A peer-reviewed version of this preprint was published in PeerJ on 3 October 2017.

Castagnola A, Jackson J, Perera OP, Oppert C, Eda S, Jurat-Fuentes JL. 2017. Alpha-arylphorin is a mitogen in the *Heliothis virescens* midgut cell secretome upon Cry1Ac intoxication. PeerJ 5:e3886 https://doi.org/10.7717/peerj.3886

Arylphorin is a mitogen in the *Heliothis virescens* midgut cell secretome upon Cry1Ac intoxication

Anais Castagnola [1], **Jerreme Jackson** [2], **Omaththage P Perera** [3], **Cris Oppert** [1], **Shigetoshi Eda** [4], **Juan Luis Jurat-Fuentes** [Corresp. 1, 2]

[1] Entomology and Plant Pathology, University of Tennessee, Knoxville, Tennessee, United States

[2] Genome Science and Technology Graduate Program, University of Tennessee, Knoxville, Tennessee, United States

[3] Southern Insect Management Research Unit, USDA-ARS, Stoneville, MS, USA

[4] Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, Tennessee, United States

Corresponding Author: Juan Luis Jurat-Fuentes
Email address: jurat@utk.edu

Insecticidal crystal (Cry) proteins produced by the bacterium *Bacillus thuringiensis* (Bt) target cells in the midgut epithelium of susceptible larvae. While the mode of action of Cry toxins has been extensively investigated, the midgut response to Cry intoxication and its regulation are not well characterized. In this work, we report the secreted proteome (secretome) of primary mature midgut cell cultures from *Heliothis virescens* larvae after exposure to Cry1Ac toxin compared to control buffer treatment. Biological activity of the Cry1Ac-induced secretome was monitored as higher proliferation and differentiation and an overall reduction in total cell mortality over time in primary *H. virescens* midgut stem cell cultures when compared to treatment with control buffer secretome. Differential proteomics identified 4 proteins with significant differences in abundance comparing Cry1Ac-treated and control secretomes. The most significant difference detected in the Cry1Ac secretome was an arylphorin protein not detected in the control secretome. Feeding of purified arylphorin to *H. virescens* larvae resulted in midgut hyperplasia and significantly reduced susceptibility to Cry1Ac toxin compared to controls. These data identify arylphorin as a protein with a putative relevant role in the midgut regeneration process in response to Cry1Ac intoxication.

Arylphorin is a mitogen in the *Heliothis virescens* midgut cell secretome upon Cry1Ac intoxication

Castagnola[1§], A., Jackson[2§], J., Perera[3], O. P., Oppert[1§], C. Eda[4], S., and J. L. Jurat-Fuentes[1*]

[1]Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996, USA

[2]Genome Science and Technology Program, University of Tennessee, Knoxville, TN 37996, USA

[3]United States Department of Agriculture- Agricultural Research Service, Southern Insect Management Research Unit, Stoneville, MS, 38776, USA

[4]Department of Forestry, Wildlife, and Fisheries, University of Tennessee, Knoxville, TN 37996, USA

[§]Current address: A.C.: ManTech International Corporation, Herndon, VA 20171, USA; J.J.: Oklahoma State University, Department of Microbiology and Molecular Genetics, Stillwater, OK 74078, USA; C.O.: Bayer CropScience, Morrisville, NC 27560, USA

Running title: Arylphorin in response to Cry1Ac

Keywords: *Bacillus thuringiensis*; *Heliothis virescens,* stem cell; midgut; regeneration; Cry1Ac, arylphorin

*To whom all correspondence should be addressed (jurat@utk.edu)

Abstract

Insecticidal crystal (Cry) proteins produced by the bacterium *Bacillus thuringiensis* (Bt) target cells in the midgut epithelium of susceptible larvae. While the mode of action of Cry toxins has been extensively investigated, the midgut response to Cry intoxication and its regulation are not well characterized. In this work, we report the secreted proteome (secretome) of primary mature midgut cell cultures from *Heliothis virescens* larvae after exposure to Cry1Ac toxin compared to control buffer treatment. Biological activity of the Cry1Ac-induced secretome was monitored as higher proliferation and differentiation and an overall reduction in total cell mortality over time in primary *H. virescens* midgut stem cell cultures when compared to treatment with control buffer secretome. Differential proteomics identified 4 proteins with significant differences in abundance comparing Cry1Ac-treated and control secretomes. The most significant difference detected in the Cry1Ac secretome was an arylphorin protein not detected in the control secretome. Feeding of purified arylphorin to *H. virescens* larvae resulted in midgut hyperplasia and significantly reduced susceptibility to Cry1Ac toxin compared to controls. These data identify arylphorin as a protein with a putative relevant role in the midgut regeneration process in response to Cry1Ac intoxication.

Introduction

Insecticidal Cry proteins synthesized by the bacterium *Bacillus thuringiensis* (Bt) are used in pesticides and produced by transgenic crops to control destructive lepidopteran and coleopteran crop pests (Sanchis, 2011). The specificity of these Cry toxins is mostly determined by their binding to specific receptors on the brush border membrane of the insect intestinal epithelium (reviewed in (Adang et al., 2014). Binding to these receptors is conducive, in most cases, to enterocyte death and subsequent disruption of the gut epithelial barrier, allowing Bt and resident bacteria to invade the hemocoel and cause septicemia (Broderick et al., 2009; Raymond et al., 2009). However, it has long been established that lepidopteran larvae can recover from exposure to Cry toxins (Dulmage and Martinez, 1973; Nishiitsutsujiuwo and Endo, 1981; Sutherland et al., 2003), and that recovery depends on a midgut regenerative response (Chiang et al., 1986; Spies and Spence, 1985). Moreover, an enhanced midgut regenerative response has been proposed as a resistance mechanism to Cry1Ac toxin in selected strains of *Heliothis virescens* (Forcada et al., 1999; Martínez-Ramírez et al., 1999), highlighting the importance of this defensive mechanism in determining susceptibility to Cry toxins. However, information on the molecular regulation of this midgut healing response to Cry toxins in insects is very limited.

