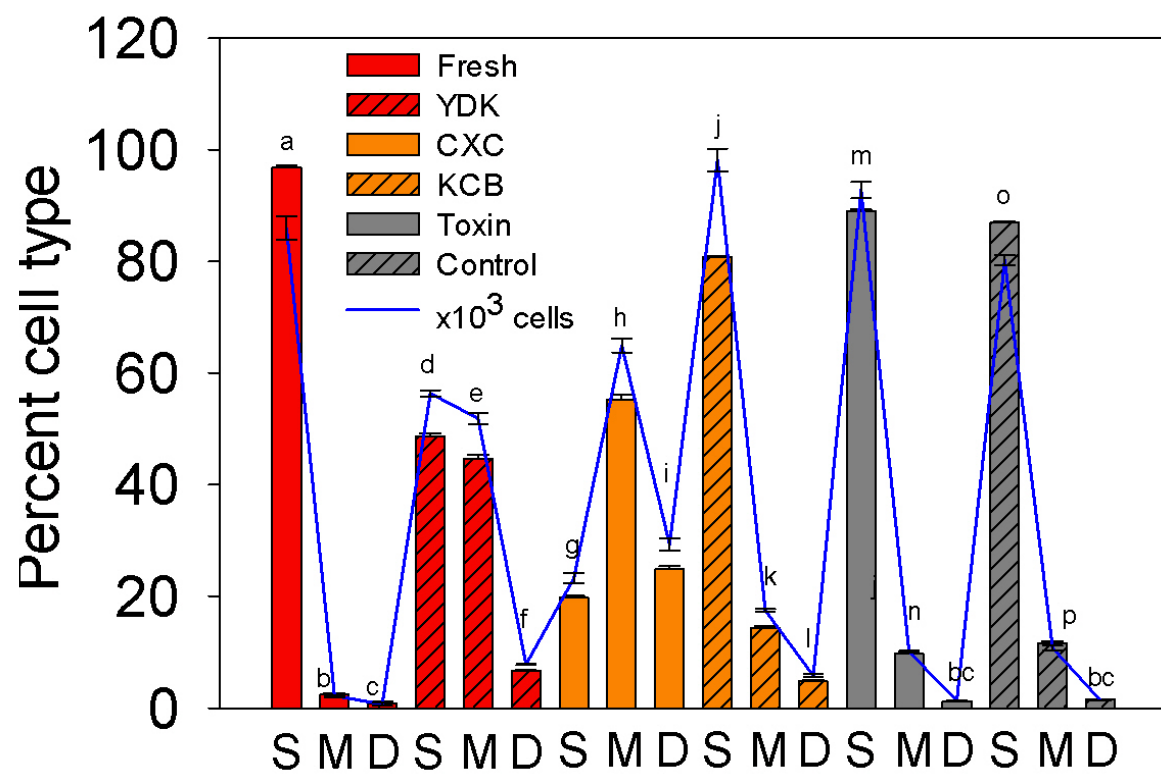
4.4.2 Stem cell bioactivity responses to mature midgut cell secretomes are distinct between resistant strains

To further characterize the midgut regenerative response to Cry intoxication in susceptible and resistant *H. virescens* larvae, we obtained the proteins secreted (secretome) by primary mature midgut cell cultures obtained from YDK, CXC, or KCB larvae. Secretomes were collected by incubating purified midgut mature cell cultures with Cry1Ac, Cry3Aa, and buffer controls. The resulting secretomes were incubated with primary midgut stem cell cultures from the corresponding strain and bioactivity measured using a flow cytometry method ([Castagnola, Eda et al. 2011](#)). There were a low proportion of mature cells present in the fresh stem cell cultures, and the Cry1Ac toxin alone could result in increased mature cell death (Fig. 13). The dying mature cells probably secreted cytokines and growth factors that induced a low percentage of stem cells to differentiate, shifting the percent cell type to a slightly larger ratio of mature cells (Fig. 13, grey bars). When considering total cell numbers for each cell type, the amount of stem cells was significantly lower after treatment with secretome.. Stem cell bioactivity results showed an increase in the amount of total cells in the cultures treated with Cry1Ac secretomes compared to Cry1Ac toxin or Grace's media controls (Fig. 13).

Secretomes from different strains caused distinct responses in the proportion of cell types in stem cell cultures. Incubation with secretome derived from YDK cells induced a decrease in the number of stem cells and a corresponding increase in the number of mature cells, while the number of dead cells more than doubled compared to controls. In contrast, incubation with secretome from CXC cells resulted in highly reduced numbers of stem cells, while the number of mature and dead cells increased more than in the case of YDK secretome. In stem cell cultures

incubated with secretome from KCB midgut cells we observed only small differences with negative controls. In all cases the total cell amounts increased, probably due to cells actively dividing in culture.

Figure 13: Changes in midgut cell types in primary stem midgut cell cultures after exposure to Cry1Ac-secretomes from susceptible and resistant *H. virescens* larvae. Primary midgut stem cell cultures were treated with the corresponding secretome from mature midgut cells exposed to Cry1Ac toxin for 12 hours, then the number of stem (S), mature (M), and dead (D) cells was quantified using a flow cytometry method. Cell type percentages (bars) were calculated from total cell numbers in each cell type (blue line) for each primary culture. Treatment with 1 µg/ml of Cry1Ac toxin (gray bars) and untreated cells are included for reference. Bars denote the standard error of the mean calculated from primary stem cell cultures derived from 45 susceptible larvae, and measurements were taken from two wells per treatment group and counted in duplicate on the flow cytometer. The power of the performed ANOVA test was $\alpha = 0.050$, normality was established with Shapiro-Wilk at a value of $P = 0.072$, and an equal variance test passed at a value of $P = 0.422$. Different letters denote statistically significant differences at $P < 0.001$.

