ABSTRACT

MAGALHAES, LEONARDO COELHO. Managing Insects and Insect Resistance: From Apple Orchards to Transcriptomics. (Under the direction of Dr. James F. Walgenbach.)

Apple is a primary fruit crop in North Carolina. The codling moth, *Cydia pomonella* (L.), and oriental fruit moth, *Grapholita molesta* (Busck), are two key apple pests in North Carolina. Management of these two insects has been mainly achieved by constant application of broad-spectrum insecticides, primarily organophosphates (more than 40 years). Due to organophosphate resistance development and regulatory actions, apple growers are transitioning to management programs that use new, reduced-risk insecticides.

This study evaluated the toxicity of nine new insecticides to eggs, larvae and adults, assessed their relative toxicity and residual activity to codling moth and oriental fruit moth. Results showed that codling moth and oriental fruit moth larvae were very susceptible to many of these new compounds. Moreover, some insecticides presented better or at least comparable results to azinphosmethyl, a long used standard material, in the residual experiments.

This transition to new chemistry insecticide could be threatened by the development of resistance or cross resistance to older insecticides. Codling moth, especially, is well-known for developing resistance to many insecticide groups in almost every apple growing region. Thus, establishing baseline susceptibility levels and using reliable bioassays are essential steps in insecticide resistance programs. There are a number of different bioassays to monitor for codling moth resistance; however, many are not applicable to new insecticides or may take months to complete.

Contact insecticides, acetamiprid and azinphosmethyl, were significantly more toxic to neonates than 4th instars. However, there was no significant difference in neonate and 4th

instar response to ingestion insecticides, chloronantrinilipole, methoxyfenozide, novaluron and spinetoram. Field colonies that presented control failures were significantly more resistant than the laboratory colony and the colony established form abandoned orchards. A diagnostic dose of 20 ug/ml (LC_{99}) was established to monitor for codling moth resistance to methoxyfenozide. These results demonstrated a novel and rapid bioassay can be used to monitor codling moth resistance to methoxyfenozide.

The use of *Bacillus thuringiensis* (*Bt*) transgenic plant technologies has practically eliminated caterpillar damage in crops; however, so far, this approach is completely ineffective against Hemiptera pest species. The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is a very destructive pest to many crops, but it has been more noticeable in Bt cotton. Furthermore, the development of resistant populations of tarnished plant bug to common insecticides makes it harder to keep pestiferous populations below economic damage.

Ideally, a transgenic approach for sucking herbivores as efficient as *Bt* crops for caterpillars, could drastically change this scenario. The digestive system is the primary interface between the insect and transgenic plants and the most important relative to the use of transgenic insecticidal proteins for insect control. The goal of this study was to develop the first 454 whole body and digestive system transcriptomes to a plant bug to enhance our current knowledge on plant-sucking insect digestion. Here we have identified eight proteins, leptin receptor, adiponectin receptor, tachykinin precursor, GPCRs, proprotein convertases that could be used as potential targets to control this pest.

Managing Insects and Insect Resistance: From Apple Orchards to Transcriptomics

by
Leonardo Coelho Magalhaes

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Entomology

Raleigh, North Carolina

2011

APPROVED BY:

James Walgenbach Chair of Advisory Committee	Michael Roe
George Kennedy	Fred Gould

BIOGRAPHY

Leonardo Coelho Magalhaes is originally from Rio de Janeiro, Brazil. At age of 7 he moved to Sao Paulo where he grew up. In 1998, he moved to Vicosa to pursue his undergraduate degree in agronomy. After a month in Vicosa he was introduced to the fascinating world of insects. In Vicosa he was under the supervision of Dr. Raul Guedes working with pest management and toxicology. After that, he quickly became interested in eventually pursuing a PhD in Entomology.

After an exchange program in Minnesota in 2002, Leonardo decided to get a M.S. in Entomology in the U.S. Right after graduating from Vicosa in 2004, he started his M.S. program in Entomology in Nebraska in the fall of the same year under the direction of Drs. Thomas Hunt and Blair Siegfried.

Then, in 2007, he started the PhD program in Entomology (minor in Biotechnology) under the orientation of Drs. James Walgenbach and Michael Roe. Currently, he is working full-time as an Entomologist in the research and discovery screening group at Bayer CropScience.

ACKNOWLEDGEMENTS

There were many people involved in this project that I have to acknowledge. First I would like to express my sincere appreciation to my graduate committee: Drs James Walgenbach, Michael Roe, Fred Gould and George Kennedy for constructive criticism, guidance during my research program and coursework. I am especially grateful to my major advisors, Drs Walgenbach and Roe for recruiting me to the Department of Entomology at NCSU and believing that I could learn molecular biology. Thanks for your guidance, constant support, willingness to allow me trying my own ideas and interest in my professional and personal growth. I could not have asked for a better combination of projects and advisors for my career goals. Dr. Roe always says that anyone can learn molecular biology and now I truly believe his words.

I would like to thank other lab members for fostering a positive, fun and interactive working environment. I thank: Drs Ana Cabrera, Brooke Bissinger, Charles Apperson, Christof Stumpf, Jaap van Kretschmar, Katalin Böröczky, Kevin Donohue, Logananthan Ponnusamy and Sayed Khalil and Fred Stell, Luma Abu-Ayyash and Nicholas Kimps for their friendship. I am particularly grateful to Ana, Brooke, Jaap, Kevin and Sayed for their willingness and patience to teach me molecular biology, positive criticism and suggestions to my project obstacles. Your support enriched this thesis.

Other members at the Mountain Horticulture Research and Extension Station were critical to this research. Elijah Meck, Monica Schiessl, Nicole Orengo, Steve Schoof, Vonny Barlow and other innumerous summer workers were always willing to assist in the rearing of the insects, conducting some of the bioassays, apple collections and colony maintenance.

I have to extend my gratitude to other members of the department and the university that provided assistance with research questions and departmental and university procedures. Joyce Taylor and Pat Bacheler made my life easier by assisting me with every paperwork and procedures I needed in the department. I have to thank Drs. Allen Cohen, Jack Bacheler and Eric Blinka for sharing their expertise about *Lygus lineolaris* collection, rearing and cotton pests in general. Drs. Nigel Deighton and Megan R. Ulmer assisted with 454 sequencing. Drs. Deborah Thompson, Charles Opperman, Elizabeth Schol and Alonso Suazo assisted with bioinformatics and RNAi work.

For funding, I have to acknowledge the North Carolina Agricultural Research Service, USDA and Cotton Inc. Funds from the Department of Entomology and the NIH/MBTP NCSU program were also greatly appreciated.

I have to express my great appreciation for my former mentors. Drs. Blair Siegfried, Raul Guedes and Thomas Hunt that continue to support my career, look after my future and provide great advices for my professional and personal goals.

I specially thank my family, my wife Marilia Oliveira and my daughter Sophia Magalhaes. Your encouragement, patience, love and constructively criticizing were vital to this project, especially during many hard moments. My daughter does not know yet (she is almost 2 years old), but her long naps at night when she was a newborn helped me to stay rested and focused in my studies. Thanks for providing the comfort that I needed to be successful during this journey.

TABLE OF CONTENTS

LIST OF TABLES……………………………………………………………………………vi

LIST OF FIGURES…………………………………………………………………………vii

CHAPTER I: Baseline Susceptibility and Residual Activity of New Insecticides to Codling Moth and Oriental Fruit Moth…………………………………………………….......1
- Abstract……………………………………………………………………………..2
- Introduction…………………………………………………………………………3
- Materials and Methods……………………………………………………………...4
- Results………………………………………………..…………………………10
- Discussion………………………………………………………………………13
- Acknowledgements…………………………………………………………….…17
- References cited..……………………………………………………………………17
- Figures and tables…………………………………………………………………21

CHAPTER II: Development of a Rapid Methoxyfenozide-Resistance Monitoring Bioassay for Codling Moth………………………………………………………………………….30
- Abstract……………………………………………………………………………31
- Introduction………………………………………………………………………..32
- Materials and Methods…………………………………………………………….34
- Results and Discussion……………………………………………………………38
- Conclusions………………………………………………………………………..42
- Acknowledgements……………………………………………………………..…43
- References…………………………………………………………………………44
- Figures and tables…………………………………………………………………49

CHAPTER III: Pyrosequencing of the Gut and Whole Body Transcriptome of the Tarnished Plant Bug, *Lygus lineolaris*, and Identification of Putative Genes Important in Insect Growth and Development…………………………………………………………….……………55
- Abstract……………………………………………………………………………56
- Introduction………………………………………………………………………..57
- Materials and Methods…………………………………………………………….60
- Results……………………………………………………………………………..64
- Discussion…………………………………………………………………………71
- Acknowledgements…………………………………………………………….…80
- References…………………………………………………………………………80
- Figures and tables…………………………………………………………………90

LIST OF TABLES

Chapter I

Table 1. Susceptibility of codling moth and oriental fruit moth eggs to 10 insecticides..22

Table 2. Codling moth and oriental fruit moth neonate susceptibility to six different insecticides……………………………………………….………………………………23

Table 3. Codling moth and oriental fruit moth larval susceptibility to nine different insecticides (larval stage exposure)…………………………….……………………..24

Table 4. Codling moth and oriental fruit moth male adult susceptibility to acetamiprid and azinphosmethyl……………………………………………………………………...25

Table 5. Mean codling moth percentage egg hatch and number of larval entries per hatched egg into apples………………………………………………………………..26

Table 6. Mean oriental fruit moth percentage egg hatch and number of larval entries per hatched eggs into apples ………………………………………………………………...28

Chapter II

Table 1. Duration of codling moth larval stages feeding on bean based artificial diet….51

Table 2. Neonate and 4th instar codling moth response to acetamiprid, azinphosmethyl, chloronantrinilipole, novaluron and spinetoram……….……………….…………………..52

Table 3. Response of laboratory population 10-d old larvae to methoxyfenozide LC_{95}...53

Table 4. Mortality of populations of codling moth at discriminating concentration of methoxyfenozide.……………………………………………...…………………...54

Chapter III

Table 1. Summary statistics for *L. lineolaris* gut and whole body assembly and annotation…………………………………………………………………………………..90

Table 2. Top 3 enzyme terms in each enzyme classification (EC)…...……...…………91

Table 3. Top5 combined InterPro predictive protein families and domains.……………94

LIST OF FIGURES

Chapter I

Figure 1. Mean codling moth number of larval entries per apples collected from chloranantrinilipole and spinetoram sprayed trees……………………………………21

Chapter II

Figure 1. Top view of a single 16-well bioassay plasticware containing 200 µl of lyophilized codling moth artificial diet……………………………………………….49

Figure 2. Comparative toxicity of methoxyfenozide to different codling moth colonies to neonate and 4th instar…………………………………………………………………50

Chapter III

Figure 1. Overview of homology searches……………………………………………95

Figure 2. Top BLAST hit species distribution for each contig…………………………..96

Figure 3. Gene ontology (GO) assigned terms…………………………………………..97

Figure 4. Alignment of *L. lineolaris* putative leptin receptor……………………………99

Figure 5. Alignment of *L lineolaris* putative adiponectin receptor…………………….100

Figure 6. Alignment of *L lineolaris* putative tachykinin precursor…………………….101

Figure 7. Alignment of *L lineolaris* putative neuropeptide Y receptor………...………102

Figure 8. Alignment of *L lineolaris* putative CAPA receptor………………………….103

Figure 9. Alignment of *L lineolaris* putative GPR 155……………………………...104

Figure 10. Alignment of *L lineolaris* putative furin…………………………………106

Figure 11. Alignment of *L lineolaris* (Ll) putative proprotein convertase (type-2)……108

Chapter I

For possible publication in *Journal of Economic Entomology*
Address Correspondence to:

James F. Walgenbach
MHCREC, 455 Research Dr.
Department of Entomology
North Carolina State University
Mills River, NC 28759
Phone: 828 684-3562
Email: jim_walgenbach@ncsu.edu

Baseline Susceptibility and Residual Activity of New Insecticides to Codling Moth and Oriental Fruit Moth

Leonardo C. Magalhaes and James F. Walgenbach*

Department of Entomology, North Carolina State University, Raleigh, NC 27695; *Address correspondence to: jim_walgenbach@ncsu.edu

Abstract: The codling moth, *Cydia pomonella* (L.), and oriental fruit moth, *Grapholita molesta* (Busck), are two key apple pests in North Carolina. Growers have relied extensively on organophosphate insecticides, primarily Guthion (azinphosmethyl), for more than 40 years to manage these pests. Due to organophosphate resistance development and regulatory actions, apple growers are transitioning to management programs that use new, reduced-risk insecticides. This study evaluated the toxicity of these new insecticides to eggs, larvae and adults, assessed their relative toxicity and the residual activity to codling moth and oriental fruit moth. A laboratory susceptible strain of both species, which were collected from commercial orchards in Henderson County, NC, in 1998, were used for all bioassays. For larval studies, insecticides were topically applied to the surface of the diet, onto which neonates were placed. Toxicity was based on two measures of mortality; 5-day mortality and development to adult stage. Results showed that codling moth and oriental fruit moth larvae were very susceptible to many of these new compounds. Moreover, some insecticides presented better or at least comparable results to azinphosmethyl in the residual experiments. However, some of these new compounds did not present ovicidal activity, which may difficult their use in apple orchards. Both the codling moth and oriental fruit moth have developed resistance to a diversity of insecticides used for their control. Thus, establishing baseline susceptibility levels is an essential first step in insecticide resistance programs for these two main apple pests in North Carolina.

Keywords: codling moth, oriental fruit moth, reduced-risk insecticides, baseline

The codling moth, *Cydia pomonella* (L.), has long been recognized as a world-wide pest of apple (Barnes 1991), and in the past decade oriental fruit moth, *Grapholita molesta* (Busck), has emerged as an important pest of apple, particularly in the eastern United States (Borchert et al. 2004a, Myers et al. 2006). Larvae of both species are internal-feeders of fruit, and, due to the high value of host crops and strict quality standards for both domestic and export markets, there is a near-zero tolerance for damage. Consequently, insecticides have played a key role in the management of these insects. While mating disruption is playing an increasingly important role in the management of these insects (Calkins and Faust 2003, Kovanci et al. 2005), insecticides have and will continue to be critical tools in managing these pests.

Organophosphate insecticides have played an important role in managing apple insect pests for ~40 years, but resistance development in codling moth (Bush et al. 1993, Knight et al. 1994, Dunley and Welter 2000, Contreras et al. 2007, Reyes et al. 2007) and oriental fruit moth (Usmani and Shearer 2001, Shearer and Usmani 2001), along with the loss of chemicals through regulatory actions, has reduced the availability of organophosphates as management tools. Consequently, the US apple industry is transitioning away from organophosphate insecticides in favor of new chemicals with more friendly environmental and human health profiles. These products represent a diversity of modes of action, and include neonicotinoids, spinosyns, anthranilic diamides, and insect growth regulators including the benzoylureas, diacylhydrazine and juvenile hormone mimics.

The successful transition from organophosphates to new chemistry insecticides for management of codling moth and oriental fruit moth is dependent, in part, on understanding

the relative toxicity of products to various life stages of insects. Knowledge of life-stage specificity is important for determining application timing in the field and for devising bioassays in resistance monitoring programs. For instance, organophosphates commonly used for control of lepidopterans in apples, such as azinphosmethyl, have activity to multiple life stages including eggs, larvae and adults. With this chemistry, insecticides are typically applied at initial egg hatch, and adults have been used for resistance monitoring bioassays (Dunley and Welter 2000). In contrast, insect growth regulators used in apple systems, such as the ecdysone agonists, tebufenozide and methoxyfenozide, are ovicidal and larvicidal, but not toxic to codling moth (Sun and Barrett 1999) or oriental fruit moth adults (Borchert et al. 2004b); although, they can exhibit sublethal effects to adults that adversely affect populations (Sun and Barrett 1999). Consequently, these insecticides are typically applied at initial egg laying rather than egg hatch (Borchert et al. 2004b), and larvae are used in resistance monitoring bioassays (Mota-Sanchez et al. 2008).

The purpose of this study was to assess the relative toxicity of these new insecticides against different life stages of the codling moth and oriental fruit moth, and to establish baseline dose-response curves of larvae to various insecticides.

Materials and Methods

Insects. The insects used in bioassays were obtained from laboratory colonies maintained at Mountain Horticultural Crops Research and Extension Center in Mills River, North Carolina. Colonies of both insects were collected from commercial orchards in Henderson County, NC, in 1998, and have been continuously reared on a bean-based diet

(Yokoyama et al. 1987).

Chemicals. The insecticides were used as commercial formulations for the ovicidal and larvicidal bioassays. The formulated insecticides were acetamiprid (Assail 70WP, Cerexagri, King of Prussia, PA), methoxyfenozide (Dow Agrosciences, Indianapolis, IN), indoxacarb (Avaunt 30WG, DuPont, Wilmington, DE), spinosad (SpinTor 2SC, Dow Agrosciences, Indianapolis, IN), chloronantrinilipole (Altacor WG, DuPont, Wilmington, DE), spinetoram (Delegate WG, Dow Agrosciences, Indianapolis, IN), thiacloprid (Calypso 4F, Bayer CropScience, Research Triangle Park, NC), novaluron (Rimon 10EC, Chemtura, Middlebury, CT) and pyryproxyfen (Esteem 35WP, Valent Richardson, TX). Azinphosmethyl (Guthion 50WP, Bayer CropScience, Research Triangle Park, NC) was also included, because it is a standard organophosphate commonly used for codling moth and oriental fruit moth in apples. Azinphosmethyl and acetamiprid were used as technical grade for the adult bioassay because it was diluted in acetone.

Ovicidal Bioassay. This bioassay followed the procedures of Borchert et al. (2004b). In summary, for each species, 10 male and 15 female pupae were placed into an oviposition chamber. The chamber consisted of a 4-liter cylindrical plastic white bucket (20 cm in diameter by 16.5 cm in height) lined with cheesecloth to prevent adults from laying eggs on the sides of the container. Each container was supplied with a sucrose solution, 5 and 10% for codling moth and oriental fruit moth, respectively. Oriental fruit moth containers were also supplied with water. Sucrose solution and water were supplied in small Petri dishes (3 cm in diameter) with cotton and lined with cheesecloth. “Golden Delicious” apples were positioned in the oviposition chambers 2-3 d after moths emerged and mated.

At least four concentrations and three replications were prepared from serial dilutions by using distilled water. A surfactant, Latron B1956 (Rohm and Haas), at the rate of 1 drop/liter was added to improve residual coverage. Fruits were dipped into insecticidal solutions or water (control) for 5 s, removed and a gentle air stream produced by a small fan dried the fruits for 2 h. Nine fruits were placed into oviposition chambers. Fruits were left overnight (± 14 hours) in the oviposition chambers and then removed. Oviposition chambers were held in a rearing room 25°C, ~60% RH, and a photoperiod of 16:8 (L:D) h. Eggs were counted and fruits were held in similar sealed plastic containers for 7 d at the same environmental conditions as above. Eggs were then examined to determine percentage of hatch.

Larval Bioassays. Toxicity was based on two measures of mortality; 5-day neonate mortality, and neonate to adult development. The five-day neonate mortality test was conducted using 20-cell plastic trays, each cell held 2 ml. Each cell was filled with 1 ml of diet, dispensed with a repeating dispenser pipette, and 100 ul aliquots of insecticide solution was topically applied to the surface of diet. A single neonate larva was placed in each cell, after which the cells were sealed with a Mylar membrane using a warm iron. At least six concentrations per insecticide that resulted in >1% and <99% mortality was tested. Distilled water was used as the control and Latron was added to all solutions at 1 drop/liter. Two pin size holes were made in each cell to allow air circulation. The trays were placed in a rearing room at 25°C, ~60% RH, and a photoperiod of 16:8 (L:D) h. Mortality was checked after 5 days and larvae were considered dead when they were unable to move when probed by a camel-hair brush. Moribund larvae were considered dead.

The neonate to adult bioassay used 20 ml plastic cups. The cups were filled with ~6

ml of bean based diet, and 200 ul of insecticide solution was topically applied to the surface of the diet once it had cooled down. After treatment with insecticide solutions, cups were placed in a laboratory hood for 6 h where a gentle stream of air dried the top of the diet after. Again three replications of at least six concentrations each per insecticide that resulted in mortality ranging from >1% to <99% was tested. As above, distilled water was used as a control and Latron was added to solutions at 1 drop/liter. Diet cups were placed in a rearing room at 25°C, ~60% RH, and a photoperiod of 16:8 (L:D) h throughout the larval development period. Mortality was based on the number of individuals that did not complete development to the adult stage.

In both assays, diet was not allowed to over-dry before larvae were placed on diet, as this could have resulted a crack on the surface or a gap between the diet and side of the cell, both of which may allow a larva to avoid contact or ingestion of test insecticides. Cells in which diet cracked on the surface or was separated from the side of the cell were discarded from the analysis. Replicates with control mortalities > 20% were also discarded.