The most detailed information on the response to Cry intoxication has been obtained in the nematode *Caenorhabditis elegans*. In this organism both the MAPK p38 and c-Jun N-terminal kinase pathways have been reported as important to the defense response against Cry5B toxin (Huffman et al., 2004). A role for the p38 kinase in midgut defense against Cry1Ab has also been proposed in the lepidopteran *Manduca sexta* (Cancino-Rodezno et al., 2010). In *Bombyx mori* larvae, the JNK and JAK-STAT pathways were found to be up-regulated in the early response to Cry1Aa intoxication (Tanaka et al., 2012). Subtractive hybridization libraries and custom microarrays detected a down-regulation of metabolic

enzymes and up-regulation of genes involved in detoxification, stress, or immune responses after intoxication of *Choristoneura fumiferana* and *M. sexta* larvae with Cry1Ab protoxin (Meunier et al., 2006; van Munster et al., 2007). Proteomic analyses of Cry intoxication in the coleopteran model *Tribolium castaneum* also detected down-regulation of metabolic and up-regulation of defensive genes (Contreras et al., 2013a) and identified the hexamerin apolipophorin III as involved in the immune response to Cry3Ba intoxication (Contreras et al., 2013b). Similar trends have been reported in larvae of *Spodoptera exigua* (Herrero et al., 2007) and *Spodoptera frugiperda* (Rodriguez-Cabrera et al., 2008) challenged with Cry1Ca toxin. In the case of *S. exigua*, specific members of a family of proteins responding to pathogens (REPAT) and arylphorin genes were found to be up-regulated in response to intoxication with a Bt-based pesticide (Hernández-Martínez et al., 2010). This activation was constitutive in larvae from a strain of *S. exigua* resistant to the Bt-pesticide. Up-regulation of arylphorin was also found in a Cry1Ab-resistant compared to a susceptible strain of *Diatraea saccharalis* (Guo et al., 2012). In contrast, exposure of *S. exigua* to a Bt toxin (Vip3Aa) with a distinct mode of action compared to Cry toxins, or exposure of *Lymantria dispar* larvae to a commercial Bt pesticide resulted in reduced arylphorin expression (Bel et al., 2013; Sparks et al., 2013). Although arylphorin has been previously shown to induce midgut stem cell proliferation (Hakim et al., 2007), the specific functional roles of REPAT and arylphorin proteins in midgut regeneration after Cry intoxication have yet to be elucidated.

Primary midgut cell cultures from lepidopteran larvae have been used as an *in vitro* model to study the molecular cues directing midgut regeneration (Hakim et al., 2010), and are capable of regeneration after intoxication with Bt toxins (Loeb et al., 2001b). A number of peptidic midgut proliferation and/or differentiation factors (MDFs) from mature cell conditioned media and hemolymph have been reported (reviewed in (Hakim et al., 2010). One of these MDFs (MDF1) was localized to mature midgut cells upon Cry intoxication

(Goto et al., 2001), yet its role in midgut healing has not been experimentally demonstrated.

Given that healing regulatory factors are secreted by stressed midgut cells, we hypothesized that proteomic analysis of the subproteome of secreted proteins (secretome) would allow the identification of proteins involved in the midgut response to injury. While midgut subproteomes from the midgut lumen (Pauchet et al., 2008) and peritrophic matrix (Campbell et al., 2008) have been characterized in *Helicoverpa armigera* larvae, the lepidopteran midgut cell secretome and its alteration during Cry1Ac intoxication has not been previously studied. We report the characterization and comparison of secretomes from *H. virescens* primary mature midgut cell cultures after treatment with activated Cry1Ac toxin versus control treatments to identify potential candidate proteins and test their involvement in regulating the gut regenerative response.

Materials and methods

Insects and toxin feeding

Eggs from the Cry1Ac-susceptible YDK strain of *H. virescens* (Gould, 1995) were kindly supplied by Dr. Fred Gould (North Carolina State University). Upon hatching, larvae were reared on artificial diet (Bio-Serv, Flemington, NJ) at 28°C on an 18L:6D photoperiod.

Fourth instar larvae were anesthetized on ice for 20 min and midguts dissected under sterile conditions and used for preparation of primary midgut cell cultures.

Toxin feeding experiments for arylphorin detection in Westerns were conducted by placing early fourth instar larvae in empty cups for 1 h before they were transferred to cups containing artificial diet contaminated with the same volume of toxin buffer (20 mM TRIS/HCl, 0.3 M NaCl pH 8.0) or with a sublethal Cry1Ac toxin dose (1 µg/ml) incorporated in the diet. Larvae were observed for feeding and the midguts were dissected from actively

feeding larvae after 2, 4, 6 and 18 hours; and then flash frozen.

Bacterial toxins

Bacillus thuringiensis var. *kurstaki* strain HD73 producing Cry1Ac toxin was obtained from the *Bacillus* Genetic Stock Center (BGSC, Columbus, OH). Bacterial culturing, toxin activation and purification were as described elsewhere (Perera et al., 2009). Purity of activated toxins was assessed by SDS-10% PAGE (data not shown), and protein concentration quantified using the Coomassie Plus Protein Assay (Pierce) with bovine serum albumin (BSA) as standard. Purified toxin samples were kept at -80°C until used (less than 6 months).

Establishment of primary midgut cell cultures

All dissections and transfers were done in the sterile environment of a biosafety cabinet. Preparation and establishment of primary midgut cell cultures were done following protocols described previously (Castagnola et al., 2010). Briefly, midguts of fourth instar *H. virescens* larvae were dissected and cleaned of food, peritrophic matrix, and Malphigian tubules using forceps and rinsing in sterile Ringer's (Barbosa, 1974) containing 0.5% (v/v) gentamicin (Invitrogen, Carlsbad, CA), 0.1% bleach, and 1x antibiotic/antimycotic (Invitrogen). Incubation media was prepared by mixing working Grace's (supplemented Grace's Insect Medium [Invitrogen] containing 1x antibiotic/antimycotic and 0.1% gentamicin) in sterile Ringer's solution in a 3:1 ratio (Loeb et al., 1984). Five to six cleaned midguts were cut in sections and incubated in 2 ml of incubation media for 90 min at room temperature. After incubation, the midgut tissue was homogenized by carefully pipetting up and down and then sieved through 70 µm 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 supernatants

discarded. The pellet in each tube contained midgut mature and stem cells and was resuspended in 1 ml of working Grace's media.

Stem cells were separated from mature cells using a density gradient (Loeb and Hakim, 1999). Briefly, samples were overlaid on 3 ml of Ficoll-Paque (GE LifeSciences, NJ) in a 15 ml conical tube, and then 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 tissue debris were discarded, and the mature cells were collected in the pellet and bottom 0.25 ml. Ficoll-Paque was eliminated from stem and mature cell samples by washing twice with incubation media (600 x *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 hemocytometer (Bright-Line, Horsham, PA). Using this procedure, we reproducibly obtained approximately 8 x 10^5 stem cells and 1 x 10^7 mature cells from 30 *H. virescens* larvae. Cells were counted in a hemocytometer and diluted to 4 x 10^5 cells/mL (stem cells) or 3.5 x 10^6 cells/mL (mature cells) with working Grace's, and kept in a sterile incubator at 26°C.

Preparation of midgut cell secretomes

Purified mature midgut cells in working Grace's were seeded (3.5 x 10^6 cells) in individual wells of a 12-well culture-treated plate (Corning, Corning, PA). The Cry1Ac toxin concentration (1 µg/ml) used as treatment to induce the midgut secretome was chosen based on inducing sublethal cytotoxicity (percent mortality 15.84 ± 0.29 compared to 8.09 ± 0.99 in buffer treatment) as measured by trypan blue staining of primary *H. virescens* mature cell cultures treated for 18 hours at 26°C. The purified Cry1Ac toxin (1 µg/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. After incubation, media supernatant containing the proteins secreted by the midgut cells was collected by centrifugation (2,000 x *g* for 20 min at 4°C). Two independent biological samples were collected and pooled to prepare each secretome used for further analyses or stored at -80° C until used for bioactivity assays as described below.