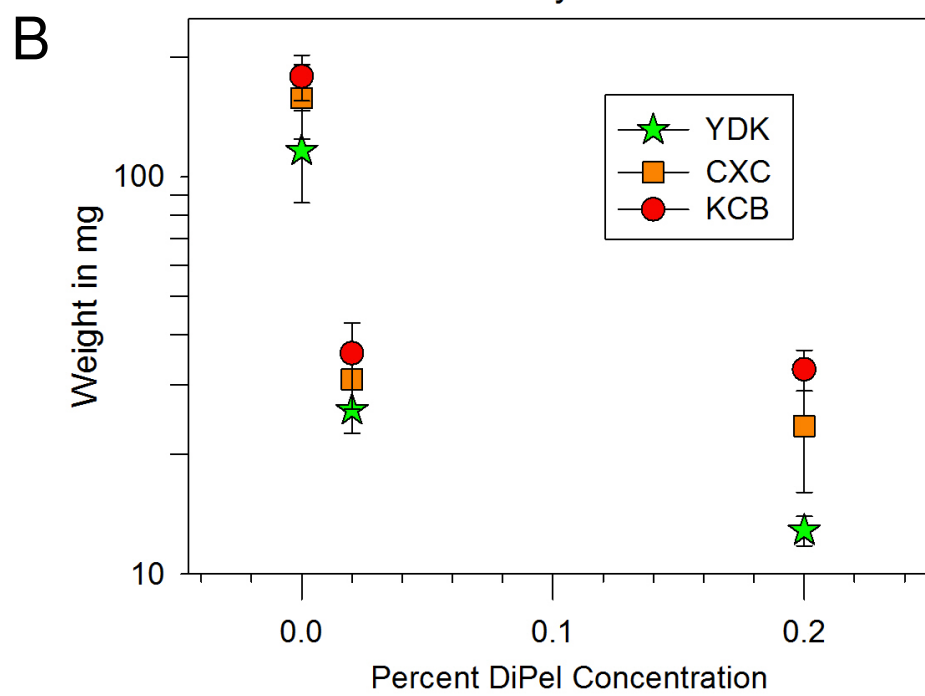
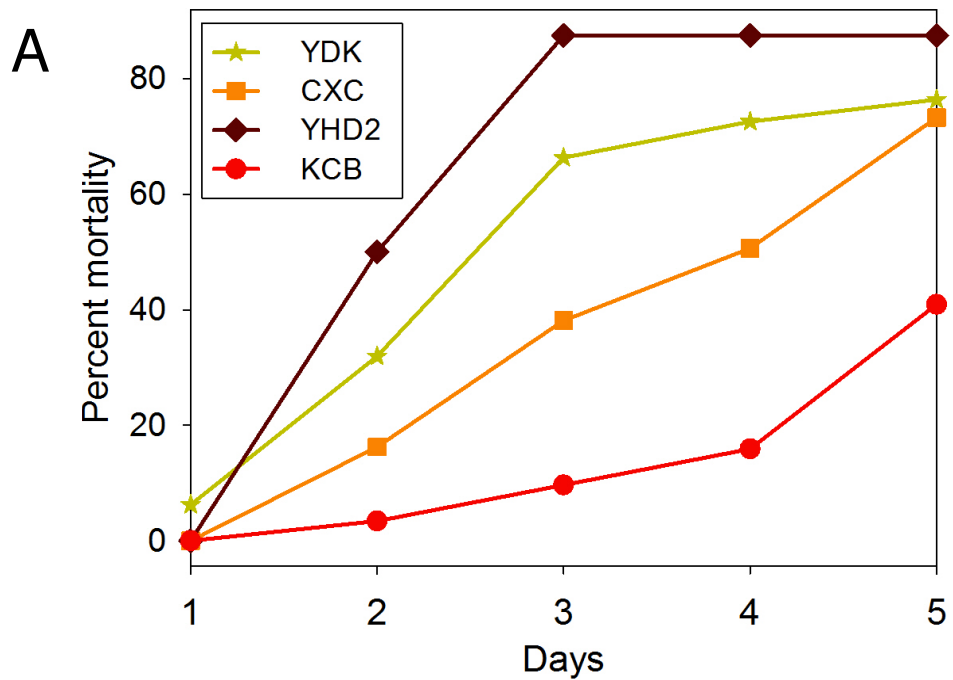


4.4.3 Secretomes from resistant larvae increase tolerance to DiPel in susceptible larvae

To obtain further evidence supporting the existence of growth factors controlling midgut regeneration in secretomes derived from mature midgut cell cultures treated with Cry1Ac toxin, we fed secretomes and increasing concentrations of the Bt-based commercial pesticide DiPel to susceptible YDK *H. virescens* larvae. The secretomes derived from midgut cells from larvae of the KCB, and CXC strains decreased larval mortality compared to the secretome from the susceptible YDK strain (Fig. 14A). As an additional control, we obtained secretome from midgut cells obtained from larvae of the YHD2-B strain of *H. virescens*. This strain has been characterized to be resistant to Cry1Ac toxin through alteration of midgut toxin receptors ([Gahan, Ma et al. 2005](#); [Gahan, Pauchet et al. 2010](#)). Treatment with YHD2-B secretome resulted in higher mortality than the secretome derived from the susceptible YDK strain. These results also correlated with larval weight, as surviving larvae fed CXC or KCB secretomes were heavier than larvae fed YDK secretome at the highest DiPel concentration tested (Fig. 14B).

Figure 14: Susceptibility to DiPel in larvae treated with Cry1Ac-induced secretomes. A)

First instar larvae were fed diet containing secretomes from YDK, CXC, KCB, or YHD2-B midgut cells as indicated and 0.2% DiPel. The percentage of larval mortality was assessed during 5 days. **B)** The weights of the survivor larvae in bioassays with larvae fed YDK, CXC, or KCB secretomes and different concentrations of DiPel (v/v) were measured in larvae surviving after 5 day incubation. Each experiment was performed with 16 larvae in standard bioassay trays and repeated once.



4.4.5 Identification of proteins from midgut cell secretomes of resistant *H. virescens* strains

The secretome was analyzed by tandem mass spectrometry and significant peptides were identified by spectral counting. Cry1Ac treated YDK, KCB and CXC secretomes were analyzed compared to control from different perspectives to draw conclusions about the influence of the mature cell secretome on successful Cry1Ac tolerance. First, all proteins were separated based upon statistical significance p values with respect to response to Cry1Ac intoxication (Table 4). Then, the statistically significant findings were separated based upon the response specific to each resistant strain (colored fonts).

The most prominent response to Cry1Ac in both susceptible and resistant strains was the increase in arylphorin expression. Proteins which were down regulated in resistant strains or up-regulated in susceptible (orange font) may give insight into toxin susceptibility. Proteins which fit into this category consisted of contig 1567 with high homology to *Bombyx mori* chymotrypsin inhibitor (accession number AAK52495.1), *Aedes aegypti* calmodulin (accession number EAT35514.1), *B. mori* calreticulin precursor (accession number NP001037075.1) and *Helicoverpa armigera* single domain allergen protein (accession number ABX39544.1). The function of the single domain allergen in the insect remains wholly unknown and was also up-regulated in the susceptible strain but not found in resistant strains. *Nasonia vitripennis* histone (accession number XP001600560.1) was the only protein identified with specificity to the KCB secretome (red font) compared to the multitude specific to CXC (purple font). One protein matching spectrin from *Pediculus humanus corporis* (accession number EEB17271.1) was up-regulated in KCB compared to YDK was a. Proteins specific for the CXC secretome matched *H. armigera* aminopeptidase (accession number AF441377), *Tribolium castaneum* citrate synthase

(accession number XP970124), *Papilio xuthus* myosin (accession number BAG30740), ribosomal proteins l7a from *Heliconius melpomene* (accession numbers ABS57440.1) and L18 from *Biston betularia*, (accession number ADO33048.1), *T. castaneum* oligopeptidase A (accession number XP971989), *Nasonia vitripennis* ribophorin II (accession number XP001601669), *Heliconius cydno cordula* catalase (accession number ADI80736.1), and an unidentified protein termed contained in contig 3 from an *H. virescens* custom transcriptome. Other proteins up-regulated in CXC compared to both YDK and KCB secretomes matched heat shock proteins from *Chilo suppressalis* (accession number ACT52824.1), and *Culex quinquefasciatus* (accession number EDS32112.1), glutamate dehydrogenase from *B. mori* (accession number NP001040245.1), and glyceraldehyde-3-phosphate dehydrogenase from *Lutzomyia longipalpis* (accession number ABV60323.1).