Adult Bioassay. Our objective in this bioassay was to compare the toxicity of the neoticiotinoid acetamiprid to the organophosphate azinphosmethyl because acetamiprid is one the few new chemistry insecticides that is acutely toxic to codling moth, and baseline acute toxicity of azinphosmethyl to codling moth is well established (Dunley and Welter 2000). The methodology of Dunley and Welter (2000) was followed for this bioassay. Briefly, one-day old male moths were placed dorsal side down on cards treated with a thin coating of Tangle-Trap sticky coating (Tanglefoot, Grand Rapids, MI), and then 1 μl of the appropriate insecticide concentration was applied to the ventral surface of the abdomen. The

insecticide was applied with a microsyringe mounted in a repeating dispenser (Hamilton, Reno, NV). Moths were cooled for 10 min at 4°C to facilitate insecticide application and moth transfer to sticky cards. Five concentrations of each pesticide were tested and three replicates of 15 moths were assayed per concentration. Technical grade insecticide dissolved in acetone was used for bioassays, and acetone alone was used as a control. After treatment, the sticky cards containing moths were placed in a growth chamber at 15 ± 2°C and 60% R.H. for 48 h. Moths were considered dead when there was no vigorous leg or wing movement after the abdomen was brushed with a fine camel-hair brush. Control mortality did not exceed 20% in any of the bioassays performed.

Residual Study. This study was conducted in 2004 and 2008 at the Mountain Research Crop Station in Mills River, NC. In 2004, six insecticide treatments and a control (water only) were each applied on 8 July and 9 August, and included acetamiprid (Assail 70WP) at 0.15 kg[ai]/ha, azinphosmethyl (Guthion 50WP) at 1.12 kg[ai] /ha, indoxacarb (Avaunt 30WDG) at 0.11 kg[ai]/ha, methoxyfenozide (Intrepid 2F) at 0.28 kg[ai]/ha, novaluron (Rimon 0.83EC) at 0.15 kg[ai]/ha and thiacloprid (Calypso 4SC) at 0.20 kg[ai]/ha. Plots consisted of five 18-y-old "Golden Delicious" apple trees. Apples for bioassays were sampled from the middle three trees. Applications were made with an airblast sprayer delivering 935 liters/ha.

To assess the residual activity of the insecticides against codling moth and oriental fruit moth eggs and neonates, 10 randomly chosen apples per treatment were removed on the day of application and at 7, 10, 14, 21 and 28 days after application. Apples were transferred to the laboratory and five fruit from each treatment were placed in separate codling moth and

oriental fruit moth oviposition buckets. The chambers followed the same design previously described in the ovicidal bioassay. Oviposition chambers were placed in a rearing room at 25°C, ~60% RH, and a photoperiod of 16:8 (L:D) h, and apples were removed from the buckets after an overnight oviposition period (about 14 h). Eggs were counted and fruits were held in similar sealed plastic containers under the same conditions as described for the oviposition bioassays. A week later the number of hatched eggs and entries were recorded.

In 2008, only the two newest insecticides were evaluated, chloronantrinilipole (Altacor 35WG) at 0.074 kg[ai]/ha and spinetoram (Delegate 25WG) at 0.23 kg[ai]/ha, and both only against codling moth. Insecticides and a water control were applied on 21 May and 5 June. The study was conducted in the same orchard as the 2004 plots, following the same procedure previously described. The ovicidal residual activity was not conducted in 2008; however, the larvicidal residual effects were evaluated as five apples per treatment were harvested and exposed to neonates; four neonates were transferred to each test apple. Apples were kept in buckets in the same conditions as described for the 2004 study.

Statistical Analysis. Mortality data were analyzed by probit analysis (Finney, 1971) using POLO-PC (LeOra Software 2002). The residual activity data were analyzed as number of entries per hatched eggs (or neonates placed on fruit in 2008) using analysis of variance (ANOVA) (PROC MIXED). For all significant ANOVA's ($P \leq 0.05$), Fisher's Protected Least Significance Difference (LSD) was performed (SAS Institute 2002) to separate differences among means. Treatment differences were considered significant ($P \leq 0.05$) or marginally significant ($0.1 \leq P > 0.05$).

Results

Ovicidal Bioassay. The three insect growth regulators, pyriproxyfen, novaluron and methoxyfenozide, all exhibited considerable ovicidal activity against codling moth with LC_{50} values <5 µg/ml (Table 1). Among the neonicotinoids, acetamiprid had greater ovicidal activity against codling moth compared with thiacloprid, with LC_{50} of 1.05 and 66 µg/ml, respectively. All remaining insecticides tested had LC_{50} values >300 µg/ml. It should be noted; however, that the estimated LC_{50} of azinphosmethyl (309 µg/ml), is about three times lower than typical field use rates (~1,000 µg/ml).

Oriental fruit moth eggs generally appeared to be more tolerant to all insecticides tested compared with codling moth; although LC_{50} values for the two insects did overlap for all insecticides except methoxyfenozide, pyriproxyfen, and acetamiprid. Novaluron and acetamiprid had the greatest activity against oriental fruit moth eggs with LC_{50} values of 7.86 and 17.28 µg/ml, respectively. Results with methoxyfenozide and pyriproxyfen were most striking, with the methoxyfenozide LC_{50} value to oriental fruit moth eggs being >100 times higher than codling moth, and the LC_{50} for pyriproxyfen to oriental fruit moth eggs being >1000 µg/ml.

Larvicidal Bioassays. In contrast to results obtained with eggs; insecticides were generally more toxic to oriental fruit moth than to codling moth neonates based on 5-d mortality counts. Methoxyfenozide was an exception, with LC_{50} values being ~8.5X higher for oriental fruit moth than codling moth (5.6 versus 0.65 µg/ml) (Table 2). LC_{50} values for the remaining insecticides were 1- to 7-fold lower for oriental fruit moth. Chloronantrinilipole and spinetoram were the most toxic to neonates of both species, with

LC_{50} values of 0.08 and 0.06 µg/ml for codling moth, and 0.04 and 0.05 µg/ml for oriental fruit moth, respectively. Spinosad was also very toxic to codling moth neonates (LC_{50} 0.97 µg/ml). Methoxyfenozide was very toxic to codling moth (LC_{50} 0.65 µg/ml), but not to oriental fruit moth (LC_{50} 5.60 µg/ml). Acetamiprid and novaluron presented moderate toxicity levels. Thiacloprid had low toxicity to codling moth (LC_{50} 9.9 µg/ml) and oriental fruit moth neonates (LC_{50} 2.02 µg/ml) and pyriproxyfen was the least toxic insecticide tested. The standard insecticide, azinphosmethyl, was moderately toxic to neonates of both insects.

When LC_{50} values were calculated based on the entire length of larval development (i.e., survivorship based on successful development to the adult stage), again chloronantrinilipole and spinetoram were, generally, most toxic to codling moth and oriental fruit moth larvae (Table 3). Furthermore, novaluron and methoxyfenozide were also very toxic to larvae of both species. Spinosad was of intermediate toxicity among the insecticides tested. Acetamiprid, thiacloprid and pyriproxyfen had opposite results for the two insects. The neonicotinoids, acetamiprid and thiacloprid, were more than 10 times more toxic to codling moth than oriental fruit moth larvae. Even more striking, pyriproxyfen was more than 100 times more toxic to codling moth than oriental fruit moth. Azinphosmethyl was the least toxic insecticide against codling moth; however, it was moderately toxic to oriental fruit moth.

Adult bioassay. Among the new chemistry insecticides used in apple for lepidopteran control, the neonicotinoids are the only group with acute toxicity to moths. Acetamiprid was slightly more toxic than azinphosmethyl to codling moth, with an LC_{50} value of 0.04 and 0.10 µg/ml, respectively (Table 4). Acetamiprid also had a lower LC_{50}

value to oriental fruit moth adults, although these values did not differ based on overlapping confidence intervals.

Residual study. Based on percentage egg hatch over time, methoxyfenozide and novaluron exhibited the longest ovicidal activity against codling moth in the 2004 study (Table 5). In both application dates, 8 July and 9 August, methoxyfenozide and novaluron consistently presented the highest ovicidal activity throughout the 28 day experiment. The neonicotinoids, acetamiprid and thiacloprid showed moderate levels of ovicidal residual activity which was similar to azinphosmethyl. Indoxacarb had the poorest ovicidal activity among the insecticides tested. Similar results were obtained regarding the larvicidal activity (entries/hatched egg), as novaluron and methoxyfenozide again had the longest residual activity. Moreover, the neonicotinoids were comparable to novaluron and methoxyfenozide, and throughout the experiment significantly reduced entries compared to the control for 28 days after treatment. Azinphosmethyl was initially toxic to neonates, but did not differ from the control after about 21 d. Indoxacarb appeared to have the shortest residual activity against larvae, with entries per hatched egg not differing from the control after 10 days.

Similar results were observed for oriental fruit moth (Table 6). Although ovicidal data was more variable for oriental fruit moth than codling moth, novaluron and methoxyfenozide generally had the longest residual activity against eggs, with thiacloprid being the only other insecticide with significant ovicidal activity for up to 10 days after treatment. Larvicidal residual activity was generally shorter against oriental fruit moth than codling moth, with no differences between insecticide treatments and the control 21 days after the 8 July application and 14 days after the 9 August application.

In the 2008 residual study where neonates were placed on apples, larvae entries in chloranantrinilipole and spinetoram treated apples did not differ from the control after 13 days, although numerically chloranantrinilipole tended to have fewer larval entries than the control (Fig. 1). However, after the second application two weeks later on 5 June, both insecticides exhibited activity for up to 42 days after treatment. Additionally, chloranantrinilipole continued to be efficient even 49 days after treatment (Fig. 1). Overall, chloranantrinilipole had a better residual activity than spinetoram against codling moth larvae.

Discussion

Organophosphate insecticides, principally azinphosmethyl, have played a key role in managing insect pests of apples for >40 years. Where resistance has not evolved, azinphosmethyl is still very effective against all life stages of codling moth and oriental fruit moth. However, organophosphate resistance and cross resistance is widespread among codling moth and oriental fruit moth populations (Bush et al. 1993, Contreras et al. 2007, Dunley and Welter 2000, Knight et al. 1994, Mota-Sanchez et al. 2008, Reyes at al. 2007, Reuveny and Cohen 2004 Usmani and Shearer 2001, Shearer and Usmani 2001), which greatly reduces its usefulness. Furthermore, many organophosphate insecticides have been lost to regulatory decisions related to reassessment under the Food Quality Protection Act, and azinphosmethyl will not be allowed for use on apples after 2012. As a result, apple growers have and will continue to transition to pest management programs that use reduced-risk and alternative insecticides. Results presented here provide information useful in

helping to devise proper use patterns of new chemistry insecticides, and provide baseline toxicity values for resistance monitoring.

Oriental fruit moth eggs were generally more tolerant to insecticides compared to codling moth eggs. This was most evident with the insect growth regulators novaluron, methoxyfenozide and pyriproxyfen, all of which were highly toxic to codling moth eggs, with LC_{50} values considerably lower than field use rates (Table 1). Among these insecticides, however, only novaluron exhibited a high degree of toxicity to oriental fruit moth eggs, and pyriproxyfen was essentially non toxic to oriental fruit moth. Borchert et al. (2004b) previously observed methoxyfenozide to be more toxic to codling moth versus oriental fruit moth eggs, but differential ovicidal response of novaluron and pyriproxyfen to these two species has not been reported. When viewed in relation to field use rates, the neonicotinoids acetamiprid and thiacloprid and the organophosphate azinphosmethyl were the only other insecticides to demonstrate relatively high levels of ovicidal activity against codling moth or oriental fruit moth.

All insecticides except pyriproxyfen were acutely toxic to both codling moth and oriental fruit moth neonates. In contrast to ovicidal results in which oriental fruit moth eggs were generally more tolerant than codling moth eggs, methoxyfenoizde was the only insecticide that was less toxic to neonates of oriental fruit moth compared to codling moth. Based on non-overlapping confidence intervals, novaluron, acetamiprid, thiacloprid and azinphosmethyl all had significantly lower LC_{50} values for oriental fruit moth versus codling moth (Table 2). The two most toxic insecticides to neonates of both species were the antharillic diamide chlorantraniliprole and the spinosyn spinetoram, which are also the two

most newly registered insecticides in apples. Mota-Sanchez et al. (2008), used a similar bioassay to ours and observed comparable dose-response data for codling moth neonates. Their estimated LC_{50} values were 5.7, 0.5 and 3 μg/ml for azinphosmethyl, methoxyfenozide and acetamiprid, respectively for their susceptible colony (Kalamazoo colony). Although, their acetamiprid LC_{50} estimate was almost two-fold higher than ours, azinphosmethyl and methoxyfenozide LC_{50}s are within the range reported here.

For larvicidal bioassays in which mortality was based on development to the adult stage, for most insecticides there was no difference in the response of the two insect species. The exception was for pyriproxyfen and the neonicotinoids acetamiprid and thiacloprid, all of which had LC_{50} values that were significantly higher for oriental fruit moth compared to codling moth. This is the opposite of what was observed in 5-d exposure studies, when LC_{50} values for acetamiprid and thiacloprid were higher for codling versus oriental fruit moth. In the 5-d bioassays, moribund larvae were scored as dead, and perhaps oriental fruit moth larvae exhibited a greater ability to recover from the initial exposure than codling moth, which would explain higher LC_{50} values for oriental fruit moth larvae in the life-time bioassays.

Field residual studies showed that the insect growth regulators with the highest ovicidal activity (methoxyfenozide and novaluron), along with chlorantraniliprole and spinetoram, demonstrated the longest field residual activity (Tables 5 and 6 and Fig. 1). Methoxyfenozide's long residual activity (up to 28 d after application for codling moth) observed here was also reported by Borchert et al. (2004b). While neither chlorantraniliprole nor spinetoram exhibited ovicidal activity, their high toxicity to larvae was evident in

laboratory and field studies. The residual activity of chlorantraniliprole was particularly long, with larval entries into fruit significantly reduced below the control for up to 49 days after two applications. The high toxicity of these two insecticides to neonates of codling moth and oriental fruit moth, combined with good residual activity may allow them to efficiently control both species.

The codling moth phenology model (Riedl et al. 1976) has long been used to predict population events such as adult emergence, egg laying and egg hatch. This information, combined with knowledge of the toxicity of insecticides to different life stages, is useful in determining the proper timing of insecticide applications. For instance, insecticides with high ovicidal activity, such as methoxyfenozide, are recommended to be applied at the initiation of codling moth oviposition (Borchert et al. 2004b), while those that have greater larvicidal than ovicidal activity are applied later at the initiation of egg hatch. For those insecticides that exhibit toxicity to both immature and adult stages, such as azinphosmethyl, the precise timing of applications is not as critical. Our results indicate that methoxyfenozide, novaluron and pyriproxyfen are all excellent ovicides against codling moth and would benefit by being applied at the initiation of egg laying. In contrast, chlorantraniliprole and spinetoram exhibited no ovicidal activity but were extremely toxic to neonates, which suggests applications coinciding with egg hatch would be most efficient. Similar to azinphosmethyl, the neonicotinoid acetamiprid exhibited toxicity to eggs, neonates and adults, which offers more leeway in terms of the timing of applications compared to chemicals active against only eggs or neonates.

There is a diversity of insecticide chemistry with different modes of action and life-

stage specificity available for management of codling moth and oriental fruit moth in apple systems. This diversity should bode well for resistance management programs. Among the insecticides evaluated in this study, resistance has been reported in the US only for azinphosmethyl (Knight et al. 1994, Dunley and Welter 2000, Mota-Sanchez et al. 2008) and methoxyfenozide (Knight et al. 2001, Mota-Sanchez et al. 2008). Monitoring for resistance is a key component of resistance management, and results of this study provide baseline data on the susceptibility and residual activity of new insecticides expected to play an important role in managing codling moth in the near future.

Acknowledgements

We thank Monica Schiessl and Nicole Orengo for assistance in rearing the insects and conducting some of the bioassays, and Steve Schoof for assisting in apple collections and colony maintenance. This research was funded, in part, by a fellowship from the NIH/MBTP NCSU program and the North Carolina Agricultural Research Service.

References Cited

Barnes, M. M. 1991. Codling moth occurrence, host race formation and damage. In L.P.S. Van der Geest and H. W. Evenhuis [eds.], Tortricid pests: their biology, natural enemies and control. Elsevier, Amsterdam, The Netherlands.

Borchert, D.M., R.E. Stinner, J.F. Walgenbach, and G.G. Kennedy. 2004a. Oriental fruit moth (Lepidoptera: Tortricidae) phenology and management with methoxyfenozide in North Carolina apples. J. Econ. Entomol. 97: 1353-1364.

Borchert, D.M., J.F. Walgenbach, G.G. Kennedy, and J.W. Long. 2004b. Toxicity and residual activity of methoxyfenozide and tebufenozide to codling moth (Lepidoptera: Tortricidae) and oriental fruit moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 97: 1342-1352.

Bush, M. R., Y. A. I. Abdel-Aal, and G. C. Rock. 1993. Parathion resistance and esterase activity in codling moth (Lepidoptera: Tortricidae) from North Carolina. J. Econ. Entomol. 86: 660-666.

Calkins, C. O., and R. Faust. 2003. Overview of areawide programs and the program of suppression of coling moth in the western USA directed by the United States Department of Agriculture-Agricultural Research Service. Pest Manag. Sci. 59: 601-604.

Contreras, E. F., M. Reyes, W. Barros, and B. Sauphanor. 2007. Evaluation of azinphos-methyl resistance and activity of detoxifying enzimes in codling moth (Lepidoptera: Tortricidae) from central Chile. J. Econ. Entomol. 100: 551-556.

Dunley, J. E. and S. C. Welter. 2000. Correlated insecticide cross-resistance in azinphosmethyl resistant codling moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 93: 955-962.

Finney, D. J. 1971. Probit Analysis. Cambridge University Press, Cambridge, UK.

Knight, A. L., J. F. Brunner, and D. Alston. 1994. Survey of azinphosmethyl resistance in codling moth (Lepidoptera: Tortricidae) in Washington and Utah. J. Econ. Entomol. 87: 285-292.

Knight, A. L., J. E. Dunley, and R. K. Jansson. 2001. Baseline monitoring of codling moth (Lepidoptera: Tortricidae) larval response to benzoylhydrazine insecticides. J. Econ. Entomol. 94: 264-270.

Kovanci, O.B., C. Schal, J.F. Walgenbach, and G.G. Kennedy. 2005. Comparison of mating disruption with pesticides for management of oriental fruit moth (Lepidoptera : Tortricidae) in North Carolina apple orchards. J. Econ. Entomol. 98: 1248-1258.

LeOra Software. 2002. POLO-PC. A user's guide to probit and logit analysis., Berkeley, CA.

Mota-Sanchez, D., J. C. Wise, R. V. Poppen, L. J. Gut and R. M. Hollingworth. 2008. Resistance of codling moth, Cydia pomonella (L.) (Lepidoptera: Tortricidae), larvae in Michigan to insecticides with different modes of action and the impact on field residual activity. Pest Manag. Sci. 64: 881-890.

Myers, C.T., L. A. Hull, and G. Krawczyk. 2006. Effects of orchard host plants on the oviposition preference of the oriental fruit moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 99: 1176-1183.

Reuveny, H., and E. Cohen. 2004. Resistance of codling moth *Cydia pomonella* (L.) (Lep., Tortricidae) to pesticides in Israel. J. Appl. Entomol. 128: 645-651.

Reyes, M., P. Franck, P. J. Charmillot, C. Ioriatti, J. Olivares, E. Pasqualini, and B Sauphanor. 2007. Diversity of insecticide resistance mechanisms and spectrum in European populations of the codling moth, *Cydia pomonella*. Pest. Manag. Sci. 63: 890-902.

Riedl, H., B. A. Croft, and A. J. Howitt. 1976. Forecasting codling moth phenology based on pheromone trap catches and physiological models. Can. Entomol. 108: 449-460.

SAS Institute. 2002. User’s manual, version 8.1. SAS Institute, Cary, NC.

Shearer, P. W., and K. A. Usmani. 2001. Sex-related response to organophosphorus and carbamate insecticides in adult Oriental fruit moth, *Grapholita molesta*. Pest Manag. Sci. 57: 822-826.

Sun, X., and B. A. Barrett. 1999. Fecundity and fertility changes in adult codling moth (Lepidoptera: Tortricidae) exposed to surfaces treated with tebufenozide and methoxyfenozide. J. Econ. Entomol. 92: 1039-1044.

Usmani, K.A., and P.W. Shearer. 2001. Susceptibility of male Oriental fruit moth (Lepidoptera: Tortricidae) populations from New Jersey apple orchards to azinphosmethyl. J. Econ. Entomol. 94: 233-239.

Yokoyama, V. Y., G. T. Miller, and J. M. Harvey. 1987. Development of oriental fruit moth (Lepidoptera: Tortricidae) on a laboratory diet. J. Econ. Entomol. 80: 272-276.