Secretome samples for 1DGel LC/MS/MS analysis were concentrated to 50-100 µl and the 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. Samples were shipped to NextGen Sciences (Ann Arbor, MI) for proteomic analysis.

Stem cell bioactivity assays

Primary *H. virescens* midgut stem cell cultures were stained with the viability stain calcein AM (Invitrogen) following manufacturer's instructions and then counted and gated using flow cytometry. Purified stem cells were seeded into individual wells of a 12-well plate at a concentration of 4 x 10^4 cells/ml and a final volume of 500 µl. Treatments of Cry1Ac or buffer-induced secretomes were applied to the wells (final well volume was 1 ml). The time zero control was measured by adding working Grace's media. After 30, 60 and 180 min of incubation time, proliferation and differentiation of cells were measured by staining for viability with calcein AM (Invitrogen) and analyzed for side scatter and green (calcein AM) fluorescence by counting 20,000 non-gated events in an LSRII flow cytometer (BD Bioscience, CA), as previously described (Castagnola et al., 2010). At least two wells for the same treatment were measured per experiment, and experiments were replicated thrice.

Proteomic analysis of primary midgut cell culture secretome

Proteins in secretome samples (10 µg) were separated by 1D SDS-10% PAGE using the NuPAGE Bis-Tris mini gel system (Invitrogen) following manufacturer's instructions, and then each of the sample lanes was 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 10 mM DTT at 60°C, and then allowed to cool to room temperature before being alkylated with 100 mM iodoacetamide. Tryptic digestion was done at 37°C for 4 hours, and reactions were stopped by addition of formic acid (0.1% final concentration). 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 at MS Bioworks (Ann Arbor, MI) using Mascot (Matrix Science, London, UK) and queried against a custom *H. virescens* transcriptome database (Perera et al., 2015) translated in the 6 possible frames. 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 as variable modifications.

Scaffold (version Scaffold_4.4.6, 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 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone

were grouped to satisfy the principles of parsimony. Normalized spectral counts were used for quantitative analysis using the Fisher's exact test as previously described (Zhang et al., 2006), using the $P<0.005$ level of significance to increase the probability of accurate detection of differences in protein levels for each of the identified proteins in the buffer or Cry1Ac-induced secretomes.

Purification of arylphorin

Hemolymph was collected from 60 pharate 5th instar *H. virescens* larvae by making a small incision at the base of the first 1st and/or 2nd proleg and collecting droplets of hemolymph into 15 mL conical tubes containing 5 mg of phenylthiourea to block hemolymph phenoloxidase activity, and maintaining on ice. After collection, hemolymph was frozen at -20°C until used (no longer than 2 months). Frozen hemolymph was thawed on ice and diluted 5-fold in 20 mM Tris pH 7.9 (buffer A). For fractionation, hemolymph was filtered (0.22 μm) and loaded onto a HiTrap Q HP column (GE Healthcare), previously equilibrated with buffer A and connected to an AKTA FPLC system (GE Healthcare). Proteins were eluted with a 0-1 M linear gradient of NaCl in 20 mM Tris pH 7.9 (buffer B) at a flow rate of 1 mL/min, collecting 1 mL fractions. To reduce the presence of smaller proteins co-purifying with α-arylphorin, fractions estimated to contain α-arylphorin (based on presence of ~70kDa band on electrophoretic observations, data not shown) were combined and filtered using an Amicon Ultra-15 mL centrifugal unit (Millipore) with a MWCO of 50 kDa. After concentration, partially purified α-arylphorin was quantified with the Coomassie Plus Protein Assay (Pierce) using BSA as the standard, and then aliquoted and maintained at -80°C until used.

Arylphorin feeding bioassays

Artificial diet was dispensed into wells of 128-well bioassay trays (both from Bio-Serv, Flemington, NJ) and left to dry in a laminar flow cabinet. A single concentration of α-arylphorin causing hyperplasia in preliminary assays (0.781 µg/mL) was prepared in 20 mM Tris, pH 7.9 and distributed (75 µL) to the dry diet surface of each well and gently swirled to ensure even coating (29.2 ng/cm^2 final dose per well). Upon drying, a single neonate larva was placed in each well and the wells were sealed with adhesive covers. Larvae were allowed to feed on diet containing α-arylphorin for five days under standard rearing conditions and then moved to diet that was surface-contaminated with Cry1Ac toxin (0.5 µg/cm^2) or toxin buffer, and mortality scored after seven days. Bioassays were conducted with 16 neonate larvae per treatment and replicated three times.

Histological sections

The number of midgut cells in larvae fed arylphorin or buffer (as described above) was determined by counting the number of nuclei incorporating DAPI (4',6-diamidino-2-phenylindole) in gut sections. Larvae (4-6 per treatment) were fixed in ice-cold Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) and stored at 4°C overnight in biopsy cassettes, and then transferred to freshly prepared 70% ethanol. Larval tissues were processed using a Tissue-Tek VIP processor (Sakura, Torrance, CA) and embedded in paraplast medium (Sigma-Aldrich, St. Louis, MO). Block sections were obtained by cutting 5 µm slices using a Micron HM355s microtome (Thermo Scientific). For DAPI staining, tissues were mounted on Superfrost Plus Slides (Fisher Scientific, Waltman, MA) following recommended guidelines (Slaoui and Fiette, 2011). Slides prepared for DAPI staining were deparaffinized through two 10 minute washes in xylene followed by a rehydration series of ethanol washes (absolute, 95%, 70%) for 5 minutes each, and finally washed twice for 5 minutes in distilled water. Tissues were permeabilized by treatment with 10 mM citrate

buffer pH 6.0 at 95-99°C for 20 minutes and rinsed in two 1-minute washes of phosphate buffered saline (PBS, 2 mM KCl,135 mM NaCl, 1.7 mM KH_2PO_4,10 mM Na_2HPO_4, pH 7.4). Blocking was performed in PBS containing 0.1% Tween-20 and 3% BSA for 1 h. After blocking, tissues were mounted in a water-based medium containing DAPI for nuclear detection. The total number of cells in each treatment was estimated by counting the number of DAPI-stained nuclei per 100 μm^2 in 3 sections from independent larvae for each treatment.