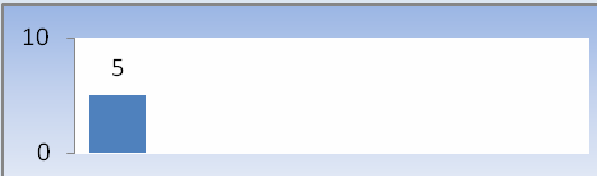
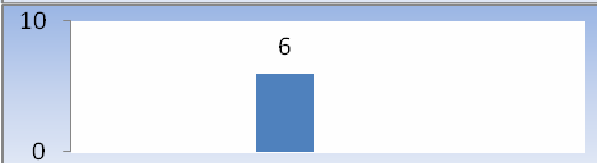
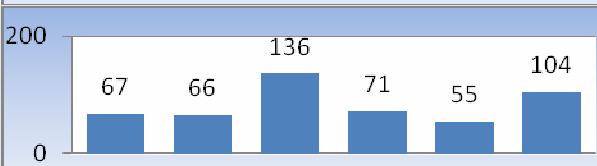
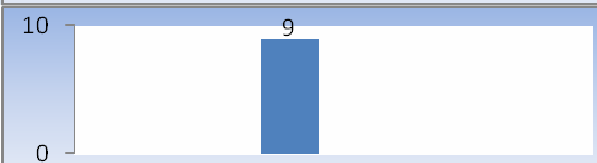
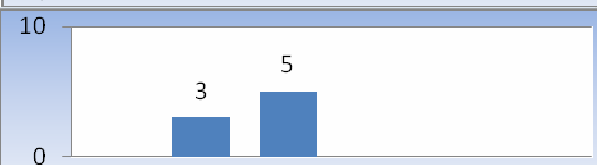
Table 3: Statistically significant proteins associated with the Cry1Ac secretomes from susceptible and resistant *H. virescens* strains. Predicted subcellular localization are secreted (S), intracellular (I), or membrane (M). Tandem mass spectrometry spectra are counted to estimate protein quantities between treatments and are graphically represented in the last column. The contents in the table are listed by the highest to lowest most statistically significant findings. Font colors denote specificity: down regulated in all resistant strains (orange), KCB (red), CXC (purple).

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
899	CAB55605	<i>Spodoptera litura</i>	Arylphorin precursor	201	0.0	S	3.00e-09	16	17	15	4		
	AAL34109.1	<i>Helicoverpa armigera</i>	Aminopeptidase N1	113		M	3.30e-08			29			
	EEB17271.1	<i>Pediculus humanus corporis</i>	Spectrin alpha chain	278		I	8.10e-07	15	25				

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
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Table 3. Continued

448	CAB55605	<i>Spodoptera litura</i>	Arylphorin precursor	175	0.0	S	1.5e-06	8	9	9			
891	XP970124	<i>Tribolium castaneum</i>	Citrate synthase	218	0.0	I	1.3e-04			107			
ABX39544.1	<i>Helicoverpa armigera</i>	Single domain major allergen 1 protein	25			S	1.0e-03	3			4	4	4
3		Unidentified	1376				3.2e-03			4			
ACT52824.1	<i>Chilo suppressalis</i>	Heat shock protein	61			I	5.9e-03	47	50	106	53	45	79

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
Table 3. Continued													
1567	AAK52495.1	<i>Bombyx mori</i>	Chymotrypsin inhibitor	122	2e-130	S	6.1e-03						
2209	BAG30740	<i>Papilio xuthus</i>	Myosin	531	0.0	I	6.1e-03						
655	ACT52824	<i>Chilo suppressalis</i>	Heat shock protein	197	0.0	I	6.8e-03						
	ABS57440.1	<i>Heliconius melpomene</i>	Ribosomal protein L7a			I	0.012						
18			Unidentified				0.012						

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
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Table 3. Continued

3349	XP971989	<i>Tribolium castaneum</i>	Oligopeptidase A	181	0.0	S	0.012						
	EDS32112.1	<i>Culex quinquefasciatus</i>	Heat shock protein, mitochondrial	60		I	0.012						
	EAT35514.1	<i>Aedes aegypti</i>	Calmodulin	17		I	0.013						
706	NP001040245	<i>Bombyx mori</i>	Glutamate dehydrogenase	172	0.0	I	0.02						
	XP001600560.1	<i>Nasonia vitripennis</i>	Histone	11		I	0.022						

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
Table 3. Continued													
151	XP001601669	Nasonia vitripennis	Ribophorin II	237	1.46987e-101	I	0.041						
3505	XP316153	Anopheles gambiae	3-oxoacid transferase 1	191	0.0	I	0.041						
	NP001037075.1	Bombyx mori	Calreticulin precursor			I	0.041						
	ABV60323.1	Lutzomyia longipalpis	Glyceraldehyde-3-phosphate dehydrogenase			I	0.041						
	ADI80736.1	Heliconius cydno cordula	Catalase			S	0.041						

Contig number	Database match (accession number)	Organism	Description	Molecular weight (kDs)	E- value	Predicted location	Fishers exact test, p-value	YDK 1Ac	KCB 1Ac	CXC 1Ac	YDK B	KCB B	CXC B
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Table 3. Continued

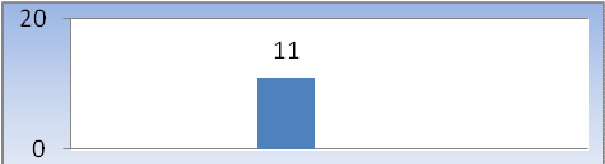
ADO33048.1

Biston betularia

Ribosomal protein L18

I

0.041



4.4.6 Changes in hexamerin expression in response to Cry1Ac *H. virescens* larval midguts

Our proteomic data suggested that in the CXC and KCB strains had higher arylphorin expression after toxin challenge compared to YDK larvae. To further test the differential regulation of arylphorin expression in susceptible and resistant larvae after intoxication with Cry1Ac or DiPel, we used qRT-PCR with primers targeting hexamerin and arylphorin contigs from an available custom *H. virescens* transcriptome. In CXC larvae, arylphorin was up-regulated after 2 hours of exposure when compared to YDK larvae (Fig. 15A). This pattern was also observed when testing CXC larvae that had been exposed to Cry1Ac diet for 2 hours but then transferred to control diet for recovery, suggesting that the increased arylphorin expression is contemporary to midgut regeneration. Arylphorin transcript levels were also higher in CXC larvae after 6 hours of continual exposure to Cry1Ac diet compared to YDK larvae. Larvae from the KCB strain presented a similar pattern of arylphorin expression, but with lower transcript levels than detected for CXC (Fig. 15B). As observed for CXC larvae in recovery bioassays, arylphorin expression in KCB larvae was similar after 2 hours of exposure to Cry1Ac or 2 hours after recovery from intoxication. In contrast to the trend similarities observed for CXC and KCB larvae after exposure to Cry1Ac toxin, arylphorin expression in these larvae after DiPel intoxication was very diverse. After a longer exposure time to DiPel, both resistant strains exhibited increased hexamerin expression, with arylphorin being preliminarily up-regulated and the rest of the hexamerins following (Fig. 16A & B). Overall, hexamerin expression was higher in resistant strain CXC (Fig. 16A) compared to KCB (Fig. 16B).