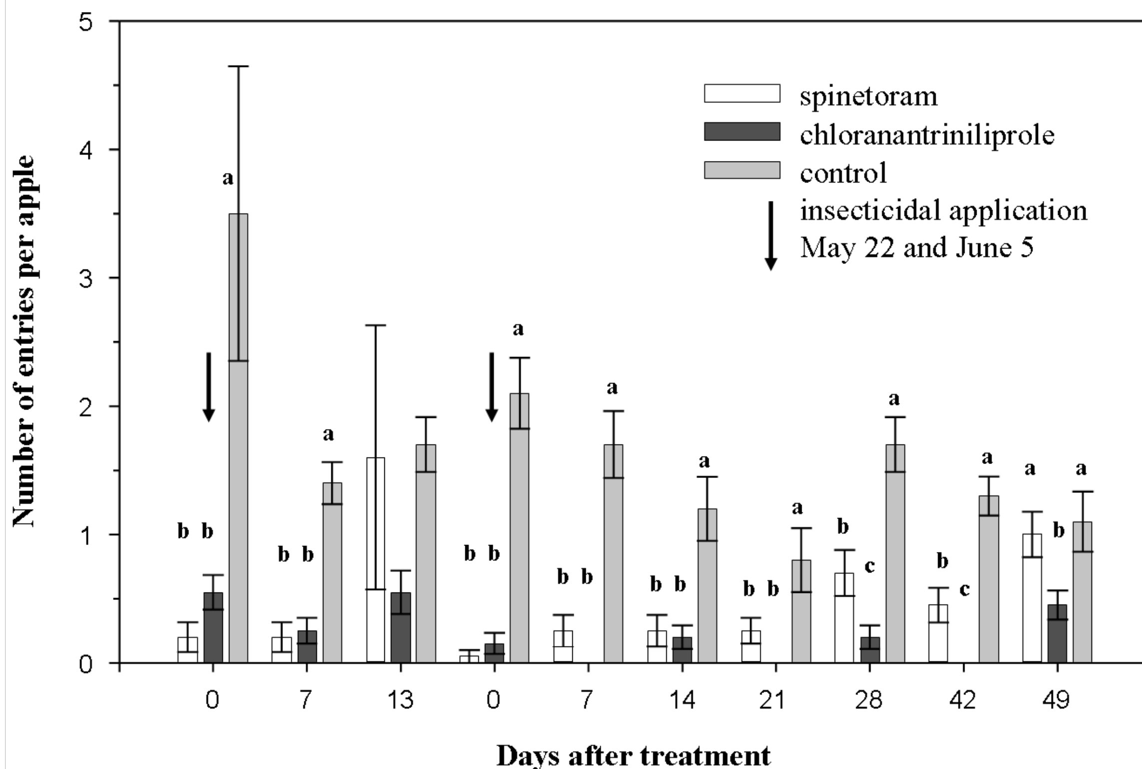


Figure 1. Mean ± SEM codling moth number of larval entries per apples collected from chloranantrinilipole and spinetoram sprayed trees. Insecticides were sprayed on 21 May and 5 June in Mills River, NC, 2008. Bars in the same day followed by the same letter are not significantly different from each other (Fisher's protected LSD, P ≤ 0.05).

Table 1. Susceptibility of codling moth and oriental fruit moth eggs to 10 insecticides. Apple fruits were treated (dipped for 5 s) before egg laying.

Species	Insecticide	*n*	Slope (± SE)	LC_{50} (95% CL) - µg/ml	χ^2	[b]µg/ml field rate
Codling moth	Novaluron	531	1.16 (0.13)	2.12 (0.95 – 4.65)	8.71	156
	Methoxyfenozide	445	1.02 (0.15)	3.00 (1.12 – 8.00)	3.79	300
	Pyriproxyfen	1113	0.32 (0.08)	0.01 (0.00 – 0.06)	0.01	108
	Chlorantraniliprole	1134	—[a]	>1000	—	67
	Spinosad	1403	—[a]	>1000	—	108
	Spinetoram	503	0.60 (0.12)	305.43 (142.90 – 734.35)	0.21	96
	Acetamiprid	372	0.74 (0.10)	1.06 (0.44 – 2.55)	22.59	120
	Thiacloprid	456	1.96 (0.67)	65.63 (22.41 – 191.95)	23.25	216
	Indoxacarb	395	0.86 (0.31)	625 (139 – 3044)	0.36	108
	Azinphosmethyl	479	1.54 (0.42)	309 (75 – 1456)	3.73	1199
Oriental fruit moth	Novaluron	567	1.06 (0.11)	7.89 (3.36 – 15.73)	5.68	156
	Methoxyfenozide	427	2.72 (0.42)	354.03 (158.78 – 799.73)	8.11	300
	Pyriproxyfen	1580	—[a]	>1000	—	108
	Chlorantraniliprole	1467	—[a]	>1000	—	67
	Spinosad	605	—[a]	>1000	—	108
	Spinetoram	1573	1.09 (0.1)	468.25 (163.19 – 1515.16)	5.31	96
	Acetamiprid	594	3.08 (0.36)	17.28 (7.55 – 34.43)	16.10	120
	Thiacloprid	534	7.68 (>8)	83.59 (37.81 – 175.96)	7.32	216
	Indoxacarb	599	—[a]	>1000[a]	—[a]	108
	Azinphosmethyl	578	1.79 (0.42)	328.91 (132.43 – 883.29)	11.35	1199

CL, confidence limit

[a]Mortality <50% at the highest concentration

[b]Recommended field rate applied at 100 gal/A

Table 2. Codling moth and oriental fruit moth neonate susceptibility to six different insecticides topically applied to the diet. Neonate (< 24 h-old) mortality was evaluated five days later.

Insect	Insecticide	*n*	Slope (± SE)	LC_{50} (95% CL) - µg/ml	χ^2
Codling moth	novaluron	420	1.76 (0.34)	5.45 (3.63 – 7.64)	0.95
	methoxyfenozide	400	2.07 (0.31)	0.65 (0.41 – 0.89)	3.13
	pyriproxyfen	380	0.03 (0.01)	58.34 (44.19 – 69.34)	1.12
	chlorantraniliprole	538	1.98 (0.32)	0.08 (0.02 – 0.14)	8.53
	spinosad	348	6.03 (0.98)	0.97 (0.63 – 1.30)	28.54
	spinetoram	556	2.81 (0.46)	0.06 (0.03 – 0.08)	5.27
	acetamiprid	562	0.75 (0.10)	1.71 (1.35 – 2.06)	3.76
	thiacloprid	321	2.48 (0.65)	9.90 (6.0 – 14.3)	7.56
	azinphosmethyl	360	5.38 (1.24)	5.26 (3.87 – 6.26)	1.02
Oriental fruit moth	novaluron	288	1.61 (0.38)	0.78 (0.30 – 1.23)	1.15
	methoxyfenozide	326	3.01 (0.54)	5.60 (2.82 – 7.96)	4.60
	Pyriproxyfen	346	—[a]	>50	—
	chlorantraniliprole	502	3.33 (0.58)	0.04 (0.01 – 0.06)	4.16
	spinetoram	280	2.18 (0.41)	0.05 (0.03 – 0.07)	2.41
	acetamiprid	424	2.06 (0.34)	0.67 (0.39 – 0.94)	1.97
	thiacloprid	319	2.09 (0.63)	2.02 (0.61 – 3.26)	0.16
	azinphosmethyl	243	2.36 (0.42)	1.65 (0.99 – 2.28)	2.33

CL, confidence limit

[a]Mortality <50% at the highest concentration

Table 3. Codling moth and oriental fruit moth larval susceptibility to nine different insecticides topically applied to the diet. Exposure started as neonate (< 24 h-old) and mortality was evaluated as adults emerged.

Species	Insecticide	*n*	Slope (± SE)	LC_{50} (95% CL) - µg/ml	χ^2
Codling moth	Novaluron	360	1.61 (0.36)	0.29 (0.18 – 0.43)	2.26
	Methoxyfenozide	360	4.44 (1.02)	0.61 (0.46 – 0.74)	2.32
	Pyriproxyfen	338	1.33 (0.31)	0.13 (0.03 – 0.24)	2.58
	Chlorantraniliprole	200	6.44 (1.70)	0.28 (0.12 – 0.29)	0.45
	Spinosad	240	4.17 (1.06)	1.51 (0.91 – 2.54)	1.91
	Spinetoram	200	3.43 (0.76)	0.11 (0.06 – 0.14)	4.47
	Acetamiprid	480	3.82 (0.86)	0.25 (0.16 – 0.38)	1.30
	Thiacloprid	360	8.99 (2.47)	0.55 (0.34 – 0.88)	4.90
	Azinphosmethyl	360	3.49 (0.80)	4.72 (2.67 – 8.38)	2.83
Oriental fruit moth	Novaluron	720	2.57 (0.36)	0.35 (0.26 – 0.46)	1.62
	Methoxyfenozide	720	3.93 (0.55)	0.44 (0.34 – 0.56)	5.38
	Pyriproxyfen	720	1.99 (0.41)	13.79 (9.88 – 18.98)	3.86
	Chlorantraniliprole	400	6.63 (1.46)	0.28 (0.22 – 0.32)	1.75
	Spinosad	720	3.34 (0.41)	0.98 (0.76 – 1.25)	1.34
	Spinetoram	400	2.98 (0.45)	0.17 (0.12 – 0.21)	0.82
	Acetamiprid	720	3.15 (0.49)	4.21 (3.14 – 5.54)	0.68
	Thiacloprid	960	1.74 (0.28)	6.47 (4.94 – 8.29)	5.72
	Azinphosmethyl	720	5.98 (0.84)	2.73 (2.10 – 3.51)	4.91

CL, confidence limit

Table 4. Codling moth and oriental fruit moth male adult susceptibility to acetamiprid and azinphosmethyl using topical bioassay.

Species	Insecticide	*n*	Slope (± SE)	LC_{50} (95% CL) - µg/ml	χ^2
Codling moth	Acetamiprid	616	1.19 (0.17)	0.04 (0.03 – 0.06)	2.87
	Azinphosmethyl	240	3.08 (0.40)	0.10 (0.08 – 0.12)	1.15
Oriental fruit moth	Acetamiprid	479	2.52 (0.32)	0.06 (0.05 – 0.08)	1.17
	Azinphosmethyl	150	2.21 (0.40)	0.10 (0.07 – 0.13)	0.25

CL, confidence limit

Table 5. Mean (± SEM) codling moth percentage egg hatch and number of larval entries per hatched egg into apples collected from trees sprayed in two different dates.

	% Hatched egg (DAT[a])						Entries/hatched egg (DAT[a])					
Insecticide	0	7	10	14	21	28	0	7	10	14	21	28
						Application date: 8 July						
Indoxacarb	42.52^{b}	46.39^{bc}	58.78^{bc}	76.63^{ab}	70.79^{b}	86.05^{a}	0.09^{b}	0.11^{b}	0.09^{ab}	0.19^{ab}	0.13	0.17^{ab}
	(13.31)	(8.16)	(11.14)	(3.87)	(5.79)	(4.41)	(0.06)	(0.04)	(0.04)	(0.08)	(0.04)	(0.06)
Methoxyfenozide	0.99^{c}	65.22^{ab}	10.43^{e}	2.73^{d}	7.95^{d}	28.68^{c}	0.0^{b}	0.0^{b}	0.04^{b}	0.0^{c}	0.05	0.05^{b}
	(0.62)	(8.70)	(5.54)	(1.43)	(3.32)	(6.05)	(0.0)	(0.0)	(0.04)	(0.0)	(0.05)	(0.05)
Novaluron	2.70^{c}	14.04^{de}	4.82^{e}	12.54^{d}	9.07^{d}	25.30^{c}	0.05^{b}	0.0^{b}	0.0^{b}	0.08^{bc}	0.2	0.09^{b}
	(1.68)	(5.37)	(2.32)	(4.03)	(6.66)	(10.37)	(0.05)	(0.0)	(0.0)	(0.04)	(0.2)	(0.06)
Acetamiprid	7.86^{c}	59.38^{ab}	39.06^{cd}	66.89^{bc}	72.63^{b}	67.17^{b}	0.05^{b}	0.06^{b}	0.0^{b}	0.07^{bc}	0.09	0.11^{b}
	(2.88)	(8.14)	(5.33)	(6.41)	(3.90)	(4.86)	(0.05	(0.04)	(0.0)	(0.02)	(0.02)	(0.03)
Thiacloprid	18.53^{c}	5.65^{e}	68.17^{ab}	61.01^{c}	74.57^{ab}	85.78^{a}	0.03^{b}	0.1^{b}	0.01^{b}	0.09^{bc}	0.03	0.04^{b}
	(14.51)	(3.24)	(9.47)	(4.23)	(4.47)	(2.97)	(0.03)	(0.1)	(0.01)	(0.06)	(0.01)	(0.02)
Azinphosmethyl	8.93^{c}	28.19^{cd}	36.09^{d}	55.64^{c}	56.90^{c}	83.54^{a}	0.0^{b}	0.0^{b}	0.21^{a}	0.11^{abc}	0.19	0.15^{b}
	(2.28)	(7.77)	(8.45)	(3.43)	(2.35)	(3.27)	(0.0)	(0.0)	(0.09)	(0.07)	(0.04)	(0.05)
Control	68.93^{a}	75.08^{a}	83.13^{a}	82.67^{a}	85.68^{a}	88.24^{a}	0.39^{a}	0.28^{a}	0.21^{a}	0.25^{a}	0.27	0.29^{a}
	(4.51)	(6.53)	(2.53)	(2.74)	(1.08)	(2.36)	(0.06)	(0.05)	(0.06)	(0.02)	(0.07)	(0.02)

Table 5. Continued

	Application date: 9 august											
Indoxacarb	31.5^{b}	63.9^{b}	75.2^{ab}	36.6^{c}	62.7^{ab}	77.5^{a}	0.04^{b}	0.2^{a}	0.09^{b}	0.1^{b}	0.06^{abcd}	0.19
	(4.3)	(7.5)	(3.5)	(10.3)	(8.8)	(6.0)	(0.04)	(0.07)	(0.03)	(0.07)	(0.03)	(0.04)
Methoxyfenozide	2.1^{c}	3.0^{d}	68.9^{b}	12.2^{d}	22.0^{c}	24.1^{b}	0.0^{b}	0.0^{b}	0.01^{c}	0.0^{b}	0.05^{bcd}	0.1
	(1.3)	(0.9)	(2.1)	(7.5)	(11.7)	(7.5)	(0.0)	(0.0)	(0.01)	(0.0)	(0.04)	(0.1)
Novaluron	4.4^{c}	31.4^{c}	5.7^{d}	9.6^{d}	7.6^{c}	23.9^{b}	0.0^{b}	0.07^{b}	0.0^{c}	0.0^{b}	0.0^{d} (0.0)	0.356
	(2.9)	(9.5)	(3.0)	(3.1)	(4.6)	(12.6)	(0.0)	(0.04)	(0.0)	(0.0)		(0.2)
Acetamiprid	19.9^{b}	36.4^{c}	18.8^{c}	69.5^{ab}	59.3^{ab}	71.9^{a}	0.03^{b}	0.03^{b}	0.0^{c}	0.03^{b}	0.1^{abc}	0.086
	(3.9)	(6.9)	(7.2)	(7.1)	(5.3)	(10.6)	(0.03)	(0.03)	(0.0)	(0.01)	(0.03)	(0.03)
Thiacloprid	25.6^{b}	60.3^{b}	3.1^{d}	61.4^{b}	48.9^{b}	71.8^{a}	0.0^{b}	0.01^{b}	0.02^{c}	0.06^{b}	0.04^{cd}	0.032
	(8.1)	(6.5)	(1.4)	(9.3)	(8.7)	(9.0)	(0.0)	(0.01)	(0.02)	(0.04)	(0.02)	(0.02)
Azinphosmethyl	5.1^{c}	57.4^{b}	29.3^{c}	53.8^{a}	79.3^{a}	64.6^{a}	0.0^{b}	0.01^{b}	0.01^{c}	0.04^{b}	0.13^{ab}	0.073
	(1.6)	(3.1)	(5.1)	(8.0)	(4.2)	(3.7)	(0.0)	(0.01)	(0.01)	(0.03)	(0.03)	(0.04)
Control	74.8^{a}	85.4^{a}	86.5^{a}	85.4^{a}	79.8^{a}	77.8^{a}	0.12^{a}	0.29^{a}	0.21^{a}	0.21^{a}	0.14^{a}	0.194
	(7.0)	(4.3)	(3.7)	(2.5)	(13.9)	(4.2)	(0.05)	(0.06)	(0.04)	(0.05)	(0.04)	(0.05)

[a]DAT – days after treatment

Insecticide rates were: acetamiprid (Assail 70WP) at 0.15 kg[ai]/ha, azinphosmethyl (Guthion 50WP) at 1.12 kg[ai] /ha, indoxacarb (Avaunt 30WDG) at 0.11 kg[ai]/ha, methoxyfenozide (Intrepid 2F) at 0.28 kg[ai]/ha, novaluron (Rimon 0.83EC) at 0.15 kg[ai]/ha and thiacloprid (Calypso 4SC) at 0.20 kg[ai]/ha. Plots were sprayed in two different dates in Mills River, NC, 2004. Means in the same column followed by the same letter are not significantly different from each other (Fisher's protected LSD, $P \leq 0.05$).

Table 6. Mean (± SEM) oriental fruit moth percentage egg hatch and number of larval entries per hatched eggs into apples collected from trees sprayed in two different dates.

	% Hatched egg (DAT[a])						Entries/hatched egg (DAT[a])					
Insecticide	0	7	10	14	21	28	0	7	10	14	21	28
Application date: 8 July												
Indoxacarb	84.2^{a}	84.9^{ab}	74.2^{a}	77.5^{a}	91.3^{a}	93.0^{a}	0.45^{ab}	0.11^{b}	0.26	0.29^{a}	0.27	0.13
	(8.5)	(4.1)	(9.5)	(9.5)	(2.4)	(2.5)	(0.17)	(0.04)	(0.08)	(0.09)	(0.1)	(0.03)
Methoxyfenozide	43.0^{c}	72.2^{b}	22.8^{bc}	76.8^{b}	22.3^{b}	38.6^{d}	0.05^{c}	0.0^{c} (0.0)	0.09	0.0^{b}	0.29	0.11
	(11.2)	(4.2)	(7.7)	(3.7)	(3.9)	(5.3)	(0.03)		(0.05)	(0.0)	(0.2)	(0.04)
Novaluron	52.3^{abc}	15.2^{d}	5.3^{c}	24.6^{b}	39.4^{b}	78.6^{bc}	0.24^{bc}	0.06^{bc}	0.2 (0.2)	0.05^{b}	0.19	0.13
	(15.1)	(4.0)	(2.8)	(6.2)	(12.9)	(5.7)	(0.03)	(0.05)		(0.03)	(0.11)	(0.05)
Acetamiprid	45.1^{bc}	75.4^{ab}	79.9^{a}	64.2^{a}	86.0^{a}	92.3^{a}	0.02^{c}	0.01^{c}	0.05	0.04^{b}	0.05	0.1
	(12.8)	(5.6)	(4.9)	(9.5)	(4.1)	(1.8)	(0.02)	(0.01)	(0.03)	(0.02)	(0.02)	(0.03)
Thiacloprid	42.3^{c}	11.2^{d}	26.9^{b}	69.9^{a}	78.9^{a}	88.7^{ab}	0.04^{c}	0.03^{bc}	0.02	0.11^{b}	0.08	0.05
	(11.6)	(5.3)	(6.7)	(7.7)	(7.4)	(2.2)	(0.04)	(0.03)	(0.02)	(0.1)	(0.03)	(0.02)
Azinphosmethyl	33.6^{c}	50.8^{c}	60.7^{a}	66.4^{a}	29.7^{b}	71.9^{c}	0.0^{c} (0.0)	0.02^{c}	0.13	0.09^{b}	0.12	0.09
	(7.4)	(5.3)	(11.1)	(6.1)	(5.8)	(3.2)		(0.02)	(0.12)	(0.04)	(0.08)	(0.04)
Control	77.8^{ab}	88.2^{a}	80.9^{a}	83.2^{a}	83.5^{a}	83.5^{ab}	0.52^{a}	0.29^{a}	0.34	0.35^{a}	0.21	0.15
	(9.9)	(3.4)	(5.9)	(5.3)	(6.0)	(2.5)	(0.13)	(0.03)	(0.03)	(0.04)	(0.04)	(0.03)

Table 6. Continued

							Application date: 9 august					
Indoxacarb	82.1[a]	86.8[a]	89.7[a]	73.4[b]	88.3[a]	88.4[a]	0.039[b]	0.165[a]	0.082[b]	0.044	0.174	0.289
	(4.3)	(4.3)	(2.4)	(5.5)	(3.3)	(4.9)	(0.03)	(0.04)	(0.01)	(0.03)	(0.04)	(0.08)
Methoxyfenozide	20.1[c]	36.1[b]	84.0[a]	17.9[c]	10.6[b]	16.9[c]	0.013[b]	0.033[b]	0.022[b]	0.218	0.011	0.194
	(4.4)	(8.2)	(1.8)	(4.5)	(10.6)	(6.3)	(0.01)	(0.03)	(0.01)	(0.20)	(0.01)	(0.09)
Novaluron	4.8[d]	66.5[a]	34.1[b]	20.8[c]	13.0[b]	49.5[b]	0.0[b]	0.038[b]	0.04[b]	0.1	0.106	0.297
	(2.3)	(13.4)	(6.7)	(4.5)	(7.2)	(11.4)	(0.0)	(0.03)	(0.04)	(0.07)	(0.1)	(0.09)
Acetamiprid	32.3[b]	77.0[a]	80.6[a]	90.8[a]	87.2[a]	80.8[a]	0.0[b]	0.03[b]	0.02[b]	0.025	0.064	0.243
	(5.5)	(6.7)	(16.0)	(2.2)	(7.4)	(4.1)	(0.0)	(0.02)	(0.02)	(0.01)	(0.02)	(0.09)
Thiacloprid	30.8[bc]	69.8[a]	40.8[b]	84.9[a]	86.3[a]	84.8[a]	0.0[b]	0.014[b]	0.058[b]	0.054	0.252	0.062
	(3.9)	(6.9)	(10.8)	(2.2)	(3.9)	(3.6)	(0.0)	(0.01)	(0.04)	(0.05)	(0.12)	(0.06)
Azinphosmethyl	30.5[bc]	68.9[a]	81.6[a]	84.0[a]	83.8[a]	33.8[bc]	0.0[b]	0.013[b]	0.078[b]	0.118	0.191	0.919
	(4.2)	(6.9)	(4.0)	(2.1)	(2.5)	(8.1)	(0.0)	(0.01)	(0.04)	(0.04)	(0.07)	(0.77)
Control	83.1[a]	87.4[a]	89.6[a]	91.4[a]	88.4[a]	76.4[a]	0.19[a]	0.243[a]	0.291[a]	0.287	0.2	0.314
	(3.4)	(3.2)	(4.6)	(1.6)	(2.9)	(6.0)	(0.03)	(0.05)	(0.03)	(0.03)	(0.03)	(0.07)

[a]DAT – days after treatment

Insecticide rates were: acetamiprid (Assail 70WP) at 0.15 kg[ai]/ha, azinphosmethyl (Guthion 50WP) at 1.12 kg[ai] /ha, indoxacarb (Avaunt 30WDG) at 0.11 kg[ai]/ha, methoxyfenozide (Intrepid 2F) at 0.28 kg[ai]/ha, novaluron (Rimon 0.83EC) at 0.15 kg[ai]/ha and thiacloprid (Calypso 4SC) at 0.20 kg[ai]/ha. Plots were sprayed in two different dates in Mills River, NC, 2004. Means in the same column followed by the same letter are not significantly different from each other (Fisher's protected LSD, $P \leq 0.05$).