Results

Secretome bioactivity in primary midgut stem cell cultures

After obtaining the proteins secreted by primary mature midgut cell cultures in response to Cry1Ac or control buffer treatment, we monitored their regenerative properties on primary *H. virescens* midgut stem cell cultures using a flow cytometry method based on differential calcein staining (Castagnola et al., 2011). Gates defined by the side scattered light and calcein fluorescence allowed monitoring of changes in the number of stem, mature, and dead cell subpopulations within each sample (Fig. 1). Freshly prepared *H. virescens* midgut stem cell cultures (time zero in Fig. 1) contained predominantly stem cells (approx. 3.5 x 10^4 cells or 85%), and a smaller proportion of mature (0.6 x 10^4 cells or 14%) and dead (0.6 x 10^3 or 1%) cells. Treatment with buffer-induced secretome resulted in a non-significant trend (Student's T-test, P = 0.074) of rapid (30 min.) reduction in the number of stem cells concomitant with a significant increase in the number of dead (Student's T-test, P <0.05) and mature cells (Student's T-test, P <0.05) (Fig. 1, open symbols). Technical limitations prevented us from determining whether the cell types accounting for the increased dead cell numbers in this treatment were stem, mature or both, but this observation may reflect the low viability in culture of midgut mature cells in the original preparation and additional mature cells originating from stem cell differentiation. After three hours, only a

small reduction in the number of dead cells (probably due to cytolysis) was observed in these cultures.

Similarly to treatment with buffer-induced secretome, a significant (Student's T-test, $P < 0.05$) initial tendency to increase the number of dead and mature cells and decrease stem cell numbers was detected in cultures treated with Cry1Ac-induced secretome (Fig. 1, black symbols). However, an important observation was that the total number of dead cells after 30 min or 1 hour was approximately half in cultures treated with Cry1Ac compared to buffer secretome treatment. After 3 hours, the number of stem cells in the Cry1Ac secretome-treated samples had recovered to similar levels observed for the initial conditions, while the number of dead cells had significantly decreased compared to 30 min (Student's t test, $P < 0.05$) and the number of mature cells remained constant. The recovery in the number of stem cells between 30 min and 3 h (Student's T-test, $P < 0.05$) was suggestive of a regenerative response to Cry1Ac secretome compared to control treatment. This response was not observed when primary midgut stem cell cultures were treated with the same Cry1Ac toxin concentration used to obtain the Cry1Ac-induced secretome (data not shown), indicating the presence of mitogens in the Cry1Ac-induced secretome.

Identification of secretome proteins and alterations in response to Cry1Ac intoxication

Proteins in buffer and Cry1Ac-induced secretomes from *H. virescens* primary mature midgut cell cultures were identified using LC/MS/MS and a custom *H. virescens* transcriptome database (Perera et al., 2015). A total of 358 proteins were identified in the secretomes (protein False Discovery Ratio [FDR] 0.0%), with 326 and 313 proteins detected in the buffer and Cry1Ac-induced secretomes, respectively. Out of the 358 identified proteins, 281 proteins were common to both secretomes, while 45 proteins were unique to the buffer, and 32 to the Cry1Ac induced secretomes, respectively. Normalized spectral counts

for all proteins detected in the Cry1Ac secretome and their corresponding values in buffer secretome are listed in Table S1. The list of identified proteins included proteins involved in physiological functions expected for the gut tissue, such as digestive enzymes, storage and transport proteins, immune and stress-related proteins, and also putative Cry toxin receptors. Intracellular proteins (ribosomal, mitochondrial and cytosolic enzymes, nucleotide-related proteins) were also commonly detected, possibly due to leakage of cell contents after cell death.

Statistical analyses of normalized spectral counts identified 4 proteins with significant different abundance (Fisher's Exact test with Benjamini-Hochberg correction; $P<0.005$) when comparing buffer to the Cry1Ac-induced secretome (Table I). Differentially present proteins included digestive enzymes (aminopeptidase and glucosidase), a protein involved in processes of cell growth and proliferation (elongation factor 1 gamma), and a storage protein (arylphorin). Both the Cry1A receptor aminopeptidase and the elongation factor 1 gamma ere unique to the buffer secretome, while only arylphorin was unique to the Cry1Ac secretome. An uncharacterized glucosidase was more abundant in the Cry1Ac secretome (Table I).

Effect of arylphorin on *H. virescens* susceptibility to Cry1Ac toxin.

Given that the only unique protein with differential levels identified in the Cry1Ac compared to buffer secretome was arylphorin, we concentrated our efforts on the putative role of this protein in the midgut response to Cry1Ac intoxication. To test the effect of arylphorin during the response to Cry1Ac intoxication, we purified arylphorin from *H. virescens* hemolymph and fed it to neonates for five days before exposing them to Cry1Ac toxin. Mass spectrometry analysis of the purified arylphorin sample supported that it was 90% pure (data not shown). Larvae feeding on this purified arylphorin sample for 5 days developed midgut hyperplasia when compared to controls (Fig. 2A). This hyperplasia

resulted from a significant increase in the number of cells per unit of midgut surface (Fig. 2B). Exposure of larvae fed buffer without arylphorin to Cry1Ac toxin (0.5 µg/cm^2) for seven days resulted in 34 ± 5% mortality. In contrast, a significant reduction (Student's t-test; P<0.05) in mortality to 15 ± 3% was detected in larvae that had been pre-exposed to arylphorin (29.2 ng/cm^2) before exposure to Cry1Ac (Fig. 2C). No significant difference was detected between mortality in control larvae (3% mortality) and larvae exposed to arylphorin for five days (Mann-Whitney Rank Sum Test used due to data failing normality test; P<0.05).

Discussion

Insect gut cells are responsible for the absorption of nutrients and secretion of proteins into the gut lumen to help regulate digestion. Previous studies on lepidopteran larval midgut proteomes have focused on proteins present in the peritrophic matrix (Campbell et al., 2008) and midgut lumen (Pauchet et al., 2008), or the identification of putative binding sites for Cry insecticidal proteins (Krishnamoorthy et al., 2007; McNall and Adang, 2003). In this study we aimed at help resolve the current lack of proteomic information on the response to noxious stimuli, more specifically the Cry1Ac toxin, in the lepidopteran midgut cells. We report on the identification of proteins secreted by primary larval midgut cell cultures of *H. virescens* and their differential secretion of proteins after exposure to Cry1Ac toxin, the most active Cry toxin against that insect (van Frankenhuyzen and Nystrom, 2015).

Primary *H. virescens* midgut cell cultures were described to undergo a regenerative process after exposure to a Cry toxin, which involved an increase in the number of differentiating cells compared to controls (Loeb et al., 2001a). This process was observed in our experiments after treatment with Cry1Ac-induced secretome as an increase in the number of mature cells concomitant with an initial decrease in the number of stem cells, consistent with stem cell differentiation, followed by an increase in the number of stem cells to initial

levels (evidence of stem cell proliferation). In contrast, the proliferative stem cell phase was not observed when treating the cell cultures with the secretome induced by buffer treatment. Based on these observations, we hypothesized the presence of growth factors in the Cry1Ac-induced secretome implicated in the regenerative process.