Figure 15: Hexamerin family of transcripts expression steadily increases under Cry1Ac intoxication with a role for arylphorin expression uniquely up-regulated. Third instar larvae from the CXC (A) and KCB (B) strains of *H. virescens* were exposed to diet containing 1 µg/ml Cry1Ac toxin for the time points indicated. Midguts were then dissected and RNA purified and used for qRT-PCR assays. Transcript levels for the same contigs in YDK (blue lines) are presented for reference. Black line denotes the transcript levels in larvae that were exposed to diet containing toxin for 2 hours and then transferred to control diet for recovery. Relative quantification of gene expression calculations were done using time zero midguts and tubulin gene expression as an endogenous control. Data shown are the mean values and corresponding standard errors calculated from each time point sample which consisted of three pooled midguts, PCR reactions were performed a minimum of three times, and using at least three technical replicates per experiment.

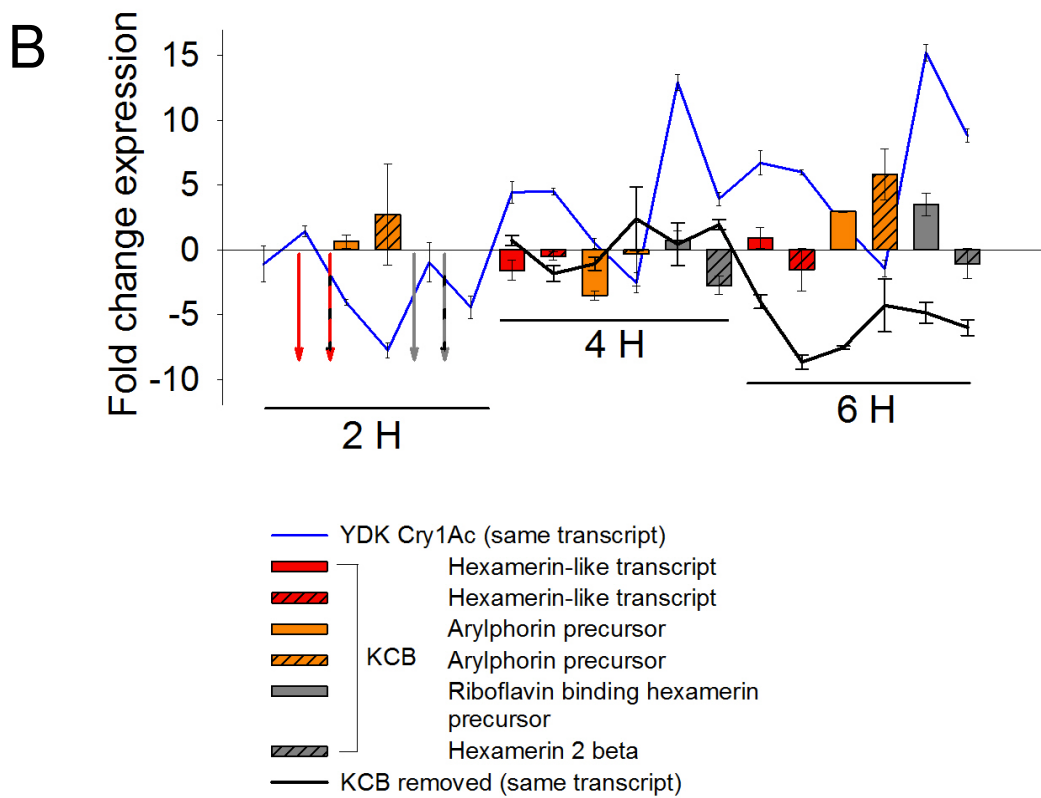
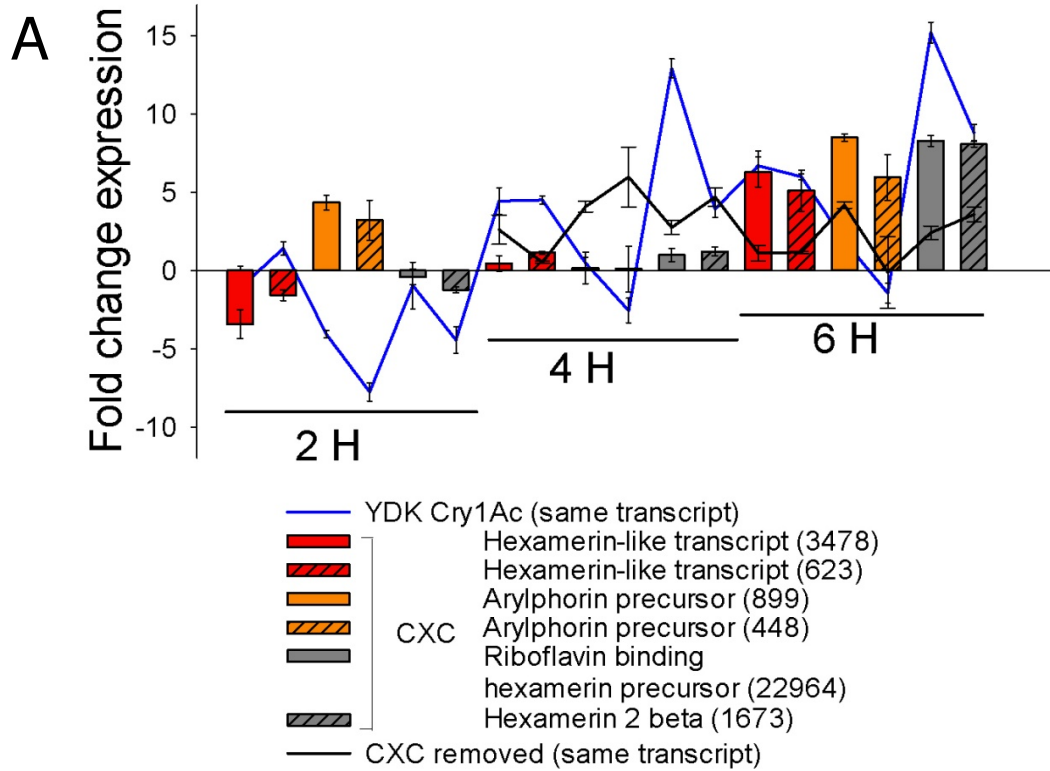
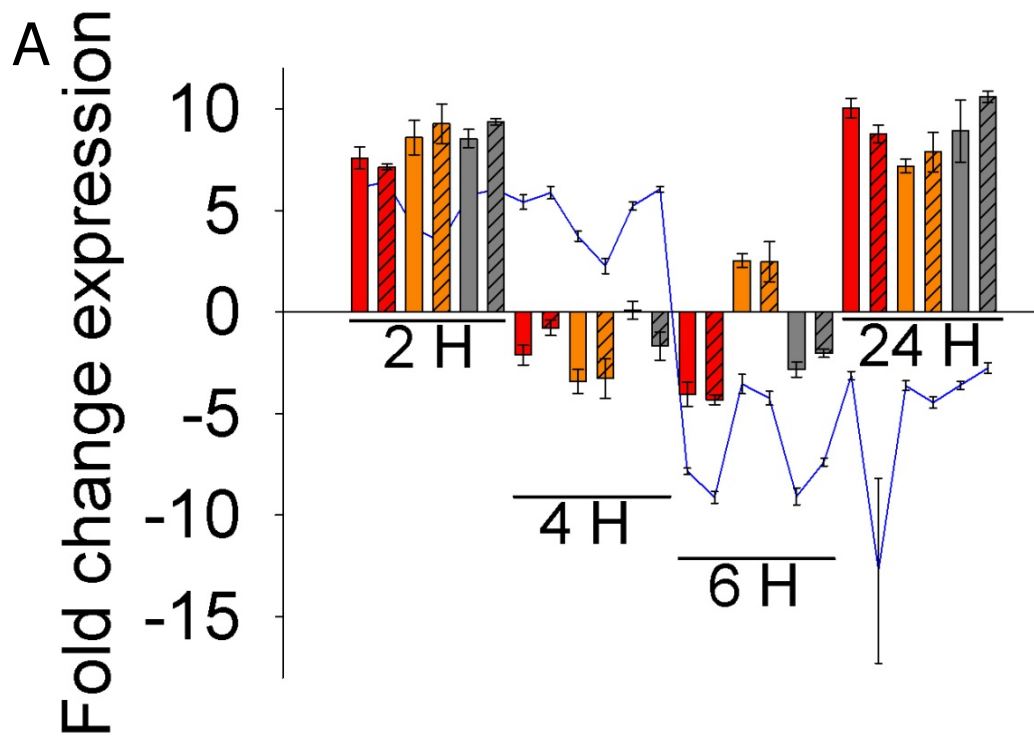
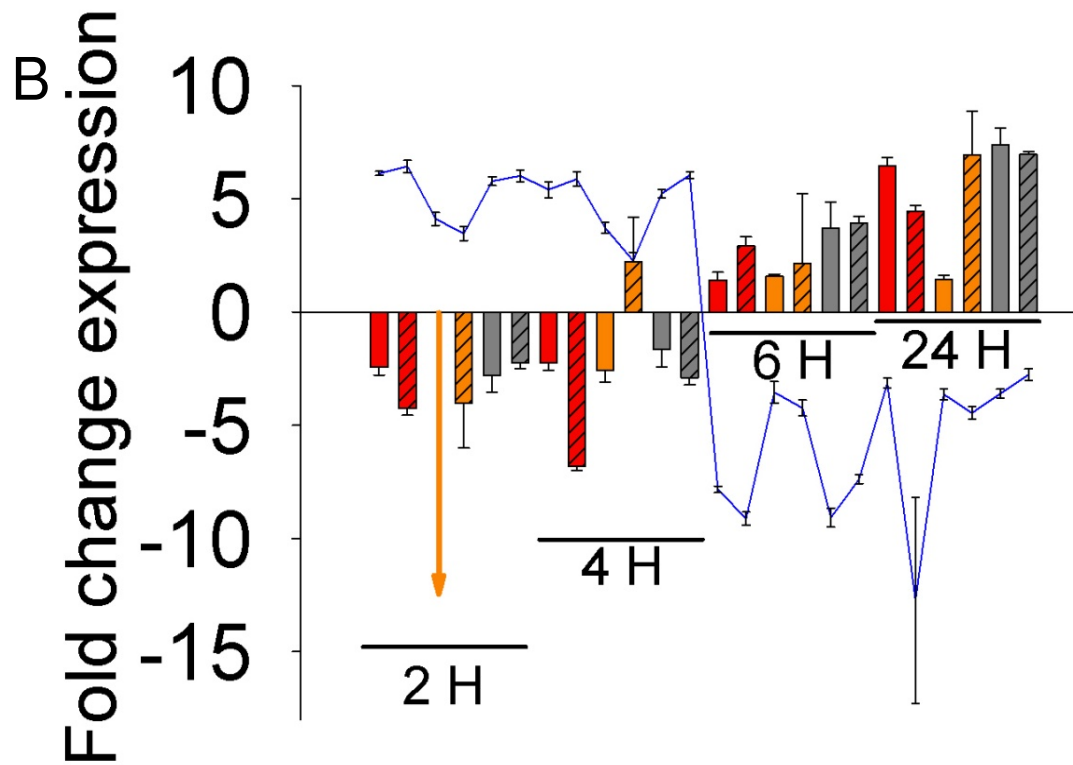