For possible publication in *Pest Management Science*

Address Correspondence to:

James F. Walgenbach
MHCREC, 455 Research Dr.
Department of Entomology
North Carolina State University
Mills River, NC 28759
Phone: 828 684-3562
Email: jim_walgenbach@ncsu.edu

Development of a rapid methoxyfenozide-resistance monitoring bioassay for codling moth

Running title: Codling moth resistance bioassay

Leonardo C. Magalhaes, Jaap B. Van Kretschmar, Vonny M. Barlow, R. Michael Roe and James F. Walgenbach*

Department of Entomology, North Carolina State University, Raleigh, NC 27695; *Address correspondence to: jim_walgenbach@ncsu.edu

Abstract:

BACKGORUND: The codling moth, *Cydia pomonella* (L.), is one of the most important apple pest worldwide. Insecticides have been used extensively to manage this pest, and codling moth has evolved resistance to many insecticide groups in almost every apple growing region. There are a number of different bioassay methods to monitor for codling moth resistance; however, many are not applicable to new insecticides or most take months to complete from the time of field collection to bioassay results. A novel 16-well plasticware bioassay plate containing lyophilized diet rehydrated with insecticide dilutions in water was developed for resistance monitoring of codling moth.

RESULTS: Contact insecticides, acetamiprid and azinphosmethyl, were significantly more toxic to neonates than 4th instars. However, there was no significant difference in LC_{50} values between neonate and 4th instar to the ingestion insecticides, chloronantrinilipole, methoxyfenozide, novaluron and spinetoram. Field colonies of codling moth collected from orchards experiencing control failures to methoxyfenozide were significantly more resistant than a susceptible laboratory colony and a colony established from an abandoned orchard. A diagnostic dose of 20 ug/ml (LC_{99}) was established to monitor for codling moth resistance to methoxyfenozide.

CONCLUSIONS: The results presented here demonstrated a novel and rapid bioassay can be used to monitor for codling moth resistance to methoxyfenozide. A single diagnostic dose is valid for methoxyfenozide and may be valid for other ingestion insecticides.

Keywords: *Cydia pomonella*, methoxyfenozide, insect resistance management, baseline, reduced-risk insecticides

1 INTRODUCTION

The codling moth, *Cydia pomonella* (L.), is recognized as one of the most important pests of apple and other pome fruits on a worldwide scale[1]. Damage caused by the codling moth is the result of larvae feeding internally within fruit, and due to the high value of host crops and strict quality standards for both domestic and export markets, there is a near-zero tolerance for damage. As a result, insecticides have been used extensively to manage this pest and resistance has developed in almost every major apple-producing region[2] and to a wide variety of chemical groups[3]. While alternatives to insecticides are playing an increasingly important role in managing this pest, such as mating disruption[4], applications of *Cydia pomonella* granulovirus[5] and even release of sterile insects[6], insecticides will likely remain an important tool used to manage this pest for the foreseeable future.

Codling moth first evolved resistance to lead arsenate in the early 20th century[7] and DDT in the early 1950's[8], and has since developed resistance to virtually every insecticide group. Resistant populations on a worldwide scale have been reported to organophosphates[9,10,11,12,13], bezoylureas including diflubenzuron[14,15,16], the diacylhydrazines tebufenozide and methoxyfenozide[17,18] and pyrethroids[16,18,19]. The occurrence of populations resistant to insecticides shortly after their appearance on the market in both the Europe[3] and the US[18] suggests that cross resistance is a potential concern for new classes of chemistry.

A number of different bioassay methods targeting adults, neonates and post-diapause larvae have been used to monitor for resistance in codling moth. A common method to monitor codling moth resistance to insecticides toxic to adults is via topical application of insecticides on males captured in pheromone sticky traps[20,21]. This has been used

predominately to monitor for azinphsomethyl resistance, but is also applicable to the neonicotinoid acetamiprid that is also toxic to adults[22]. Unfortunately this bioassay method is not applicable to many new insecticides that are not directly toxic to adults, such as insect growth regulators, the spinosyns and anthranilic diamides. These insecticides target either eggs and/or larvae; and therefore, require bioassays that expose immature stages

Other systems have been used to expose codling moth larvae to insecticides for resistance monitoring programs. Topical application to fully developed post-diapause larvae was used to monitor for tebufenozide resistance in field populations[17]. For this bioassay, diapausing larvae are collected in corrugated cardboard strips placed around the tree trunk, and then larvae are exposed to a minimum 3-month cold treatment to complete diapause development. Bioassays using neonates commonly consist of exposing individuals to insecticide either topically applied[18] or incorporated[12] into artificial diet. To obtain a sufficient number of neonates for dose-response experiments, it is generally required that laboratory colonies be established from field populations, which may require two to three months. Additionally, establishment of a wild population in the laboratory is laborious and unpredictable. Due to this time lag, results obtained from current larval resistance monitoring bioassay systems can often be used only in the next field season.

Resistance monitoring is an essential component to insecticide resistance management programs. Reliable bioassay methods and baseline data on pest susceptibility are critical to such programs. The objective of this study was to develop a rapid bioassay to monitor for codling moth resistance that does not require the establishment of wild populations in the laboratory or synchronization of larvae collected from the field. This

bioassay was based on an easy-to-use, compact, portable kit developed to monitor resistance of lepidopteran pests to *Bacillus thurigiensis* (Bt) toxins in cotton and other crops[23,24].

2. MATERIALS AND METHODS

2.1 Codling moth populations

A laboratory susceptible strain (Lab-s) and four wild *C. pomonella* populations (Clear Creek abandoned, Gilbert, Holt and Owensby) were used for bioassays. The colonies were maintained at the Mountain Horticulture Research and Extension Center in Mills River, North Carolina. The larvae were reared on artificial bean based diet [25]. Adults were fed a 5% sugar solution and water. Adults were kept in separate cages where eggs were laid on wax paper and removed every other day.

Every colony was established with larvae collected from infested apple orchards in Henderson County, North Carolina. The laboratory standard susceptible colony was established in 1998 and it has been maintained for >100 generations without any insecticide exposure. Gilbert, Holt and Owensby colonies originated from commercial orchards with a known history of methoxyfenozide control failure and they are assumed to be resistant. The Clear Creek abandoned colony was established from two abandoned orchards that had not been managed for >10 years and were located ≥3 km from the closest commercial orchards. Thus, the abandoned colony is assumed to be susceptible. These colonies were established in 2007 and 2008 and resistant colonies have been maintained under low selective pressure by applying topically low doses (5 to 25 ppm) of methoxyfenozide to the diet to prevent them from losing resistance.

2.2 Chemicals

Commercial formulations of insecticides were used for all bioassays and included acetamiprid (Assail 70WP, Cerexagri, King of Prussia, PA), azinphosmethyl (Guthion 50WP, Bayer CropScience, Research Triangle Park, NC), chloranantrinilipole (Altacor WG, DuPont, Wilmington, DE), methoxyfenozide (Dow Agrosciences, Indianapolis, IN), novaluron (Rimon 10EC, Chemtura, Middlebury, CT) and spinetoram (Delegate WG, Dow Agrosciences, Indianapolis, IN). A surfactant, Latron B1956 (Rohm and Haas), at the rate of 1 drop/liter, was added to all insecticide solutions.

2.3 Neonate and 4th instar bioassays

Recessed sixteen-well bioassay white plasticware[26] (102 mm by 99 mm) containing lyophilized codling moth diet was used for the bioassays[24]. Lyophilized plates were stored in sealed plastic bags with desiccant until further use. In each well, 200 µl of codling moth diet was dispensed in the well's subspace. Codling moth diet water content was consistently measured after lyophilization and it was determined to be about 80% by weight. Therefore, 160 µl of distilled water (control) or insecticidal solution was applied to rehydrate the meal pad. Meal pads were allowed to rehydrate for at least 30 min before the assays were performed.

To determine the length of time required for larvae to develop to the 4th instar on bean based diet, neonates were individually placed in 20 ml plastic cups containing ~6 ml of diet, and monitored daily for molts. Diet cups were placed in a rearing room at 25°C, ~60% RH,

and a photoperiod of 16:8 (L:D) h. A total of 7.8 (±0.09) days were required for larvae to complete development from instars 1 through 3 (Table 1). Hence, for bioassays using 4 instars, larvae were reared for 8 days on diet before use in bioassays.

For bioassays, either a single neonate (<24 h old) or 4th instar was carefully transferred with a fine camel-hair brush to each well. After larvae were transferred, the plate was sealed with a clear, adhesive, plastic, breathable cover (CD International, Pitman, NJ, USA). For 4th instar bioassays, larvae were reared individually in 20 ml plastic cups and extracted after 8 days and transferred to the 16-well bioassay plate. Bioassay plates were kept inside a plastic container with wet paper towel at the bottom to conserve humidity and prevent meal pads from desiccating. The container had a screen on the lid to allow air exchange. Plates were kept in a rearing room at 25±1°C, 50±6% RH, 16:8 (L:D) h and mortality data was recorded 4 days post treatment. Larvae were considered dead when they were unable to move when probed by a camel hair brush. Moribund larvae were considered dead. Control mortality did not exceed 20% in the replicates used in this study. For each bioassay, larvae were exposed to a water control and at least five serial concentrations with mortality ranging from ~15 to 95%, with at least three replicates per concentration. A replication consisted of 16 larvae.

2.4 Bioassay validations

Diagnostic doses offer the advantage of detecting resistance using fewer individuals in a shorter period of time. Exposure of a population to a LC_{99} value may allow some susceptible individuals to survive, and thus allows resistance detection at early stages. The diagnostic

concentration chosen for methoxyfenozide was 20 µg/ml. This concentration was based on neonate and 4th instar dose response curves from the susceptible colonies (laboratory and Clear Creek abandoned). This concentration was within the LC_{99} 95% confidence intervals for the 4 dose response curves (neonate and 4th instar for lab-s and Clear Creek abandoned colonies). This concentration was also within the lower confidence interval limits for the susceptible populations. As a result, it should detect resistance development at early stages. Diagnostic concentrations for the other insecticides commonly used to control codling moth will likely be established in the future.

Insecticide-free "Red Delicious" apples were used for the bioassay validation by infesting them with codling moth neonates from different laboratory colonies. Apples were used as the food source because this monitoring bioassay was designed to be used to detect resistance from field larvae extracted straight from infested apples.

To rear codling moth larvae in apples, a 4-liter plastic bucket was filled with pruning apples, and neonates hatching from eggs were placed on apples. Buckets were placed at 25±2 °C. Larvae were extracted from the apples between 10 and 20 d after infestation (3rd to 5th instar) for use in bioassays. They were then transferred to bioassay plates containing the methoxyfenozide diagnostic concentration (20 µg/ml) or distilled water (control). Bioassay plates were kept at the same conditions described above.

2.5 Statistical analysis

Dose-response mortality data were analyzed by probit analysis[27] using POLO-PC (LeOra Software 2002) and corrected using Abbott's formula[28]. LC_{50s} with overlapping 95%

confidence limits were not considered statistically different. The validation resistance evaluation studies in which populations of larvae were exposed to a discriminating dose of methoxyfenozide (20 µg/ml) were analyzed by using a one-sided Z test at 95% confidence level with correction for continuity[29]. Response was considered statistically significant if $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Neonate and 4th instar response to insecticides

Neonate and 4th instar response to contact insecticides (acetamiprid and azinphosmethyl) were statistically different. Based on LC_{50} values, acetamiprid and azinphosmethyl were 4.3 and 2.6 times more toxic to neonates than 4th instars, respectively. In contrast, there was no significant difference in neonate and 4th instar response to ingestion insecticides (chloronantrinilipole, novaluron and spinetoram), with 95% confidence limits of LC_{50} values overlapping (Table 2). This differential response to contact and ingestion insecticides may be explained by amount of diet (and toxicant) consumed and the size of larvae. While neonates fed less; and therefore, ingested less toxicant than larger larvae (4th instars often consumed the whole meal pad during the 4-day bioassay), on a per weight basis the two instars may have equivalent amounts of insecticide, and thus, a similar dose-response to ingestion insecticides. In contrast, among other factors, contact insecticides are affected by surface-to-volume ratio. Smaller larvae have a larger surface area to volume ratio; thus, insecticides may be absorbed faster. Moreover, smaller larvae have thinner cuticle, which makes it easier for insecticide to penetrate.

Overall, chloronantrinilipole and spinetoram were the most toxic insecticides to neonate and 4th instar codling moth. Magalhaes et al. [22], also observed these insecticides to be the most toxic to neonates among 10 different insecticides examined to both codling moth larvae and oriental fruit moth (*Grapholita molesta* (Busck)) larvae, another important tortricid apple pest in the east U.S. Although their LC_{50} values were lower, they used a 5-d bioassay and the insecticides were topically applied to the diet. Moreover, Jones et al. [30] showed chloronantrinilipole and spinetoram were more toxic to oriental fruit moth larvae than acetamiprid and spinosad.

3.2 Neonate and 4th instar response to methoxyfeozide

Similar to other ingestion insecticides, the response of lab colony neonates and 4th instars to methoxyfenozide was not significantly different, with 95% CL of LC_{50} values being 0.9 – 1.5 and 1.0 – 3.3 µg/ml for neonates and 4th instars, respectively (Fig. 2). This same trend also held for the susceptible Clear Creek abandoned colony and three different resistant colonies (Holt, Owensby and Gilbert). As expected, the dose-response curves of resistant colonies were shifted to the right at relatively the same proportion for neonate and 4th instar. The LC_{50} 95% CL for neonates and 4th instar overlapped for each colony; Clear Creek abandoned: 1.5 - 3.1 and 0.8 – 2.0 µg/ml, Holt: 18.8 - 47.5 and 21.4 - 113.3 µg/ml and Gilbert: 3.8 - 17.6 and 10.7 - 20.9 µg/ml, respectively.

This observation was also reported by Knight et al.[31] using methoxyfenozide and tebufenozide incorporated to the diet. The majority of the bioassays performed had LC_{50} fiducial limits overlapping for the two benzoylhydrazine insecticides tested against different

codling moth larval stages. The only significant susceptibility differences were reported for 5th instars, codling moth last larval instar.

The Gilbert colony appeared to be less resistant to methoxyfenozide compared to the Owensby and Holt colonies. Based on comparisons of LC_{50} values of the lab colony, the neonates and 4th instars exhibited 8.0 and 6.6-fold resistance, respectively, while neonates and 4th instars of the Holt colony were 24.0- and 16.7-fold resistant. Fourth instars of the Owensby exhibited an intermediate level of resistance with 12.4-fold resistant.

All three resistant colonies originated from commercial orchards that reported previous control failures. Methoxyfenozide was widely adopted in North Carolina as a replacement to old organophosphates. In these resistant orchards, methoxyfenozide was the only insecticide used to control codling moth for at least five consecutive years before observations of control failures. This resistance may also have been at least partially due to cross-resistance to organophosphates. Mota-Sanchez et al.[18] hypothesized that this may be the explanation for methoxyfenozide resistance reports in Michigan. This observation is further supported by similar studies demonstrating cross-resistance between tebufenozide (another benzoylhydrazine insecticide) and organophosphates for other tortricid pests of tree fruits in New York[32], Michigan[33] and New Zealand[34].

A diagnostic cocentration of 20 ug/ml for all life stages was chosen because it was within LC_{99} 95% confidence limits for neonates and 4th instars for both susceptible colonies – i.e., the lab and Clear Creek abandoned colonies. Applying this diagnostic concentration to the resistant colonies, it would be expected to result in 60 - 70% mortality in the Gilbert Colony and ~40% mortality in the Holt and Owensby colonies (Fig. 2).

3.3 Bioassay validations

One potential concern about the use of this bioassay system with larvae collected from the field is the food source of larvae used in bioassay. Our bioassays used larvae reared on lima bean based diet, and it is unknown if larvae reared on apples respond to insecticides in the same manner. However, we observed no difference in the response of 10-day old larvae reared on lima bean diet versus 'Red Delicious' apples (Table 3). Jones et al.[30] compared oriental fruit moth reared on "Gala" apple and the same lima bean based artificial diet used here and they reached the same conclusion.

The diagnostic concentration validation results using 10 to 15 day old larvae are presented in table 4. As expected, susceptible laboratory strain larval mortality at the discriminating concentration did not significantly differ from 99% ($P = 0.74$ and 0.57, 10 and 15 d, respectively). Moreover, mortality with the chlorantraniliprole selection colony (10 d-old larvae) did not significantly differ from 99% mortality. Surprisingly, a spinetoram selection colony exhibited mortality of only 88.3%, which was significantly different from 99%, ($P < 0.001$). All resistant colonies had mortality rates significantly lower than 99%, regardless of larval age. Furthermore, the level of mortality was related to LC_{50} values in dose-response studies, with the Gilbert colony, the least resistant, having higher mortality (93.8%) compared to the more resistant Owensby colony (31.3%). Control mortality for the Owensby colony with 20-d old larvae (5th instar) was very high, 33.33%. In contrast, larvae mortality was significantly lower for young larvae across all different colonies.

Spinetoram is a new insecticide in the spynosin group, similar to its precursor, spinosad. To date there is no report of spynosin field resistance in apple orchards. However,

Mota-Sanchez et al.[18] reported higher spinosad tolerance for two organophosphate and pyrethroid resistant populations. They attributed this to possible cross resistance, because spinosad was rarely used in these orchards. Methoxyfenozide and tebufenozide have been shown to exhibit cross-resistance to organophosphates[18,33]. Thus, cross-resistance between methoxyfenozide and spinetoram is a possibility.

Although we tested codling moth populations from only three commercial orchards for resistance to methoxyfenozide, based on control failures in numerous other orchards we suspect fairly widespread resistance to this insecticide in North Carolina. This is additional evidence of this pest's ability to rapidly develop resistance, the importance of resistance monitoring and implementing resistance management programs. A decade ago, there were few effective alternatives to organophosphate insecticides for codling moth control, but there are currently numerous insecticides with different modes of action that are effective options[22]. We have observed the loss of methoxyfenozide resistance in several laboratory colonies that were reared under no selection pressure for a minimum of 7 generations (unpublished data). Knight et al.[31] also observed a decline in the methoxyfenozide LC_{50} of their Walla-99 population after two generations. This suggests that methoxyfenozide resistance is not fixed, and that this insecticide could potentially provide effective control after several years of nonuse.

4 CONCLUSIONS

The results presented here demonstrated this novel and rapid bioassay system can be used to monitor codling moth resistance to methoxyfenozide. The lyophilization of the meal pad

allows proper distribution of the chemical or toxin evenly throughout the diet after rehydration [24,35,36]. In spite of using a different diet than originally designed for this bioassay plate (cotton Lepidopteran pests), this bioassay works equally well with codling moth diet as both the water content and the visual aspect after dehydration were very similar for the two diets. However, this system should not be used against 5th instars due to a lack of a uniform and precise response. Fifth instar is the last larval stage, and as a result the physiology and behavior may change dramatically; which may influence insecticide uptake. Moreover, the 200 µl meal pad cannot support a full-grown larva for the duration of the experiment. A 5th instar can consume the meal pad in less than a day, reducing the amount of toxin ingested to less than that needed to cause a lethal response.

A single diagnostic dose was valid for methoxyfenozide, and we suspect several other ingestion insecticides. Consequently, there is no need to synchronize larval development for this bioassay. This new bioassay offers several additional advantages over other systems, including ease of use, portability, and the ability to use larvae collected directly from apples in the field. Diagnostic doses for other commonly used insecticides should be easy to establish in the future, particularly for ingestion insecticides for which we suspect an age-independent diagnostic will prove to be valid.

ACKNOWLEDGEMENTS

The authors wish to thank Monica Schiessl and Nicole Orengo for rearing the insects and conducting some bioassays. Steve Schoof assisted with population collections. Traineeship support was provided by NIH/MBTP NCSU program. This research was funded, in part, by a USDA-RAMP grant, and the North Carolina Agricultural Research Service.

REFERENCES

1 Barnes MM, Codling moth occurrence, host race formation and damage. In L.P.S. Van der Geest and H. W. Evenhuis [eds.], Tortricid pests: their biology, natural enemies and control. Elsevier, Amsterdam, The Netherlands (1991).

2 Contreras EF, Reyes M, Barros W and Sauphanor B, Evaluation of azinphos-methyl resistance and activity of detoxifying enzimes in codling moth (Lepidoptera: Tortricidae) from central Chile. J. Econ. Entomol. **100**:551-556 (2007).