Analysis of the control and Cry1Ac-induced secretomes identified proteins expected to be produced and secreted by enterocytes (Supplementary Table 1), including digestive enzymes (proteases, glucosidases and lipases), storage and transport proteins (transferrin, apolipophorin, arylphorin, fatty acid-binding protein…), proteins involved in defense reactions (phenoloxidase, esterases, glutathione S-transferases…), and enzymes involved in diverse gut physiological processes (phosphatases, dehydrogenases, deaminases…). The relevant abundance of putative intracellular proteins detected in both secretomes probably represents the release of cellular contents into the media after enterocyte death. Proteins unique to the buffer secretome included Cry1A toxin receptor aminopeptidase and elongation factor 1 gamma. Shedding of GPI-anchored proteins such as aminopeptidases from the midgut cell surface has been previously reported in midgut cells of *Lymantria dispar* after exposure to Cry1Ac, probably as a way to reduce available levels of Bt receptors on the midgut cell surface (Valaitis, 2008). This observation is in contrast to the increased Cry1A toxin receptor aminopeptidase levels observed in the buffer secretome. While it needs to be considered that the epithelial polarization missing in primary cell cultures may affect which GPI-anchored proteins are shed, we currently do not have an explanation for the unique shedding of a putative Cry1Ac receptor aminopeptidase after treatment with control buffer but not during exposure to Cry1Ac. One possibility could be that the shedding of selected GPI-anchored proteins may be a natural process independent of exposure to Cry proteins.

It is also challenging to interpret the detected changes in abundance for other proteins, such as family 31 glucosidase and elongation factor 1 gamma. Increased glucosidase levels

during exposure to Cry1Ac compared to buffer may reflect an increased catabolism in cells exposed to Cry proteins. This hypothesis is supported by the increased expression of glycosyl hydrolases and other enzymes involved in catabolism reported for *Tenebrio molitor* larvae exposed to Cry3Aa (Oppert et al., 2012). In contrast, reduced expression of catabolic enzymes has been reported after exposure of lepidopteran larvae to Cry toxins (van Munster et al., 2007), including levels of a glycosyl hydrolase family 31 protein in *Spodoptera frugiperda* after exposure to Cry1Ca toxin (Rodriguez-Cabrera et al., 2008). The discrepancy between observations described here and previous reports in Lepidoptera may be related to the differences between *in vivo* and *in vitro* systems used in analyzing responses to Cry toxins. In line with the proposed increased catabolism, the reduced levels of elongation factor 1 gamma protein in Cry1Ac secretome may be indicative of reduced protein biosynthesis, although a role in cytoskeleton reorganization has also been proposed for this protein (Shiina et al., 1994). Levels of elongation factor 1 gamma are increased in actively proliferating cells, such as those in culture (Sanders et al., 1992) or in gastric tumors (Mimori et al., 1995). Consequently, it is possible that the reduced levels detected for this protein in the Cry1Ac compared to buffer secretome represent a reduction in anabolism concomitant with the proposed increased catabolism in cells exposed to Cry proteins.

Of the four proteins identified to be differentially present in buffer and Cry1Ac induced secretomes, arylphorin was the only protein unique to the Cry1Ac secretome, suggestive of a relevant role in response to intoxication. While traditionally considered a storage protein produced by the fat body, arylphorin has been shown to stimulate midgut stem cell proliferation (Blackburn et al., 2004; Hakim et al., 2007; Smagghe et al., 2005), and its production in the lepidopteran midgut epithelium is also established (Palli and Locke, 1987; Tang et al., 2010). Moreover, there is growing evidence supporting a role for arylphorin in insect immunity. For instance, increased arylphorin gene expression was previously detected

in response to infection with bacteria (Freitak et al., 2007) and parasites (Kunkel et al., 1990). These increased arylphorin levels could result in midgut hyperplasia, as previously described (Smagghe et al., 2003) and as observed in the present study. Increased levels of hexamerins, such as arylphorin were proposed to sequester Cry1Ac toxin to the gut lumen in *H. armigera* (Ma et al., 2005). In contrast, reduced levels of arylphorin transcripts were detected upon exposure of *S. exigua* to Vip3Aa or *L. dispar* to Bt var. *kurstaki* (Bel et al., 2013; Sparks et al., 2013). These observed discrepancies in the regulation of arylphorin may represent differences in the mode of action of Cry versus Vip3Aa toxins and the effect of Bt spores versus purified Cry proteins on midgut cells. Alternatively, it is possible that the mitogenic effect of arylphorin will depend on its relative concentration, as previously reported (Hakim et al., 2007). This would also help explain why we detected relatively lower levels of arylphorin in *H. virescens* larvae treated with Cry1Ac compared to controls. It also remains to be investigated whether the observed delayed production of arylphorin in Cry1Ac-treated *H. virescens* larvae compared to controls is a consequence of delayed development induced by intoxication or of a carefully regulated healing response.

Our data from feeding bioassays support that midgut hyperplasia induced by arylphorin is relevant to increase survival during exposure to Cry1Ac. Increased arylphorin expression was also detected in *S. exigua* resistant to a *B. thuringiensis* pesticide, yet this increase was not concomitant with enhanced midgut regeneration (Hernández-Martínez et al., 2010). Given that enhanced midgut regeneration was previously hypothesized as a resistance mechanism to Cry1Ac in *H. virescens* (Forcada et al., 1999; Martínez-Ramírez et al., 1999), the potential involvement of arylphorin in this response needs to be evaluated.

The identification of genes involved in the insect midgut defensive response to pathogens allows the development of strategies aimed at hindering this response for insecticidal use. The present study provides a first list of proteins that are differentially

released by midgut cells *in vitro* in response to Cry1Ac intoxication. *In vivo* evidence is also provided for the relevance of one of the identified proteins, arylphorin, in defense against Cry1Ac intoxication. Given the observed mitogenic effect of arylphorin on midgut stem cells, the role of this protein in defense against infection with alternative pathogens or xenobiotics affecting the midgut epithelium is predicted.

Acknowledgements

This work was supported by award National Science Foundation Career Advancement Award number IOS-0718807. Partial funding was provided by Biotechnology Risk Assessment Grant Program competitive grant No. 2010-33522-21700 from the USDA National Institute of Food and Agriculture and a DuPont Young Professor Award from DuPont to JLJ-F. The authors would also like to acknowledge the Postdoctoral Program for Excellence in Research and Teaching (PERT) at The University of Arizona Center for Insect Science, funding through the National Institute of Health training grant number 1K12 GM 00708, IRACDA Program, for supporting A. Castagnola during the time of the writing of this manuscript.

References

Adang, M., Crickmore, N., Jurat-Fuentes, J. L., Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: T. S. Dhadialla, S. Gill, Eds.), Advances in Insect Physiology Vol. 47: Insect Midgut and Insecticidal Proteins. Academic Press, San Diego, CA, 2014, pp. 39-87.

Barbosa, P., 1974. Manual of basic techniques in insect histology. Autumn publishers, Amherst, Mass.