Figure 16: Hexamerin family of transcripts investigated under DiPel intoxication from *H. virescens* resistant strains is diverse. Third instar larvae from the CXC (A) and KCB (B) strains of *H. virescens* were exposed to diet containing 1 µg/ml Cry1Ac toxin for the time points indicated. Midguts were then dissected and RNA purified and used for qRT-PCR assays. Transcript levels for the same contigs in YDK (blue lines) are presented for reference. Relative quantification of gene expression calculations were done using time zero midguts and tubulin gene expression as an endogenous control. Data shown are the mean values and corresponding standard errors calculated from each time point sample which consisted of three pooled midguts, PCR reactions were performed a minimum of three times, and using at least three technical replicates per experiment.



- YDK DiPel
- Hexamerin-like transcript (3478)
- ▨ Hexamerin-like transcript (623)
- Arylphorin precursor (899)
- ▨ Arylphorin precursor (448)
- Riboflavin binding hexamerin precursor (22964)
- ▨ Hexamerin 2 beta (1673)



- YDK DiPel
- Hexamerin-like transcript (3478)
- ▨ Hexamerin-like transcript (623)
- Arylphorin precursor (899)
- ▨ Arylphorin precursor (448)
- Riboflavin binding hexamerin precursor (22964)
- ▨ Hexamerin 2 beta (1673)

4.4.7 Resistant strains of *H. virescens* have decreased fitness compared to susceptible larvae

We detected differential expression of hexamerin gene expression after Bt intoxication, and because these genes have a relevant role in larval development, and secretomes had a lethal effect on susceptible larvae and susceptible stem cells, we were interested in testing whether these differences potentially resulted in fitness costs in resistant larvae. To test this hypothesis we compared fitness parameters between larvae of the YDK, CXC, KCB, and YHD2-B strains. Parameters investigated were fourth instar larvae weight, time to pupation or duration of larval stage, pupal weight, and pupation duration or days to emergence. The duration of the larval (Fig. 17A) and pupal (Fig. 17B) stages was significantly longer in resistant strains compared to susceptible insects. Larval weights were higher in resistant strain YHD2-B compared to resistant strains KCB and CXC, although larvae from all three resistant strains were smaller than YDK larvae (Fig. 17C). The same finding was observed for pupal weights (Fig. 17D). In general, we observed longer developmental times and smaller larvae in resistant compared to susceptible strains.