3 Reyes MP, Franck PJ, Charmillot C, Ioriatti J, Olivares E, Pasqualini and Sauphanor B, Diversity of insecticide resistance mechanisms and spectrum in European populations of the codling moth, *Cydia pomonella*. Pest. Manag. Sci. **63**:890-902 (2007).

4 Calkins CO and Faust R, Overview of areawide programs and the program of suppression of coling moth in the western USA directed by the United States Department of Agriculture-Agricultural Research Service. Pest Manag. Sci. **59**:601-604 (2003).

5 Arthurs SP, Lacey LA and Miliczky ER, Evaluation of the codling moth granulovirus and spinosad for codling moth control and impact on non-target species in pear orchards. Biol. Control. **41**:99-109 (2007).

6 Bloem KA, Bloem S and Carpenter JE, Impact of moth suppression/eradication programmes using the sterile insect technique or inherited sterility. In: Sterile insect technique. Principles and practice in area-wide integrated pest management. Ed. by Dyck VA, Hendrichs J, Robinson AS, Springer, Dordrecht, 677–700 (2005).

7 Hough WS, Relative resistance to arsenical poisoning of two codling moth strains. J. Econ. Entomol. **221**:325-329 (1928).

8 Cutwright CR, A codling moth population resistance to DDT. J. Econ. Entomol. **47**:189-190 (1954).

9 Bush MR, Abdel-Aal YAI and Rock GC, Parathion resistance and esterase activity in codling moth (Lepidoptera: Tortricidae) from North Carolina. J. Econ. Entomol. **86**:660-666 (1993).

10 Knight AL, Brunner JF and Alston D, Survey of azinphosmethyl resistance in codling moth (Lepidoptera: Tortricidae) in Washington and Utah. J. Econ. Entomol. **87**:285-292 (1994).

11 Dunley JE and Welter SC, Correlated insecticide cross-resistance in azinphosmethyl resistant codling moth (Lepidoptera: Tortricidae). J. Econ. Entomol. **93**:955-962 (2000).

12 Reuveny H and Cohen E, Resistance of codling moth Cydia pomonella (L.) (Lep., Tortricidae) to pesticides in Israel. J. Appl. Entomol. **128**:645-651 (2004).

13 Soleno J, Anguiano L, de D'Angelo AP, Cichón L, Fernández D and Montagna C, Toxicological and biochemical response to azinphos-methyl in Cydia pomonella L. (Lepidoptera: Tortricidae) among orchards from the Argentinian Patagonia. Pest Manag Sci **64**:964–970 (2008).

14 Moffitt HR, Westigard PH, Mantey KD and Van de Baan HE, Resistance to diflubenzuron in the codling moth (Lepidoptera: Tortricidae). J Econ Entomol **81**:1511–1515 (1988).

15 Sauphanor B, Bouvier JC and Brosse V, Spectrum of insecticide resistance in Cydia pomonella (Lepidoptera: Tortricidae) in southeastern France. J Econ Entomol **91**:1225–1231 (1998).

16 Sauphanor B, Brosse V, Bouvier JC, Speich P, Micoud A and Martinet C, Monitoring resistance to diflubenzuron and deltamethrin in French codling moth populations (Cydia pomonella). Pest Manag Sci **56**:74–82 (2000).

17 Ioriatti C, Tasin M, Charmillot PJ, Reyes M and Sauphanor B, Early detection of resistance to tebufenozide in field populations of Cydia pomonella L.: methods and mechanisms. J Appl Entomol **131**:453–459 (2007).

18 Mota-Sanchez DJC, Wise RV, Poppen LJ, Gut and Hollingworth RM, Resistance of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), larvae in Michigan to insecticides with different modes of action and the impact on field residual activity. Pest Manag. Sci. **64**:881-890 (2008).

19 Sauphanor B, Cuany A, Bouvier JC, Brosse V, Amichot M and Bergé JB, Mechanism of resistance to deltamethrin in Cydia pomonella (L.) (Lepidoptera: Tortricidae). Pestic Biochem Physiol **58**:109–117 (1997).

20 Varela LG, Welter SC, Jones VP, Brunner JF and Riedl H, Monitoring and characterization of insecticide resistance in codling moth (Lepidoptera: Tortricidae) in four western states. J Econ Entomol **86**:1–10 (1993).

21 Knight AL, Brunner JF and Alston D, Survey of azinphosmethyl resistance in codling moth (Lepidoptera: Tortricidae) in Washington and Utah. J Econ Entomol **87**:285–292 (1994).

22 Magalhaes LC, Walgenbach JF, Baseline susceptibility and residual activity of new insecticides to codling moth and oriental fruit moth. J. Econ. Entomol. *in review*

23 Ro RM, Bailey WD, Gould F, Kennedy GG and Sutula CL, U.S. Patent Number: 6,517,856. Insecticide Resistance Assay (2003).

24 van Kretschmar JB, Roe RM, Bailey WD, Thompson GD and Sutula CL, Feeding disruption tests (FDT) for monitoring larval lepidopteran resistance to Cry1Ac, Cry1F, and Cry1Ab. Crop Protect. *In review*.

25 Yokoyama VY, Miller GT and Harvey JM, Development of oriental fruit moth (Lepidoptera: Tortricidae) on a laboratory diet. J. Econ. Entomol. **80**:272-276 (1987).

26 Bailey WD, Brownie C, Bacheler JS, Gould F, Kennedy GG, Sorenson CE and Roe RM, Species diagnosis and *Bacillus thurigiensis* resistance monitoring of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) field strains from the southern United States using feeding disruption bioassays. J. Econ. Entomol. **94**:76-85 (2001).

27 Finney DJ, Probit Analysis. Cambridge University Press, Cambridge, UK (1971).

28 Abbott WS, A method of computing the effectiveness of an insecticide. J. Econ. Entomol. **18**:265-267 (1925).

29 Roush RT and Miller GL, Considerations for design of insecticide resistance monitoring programs. J. Econ. Entomol. **79**:293–298 (1986).

30 Jones MM, Robertson JL, Weinzierl RA, Susceptibility of Oriental Fruit Moth (Lepidoptera: Tortricidae) Larvae to Selected Reduced-Risk Insecticides. J. Econ. Entomol. **103**:1815-1820 (2010).

31 Knight AL, Dunley JE and Jansson RK, Baseline monitoring of codling moth (Lepidoptera: Tortricidae) larval response to benzoylhydrazine insecticides. J. Econ. Entomol. **94**:264-270 (2001).

32 Waldstein DE, Reissig WH, Agnello AM and Scott JG, Susceptibility of obliquebanded leafroller (Lepidoptera: Tortricidae) populations from commercial apple orchards and an unsprayed habitat in New York to tebufenozide. J Econ Entomol **92**:1251–1255 (1999).

33 Ahmad M, Hollingworth RM and Wise JC, Broad-spectrum insecticide resistance in obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) from Michigan. Pest Manag Sci **58**:834–838 (2002).

34 Wearing CH, Cross-resistance between azinphos-methyl and tebufenozide in the greenheaded leafroller, *Planotortrix octo*. Pestic Sci **54**:203–211 (1998).

35 Roe RM, Long S, Cawsey S, Bacheler JS, Sorenson CE, Hoffman N and Sutula CL, New commercial feeding disruption bioassay kit for species and insecticide resistance diagnosis in the tobacco budworm and cotton bollworm in cotton. *In* Proceedings Beltwide Cotton Conferences. National Cotton Council, Memphis, TN (2002).

36 Bailey WD, Feeding disruption bioassays: The application of novel diets to assessments of insecticide toxicity and resistance. Ph.D. Dissertation, North Carolina State University, Raleigh. 156 p (1999).

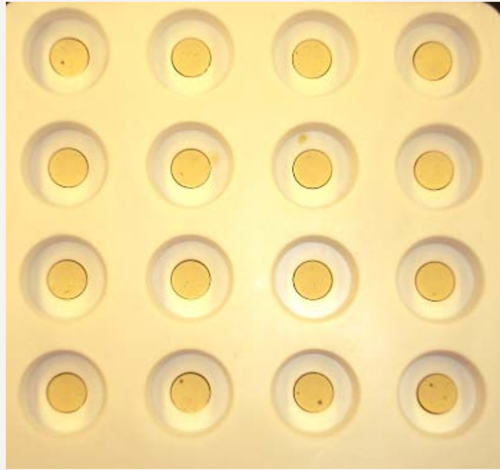


Figure 1. Top view of a single 16-well bioassay plasticware containing 200 µl of lyophilized codling moth artificial diet.

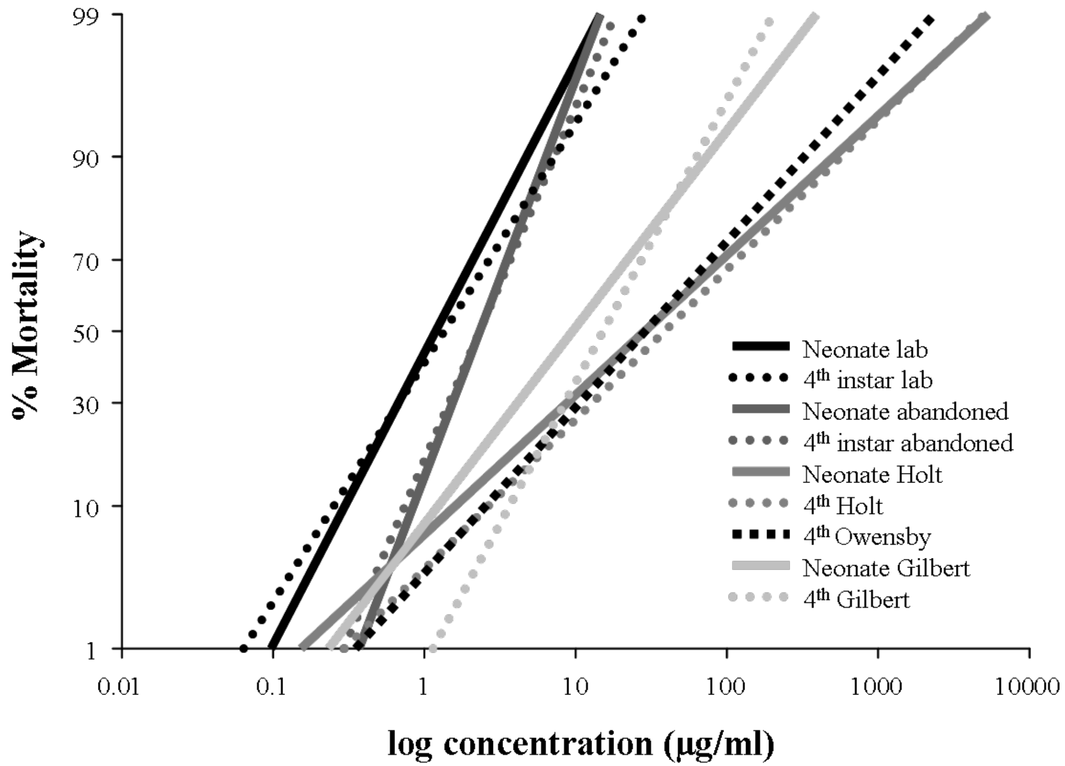


Figure 2. Comparative toxicity of methoxyfenozide to different codling moth colonies to neonate and 4th instar: laboratory and Clear Creek abandoned (susceptible) and Holt, Owensby and Gilbert (resistant). Mortality was recorded after 4 day.

Table 1. Duration of codling moth larval stages (days ± SEM) (colonies pooled) feeding on bean based artificial diet.

	Average stage duration				Average larval duration (I – III)
n	I	II	III	IV	
100	3.29 ± 0.09	2.52 ± 0.05	2.03 ± 0.07	2.51 ± 0.09	7.84 ± 0.09

Table 2. Neonate and 4th instar codling moth (laboratory colony) response to acetamiprid, azinphosmethyl, chloronantrinilipole, novaluron and spinetoram. Mortality was recorded after 4 days. Larvae were reared on artificial diet.

	Larval stage							
	Neonate				4th instar			
Insecticide	*n*	Slope (± SE)	LC_{50} (95% CL) (µg/ml)	χ^2	*n*	Slope (± SE)	LC_{50} (95% CL) (µg/ml)	χ^2
Contact								
Acetamiprid	502	4.11 (± 0.66)	0.26 (0.21 – 0.30)	2.64	414	1.37 (± 0.16)	1.12 (0.73 – 1.65)	8.71
Azinphosmethyl	758	5.11 (± 0.56)	0.61 (0.54 – 0.67)	1.32	672	5.37 (± 0.59)	1.57 (1.30 – 1.82)	5.03
Ingestion								
Chloronantrinilipole	409	1.84 (± 0.22)	0.20 (0.10 – 0.31)	9.43	463	1.77 (± 0.21)	0.19 (0.14 – 0.24)	3.20
Novaluron	1054	1.27 (± 0.09)	1.08 (0.76 – 1.47)	7.83	849	2.07 (± 0.19)	1.16 (0.79 – 1.58)	3.34
Spinetoram	989	1.64 (± 0.14)	0.09 (0.06 – 0.15)	11.95	480	2.56 (± 0.27)	0.16 (0.11 – 0.20)	5.94

CL – confidence limit

Table 3. Response of laboratory population 10-d old larvae to methoxyfenozide LC_{95} (10 ppm) after larvae were reared either on diet or "red delicious" apple. Mortality was recorded after 4 days.

Food source	*n*	% Mortality	% Control mortality	P
Diet	48	89.58	2.08	0.92
Apple	39	92.31	10.42	0.65

Table 4. Mortality of populations of codling moth at discriminating concentration of methoxyfenozide (LC_{99} - 20 µg/ml) reared in "red delicious" apples. Mortality was recorded after 4 days.

Susceptibility	Colony	Larval age (days)	*n*	% Mortality	% Control mortality
Susceptible	Laboratory	10	121	99.17	11.76
		15	64	98.44	22.50
	Altacor selec.	10	64	100.00	4.17
	Delegate selec.	11	54	83.33*	2.08
Resistant	Gilbert	10	48	93.75*	6.45
	Owensby	10	64	31.25*	4.84
		15	118	55.08*	18.75
		20	12	50.00*	33.33[+]

*Mortality is significantly different (P < 0.05) from the diagnostic concentration by the one-sided Z test at 95% CL with correction for continuity.

[+]Control mortality too high. Bioassay was discarded.

For publication in *Insect Biochemistry and Molecular Biology*

Send Correspondence to:

Dr. R. Michael Roe
Department of Entomology
Campus Box 7647
North Carolina State University
Raleigh, NC 27695-7647
919-515-4325 (phone/fax)
michael_roe@ncsu.edu

Pyrosequencing of the gut and whole body transcriptome of the tarnished plant bug, *Lygus lineolaris*, and identification of putative genes important in insect growth and development

Leonardo C. Magalhaes, Jaap B. Van Kretschmar, Kevin V. Donohue and R. Michael Roe*

Department of Entomology, Campus Box 7647m North Carolina State University, Raleigh, NC 27695-7647, USA

*Address correspondence to: michael_roe@ncsu.edu (R.M. Roe)

Abstract

Tarnished plant bug, *Lygus lineolaris*, can be found on more than 100 crops of economic importance. Due to the introduction of *Bt* cotton, lepidopteran pests were practically eliminated and pierce-sucking insects, such as *L. lineolaris*, emerged as new pests. Resistance to common insecticide further exacerbates the problem. Thus, an effective transgenic approach to control plant bugs is desired. The first 454 separate transcriptomes from gut and whole body of the tarnished plant bug are presented here. It was sequenced 116,163,527 total bases from two one-half runs on 454. The assembly resulted in 3,549 and 6,970 contigs with an average of 349 and 392 bp for the gut and whole body, respectively. Overall transcriptomes analysis was organized according to the Gene Ontology (GO), enzyme commission (EC) and InerPro (IPR) using the blast2go program. Here we further characterize eight putative proteins and receptors (leptin receptor, adiponectin receptor, tachykinin precursor, GPCRs (neuropeptide, CAPA and 155 receptors) and proprotein convertases) involved with insect growth and development. Their homology with the same proteins in other taxa and potential as targets to control *L. lineolaris* will be discussed. The use of these new technologies and better understanding of plant-sucking insect digestion will be critical to the sustainability of transgenic crops where plant bugs are an emerging pest and agriculture in general.

Keywords: 454 pyrosequencing, *Lygus lineolaris*, leptin receptor, adiponectin receptor, G protein-coupled receptor, neuropeptide Y receptor, CAPA receptor, tachykinin precursor, proprotein convertase, furin.

1. Introduction

The use of *Bacillus thuringiensis* (*Bt*) transgenic plant technologies has revolutionized agriculture and changed traditional thinking about insect control. While this technology has practically eliminated caterpillar damage in crops, such as cotton, corn and others, so far, this approach is completely ineffective against Hemiptera pest species. Among this insect group, the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is one of the most destructive pests. It has been found on more than 300 plants, and it is considered the most common and abundant mirid species in North America (Coulson, 1987). It is a pest on more than 100 crops of economic importance, which includes fiber, fruit, vegetable and seed crops (Young, 1986).

Since the introduction of *Bt* cotton in the mid 1990's, its adoption continues to expand worldwide. As a result, the bollworm and budworm complex has virtually disappeared in *Bt* cotton areas. Additionally, the success of the boll weevil eradication program in the US reduced considerably the application of broad spectrum insecticide on cotton. Consequently, the tarnished plant bug, which was indirectly controlled by insecticides targeting other pests (Hardee and Bryan, 1997) and rarely reached economic damage during flowering, has become a threat to cotton in the U.S. Currently, *L. lineolaris* is the dominant pest in the Midsouth states in the US (Musser et al., 2009). Together with *Lygus hesperus*, the tarnished plant bug is consistently the first or second most important pest among arthropod agents of cotton yield loss across the U.S (Williams, 2008, 2009, 2010). Therefore, insecticide applications in cotton production due to plant bugs have been

increasing sharply, reaching 7.5 insecticide applications in the Delta region of Mississipi in 2007 (Williams, 2008). A similar problem has been reported in China. The "plant bug complex" replaced *Helicoverpa armigifera* (Hubner) as dominant pest species on *Bt* cotton, and now transgenic cotton has become a source for plant bugs to infest other crops instead of a dead-end trap crop (Lu et al., 2010). Furthermore, the development of resistant populations of tarnished plant bug to common insecticides used in its control in the US Midsouth has been widely documented (Snodgrass and Gore, 2007) and threatens the sustainability of cotton production using genetically engineered cotton as insecticide applications are needed more often.

In the past, tarnished plant bugs fed on cotton primarily during the prebloom period; however, in the last 15 years, significant changes in cotton production has altered this pattern and multiple generations survive on cotton for more than 2 months (Musser et al., 2009). Adult and nymph tarnished plant bugs feed on cotton, mainly during the flowering period. Among other injuries, *L. lineolaris* impacts cotton by releasing salivary toxins that dissolve plant tissue. This causes flowers and small squares to abort or develop into malformed bolls (Layton, 2000). Feeding on young bolls also cause injury that reduces yield (Greene et al., 1999).

The success of transgenic cotton for the effective control of lepidopteran pests and the development of secondary sucking pests like plant bugs, suggests that a transgenic approach for sucking pests is needed. The digestive system is the primary interface between the insect and plants and provides the best site for the use of transgenic insecticidal proteins or dsRNA for the control of these pests. Also, our knowledge of digestive physiology and mechanisms

for the regulation of digestion in sucking pests is an understudied area. A better understanding digestion, absorption, excretion and water balance will be essential to the development of transgenic control of tarnished plant bug (Allen, 2007). As of January 2011, only 698 nucleotides, 405 expressed sequence tags (ESTs) and 305 proteins from *Lygus* sp. were available in GenBank. Most *L. lineolaris* genes available are microsatellites and COSI/COSII genes. The most relevant *L. lineolaris* transcriptome work published so far is that of Allen (2007) where 276 unique ESTs were described. It is clear that the current genetic information is limited compared to other insect pest species.

New opportunities have become available to study emerging pests to elucidate possible new targets for insect control from advances in high throughput DNA sequencing, solid surface DNA synthesis, methods to monitor global gene expression, bioinformatics and gene silencing. 454 pyrosequencing is a fast and reliable approach to sequence thousands of ESTs from specific tissues or whole organisms that greatly improved the number and depth of transcripts when compared to older sequencing technology (e.g. Sanger). 454 pyrosequencing technology has been used to study transcriptomes for a wide variety of insects and other arthropods with different objectives, and often leads to identification of new proteins (Donohue et al. 2010, Hahn et al., 2009, Pauchet et al., 2009 and 2010, Zagrobelny et al., 2009 and Zou et al., 2008). The goal of this study was to develop the first 454 whole body and digestive system transcriptomes to a plant bug to enhance our current knowledge of plant-sucking insect digestion.

2. Materials and methods

2.1. Insects

Tarnished plant bug adults (males and females) were collected with a sweep net from alfalfa in June and July of 2008 at the Lake Wheeler Road Field Laboratory (North Carolina State University, Raleigh, NC). Collected insects were kept in 1-gallon plastic tubs covered with one layer of cheesecloth, fed on artificial NI diet (Cohen, 2000), and held in a growth chamber (Percival Scientific Model I-66NL; Percival Scientific, Inc., Perry, IA) at 27 ± 1° C, 65% relative humidity, 14 hours light: 10 hours dark until needed for dissections. Insects were kept for at least two days in the plastic tubs to reduce the likelihood that plant material would be present in the gut at the time of RNA extraction from the insect. It is unlikely RNA from the diet was extracted together with *L. lineolaris* RNA as the diet was cooked. Furthermore, RNAses present in the diet would also contribute to RNA degradation. Before dissections, tarnished plant bug specimens were taken to a specialist at the North Carolina State University Insect Museum and confirmed as *Lygus lineolaris*.