Bel, Y., Jakubowska, A. K., Costa, J., Herrero, S., Escriche, B., 2013. Comprehensive analysis of gene expression profiles of the beet armyworm *Spodoptera exigua* larvae challenged with *Bacillus thuringiensis* Vip3Aa Toxin. PLoS One. 8**,** e81927.

Blackburn, M. B., Loeb, M. J., Clark, E., Jaffe, H., 2004. Stimulation of midgut stem cell proliferation by *Manduca sexta* α-arylphorin. Arch. Insect Biochem. Physiol. 55**,** 26-32.

Broderick, N. A., Robinson, C. J., McMahon, M. D., Holt, J., Handelsman, J., Raffa, K. F., 2009. Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary

across a range of Lepidoptera. BMC Biol. 7, 11.

Campbell, P. M., Cao, A. T., Hines, E. R., East, P. D., Gordon, K. H., 2008. Proteomic analysis of the peritrophic matrix from the gut of the caterpillar, *Helicoverpa armigera*. Insect Biochem. Mol. Biol. 38, 950-8.

Cancino-Rodezno, A., Alexander, C., Villasenor, R., Pacheco, S., Porta, H., Pauchet, Y., Soberon, M., Gill, S. S., Bravo, A., 2010. The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. 40, 58-63.

Castagnola, A., Eda, S., Jurat-Fuentes, J. L., 2010. Monitoring stem cell proliferation and differentiation in primary midgut cell cultures from *Heliothis virescens* larvae using flow cytometry. Differentiation. 81, 192-198.

Castagnola, A., Eda, S., Jurat-Fuentes, J. L., 2011. Monitoring stem cell proliferation and differentiation in primary midgut cell cultures from *Heliothis virescens* larvae using flow cytometry. Differentiation. 81, 192-198.

Chiang, A. S., Yen, D. F., Peng, W. K., 1986. Defense reaction of midgut epithelial cells in the rice moth larva (*Corcyra cephalonica*) infected with *Bacillus thuringiensis*. J. Invertebr. Pathol. 47, 333-339.

Contreras, E., Rausell, C., Real, M. D., 2013a. Proteome response of *Tribolium castaneum* larvae to *Bacillus thuringiensis* toxin producing strains. PLoS One. 8, e55330.

Contreras, E., Rausell, C., Real, M. D., 2013b. *Tribolium castaneum* Apolipophorin-III acts as an immune response protein against *Bacillus thuringiensis* Cry3Ba toxic activity. J Invertebr Pathol. 113, 209-13.

Dulmage, H. T., Martinez, E., 1973. The effects of continuous exposure to low concentrations of the δ endotoxin of *Bacillus thuringiensis* on the development of the tobacco budworm, *Heliothis virescens*. J. Invertebr. Pathol. 22, 14-22.

Forcada, C., Alcacer, E., Garcera, M. D., Tato, A., Martinez, R., 1999. Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: proteolytic and SEM study of the larval midgut. Arch. Insect Biochem. Physiol. 42, 51-63.

Freitak, D., Wheat, C. W., Heckel, D. G., Vogel, H., 2007. Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. BMC Biol. 5, 56.

Goto, S., Takeda, M., Loeb, M. J., Hakim, R. S., 2001. Immunohistochemical detection of a putative insect cytokine, midgut differentiation factor 1 (MDF-1) in midgut columnar cells of *Heliothis virescens*. Invertebr Reprod Dev. 40, 117-124.

Gould, F., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545-1559.

Guo, Z., Cheng Zhu, Y., Huang, F., Luttrell, R., Leonard, R., 2012. Microarray analysis of global gene regulation in the Cry1Ab-resistant and Cry1Ab-susceptible strains of *Diatraea saccharalis*. Pest Manag. Sci. 68, 718-30.

Hakim, R. S., Baldwin, K., Smagghe, G., 2010. Regulation of midgut growth, development, and metamorphosis. Ann. Rev. Entomol. 55, 593-608.

Hakim, R. S., Blackburn, M. B., Corti, P., Gelman, D. B., Goodman, C., Elsen, K., Loeb, M. J., Lynn, D., Soin, T., Smagghe, G., 2007. Growth and mitogenic effects of arylphorin *in vivo* and *in vitro*. Arch. Insect Biochem. Physiol. 64, 63-73.

Hernández-Martínez, P., Navarro-Cerrillo, G., Caccia, S., de Maagd, R. A., Moar, W. J., Ferré, J., Escriche, B., Herrero, S., 2010. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. PLoS ONE. 5, e12795.

Herrero, S., Ansems, M., Van Oers, M. M., Vlak, J. M., Bakker, P. L., de Maagd, R. A., 2007. REPAT, a new family of proteins induced by bacterial toxins and baculovirus

infection in *Spodoptera exigua*. Insect Biochem. Mol. Biol. 37**,** 1109-18.

Huffman, D. L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, F. G., Aroian, R. V., 2004. Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. Proc. Natl. Acad. Sci. USA. 101**,** 10995-1000.

Keller, A., Nesvizhskii, A. I., Kolker, E., Aebersold, R., 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal. Chem. 74**,** 5383-92.

Krishnamoorthy, M., Jurat-Fuentes, J. L., McNall, R. J., Andacht, T., Adang, M. J., 2007. Identification of novel Cry1Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. Insect Biochem. Mol. Biol. 37**,** 189-201.

Kunkel, J. G., Grossniklausbuergin, C., Karpells, S. T., Lanzrein, B., 1990. Arylphorin of *Trichoplusia ni*- Characterization and parasite-induced precocious increase in titer. Arch. Insect Biochem. Physiol. 13**,** 117-125.

Loeb, M., Hakim, R. S., 1999. Cultured midgut cells of *Heliothis virescens* (Lepidoptera): fibronectin and integrin β_1 immunoreactivity during differentiation *in vitro*. Invertebr. Repr. Dev. 35**,** 95-102.

Loeb, M., Martin, P., Narang, N., Hakim, R., Goto, S., Takeda, M., 2001a. Control of life, death, and differentiation in cultured midgut cells of the lepidopteran, *Heliothis virescens*. In Vitro Cellular & Developmental Biology - Animal. 37**,** 348-352.

Loeb, M. J., Brandt, E. P., Birnbaum, M. J., 1984. Ecdysteroid production by testes of the tobacco budworm, *Heliothis virescens*, from last larval instar to adult. J. Insect Physiol. 30**,** 375-381.

Loeb, M. J., Martin, P. A., Hakim, R. S., Goto, S., Takeda, M., 2001b. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. J. Insect Physiol. 47**,** 599-606.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R., Schmidt, O., 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? Insect Biochem. Mol. Biol. 35**,** 729-379.

Martínez-Ramírez, A. C., Gould, F., Ferré, J., 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera : Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. Biocontrol Sci. Technol. 9**,** 239-246.

McNall, R. J., Adang, M. J., 2003. Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. Insect Biochem. Mol. Biol. 33**,** 999-1010.