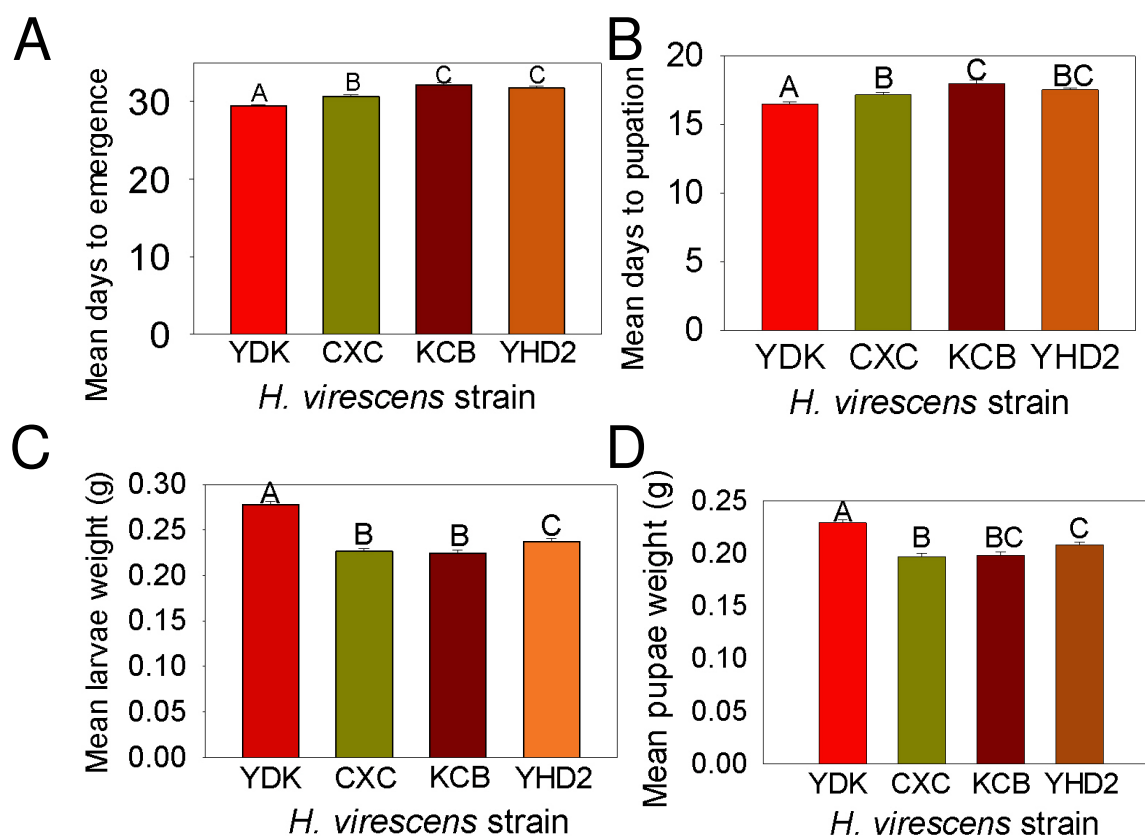


Figure 17: Developmental time and weights of larvae and pupae from susceptible (YDK) and resistant (CXC, KCB, YHD2-B) strains of *H. virescens*. Neonate larvae from each strain were reared in artificial diet, and the length of the larval (A) and pupal (B) stage monitored. Larval (C) and pupal (D) weights were measured using a metric scale. The data presented are the means calculated from two independent assays performed with 90 larvae from each strain (n=180). A Kruskal-Wallis ANOVA on ranks test was performed to test for significant differences. Different letters denote statistically significant differences at a $P < 0.001$.

4.5 Discussion

Although Bt toxins are popular safer alternatives to chemical pesticides, a major concern with the increasing use of Bt products and transgenic crops expressing Bt toxins is the development of insect resistance. The ability to delay and manage resistance has become a crucial tool when protecting the economic viability of Bt products. One strategy that has been used to delay resistance emergence is the use of diverse toxins within the same Bt product. The finding and identification of specific midgut receptors and their capacity to irreversibly bind to one particular toxin has encouraged the development of multi-toxin component Bt products. While alteration of toxin binding to these midgut receptors is the most well characterized resistance mechanism to Bt toxins ([Ferre and Van Rie 2002](#)), there are instances of insect resistance to diverse Bt toxins resulting from mechanisms which are not related to alterations in toxin binding or midgut receptors ([Jurat-Fuentes, Gould et al. 2003](#)).

In the case of the CXC and KCB strains, it was previously established that toxin binding properties were not different from susceptible strains ([Jurat-Fuentes, Gould et al. 2003](#)). Based on preliminary evidence obtained from electron microscopy, a mechanism based on enhanced midgut regeneration was proposed to explain resistance to diverse Bt toxins in these strains ([Forcada, Alcacer et al. 1999](#); [Martinez-Ramirez, Gould et al. 1999](#)). However, the molecular mechanisms underlying this observed phenomena have not been characterized.

Midgut regeneration has been previously measured using *in vitro* cell cultures ([Loeb, Martin et al. 2001](#)). Because midgut stem cells divide and mature cells do not, it is assumed that the cell divisions in mixed midgut cell cultures occur as less abundant stem cells are cued to proliferate by factors provided by dying mature cells. Based on previous reports of damaged

midgut cells from resistant strains being able to heal faster or more efficiently than *Bt*-susceptible strains ([Forcada, Alcacer et al. 1999](#)), we hypothesized that specific growth factors were responsible to trigger this enhanced response in resistant insects. The observed differential proliferation and differentiation effects in stem cells induced by secretomes from susceptible and resistant larvae, as well as the increased DiPel tolerance induced by secretomes from resistant larvae support the existence of growth factors that induce an enhanced regenerative response in secretomes from CXC and KCB larvae. Interestingly, the secretome from YHD2-B larvae failed to increase tolerance to DiPel, in agreement with the resistant mechanism in this strain being related to alteration of toxin receptors ([Gahan, Gould et al. 2001](#)).