2.2. Dissections and tissue homogenization

Dissections to remove the digestive system were conducted in 10 mM sodium phosphate buffer, pH 7.4. In this study, the digestive system was considered the pharynx through the hindgut. The salivary glands were not included in this extraction. We were

extremely careful to remove most of the malphigian tubules, ovaries and other tissues attached to the digestive system. Males and females were homogenized as we could observe eggs and ovaries in some specimens during dissection. Once the digestive system was removed from the insect, it was immediately transferred to microcentrifuge tubes containing 200 ul of TRI Reagent (Sigma-Aldrich, St. Louis, MO). The tubes were kept on dry ice throughout the time of dissections to avoid RNA degradation. Upon completion of the dissections each day, the tissue collected was homogenized for 5 s with a hand held mortar and pestle (Fisher, Pittsburg, PA) at room temperature, and the microcentrifuge tubes were stored at -80°C until needed again. This procedure was repeated each day with new material until adequate amounts of tissues were obtained for cDNA library construction. Digestive systems from approximately 30 insects were used for the preparation of RNA.

The whole insect homogenization was performed by placing 8 adults per culture tube (17 X 100 mm) (Fisher), adding 2.5 ml of Tri Reagent per tube, and immediate homogenization for 1 min at room temperature using a Brickmann (Westbury, NY) Polytron. RNA was extracted immediately after homogenization. Approximately 16 insects were used for RNA extraction and sequencing.

2.3. cDNA preparation and 454 sequencing

Frozen tissues in TRI Reagent were allowed to thaw on ice before cDNA preparation. Total RNA from whole plant bugs and pooled digestive system tissues were separately extracted in TRI Reagent according to the manufacturer's recommendations. RNA pellets

were rehydrated in 100 µM aurintricarboxylic (Hallick et al., 1977). Approximately 3 µg of total RNA from each group was pooled, and mRNA was isolated using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Purified mRNA was ethanol precipitated, rehydrated in 2 µl and combined with 10 picomoles of modified 3' reverse transcription primer (Beldade et al. 2006) and 10 picomoles SMART IV oligo (Zhu et al. 2001). The resulting 4 µl were incubated at 72°C for 2 min and then combined with the following reagents on ice: 1 µl RNase Out (40 U/µl), 2 µl 5X first strand buffer, 1 µl 20 mM DTT, 1 µl dNTP mix (10 mM each) and 1 µl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated at 42°C for 90 min then diluted to 30 µl with TE buffer (10 mM Tris HCL pH 7.5, 1 mM EDTA) and stored at -20°C until further use. The second strand was synthesized with the following thermal cycling conditions: 94°C for 2 min followed by 20 cycles of 94°C for 20 sec, 65°C for 20 sec and 68°C for 6 min using a modified 3' PCR primer (Beldade et al. 2006). The first PCR reaction was conducted, and 5 µl aliquots from different cycles were analyzed on a 1% agarose gel to optimize the number of cycles. The optimization was necessary to avoid an over-representation of the most abundant transcripts. Five reactions were then conducted at the optimized number of cycles to produce sufficient quantities of cDNA at a maximum length for 454 library preparation. The contents were combined, and the cDNA was purified using a PCR purification kit (Qiagen) according to the manufacturer's recommendations.

The cDNA library was prepared with appropriate kits (Roche, Indianapolis, IN; Qiagen) for pyrosequencing on the GS-FLX sequencer (Roche) at the NCSU Genome Research Lab, according to the manufacturer's recommendations which were described

previously (Margulies et al. 2005). cDNA was not normalized and oligodT was used. Small changes from the protocol were performed to capture as much data as possible according to Donohue et al. (2010). We conducted whole body on one-half plate and digestive system on the other 454 half plate.

2.4. Bioinformatics

Removal of primer sequence contamination and assembly of GS-FLX sequencing reads were carried out with GS Assembler ver. 1.1.02.15 (Roche) using default parameters as follows: seed step, 12; seed length, 16; seed count, 1; minimum overlap length, 40; minimum overlap identity, 90%; alignment identity score, 2; and alignment difference score, -3. Assembled contiguous sequences (minimum 100 nucleotides), herein referred to as contigs, were initially identified using the Tera-BLASTX algorithm with DeCypher (TimeLogic) against Genbank non-redundant (nr) and expressed sequence tag (est) databases (downloaded June 2008). Specific contigs presented in detail here were further analyzed and confirmed against Genbank non-redundant (nr) and expressed sequence tag (est) databases in July 2010.

Blast2go v 2.4.3 (Conesa et al., 2005) was used to provide an overall analysis of both transcriptomes. Analyses included BLAST homology searches, functional annotation by GO (www.geneontology.org), InterPro terms (InterProScan, EBI) and enzyme classification codes (EC). The analysis was performed in May 2010. Contigs were BLAST**X** against NCBI nonredundant database (*E*-value cut-off = 10^{-3}). Then, the program performed GO mapping based on homology with NCBI's QBLAST and GO annotation (*E*-value 10^{-3},

annotation cut-off 20, GO weight 5). GO Slim (goslim_generic.obo), EC codes and InterPro searches (remotely via the InterProEBI web server) were used to improve the annotation. InterPro identifies any common family and function domains. Sequence alignments were constructed with Vector NTI v 10.3.0 (Invitrogen, Carlsbad, CA). The whole body library is the digestive system transcriptome assembled with the whole body transcriptome. As a result, the sequences used for the alignments (Figs. 4 - 11) were from the whole body library as they were larger sequences.

3. Results

3.1. Transcriptomes sequencing and annotation

A total of 116,163,527 total bases from two one-half runs on 454 were obtained. This represented 229,919 and 262,555 reads of which 168,069 and 232,058 reads were assembled into contigs of the *L. lineolaris* gut and whole body transcriptomes, respectively. The average read length was 208.5 and 233.1 bp, respectively (Table 1). The assembly resulted in 3,549 and 6,970 contigs with an average of 349 and 392 bp for the gut and whole body, respectively. The smallest contig was 55 bp and the largest was 3466 bp in length. There were 62,484 sequences that could not be assembled (singletons) among both transcriptomes (Table 1).

Except by EC code, both transcriptomes presented similar annotation results. Using a cut-off *E*-value of 10^{-3}, the BLASTx search against the non-redundant NCBI protein database

returned around 45% of all contigs in both transcriptomes with at least one blast hit below the BLAST cut-off (Table 1). Less than 35% of the contigs had at least one GO term assigned and around 4% with at least one InterPro (IPR) scan result. The gut transcriptome had significantly more contigs with at least one EC code when compared to the total number of contigs in both libraries, i.e., 16.4 and 7.6%, for the gut and whole body, respectively (Table 1). All sequences discussed in further detail in this paper have an *E*-value of 5 *e*-09 or lower. Other contigs functions were not examined.

3.2. Overall transcriptomes analysis and classifications

Fig. 1 shows the *E*-value and similarity distributions of the *L. lineolaris* BLAST matches against the non-redundant database. Similar distributions are observed for the gut and whole body transcriptomes. The top BLAST hits are to a wide variety of insects and other organisms (Fig. 2). *L. lineolaris* sequences were most similar to *Tribolium castaneum*, *Acyrthosiphon pisum* and *Pediculus humanus*, where the genome was available. The green aphid (*A. pisum*) with an available genome is phylogenically the closest species to *L. lineolaris*. In addition, there were also many hits to Hymenopteran, which include a few species with genomes available. A few hits were to *L. lineolaris* and other *Lygus* species sequences available prior to our research (Fig. 2).

The gene ontology (GO), the enzyme code (EC) and InterPro database provide a general classification of the predicted proteins. Overall, the GO analysis (level 2) was very similar for both transcriptomes (Fig. 3). However, there were significantly more contigs

involved with metabolic process in the gut transcriptome (Fig. 3A); in contrast, there were significantly more sequences involved with developmental process in the whole body transcriptome (Fig. 3B). Basically, there were no differences in cellular components terms (Figs. 3C and D). The whole body library had more hits to translation and transcription regulator activity GO terms (Figs. 3E and F).

Related to enzymes, hydrolases were the most common found in both transcriptomes, accounting for relatively half of all enzymes assigned an EC code (Table 2). According to the InterPro database, there are many proteins in the *L. lineolaris* transcriptomes involved in protein digestion (peptidases and hydrolases). Proteins that metabolize xenobiotics are also represented with 7 sequences classified as Cytochrome P450s (Table 3).

3.3. Leptin receptor

An incomplete leptin receptor, N-terminally truncated, was identified from the *L. lineolaris* transcriptome. This 313 bp sequence codes for a 51-residue protein. The best homologous sequences were identified from *Rhodnius prolixus* (AAQ20841.1; BLAST score, 63.9; *E*-value, 6 *e*-9), *Acyrthosiphon pisum* (NP_001156269.1; BLAST score, 47.4; *E*-value, 7 *e*-4) and *Nasonia vitripennis* (XP_001605479.1; BLAST score, 47; *E*-value, 8 *e*-4) leptin receptor-like proteins (Fig. 4). This *L. lineolaris* sequence contains 34 residues (from residues 5 to 38) that matches a vacuolar protein sorting 55 (Vps55; pfam04133) motif partially with a poor *E*-value of 0.00456. *R. prolixus* transcript was the closest match with 60% identical aminoacids in the aligned region. The vast majority of the top 30 matches

with assigned function were also leptin receptor. Although the *E*-values were poor (> 10^{-3}), the protein query against the nr GenBank database resulted in matches from other invertebrates, fishes, birds and mammals as well.

3.4. Adiponectin receptor

A 299 bp sequence with no start or stop codon was translated into a 99-residue protein, putatively classified as an adiponectin receptor. The highest match is to the *N. vitripennis* adiponectin receptor 1 (NP_001153422.1; BLAST score, 127; *E*-value, 6 *e*-28). The top 25 matches are also to insects' adiponectin receptors or unnamed proteins. The following 75 top matches by BLASTp returned as adiponectin receptor from fishes, mammals and other vertebrates with good *E*-values (< 7 *e*-06) which indicate these peptides are highly conserved across diverse taxa. Fig. 5 shows the *L. lineolaris* putative adiponectin receptor transcript aligned with other adiponectin receptors in different insect taxa. They are all 60% or more identical in the aligned region.

3.5. Tachykinin precursor

A tachykinin precursor was identified from the tarnished plant bug library based on a 262 bp transcript. This sequence codes a 86-residue protein very similar to the *R. prolixus* tachykinin precursor (ACS45389.1, BLAST score, 101; e-value, 2 e-20; identity, 66%). Other top matches include tachykinin precursor within Insecta and other arthropods. The *L.*

lineolaris tachykinin precursor is compared to other insect and crustacean sequences in Fig. 6. This protein is very conserved among Arthropod groups. They are more than 60% identical in the aligned region.

3.6. G protein coupled receptors (GPCRs) (neuropeptide Y receptor, CAPA receptor and 155)

In this transcriptome we have identified four sequences as G protein coupled receptors (GPCRs), a large transmembrane receptor family that regulates many physiological processes. A partial putative neuropeptide Y receptor was identified from a 358 bp sequence that coded a 3' truncated 113-residue peptide. This incomplete sequence contains a partial 7 transmembrane receptor rhodopsin family (7tm_1 motif; Pfam PF00001) from residues 49 to 118 with a significant match (*E*-value, 7.15 e-09) and a partial G protein-coupled chemokine receptor-like protein (PHA03087) family from residues 25 to 118 with also a good match (E-value, 3.12 e-04). The top match is to a putative class A rhodopsin-like G-protein coupled receptor to the body louse *P. humanus corporis* (XP_002427965.1; BLAST score, 139; *E*-value, 2 *e*-31). In the overlap region, sequences are 65% identical. The majority of the other top 100 returned results with known description were also neuropeptide Y receptors from vertebrate and non-vertebrate with significant *E*-values (*E*-values < 9 *e*-12). An alignment of *L. lineolaris* neuropetide Y receptor to other neuropeptide Y receptors matches among insect is shown in Fig. 7.

Two sequences were assigned as CAPA receptors in the *L. lineolaris* library. They

were named CAPA 1 and 2 according to their contig order in the library and do not imply any higher physiological importance or hierarchy. The contigs are 259 and 623 bp long for CAPA 1 and 2, respectively. As they may be a part of the same gene, the two sequences were aligned together with other top CAPA receptor-like proteins hits known from insects (Fig. 8). The best match was to a putative G-protein coupled octopamine receptor to the human body louse (XP_002426611.1; BLAST score, 322; *E*-value, 2*e*-86). However, the top 10 matches with a named gene resulted in a CAPA receptor. Neither transcripts have the start or stop codons. CAPA 2 is located towards the 5' end of the gene and CAPA 1 seems to be located right after CAPA 2 (Fig. 8). CAPA 1 translates to a 70-residue protein and CAPA 2 to a 204-residue peptide. Several partial motifs were identified in the combined aligned sequences: seven-transmembrane G-protein-coupled receptor class (7TM_GPCR; Pfam PF10328), G protein-coupled chemokine receptor-like protein (PHA03087), CC chemokine receptor-like protein (PHA02638), chemokine receptor-like protein (PHA02834) and the DNA packaging protein UL33 (PHA03235). Additionally, the 7tm_1 motif, a common motif among GPCRs was also identified, and the overlap region was highly significant (*E*-value, 1.24 *e*-32).

The last GPCR identified was neuropeptide 155. This 528 bp transcript translates to a 173-truncated peptide. The majority of the top 60 hits were to unknown insect proteins. However, this *L. lineolaris* contig had good matches to known GPCRs 155 sequences from a wide variety of animal taxa (*E*-value, < 6 *e*-12). An aligment to some of these sequences demonstrates a high degree of similarity (> 50%) (Fig. 9). No motif could be identified from this sequence.

3.7. Proprotein convertase (furin and type-2)

Three proprotein convertase sequences were sequenced. Two sequences were putatively identified as furin and another one as type-2. The two sequences were analyzed (BLASTx) separately; however they were combined for an alignment with other insect furin peptides as they may be a part of the same gene. The first sequence is 268 bp in length and code an 89 amino acid partial protein. The second sequence is about the same size (269 bp; 89 residue peptide). Sequence two is located towards the 5' end. It seems they are separated by 211 amino acids (Fig. 10).

The sequences combined contain multiple proprotein convertase domains and families. Peptidases S8 15 (cd07498) and peptidases S8 autotransporter serine protease like (cd04848) families were not significant (E-values, 1.03e-03and 8.60e-03, peptidases S8 15 and peptidases S8 autotransporter, respectively). However, peptidases S8 Protein convertases kexins furin-lik (cd04059), peptidase S8 (Pfam PF00082), and proprotein (Pfam PF01483) domains were highly significant (E-values < 2.54 *e*-14). Additionally, the sequence contains the catalytic triad, Asp, His and Ser. The C-terminal to the p protein domain spans from residues 90-145 (Fig. 10). The best match in the GenBank nr database is *P. humanus corporis* putative endoprotease furin (BLAST score, 181; *E*-value, 4 *e*-44; Identity, 94%).

The third sequence had the majority of the best 100 hits as type 2 proprotein convertase. All the hits had a significantly match ranging from *E*-value 2*e*-09 to 9*e*-35. This truncated contig is 268 bp long which translates to 89 amino acid peptide in length. The

multiple matches to other proprotein convertase (type 2) and the alignment to the top hits of the same protein from other insects (Fig. 11) support the identification of this protein.

4. Discussion

4.1. Transcriptome overall analysis

As of January 2011, there were reported only 123 nucleotide sequences, 381 expressed sequence tags (ESTs) and 175 protein sequences in GenBank from *Lygus lineolaris*. Among these 60 nucleotide sequences, many are microsatellites and ribosomal genes. Related to ESTs, 276 of the 381 were described in the most relevant tarnished plant bug transcriptome published (Allen, 2007) up to the study presented here. Although the economic and medical significance of hemipterans are a growing problem, our current knowledge of the molecular biology of true bugs is minimal compared to other insect orders. The present study was equivalent to a "quantum jump" in advancing the molecular biology of this group and produced the first large transcriptome from any sucking insect of importance and the second large gut transcriptome from any insect. Here, we have identified more than 9,500 sequences in two 454 half-runs. More than half of these ESTs (ranging from 94 to 2344 bp) are novel with not a single match by BLASTx; which indicates our understanding of gene expression, in general, relative to insect, but especially in sucking pests, is in its infancy.

Since it was first published in 2005 (Margulies et al., 2005), 454 sequencing

technology has been widely adopted (Rothberg and Leamon, 2008). Although it does not produce reads as large as Sanger-based capillary electrophoresis technology yet (up to 700 bp), 454 pyrosequencing has democratized sequencing and has evolved rapidly. As massive parallel sequencing became cheaper, individual laboratories were able to submit samples to perform complete genome or transcriptome studies (Rothberg and Leamon, 2008). Futhermore, pyrosequencing technology significantly shortens data acquisition as many hurdles of Sanger sequencing are eliminated (e.g. molecular cloning, colony picking) (Zou et al., 2008).

Specifically to tarnished plant bug transcriptome described here, we have classified several novel genes within *L. lineolaris* and Hemiptera. So far, a single Hemiptera has had its genome sequenced, the green aphid *A. pisum*. Still, this is insufficient for such a large order. The kissing bug, *R. prolixus*, should have its genome available soon and this genome could greatly improve the identification of our ESTs as this species is within the same suborder (Heteroptera) as the tarnished plant bug.

4.2. Energy control and storage

Leptin and adiponectin are two hormones recently discovered in vertebrates (Zhang et al., 1994 and Scherer et al., 1995). Although it is produced by other tissues, leptin and adiponectin in vertebrates are mainly produced by adipose tissue (Galic et al., 2010). They both contribute to vertebrate overall metabolism, energy control and lipid processing.

Leptin is involved in the balance of the overall availability of energy. Feeding

increases insulin production which increases leptin levels; fasting increases counter-regulatory hormones, which decrease leptin (Myers, 2004). In fasting, leptin levels decrease enhancing appetite and reducing energy utilization; adequate energy stores produce the opposite effects, i.e., leptin levels rise and energy can be used (Myers, 2004). Leptin regulates other physiological functions in vertebrates (e.g. puberty and reproduction); however, it is better known for its function in lipid metabolism (Reidy and Weber, 2000). Adiponectin controls energy utilization, glucose and fatty acid oxidation by activating AMP-activated protein kinase (AMPK) (Kubota et al., 2007). Adiponectin seems to be a key regulator of reproduction as well (Brochu-Gaudreau et al., 2010). In insects, adiponectin is produced in the corpora cardiaca and it is known to mobilize energy (lipids) into the haemlymph to be transported to different tissues (Gade et al., 2004).

So far, six isoforms of the leptin receptor (Lee et al., 1996) and three of the adiponectin receptor (AdipoR1, AdipoR2 and T-cadherin) have been reported (Brochu-Gaudreau et al., 2010). AdipoR1 and R2 present an opposite topology where the N terminus is internal and the C terminus is located outside the cell to G protein-coupled receptors (GPCRs). R1 and R2 have the common seven transmembrane domains and are structurally conserved among a wide variety of taxa (Yamauchi et al., 2003). The small *L. lineolaris* leptin and adiponectin receptor ESTs identified here (Figs. 4 and 5) prevent us from classifying them to a specific isoform. The leptin receptor presented here seems to be homologous to receptor 1 in vertebrates. Nevertheless, *E*-values were marginal (>1) for the assignment of function, and further work, such as 5’ and 3’ PCR races and further sequencing, will be necessary to extend this transcript to obtain a better alignment, *E*-value,

to validate the function of these ESTs to specific adiponectin receptors. Most of the adiponectin receptor top hits indicate the *L. lineolaris* adiponectin receptor presented in this study is an AdipoR2. Yet, there are many good matches to AdipoR1. These two receptors are 67% identical (Brochu-Gaudreau et al., 2010) making the correct identification of this 99-residue adiponectin receptor from the plant bug difficult without additional sequence information.

In the silk moth, AdipoR seems to be expressed in a wide variety of tissues: Malpighian tubules, fat body, testis, ovary, blood, silk gland and midgut (Zhu et al., 2008). However, AdipoR was highly expressed in the Malpighian tubules. Zhu et al. (2008) hypothesized this receptor may be involved with lipid metabolism, as is in vertebrates, and detoxification of compounds. Furthermore, they were able to separate AdipoRs into four different clades: vertebrates, insects, nematodes and Fungi. The *L. lineolaris* AdipoR reported here seems to support the insect clade as the first top 20 hits were to insect adiponectin receptors and other insects unknown proteins (data not shown).

Until now leptin and adiponectin have not been found in insects. Still, in GenBank as of June 2010, 71 adiponectin-like receptors and 3 leptin-like receptors were identified from insects in the protein database. Thus, it may be too soon to state these proteins are not found in insects. Furthermore, leptin is associated with neuropeptide Y which has been reported here (Fig. 7). Neuropeptide Y in vertebrates stimulates appetite (Ahima et al., 1996). An increase of leptin levels decrease neuropeptide Y concentration which consequently decreases appetite (Reidy and Weber, 2000). Therefore, if present, it could be hypothesized leptin and adiponectin have similar function in insects as in vertebrates. Still, homologous

receptors do not mean they have the same function in vertebrates and insects, and further studies will be necessary to better understand the role(s) of these receptors among insect groups.