Meunier, L., Prefontaine, G., Van Munster, M., Brousseau, R., Masson, L., 2006. Transcriptional response of *Choristoneura fumiferana* to sublethal exposure of Cry1Ab protoxin from *Bacillus thuringiensis*. Insect Mol. Biol. 15**,** 475-483.

Mimori, K., Mori, M., Akiyoshi, T., Tanaka, S., Sugimachi, K., 1995. The overexpression of elongation factor 1 gamma mRNA in gastric carcinoma. Cancer. 75**,** 1446-1449.

Nishiitsutsujiuwo, J., Endo, Y., 1981. Mode of action of *Bacillus thuringiensis* delta-endotoxin - Effect on *Galleria mellonella* (Lepidoptera, Pyralidae). Applied Entomology and Zoology. 16**,** 79-87.

Oppert, B., Dowd, S. E., Bouffard, P., Li, L., Conesa, A., Lorenzen, M. D., Toutges, M., Marshall, J., Huestis, D. L., Fabrick, J., Oppert, C., Jurat-Fuentes, J. L., 2012. Transcriptome profiling of the intoxication response of *Tenebrio molitor* larvae to *Bacillus thuringiensis* Cry3Aa protoxin. PLoS ONE. 7**,** e34624.

Palli, S. R., Locke, M., 1987. The synthesis of hemolymph-proteins by the larval midgut of an insect *Calpodes ethlius* (Lepidoptera, Hesperiidae). Insect Biochem. 17**,** 561-572.

Pauchet, Y., Muck, A., Svatos, A., Heckel, D. G., Prei, S., 2008. Mapping the larval midgut lumen proteome of *Helicoverpa armigera*, a generalist herbivorous insect. J. Proteome Res. 7**,** 1629-1639.

Perera, O. P., Shelby, K. S., Popham, H. J., Gould, F., Adang, M. J., Jurat-Fuentes, J. L., 2015. Generation of a transcriptome in a model lepidopteran pest, *Heliothis virescens*, using multiple sequencing strategies for profiling midgut gene expression. PLoS One. 10**,** e0128563.

Perera, O. P., Willis, J. D., Adang, M. J., Jurat-Fuentes, J. L., 2009. Cloning and characterization of the Cry1Ac-binding alkaline phosphatase (HvALP) from *Heliothis virescens*. Insect Biochem. Mol. Biol. 39**,** 294-302.

Raymond, B., Johnston, P. R., Wright, D. J., Ellis, R. J., Crickmore, N., Bonsall, M. B., 2009. A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae. Environmental microbiology. 11**,** 2556-63.

Rodriguez-Cabrera, L., Trujillo-Bacallao, D., Borras-Hidalgo, O., Wright, D. J., Ayra-Pardo, C., 2008. Molecular characterization of *Spodoptera frugiperda-Bacillus thuringiensis* Cry1Ca toxin interaction. Toxicon. 51**,** 681-92.

Sanchis, V., 2011. From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. Agron. Sustain. Dev. 31**,** 217-231.

Sanders, J., Maassen, J. A., Moller, W., 1992. Elongation factor-1 messenger-RNA levels in cultured cells are high compared to tissue and are not drastically affected further by oncogenic transformation. Nucleic Acids Res. 20**,** 5907-10.

Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A., Nishida, E., 1994. Microtubule severing by elongation factor 1 alpha. Science. 266**,** 282-5.

Slaoui, M., Fiette, L., Histopathology procedures: from tissue sampling to histopathologicale evaluation drug safety evaluation. In: J.-C. Gautier, (Ed.). Humana Press, 2011, pp. 69-82.

Smagghe, G., Vanhassel, W., Moeremans, C., De Wilde, D., Goto, S., Loeb, M. J., Blackburn, M. B., Hakim, R. S., 2005. Stimulation of midgut stem cell proliferation and differentiation by insect hormones and peptides. Ann. N. Y. Acad. Sci. 1040**,** 472-475.

Smagghe, G. J., Elsen, K., Loeb, M. J., Gelman, D. B., Blackburn, M., 2003. Effects of a fat body extract on larval midgut cells and growth of lepidoptera. In Vitro Cell Dev Biol Anim. 39**,** 8-12.

Sparks, M. E., Blackburn, M. B., Kuhar, D., Gundersen-Rindal, D. E., 2013. Transcriptome of the *Lymantria dispar* (gypsy moth) larval midgut in response to infection by *Bacillus thuringiensis*. PLoS One. 8**,** e61190.

Spies, A. G., Spence, K. D., 1985. Effect of sublethal *Bacillus thuringiensis* crystal endotoxin treatment on the larval midgut of a moth, *Manduca sexta*. Tissue and Cell. 17**,** 379-394.

Sutherland, P. W., Harris, M. O., Markwick, N. P., 2003. Effects of starvation and the *Bacillus thuringiensis* endotoxin Cry1Ac on the midgut cells, feeding behavior, and growth of lightbrown apple moth larvae. Ann. Entomol. Soc. Am. 96**,** 250-264.

Tanaka, S., Yoshizawa, Y., Sato, R., 2012. Response of midgut epithelial cells to Cry1Aa is toxin-dependent and depends on the interplay between toxic action and the host apoptotic response. FEBS J. 279**,** 1071-1079.

Tang, B., Wang, S., Zhang, F., 2010. Two storage hexamerins from the beet armyworm *Spodoptera exigua*: Cloning, characterization and the effect of gene silencing on survival. BMC Mol. Biol. 11**,** 65.

Valaitis, A. P., 2008. *Bacillus thuringiensis* pore-forming toxins trigger massive shedding of GPI-anchored aminopeptidase N from gypsy moth midgut epithelial cells. Insect Biochem. Mol. Biol. 38**,** 611-618.

van Frankenhuyzen, K., Nystrom, C., The *Bacillus thuringiensis* toxin specificity database. 2015.

van Munster, M., Prefontaine, G., Meunier, L., Elias, M., Mazza, A., Brousseau, R., Masson, L., 2007. Altered gene expression in *Choristoneura fumiferana* and *Manduca sexta* in response to sublethal intoxication by *Bacillus thuringiensis* Cry1Ab toxin. Insect Mol. Biol. 16**,** 25-35.

Zhang, B., VerBerkmoes, N. C., Langston, M. A., Uberbacher, E., Hettich, R. L., Samatova, N. F., 2006. Detecting differential and correlated protein expression in label-free shotgun proteomics. Journal of Proteome Research. 5**,** 2909-2918.

Table I: Identified proteins with significant differences in abundance between buffer and Cry1Ac secretomes (Fisher's Exact Test; $P<0.005$). The weighted spectral levels detected for each protein in the buffer and Cry1Ac induced secretomes are shown.