A number of growth factors promoting midgut cell culture regeneration have been reported, including AlbuMAX II ([Castagnola, Eda et al. 2011](#)), fetal bovine serum ([Nishino and Mitsuhashi 1995](#)), ecdysteroids, alpha-arylphorin, MDFs 1-4, bombyxin, and calcium ions ([Loeb 2010](#)). The results from our study indicate a specific role for arylphorin in midgut regeneration in response to toxicity thus correlating Bt toxin damage and induced stem cell activity. Arylphorin was expressed in higher amounts in resistant strains when measuring midgut tissue transcript abundance in response to Cry intoxication but was similarly expressed by mature cells in susceptible and resistant secretomes. This may be due to two factors. In an *in vivo* system crosstalk between tissue types could enhance hexamerin response to intoxication or in cell culture only minimal changes in arylphorin titers are needed for an enhanced regenerative/mitogenic affect.

The regenerative properties, midgut histopathological structure, and secretome protein content in the susceptible strain was used for comparison to resistant strains in response to the Bt

toxin Cry1Ac. Significant proteins identified by mass spectrometry, which were identified in resistant but not in susceptible-derived secretomes were aminopeptidase in CXC, histones in KCB, and down-regulation of calcium binding proteins in both CXC and KCB. The shedding of aminopeptidase, which is a known Bt receptor in *H. virescens* ([Luo, Sangadala et al. 1997](#)), may provide a population of non-functional Bt receptors ([Valaitis 2008](#)). The increased levels of this protein in CXC may also relate to the abundance of mature cells and enhanced differentiation in stem cell bioactivity assays. The availability of histones present in the secretome may relate to the increased stem cell proliferation found in bioactivity assays, since histone activity is commonly used as proliferation marker ([Park and Takeda 2008](#)). Because available calcium is known to enhance proliferative activity in midgut cells ([Loeb 2005](#)), the down-regulation of proteins like calmodulin and calreticulin which have known calcium binding properties ([Forest, Swulius et al. 2008](#)), could inhibit regenerative capabilities in susceptible strains.

Generally, the increased arylphorin expression could be described as a midgut defense and regenerative response. The midgut has been previously reported to act as a sensing organ to trigger increased arylphorin in the hemocoel in response to bacteria presence ([Freitak, Heckel et al. 2009](#)), and synthesis of arylphorin by midgut cells has been previously demonstrated ([Palli and Locke 1987](#)). Both susceptible and resistant strains had arylphorin identified as differentially secreted in response to Cry1Ac toxin, although at different levels. While it is possible that arylphorin may have a role in defense but not in enhanced regeneration, it has been previously shown that small differences in arylphorin levels can have drastic effects on stem cell response ([Blackburn, Loeb et al. 2004](#)). Thus, it is possible that the small differences in arylphorin levels between susceptible and resistant larvae detected in our proteomic and

genomic assays may result in important differences in stem cell-induced regeneration. Taking into account that susceptible midgut epithelium had characteristic signs of autophagy and stress ([Sousa, Santos et al. 2010](#)) after Cry1Ac intoxication compared to midguts from resistant strains, it is plausible that arylphorin could exert different responses in midgut regeneration between strains. Thus, resistant strains could use arylphorin during regeneration while susceptible strains are incapable of regeneration due to lack of additional necessary factors.

Both CXC and YDK secretomes caused enhanced stem cell differentiation in stem cell bioactivity assays. The contents of CXC and YDK secretome account for this effect. Both of mature cell secretomes from these strains had increased protease expression compared to KCB. Autophagous-type vacuolization was observed in midgut cross sections from both CXC and YDK strains. The correlating finding in the secretome which can account for this observation is the number of apoptotic markers present in both CXC and YDK secretomes but not KCB. Proteins in the YDK and CXC secretome indicated simultaneous activation of apoptosis and suppression of autophagy. For instance, the YDK secretome contained secretory trypsin inhibitor, which prevents autophagy ([van Hoef, Breugelmans et al. 2011](#)) and the activation of prophenyloxidase pathways with serpin expression ([Jiang, Vilcinskas et al. 2010](#)). Although there were enzymes present in the CXC secretome to suppress autoimmune imbalance, such as ROS-inactivating catalase and oligopeptidase inactivating small peptides, there were less apoptotic markers in CXC compared to YDK. There are previous studies which correlate Bt toxin tolerance to efficient proteolytic processing of toxin (over processing) and protoxin (under activation) to result in resistance ([Forcada, Alcacer et al. 1996](#); [Karumbaiah, Oppert et al. 2007](#)). It is possible that the balance between apoptosis and autophagy is similar in CXC. Apoptosis up-

regulation could result in massive midgut shutdown providing a population of non-functional receptors in the form of dead cells. Suppressing apoptosis by quick expression of catalases and oligopeptidases could stop this process when toxin is no longer present. There are other mechanisms within the insect which coordinate proliferation and apoptosis through ecdysteroids ([Champlin and Truman 1998](#)), this may be a similar feedback mechanism in the midgut in the presence of Bt toxins.

We were unable to obtain identification of many KCB secretome proteins unique to Cry1Ac intoxication. In midgut H&E staining we detected an increased abundance of goblet cells in KCB compared to YDK and CXC midguts. According to the stem cell bioactivity assays, the KCB secretome induced enhanced stem cell proliferation. Goblet cells have vacuolar type ATPase ion pumps, which account for ion homeostasis in the midgut ([Wieczorek, Beyenbach et al. 2009](#)). Thus, it is possible that differences in ion abundance may cause enhanced stem cell proliferation ([Chamberlin, Gibellato et al. 1997](#)) in intoxicated KCB midguts.

Changing Bt crop refuge requirements and single toxin crop varieties in proximity to pyramided crop varieties raises questions related to resistant mechanisms not involving toxin receptor alterations. Refuge allows rare and less fit resistant insects to mate with larger populations of susceptible insects ([Daniell 2002](#)). It is well established that Cry toxin resistance is often associated with high fitness costs ([Gassmann, Carriere et al. 2009](#)). It has been established that the fitness costs alone are enough to mitigate the emergence of resistance ([Sayyed, Ahmad et al. 2008](#)). Therefore, we were interested in estimating potential fitness costs associated with strains with enhanced midgut regeneration. Also, when resistant secretomes were exposed to susceptible larvae some level of lethality was observed. This observation

relates to the detected increased dead cell numbers in stem cell cultures treated with secretomes from resistant strains. The fitness parameters measured were length of time until pupation and duration of the pupae life stage because of ecdysone-mediated expression of arylphorin ([Manohar, Gullipalli et al. 2010](#)). The stimulatory affect of excess ecdysone increasing arylphorin expression were analogous with previous reports supporting that caloric restriction, longer lifespan, and increased arylphorin are coordinated in lepidopteran insects ([Li, Chen et al. 2009](#)).

5 Conclusions and outlook

This dissertation provides an in-depth analysis of the regenerative mechanisms in the midgut of the common lepidopteran agricultural pest, *Heliothis virescens*. Although reports on midgut regeneration and its importance to resistance to Cry toxins have been previously reported ([Gould, Martinez-Ramirez et al. 1992](#); [Forcada, Alcacer et al. 1999](#)), our work represents the first attempt at characterizing the molecular cues involved in regulation of this process. The central focus of our project was to investigate midgut response and functional regeneration in both susceptible and laboratory-selected resistant strains using a multifaceted molecular biology approach. The work described in this dissertation combines development of a novel midgut regeneration monitoring assay (Chapter 2) with other molecular biology methodologies such as proteomics, real time PCR and histopathological analysis.