4.3. Tackykinin related peptides

Tachykinin is one of a few neuropeptides that is highly conserved among vertebrate and invertebrate groups (Van Loy et al., 2010). Tachykinins are abundant brain-gut peptides in vertebrates with many physiological roles. These multifunctional peptides are mainly involved with the process of sensory stimulation, control of motor activities/muscle contraction (including cardiovascular) and gut motility on both central and peripheral tissues in vertebrates (Scherkenbeck and Zdobinsky, 2009). Unlike vertebrate and a few invertebrate tachykinins that contain the aminoacid sequence -FXGLM-NH_2, the majority of invertebrate tachykinins have the conserved C-terminal FXGYR-NH_2 (Scherkenbeck and Zdobinsky, 2009). Because of this and other differences, invertebrate tachykinins are usually called tachykinins related peptides (TKRPs). Although the biological roles of TKRPs is not as well understood as tachykinins in vertebrates, in recent years, it has been found TKRPs have multiple endogenous functions in insects including diuresis (Skaer et al., 2002), embryonic development (Winther et al., 2006), gut and oviduct contraction (Schoofs et al., 1990), regulation of hormone release and neuron polarization (Satake et al., 2003).

Since it was first identified from locusts in 1990 (Schoofs et al., 1990), multiple TKRPs have been found in many insects. The American cockroach has the largest number of

TKRPs identified so far, 13 (Predel et al., 2005). Different isoforms may be redundant; however, some have tissue specific functions (Muren and Nassel, 1997). As with many peptides, TKRPs have a precursor that is cleaved after translation to generate the mature peptide. Most peptides are 7-11 aminoacids and usually there are many TKRPs in a single species encoded by one gene (Satake et al., 2003).

Here we identified a *L. lineolaris* TKRP precursor (Fig. 6). It is likely there are many TKRPs on this precursor as this contig is truncated on both the 3' and 5' ends. This was not the first report of TKRP among hemipterans. Neupert et al. (2009) sequenced six TKRPs from the southern green stink bug, *Nezara viridula*. Neupert et al. identified multiple TKRPs isoforms from another six Hemiptera based on the *N. viridula* TKRPs. Furthermore, the kissing bug, *Rhodnius prolixus*, has at least two TKRPs that are expressed throughout the central nervous system and fine processes of the hindgut. Although *L. lineolaris* TKRPs and/or its precursor were not identified in the gut library, it is very unlikely TKRPs are not expressed in this insect gut. Further research would be necessary to investigate if *L. lineolaris* TKRPs present similar tissue expression to other hemipterans in the hind gut and central nervous system.

4.4. G protein-coupled receptor

G protein-couple receptors (GPCRs) are receptors localized on cell membranes. They have typical seven transmembrane domains, a N terminus located outside the cell and a C terminus positioned inside the cell. GPCR is a large protein superfamily which may

compose 1-2% of the animal genome. Once the natural ligand (neurohormone) binds, a series of intracellular messages is initiated which have central roles in reproduction, development, feeding, behavior and growth (Hauser et al., 2006). Insects have usually 50-80 neurohormone GPCRs (Grimmelikhuijzen et al., 2007).

Neuropeptide Y (NPY) is a very abundant neuropeptide in the brain of mammals (Allen, 1990). It plays an important role in appetite, digestion and metabolism among others (Pedrazzini et al., 2003). So far, invertebrates are not known to have NPY. However, there are many NPY like peptides and neuropeptide F (in the NPY family) that have been identified in a variety of insects which have the same roles as NPY in mammals. There are several NPY receptor subtypes that are localized in different areas of the central nervous system. As a result, they may have different roles. In the kissing bug, *Triatoma infestans*, a high concentration of the NPY receptors L1 and IR were found in areas of the central nervous system responsible for visual and chemosensory pathways (Sttembrini et al., 2003). It is too early to determine if the putative NPY sequence presented here has the same functions as in the kissing bug. Further research using immunohistochemistry and other techniques may help in elucidating the function(s) of NPY in plant bugs.

CAPA peptides are neuro hormones normally produced by the abdominal ventral nerve cord. In the hemipteran the southern green stink bug, *Nezara viridula* as well as in other insect orders, CAPA cells are typically located in the first three abdominal neuromeres. The hormone is released into the hemolymph to control visceral tissues function. The CAPA gene encodes for a prehormone that is cleaved to at least two periviscerokinins and a single pyrokinin (Predel et al., 2006). The kissing bug, *R. prolixus*, seems to be unique among the

insects studied so far where two CAPA genes have been identified (Neupert et al., 2010). Due to their breathing system, water and ion regulation is essential to insects. CAPA hormones play important roles in muscle contraction and diuresis/antidiuresis processes (Predel and Wegener, 2006) by acting directly on Malpighian tubules and urine production. In *R. prolixus*, CAPA receptors are expressed in the upper secretory segments of the Malpighian tubules, anterior gut, posterior midgut and hindgut (Paluzzi et al., 2010). Here, we have identified two CAPA receptor transcripts in the *L. lineolaris* whole body library. Although, we could not find a CAPA receptor in the gut library, one contig had a few top matches to the CAPA receptor and indicate these receptors may also be expressed in the plant bug gut as in *R. prolixus*.

4.5. Hormone maturation

Many peptidic hormones are expressed as larger polypeptides and must be processed to produce the mature functional hormone. Proprotein convertases (PC) are responsible for this process. So far, seven groups of PC have been identified in vertebrates. Usually these proteases process the hormone precursor by proteolytic cleavage after paired basic amino acids. Then, the C-terminal may be removed, followed by α-amidation. Because of the critical, general function of this enzyme in neuroendocrinology, PCs may be an important target to regulate neuropeptides and their effects and could be of interest for the development of new insect control methods (Donohue et al., 2010, Rayburn et al., 2009).

In this study we have identified three PC contigs from *L. lineolaris*, two are PC type 2

and one furin. Furin cleaves precursor on the C-terminal on a polybasic cleavage site and is expressed in every tissue tested in mammals (Rockwell et al., 2002). In *Drosophila melanogaster*, a PC2 gene, *amon*, is required during embryogenesis, early larval development (Rayburn et al., 2003) and pupal development (Rayburn et al., 2009). Moreover, furin homologs have been identified in *D. melanogaster* (De Bie et al., 1995) and *A. aegyptii* (Chen and Raikhel, 1996) among other insects.

In conclusion, 454 pyrosequencing is a new technology that can rapidly and significantly increase our understanding of gene expression and genomics of an organism. As far as we know, we present here the first large transcriptome study of any insect in the Hemiptera. Plant bugs are important emerging pests in many cotton producing areas. Although transgenic approachs to control lepidopteran pests have greatly changed agriculture, a similar approach is not available to control plant bugs or sucking pests in general. Here we have identified many potential targets to control *L. lineolaris*. For example, tachykinin has many physiological roles and could be used for insect control (Scherkenbeck and Zdobinsky, 2009). GPCRs have likewise been proposed as insect control targets (Grimelikhuijzen et al., 2007) and have been the focus for new pharmaceutical therapies for a variety of human diseases (Fredriksson et al., 2003). Insecticides could be developed to reach such targets using applications with RNAi, synthetic proteins or traditional chemical methods. Although we could not identify SID-1 or SID-2 genes responsible for dsRNA transport in different cells of *C. elegans* or any homologous messages for the RNAi amplification pathway, we found six contigs with matches to scavenger receptors (data not shown). Scavenger receptors may play a critical role in dsRNA uptake in

insects (Huvenne and Smagghe, 2010). The use of these new technologies will be critical to the sustainability of transgenic crops where plant bugs are an emerging pest and agriculture in general.

Acknowledgements

Financial support was provided from grants to R.M.R. from Cotton Inc. Support provided to LCM from NIH/MBTP NCSU program was greatly appreciated. JBvK was also supported by a graduate teaching assistantship in the NCSU Dept. of Entomology. We thank Erik Blinka, Jack Bacheler and Allen Cohen for explanations on *L. lineolaris* biology and discussions on collection methods and locations. Nigel Deighton and Megan R. Ulmer assisted with 454 sequencing. Deborah Thompson, Ana Cabrera, Brook Bissinger and Sayed Khalil provided valuable input throughout this project, and their help was truly appreciated.

References

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., Flier, J.S., 1996. Role of leptin in the neuroendocrine response to fasting. Nature 382, 250–252.

Allen, J.M. 1990. Molecular structure of neuropeptide Y. Ann. N.Y. Acad. Sci. 611, 86–98.

Allen, M.L. 2007. Expressed sequenced tags from *Lygus lineolaris* (Hemiptera: Miridae), the tarnished plant bug. Genet. Mol. Res. 6, 206-213.

Beldade, P., Rudd, S., Gruber, J.D., Long, A.D. 2006. A wing expressed sequence tag resource for *Bicyclus anyana* butterflies, an evo-devo model. BMC Genomics 7, 130-146.

Brochu-Gaudreau, K., Rehfeldt, C., Blouin, R., Bordignon, V., Murphy, B.D., Palin, M.F. 2010. Adiponectin action from head to toe. Endocr. 37, 11-32.

Chen, J.S., Raikhel, A.S. 1996. Subunit cleavage of mosquito pro-vitellogenin by a subtilisin-like convertase. Proc. Natl. Acad. Sci. USA. 93, 6186–6190.

Cohen, A.C. 2000. New oligidic production diet for *Lygus hesperus* Knight and *L. lineolaris* (Palisot de beauvois). J. Entomol. Sci. 35, 301-310.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Coulson, J.R. 1987. Economic importance and biological control of *Lygus* and *Adelphocoris* in North America. p. 1-12. *In* R.C. Hedlund and H.M. Graham (eds.) USDA-ARS National Technical Information Service, ARS-64. Springfield, VA, USA.

De Bie, I., Savaria, D., Roebroek, A.J.M., Day, R., Lazure, C., Van de Ven, W.J.M., Seidah, N.G. 1995. Processing specificity and biosynthesis of the *Drosophila melanogaster* convertases dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2. J. Biol. Chem. 270, 1020–1028.

Donohue, K.V., Khalil, S.M.S, Ross, E., Grozinger, C.M., Sonenshine, D.E., Roe R.M. 2010. Neuropeptide signaling sequences identified by pyrosequencing of the American dog tick synganglion transcriptome during blood feeding and reproduction. Insect Biochem. Mol. Biol. 40, 79-90.

Fredriksson, R., Lagerström, M.C., Lundin, L.G., Schiöth, H.B. 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol. Pharmacol. 63, 1256–1272.

Gade, G., Auerswald, L., Predel, R., Marco, H.G. 2004. Substrate usage and its regulation during flight and swimming in the backswimmer, *Notonecta glauca*. Physiol. Entomol. 29, 84–93.

Galic, S., Oakhill, J.S., Steinberg, J.R. 2010. Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol. 316, 129-139.

Greene, J. K., Turnipseed S.G., Sullivan, M.J., Herzog, G.A.. 1999. Boll damage by southern green stink bug (Hemiptera: Pentatomidae) and tarnished plant bug (Hemiptera: Miridae) caged on transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol. 92, 941-944.

Grimmelikhuijzen, C.J.P., Cazzamali, G., Williamson, M., Hauser, F. 2007. The promise of insect genomics. Pest Manag. Sci. 63, 413-416.

Hahn, D.A., Ragland, G.J., Shoemaker, D.D., Denlinger, D.L. 2009. Gene discovery using massively parallel pyrosequencing to develop ESTs for the flesh fly *Sarcophaga crassipalpis*. BMC Genomics 10, 234-243.

Hallick, R.B., Chelm, B.K., Gray, P.W., Orozco Jr., E.M. 1977. Use of aurintricarboxylic acid as an inhibitor of nucleases during nucleic acid isolation. Nucleic Acids Res. 4, 3055–3064.

Hardee, D.D. and Bryan, W.W. 1997. Influence of *Bacillus thuringiensis* transgenic and nectariless cotton on insect populations with emphasis on the tarnished plant bug (Heteroptera: Miridae). J. Econ. Entomol. 90, 663–668.

Hauser, F., Cazzamali, G., Williamson, M., Blenau,W., Grimmelikhuijzen, C.J., 2006. A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. Prog. Neurobiol. 80, 1-19.

Huvenne, H., Smagghe, G. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. J. Insect Physiol. 56, 227-235.

Kubota, N., Yano, W., Kubota, T., Yamauchi, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Okamoto, S., Shiuchi, T., Suzuki, R., Satoh, H., Tsuchida, A., Moroi, M., Sugi, K., Noda, T., Ebinuma, H., Ueta, Y., Kondo, T., Araki, E., Ezaki, O., Nagai, R., Tobe, K., Terauchi, Y., Ueki, K., Minokoshi, Y., Kadowaki, T., 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6, 55–68.

Layton, M.B. 2000. Biology and damage of the tarnished plant bug, *Lygus lineolaris*, in cotton. Southwest. Entomol. Suppl. 23, 7-20.

Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darishzadeh, J., Lee, J.L., Friedman, J.M. 1996. Abnormal splicing of the leptin receptor in diabetic mice. Nature 379, 632–635.

Lu, Y., Wu, K., Jiang Y., Xia, B., Li, P., Feng, H., Wychuys, K.A.G., Guo, Y. 2010. Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt cotton in China. Science 328, 1151-1154.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.-J., Chen, Z., Dewell, S.B., de Winter, A., Drake, J., Du, L., Fierro, J.M., Forte, R., Gomes, X.V., Goodwin, B.C., He, W., Helgesen, S., Ho, C.H., Hutchinson, S.K., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.-B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lee, W.L., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu., H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Reifler, M., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., W 487 einer, M.P., Willoughby, D.A., Yu, P., Begley, 488 R.F. and Rothberg, J.M. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376-380.

Muren, J.E., Nassel, D.R. 1997. Seven tachykinin-related peptides from the brain of the Madeira cockroach: evidence for tissue specific expression of isoforms. Peptides. 18, 7–15.

Musser, F.R., Lorenz, G.M., Stewart, S.D., Bagwell, R.D., Leonard, B.R., Catchot, A.L., Tindall, K.V., Studebaker, G.E., Akin, D.S., Cook, D.R., Daves, C.A. 2009. Tarnished plant bug (Hemiptera: Miridae) thresholds for cotton before bloom in the Midsouth of the United States. J. Econ. Entomol. 102, 2109-2115.

Myers Jr., M.G. 2004. Leptin receptor signaling and the regulation of mammalian physiology. Recent Prog. Horm. Res. 59, 287-304.

Neupert, S., Russel, W.K., Russel, D.H., Predel, R. 2010. Two capa-genes are expressed in the neuroendocrine system of *Rhodnius prolixus*. Peptides 31, 408-411.

Neupert, S., Russell, W.K., Russell, D.H., Lopes Jr., J.D., Predel, R., Nachman, R.J. 2009. Neuropeptides in Heteroptera: Identification of allatotropin-related peptide and tachykinin-related peptides using MALDI-TOF mass spectrometry. Peptides 30, 483-488.

Paluzzi, J.P., Park, Y., Nachman, R.J., Orchard, I. 2010. Isolation, expression analysis, and functional characterization of the first antidiuretic hormone receptor in insects. PNAS 107, 10290-10295.

Pauchet, Y., Wilkinson, P., van Munster M., Augustin, S., Pauron, D., ffrench-Constant R.H. 2009. Pyrosequencing of the midgut transcriptome of the poplar leaf beetle *Chrysomela tremulae* reveals new gene families in Coleoptera. Insect Biochem. Mol. Biol. 39, 403-413.

Pauchet, Y., Wilkinson, P., Vogel, H., Nelson, D.R., Reynolds, S.E., Heckel, D.G., ffrench-Constant, R.H. 2010. Pyrosequencing the *Manduca sexta* larval midgut transcriptome: messages for digestion, detoxification and defence. Insect Mol. Biol. 19, 61-75.

Pedrazzini, T., Pralong, F., Grouzmann, E. 2003. Neuropeptide Y: the universal soldier. Cell. Mol. Life Sci. 60, 350–77.

Predel, R., Wegener, C. 2006. Biology of the CAPA peptides in insects. Cell. Mol. Life Sci. 63, 2477–90.

Predel, R., Russell, W.K., Neupert, S., Russell, D.H., Esquivel, J.F., Nachman, R.J. 2006. Identification of the first neuropeptides from the CNS of Hemiptera: CAPA peptides of the southern green stinkbug *Nezara viridula* (L). Peptides 27, 2670–7.

Predel, R., Neupert, S., Roth, S., Derst, C., Nässel, D.R. 2005. Tachykinin-related peptide precursors in two cockroach species. Molecular cloning and peptide expression in brain neurons and intestine. FEBS J. 272, 3365–3375.

Rayburn L.Y.M., Rhea, J., Jocoy, S.R., Bender, M. 2009. The proprotein convertase *amontillado (amon)* is required during *Drosophila* pupal development. Dev. Biol. 333, 48–56.

Rayburn, L.Y.M., Gooding, H.C., Choksi, S.P., Maloney, D., Kidd, A.R., Siekhaus, D.E., Bender, M. 2003. *amontillado*, the drosophila homolog of the prohormone processing protease PC2, is required during embryogenesis and early laval development. Genetics 163: 227-237.

Reidy, S.P., Weber, J.M. 2000. Leptin: an essential regulator of lipid metabolism. Comp. Biochem. Physiol. 125, 285-297.

Rothberg, J.M., and Leamon, J.H. 2008. The development and impact of 454 sequencing. Nature Biotechnology 26, 1117-1124.

Satake, H., Kawada, T., Nomoto, K., Minakata, H. 2003. Insight into Tachykinin-related peptides, their receptors, and invertebrate tachykinins: a review. Zool. Sci. 20: 533–49.

Scherer, P.E., Williamn, S., Fogliano M., Baldini, G., Lodish, H.F. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J. Biol. Chem. 270, 26746-26749.

Scherkenbeck, J., and Zdobinsky, T. 2009. Insect neuropeptides: Structures, chemical modifications and potential for insect control. Bioorg. Med. Chem. 17, 4071-4084.

Schoofs L., Holman G.M., Hayes T.K., Nachman R.J., De Loof A. 1990. Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. FEBS Lett. 261, 397–401.

Skaer, N.J.V., Nassel, D.R., Maddrell, S.H.P., Tublitz N.J. 2002. Neurochemical fine tuning of a peripheral tissue: peptidergic and aminergic regulation of fluid secretion by Malpighian tubules in the tobacco hawkmoth *M. sexta*. J. Exp. Biol. 205, 1869–1880.

Snodgrass, G.L., and Gore, J.. 2007. Status of insecticide resistance for the tarnished plant bug, pp. 56-61. In Proceedings, Beltwide Cotton Conferences, 9-12 January 2007, New Orleans, LA. National Cotton Council, Memphis, TN.

Van Loy, T. Vandersmissen, H.P., Poels, J., Van Hiel, M.B., Verlinden, H., Broeck, J.V. 2010. Tachykinin-related peptides and their receptors in invertebrates: A current view. Peptides 31, 520-524.

Williams, M.R. 2008. Cotton insect losses-2007. Proceed. Beltwide Cotton Conf. National Cotton Council National Cotton Council, Memphis, TN.

Williams, M.R. 2009. Cotton insect losses-2008. Proceed. Beltwide Cotton Conf. National Cotton Council National Cotton Council, Memphis, TN.

Williams, M.R. 2010. Cotton insect losses-2009. Proceed. Beltwide Cotton Conf. National Cotton Council National Cotton Council, Memphis, TN.

Winther, A.M., Acebes, A., Ferrus, A. 2006. Tachykinin-related peptides modulate odor perception and locomotor activity in *Drosophila*. Mol. Cell. Neurosci. 31, 399–406.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T., 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762–769.

Young, O.P. 1986. Host plants of the tarnished plant bug, *Lygus lineolaris* (Heteroptera: Miridae). Ann. Entomol. Soc. Am. 79, 747–762.

Zagrobelny, M., Scheibye-Alsing, K., Jensen, N.B., Moller, B.L., Gorodkin, J., Bak, S. 2009. 454 pyrosequencing based transcriptome analysis of Zygaena filipendulae with focus on genes involved in biosynthesis of cyanogenic glucosides. BMC Genomics 10, 574-589.

Zhang, K., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425-432.

Zou, Z., Najar, F., Wang, Y., Roe, B., Jiang, H. 2008. Pyrosequence analysis of expressed sequence tags for *Manduca sexta* hemolymph proteins involved in immune responses. Insect Biochem. Mol. Biol. 38, 677-682.

Zhu, M., Chen, K., Wang, Y., Guo, Z., Yin, H., Yao, Q., Chen, H. 2008. Cloning and partial characterization of a gene in *Bombyx mori* homologous to a human adiponectin receptor. Acta Biochim. Pol. 55, 241-249.

Zhu, Y.Y., Machleder, E.M., Chenchik, A., Li, R., Siebert, P.D. 2001. Reverse transcriptase template switching: A SMART approach for full-length cDNA library construction. Biotechniques 30, 892-897.

Table 1. Summary statistics for *L. lineolaris* gut and whole body assembly and annotation.