Contig	AA[a]	#P[b]	Protein	E value	Accession #	Species	Id[c]	Cov[d]	Buffer	Cry1Ac	*P*[e]
997	747	26	Cry1A toxin receptor A	0.0	AAF08254.1	*Heliothis virescens*	98%	93%	67	0	<0.0001
2013	688	7	Arylphorin	0.0	AEO51737.1	*Helicoverpa armigera*	85%	96%	0	10	0.0010
2924	630	2	Uncharacterized family 31 glucosidase KIAA1161-like	0.0	XP_013174005.1	*Papilio xuthus*	71%	98%	26	53	0.0017
6027	363	7	Elongation factor 1 gamma	3e-168	NP_001298516.1	*Papilio polytes*	75%	79%	9	0	0.0019

[a]Number of amino acids in the translated contig.
[b]Number of exclusive unique peptides detected for the identified protein by mass spectrometry.
[c]Identity percentage between the translated contig and the NCBI protein match.
[d]Percentage of the translated contig sequence covered by the matched NCBI protein.
[e]Fisher's test probability estimate of comparisons between Buffer-Cry1Ac secretomes. Significance was considered at $P<0.005$.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2878v1 | CC BY 4.0 Open Access | rec: 16 Mar 2017, publ: 16 Mar 2017

640 **Figure legends**

641 **Figure 1**: Response in *H. virescens* midgut stem cell cultures in response to Cry1Ac or
642 buffer-induced secretomes. Primary midgut stem cell cultures were prepared and treated
643 with Cry1Ac or toxin buffer as a control. Cell type composition after indicated time
644 intervals was determined using a fluorescence-based method, with events gated into
645 individual cell type populations (Castagnola et al., 2011). Shown are the total mean number
646 of cells of a specific type and the corresponding standard error calculated from four
647 independent measurements. The symbol used for each cell type and treatment is indicated in
648 the figure.

649

650 **Figure 2**: Testing of the mitogenic effect of arylphorin in *H. virescens* larvae and its effect
651 on susceptibility to Cry1Ac. A) Histological examination of midgut epithelial tissues from
652 *H. virescens* larvae after feeding for 5 days on diet contaminated with control buffer (A) or
653 29.2 ng/cm^2 of purified arylphorin (B). After midgut dissection, tissues were embedded and
654 stained with hematoxylin and eosin (H&E). C) Total number of cells per 100 µm^2 of midgut
655 epithelial tissue in control or after treatment with 29.2 ng/cm^2 of arylphorin for 5 days, as
656 indicated. Small bars denote standard error of the mean for each treatment obtained from
657 sections counted from three independent midgut tissues; different letters for each column
658 denote statistically significant differences ($P< 0.05$, Student's t -test) among treatments. D)
659 Percentage mortality of *H. virescens* larvae exposed to meridic diet (----) or 29.2 ng/cm^2 of
660 purified arylphorin (Arylphorin) for five days, and then to diet containing 0.5 µg/cm2
661 Cry1Ac toxin (Cry1Ac) for seven days. Bars denote standard error of the mean for each
662 treatment of 16 larvae and calculated from three bioassay replicates; statistically significant
663 differences between treatment and control groups are denoted by different letters for each
664 column (P< 0.05, One-way ANOVA).

665

Figure 1

Influence of secretomes on primary midgut cell cultures

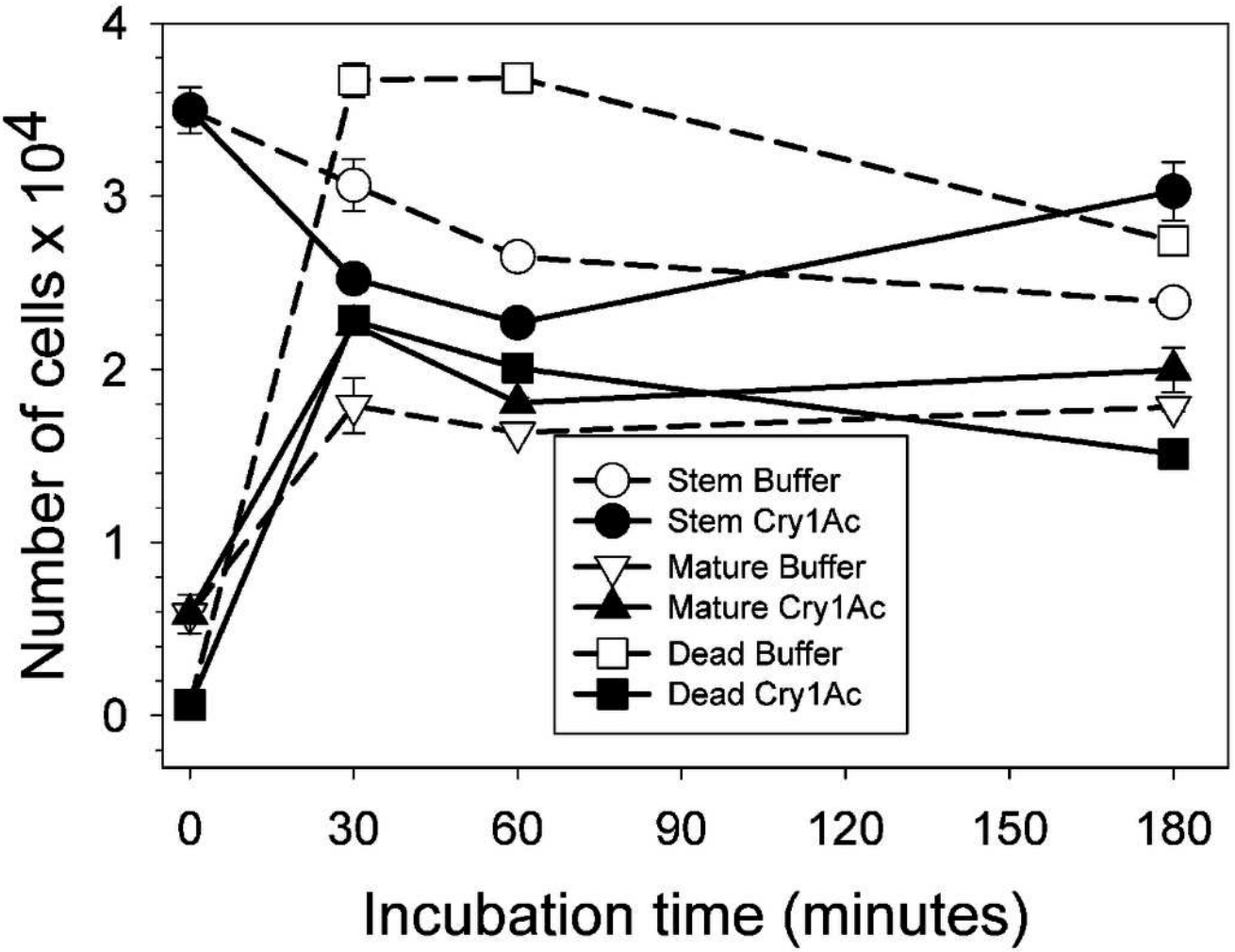

Figure 2

Mitogenic effect of arylphorin and effect on susceptibility to Cry1Ac toxin