Insect midgut regeneration in lepidopteran pests has been predominantly assessed using *in vitro* primary midgut cell cultures ([Hakim, Baldwin et al. 2001](#); [Loeb, Martin et al. 2001](#)). Prior to the development of our differential fluorescent flow cytometric assay to distinguish stem and mature midgut cell types, proliferation and differentiation events were typically measured by gross morphological attributes alone. By utilizing this multi-faceted approach we identified commercially available growth factors, provide evidence that growth factors are present in secretomes from midgut cell cultures, and demonstrate differences in midgut cell culture regeneration in susceptible and resistant strains of the agricultural pest *H. virescens*.

While much is known about single gene alterations which correlate to the toxin-receptor binding reductions in *H. virescens* ([Gahan, Ma et al. 2005](#)) and this knowledge has led to design of screening techniques ([Carlos, Perera et al. 2008](#)), screening techniques for alternative

resistance mechanisms are limited. This is a unique time for Bt-based transgenic technology with opportunity for non-traditional resistant mechanisms to emerge. There are transgenic crops expressing single insecticidal Cry proteins in proximity to crops incorporated with multiple Cry and Vip toxins. There are changing refugia requirements where non-contiguous refuge can be incorporated within crop environments. Changing Bt transgenic crop industry regulations and their impacts were reviewed in the first chapter. All of these aforementioned factors increase the risk of the development of broad spectrum resistance in target pests. Although this resistance strategy improves grower compliance issues, mixed refugia increases the selection pressure in a crop environment and decreases the potential recessiveness of new resistance alleles.

The small subunit of the hexamerin protein arylphorin, often termed alpha-arylphorin, has been discussed previously to stimulate stem cell multiplication in the midgut ([Blackburn, Loeb et al. 2004](#)). After multiple arylphorins were identified from the midgut mature cell secreted proteome, we confirmed the up-regulation of arylphorin after Cry1Ac intoxication using real time PCR (Chapter 3 & 4). The real time PCR assays demonstrated that the two Bt-resistant *H. virescens* strains have unique hexamerin-related expression profiles compared to susceptible larvae. The CXC secretome had higher abundance of non-functional Bt binding sites, secreted proteins, and midgut proteases. In contrast, the KCB secretome lacked a Cry1Ac-induced unique secretome alluding to diverse resistance mechanism between strains.

Considering the overlap between the phasing out of single toxin Bt crops and the commercialization of transgenic plants co-expressing diverse Bt toxins, there is a rare opportunity for uncharacterized resistance mechanisms to emerge in the field; especially in the case of a highly mobile crop pest *H. virescens*. These factors give new precedence to midgut

regeneration mechanisms in *H. virescens* strains KCB and CXC where toxin receptor binding is not diminished. Prior to this study, there was limited molecular information available to develop screening techniques for midgut healing responses. Consequently, the commercial significance of an insect pest having enhanced regeneration to Cry toxins has never been put into dialogue. In this work, we compare the healing response, regeneration, and enhanced regeneration of midgut cell cultures between lab-selected resistant strains KCB and CXC compared to susceptible strain YDK. We found that secretomes from resistant insects caused tolerance to DiPel in susceptible larvae. Proteomic profiling of the midgut mature cell secretome implicates arylphorin due to high statistical significance and Cry1Ac specificity. But dissimilar stem cell divisions induced by resistant strain secretomes raise questions about other unidentified proteins or potential lipid secretome candidates. Therefore, our research as a first report of the midgut mature cell secretome contributes to the characterization of the elusive enhanced midgut regeneration response which can result in resistance to diverse Bt toxins. Apart from the importance for Bt research, this is the first investigation of secreted proteins from epithelial midgut cells in culture. Also there were proteins identified which were specific to Cry3Aa protoxin treatment indicating that midgut cells may respond to a toxin that does not kill larvae.

To expand upon the work in this chapter, functional assays targeting the midgut arylphorin could be performed in the two resistant strains CXC and KCB. Based on my findings on the role of arylphorin in midgut regeneration and the high levels of arylphorin expression during starvation and intoxication, it would be expected that knockdown of arylphorin expression would result in decreased midgut regeneration and alterations in larval development. This hypothesis is also supported by results from knockdown of hexamerins in *Spodoptera*

exigua larvae ([Tang, Wang et al. 2010](#)). In that report, hexamerins were found to be expressed by diverse tissues and to have a role in both metamorphosis and response to starvation. Alterations in expression of midgut arylphorin in CXC and KCB larvae may also explain lower larval weight compared to YDK or YHD2-B. This observation may suggest that resistance costs associated to toxin receptor alterations may differ from costs resulting from alterations in midgut regeneration in resistant larvae. It would also be interesting to attempt a linkage analysis between increased midgut arylphorin expression levels and resistance to diverse Cry toxins. Recent studies suggest that developmental penalties associated with sublethal doses of toxin in *H. armigera* can be inherited epigenetically ([Rahman, Glatz et al. 2011](#)), which suggests that inheritance of resistance alleles associated with a broad spectrum tolerance to diverse Bt toxins may be more complex than single gene alterations that have been reported in cases of resistance due to reduced toxin binding to midgut receptors..

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Vita

Anaïs Severiana Castagnola was born on October 29th, 1982, in a quiet beach community of Southern California. Her early academic accomplishments, particularly in science, provided her acceptance to several top tier undergraduate institutions primarily in the Northeast; she selected Rosemont College and Smith College. After a summer semester of organic chemistry classes at Emory University, she found her academic home in the Southeast and completed her undergraduate degree at Kennesaw State University (KSU), earning a Bachelors of Science in Biotechnology. The undergraduate research exposure motivated her to join the Entomology and Plant Pathology Department at the University of Tennessee, Knoxville, in the Fall of 2007 with a research assistantship. As a graduate research and marketing assistant, Anaïs performed promotion and recruitment duties for the department, competed with the debate team at the Entomological Society for America meetings, and worked as a marketing consultant for the University of Tennessee Research Foundation's biotechnologies. Her research and work has won multiple awards. At the Society for Invertebrate Pathology's international meetings she earned an Honorable Mention for a poster competition in 2008, student travel award in 2009, and a second place prize in the student poster competition in 2011. At the Entomological Society for America meetings her debate team attained a win in 2009 and she earned second place President's Prize in the student poster competition in 2009. Her scientific research contributes to the field of *Bacillus thuringiensis* (Bt) insecticide use in agriculture, specifically the molecular characterization of midgut regeneration in response to Bt intoxication. Anaïs successfully completed her doctoral degree in the Fall of 2011 and looks forward to a challenging and promising future associated with scientific research and biotechnology endorsement.