	Gut	Whole body
Assembly		
Total number of reads	229,919	262,555
Average read length	208.5 bp	233.1 bp
Total number of singlets[1]	62,484	
Total number of contigs	3,549	6,970
Average contig length (range)	349 (55 – 2415 bp)	392 (100 - 3466 bp)
Annotation		
Number of contigs with a blast hit[2]	1,605 (45.22%)	3,126 (44.85%)
Number of contigs with at least one GO term[3]	1,233 (34.74%)	2,196 (31.50%)
Number of contigs with at least one EC code[4]	581 (16.37%)	533 (7.64%)
Number of contigs with at least one IPR[5]	152 (4.28%)	294 (4.22%)

[1]Singlet - unassembled sequence

[2]blast hit cutoff was $e\text{-}10^{-3}$

[3]Classification of a gene and gene products according to the Gene Ontology (GO) consortium

[4]Enzyme commission (EC) number classifies enzymes based on the reaction they catalyze

[5]InterPro (IPR) classifies proteins based on sequences to known domains, repeats and important sites.

Table 2. Top 3 enzyme terms in each enzyme classification (EC): oxidoreductase, transferases, hydrolases, lyases, isomerases and ligases for the *L. lineolaris* gut and whole body transcriptomes (*E*-values < 2.8*e*-4).

Enzyme	Sub-class description	Number of contigs	
		Gut	Whole Body
Oxidoreductases (EC: 1.x.x.x)	EC: 1.1.x.x - acting on the CH-OH group of donors	17	25
	EC: 1.6.x.x - acting on NADH or NADPH	22	14
	EC: 1.14.x.x - acting on paired donors, with incorporation or reduction of molecular oxygen	10	7
	Others*	41	47
	Total	90 (15.08%)	93 (16.34%)
Transferases (EC: 2.x.x.x)	EC: 2.3.x.x - acyltransferases	16	17
	EC: 2.4.x.x - glycosyltransferases	13	9
	EC: 2.7.x.x - transferring phosphorus-containing groups	51	70
	Others*	27	23
	Total	107 (17.92%)	119 (20.91%)
Hydrolases (EC: 3.x.x.x)	EC: 3.2.x.x - glycosylases	63	22
	EC: 3.4.x.x – acting on peptide bonds (peptidases)	110	76
	EC: 3.6.x.x - acting on acid anhydrides	113	141
	Others*	48	40
	Total	334 (55.95%)	279 (49.03%)
Lyases (EC: 4.x.x.x)	EC: 4.1.x.x – carbon-carbon lyases	9	7
	EC: 4.2.x.x – carbon-oxygen lyases	13	6
	EC: 4.4.x.x - carbon-sulfur lyases	2	3
	Others	1	3
	Total	25 (4.19%)	19 (3.34 %)

Table 2. Continued

Isomerases (EC: 5.x.x.x)	EC: 5.2.x.x - cis-trans-Isomerases	6	4
	EC: 5.3.x.x - intramolecular isomerases	4	4
	EC: 5.4.x.x – intramolecular transferases (mutases)	2	2
	Others*	5	6
	Total	17 (2.85%)	16 (2.81%)
Ligases (EC: 6.x.x.x)	EC: 6.1.x.x - forming carbon—oxygen bonds	7	14
	EC: 6.2.x.x - forming carbon—sulfur bonds	1	5
	EC: 6.3.x.x - forming carbon—nitrogen bonds	14	23
	Others*	2	1
	Total	24 (4.02%)	43 (7.56%)

*Other oxidoreductase classifications for the gut library include: 1.2.x.x (6), 1.3.x.x (3), 1.4.x.x (3), 1.5.x.x (1), 1.8.x.x (8), 1.9.x.x (6), 1.10.x.x (4), 1.11.x.x (7), 1.13.x.x (1) and 1.15.x.x (3). Other oxidoreductase classifications for the whole body library include: 1.2.x.x (7), 1.3.x.x (6), 1.4.x.x (2), 1.5.x.x (4), 1.8.x.x (5), 1.9.x.x (5), 1.10.x.x (6), 1.11.x.x (7), 1.13.x.x (1) and 1.15.x.x (4). Other transferase classifications for the gut library include: 2.1.x.x (7), 2.2.x.x (1), 2.5.x.x (13), 2.6.x.x (5) and 2.8.x.x (1). Other transferase classifications for the whole body library include: 2.1.x.x (6), 2.5.x.x (8), 2.6.x.x (6) and 2.8.x.x (3). Other hydrolase classifications for the gut library include: 3.1.x.x (36), 3.3.x.x (2) and 3.5.x.x (10). Other hydrolase classifications for the whole body library include: 3.1.x.x (31), 3.3.x.x (1) and 3.5.x.x (8). Other lyases classifications for the gut library include: 4.99.x.x (1). Other lyases classifications for the whole body library include: 4.6.x.x (2) and 4.99.x.x (1). Other isomerases classifications for the gut library include: 5.1.x.x (2) and 5.99.x.x (3). Other isomerases classifications for the whole body library

Table 2. Continued

include: 5.1.x.x (1) and 5.99.x.x (5). Other ligases classifications for the gut library include: 6.4.x.x (1) and 6.5.x.x (1). Other ligases classifications for the whole body library include: 6.4.x.x (1). Number of contigs is in parenthesis.

Table 3. Top5 combined InterPro predictive protein families and domains in the *L. lineolaris* after individual gut and whole body transcriptomes analysis.

Family			Domain		
InterPro number	Frequency	Description	InterPro number	Frequency	Description
IPR013128	10	Peptidase C1A, papain	IPR013781 and IPR017853	12	Glycoside hydrolase, catalytic core
IPR001128	7	Cytochrome P450	IPR007087	10	Zinc finger, C2H2-type
IPR001461	5	Peptidase A1	IPR000668	8	Peptidase C1A, papain C-terminal
IPR015643	5	Peptidase C1A, cathepsin B	IPR012335	6	Thioredoxin fold
IPR001360	4	Glycoside hydrolase, family 1	IPR009007	5	Peptidase aspartic, catalytic

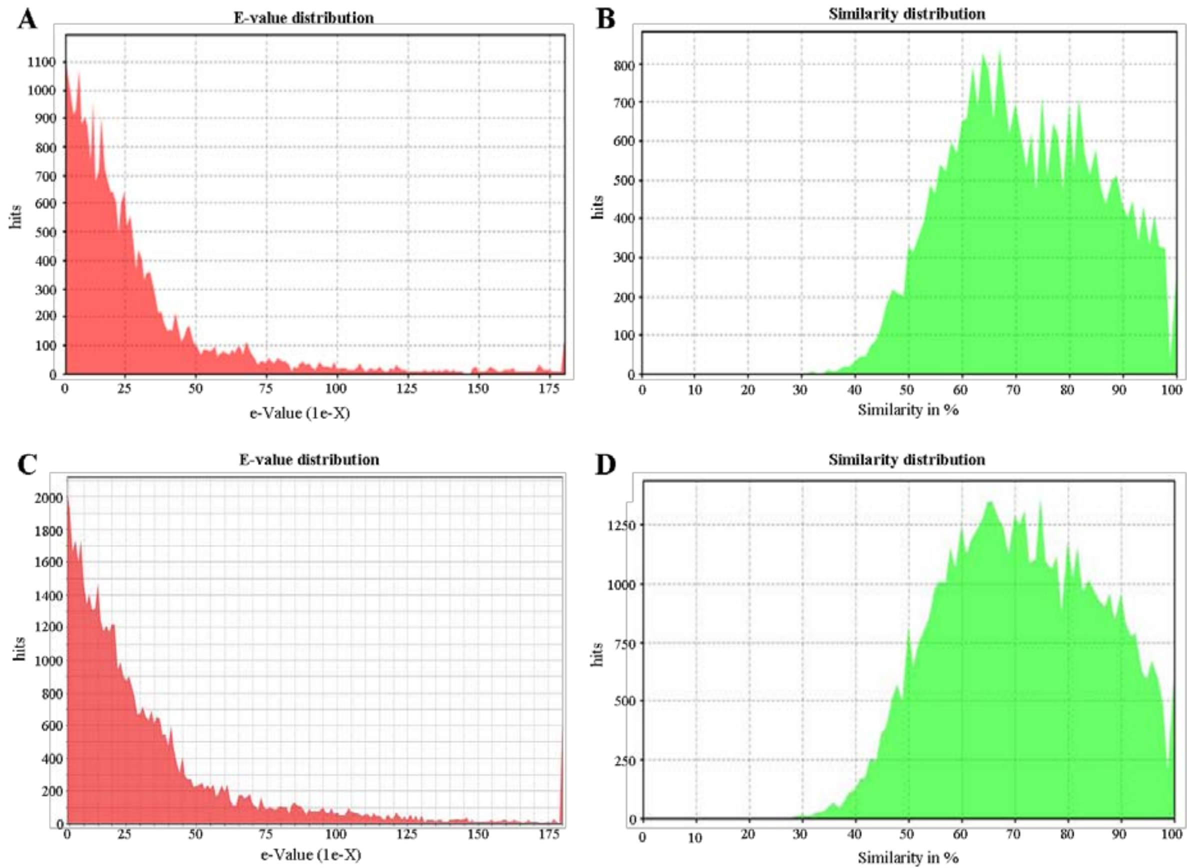


Figure 1. Overview of homology searches (BLASTx, *E*-value cut-off 10^{-3}) of *L. lineolaris* adult 454 data against the non-redundant database at NCBI. (A) *E*-value distribution of the top BLAST hit for each contig in the gut library. (B) Similarity distribution of the top BLAST hit for each contig in the gut library. (C) *E*-value distribution of the top BLAST hit for each sequence in the whole body library. (D) Similarity distribution of the top BLAST hit for each sequence in the whole body library.

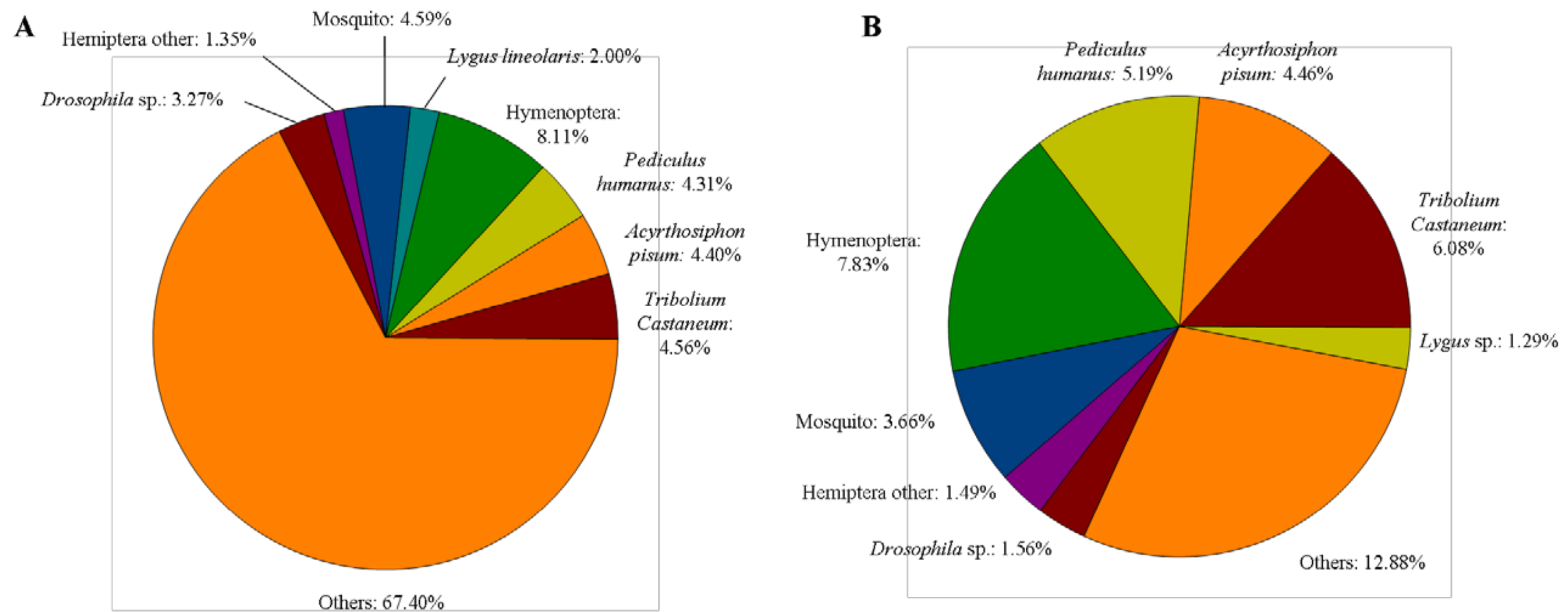
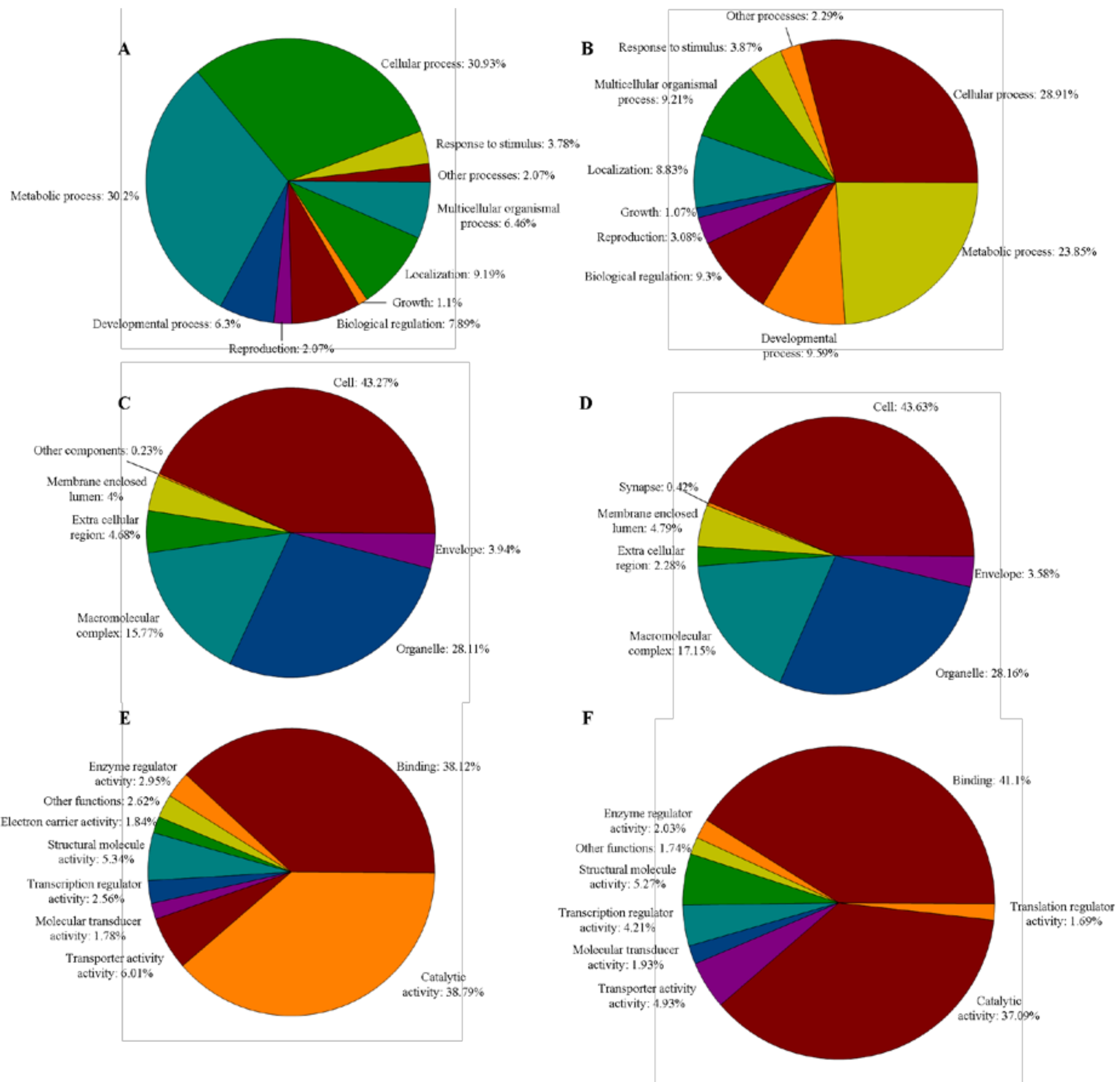


Figure 2. Top BLAST hit species distribution for each contig. (A) Gut library (3,549 contigs) and (B) whole body library (6,970 contigs). Note that *Acyrthosiphon pisum* (the green aphid), the closest species to *L. lineolaris* with the genome available, had a significant number of top hits. Also, many top hits are to *L. lineolaris* and *L. herpesrus* sequences already available at GenBank.

Figure. 3. Gene ontology (GO) assigned terms for the *L. lineolaris* gut and whole body transcriptomes. (A) Gut library biological process terms at level 2. Other processes include: biological adhesion (9), immune system process (17), locomotion (5), multi-organism process (12), pigmentation (6), rhythmic process (8) and viral reproduction (4). (B) Whole body library biological process terms at level 2. Other processes include: biological adhesion (22), immune system process (23) and multi-organism process (18). (C) Gut library cellular component terms at level 2. Other components include: synapse (4) and virion (1). (D) Whole body library cellular component terms at level 2. (E) Gut library molecular function terms at level 2. Other functions include: antioxidant activity (17), nutrient reservoir activity (8) and translation regulator activity (22). (F) Whole body library molecular function terms at level 2. Other functions include: antioxidant activity (12), electron carrier activity (23) and nutrient reservoir activity (1). A total of 1,233 GO terms has been assigned to gut library and 2,196 terms to whole body library. More than one GO term can be assigned to one sequence. Number of contigs is in parenthesis.


A
Cellular process: 30.93%
Response to stimulus: 3.78%
Other processes: 2.07%
Multicellular organismal process: 6.46%
Localization: 9.19%
Growth: 1.1%
Biological regulation: 7.89%
Reproduction: 2.07%
Developmental process: 6.3%
Metabolic process: 30.2%
B
Other processes: 2.29%
Response to stimulus: 3.87%
Multicellular organismal process: 9.21%
Cellular process: 28.91%
Localization: 8.83%
Growth: 1.07%
Reproduction: 3.08%
Biological regulation: 9.3%
Metabolic process: 23.85%
Developmental process: 9.59%
C
Cell: 43.27%
Other components: 0.23%
Membrane enclosed lumen: 4%
Extra cellular region: 4.68%
Envelope: 3.94%
Macromolecular complex: 15.77%
Organelle: 28.11%
D
Cell: 43.63%
Synapse: 0.42%
Membrane enclosed lumen: 4.79%
Extra cellular region: 2.28%
Envelope: 3.58%
Macromolecular complex: 17.15%
Organelle: 28.16%
E
Enzyme regulator activity: 2.95%
Binding: 38.12%
Other functions: 2.62%
Electron carrier activity: 1.84%
Structural molecule activity: 5.34%
Transcription regulator activity: 2.56%
Molecular transducer activity: 1.78%
Transporter activity activity: 6.01%
Catalytic activity: 38.79%
F
Binding: 41.1%
Enzyme regulator activity: 2.03%
Other functions: 1.74%
Structural molecule activity: 5.27%
Transcription regulator activity: 4.21%
Translation regulator activity: 1.69%
Molecular transducer activity: 1.93%
Transporter activity activity: 4.93%
Catalytic activity: 37.09%

```
Ll (1) ------------TPIKAPTIDWGAAFLTITGDIVVYITYLGFFTLVYQEDHNYAIWIFGFSFN
Rp (78)VSSFALPVVLARAPVTKPAIEWGACYLTLTGNIVVYITYLGFFVTLYQDDSDYNMW-------
Ap (78)VSSFALPIIMAR----VNAIAWTACNFTLCGNIVVYLTFIGFFLTLYQEDTDYSLW-------
Nv(101)VSSFALPIVLARSPMDNPVIQTGACYLTLAGNVVVYLTIIGFFLAFDHEDSDYSMW-------
```

Figure 4. Alignment of *L. lineolaris* putative leptin receptor (Ll) with *R. prolixus* (Rp; AAQ20841.1) leptin receptor-like protein and predicted *A. pisum* (Ap; NP_001156269.1) and *N. vitripennis* (Nv; XP_001605479.1) leptin receptor-like proteins. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues. The Vps55 domain is indicated by boxed residues.

```
Ll (1) ---PALHEVQDFLNDDDSCMLEEEEGYHGCPLPSTPIDPTLFDKEMVEAFKS---S---GFGEMMHNARQPAEDFVRKVWEASWSVCNFRHLPDWLQDND
Nv(38) SDIPGLHEVKELLEDDDTSCLAEEEDGVGCPLPSTPEDDRLLDCEMTEVLKAGVLSDEIDLGALAHNAAEQAEEFVRKVWEASWKQCHFRHLPSWLQDND
Aa(51) EEDDSLASEDMDLLDDDDELEEEEDDGVGCPLPSTPEDTQLLEAEMTEVLKAGVLSDEIDLGALAHNAAEQAEEFVRKVWEASWKVCHFKHLPAWLQDND
Bm (5) AGKDGIPMRRALPLKWTSSTKSSQKKKKDVRCLQHRRINICWMPKWLKFLKAGVLSDEIDLGALAHNAAEQAEEFVRKVWEASWNVCHFRHLPRWLQDND

Ll (92) YLHNGHRP---------
Nv(138) FLHAGHRPPLPSFYACF
Aa(151) FLHKGHRPPLPSFSACF
Bm(105) YLHKGHRPPLPSFSACF
```

Figure 5. Adiponectin receptor is highly conserved among insect groups. Alignment of *L lineolaris* putative adiponectin receptor (Ll) with *N. vitripennis* (Nv; NP_001153422.1), *Aedes aegypti* (Aa; XP_001649426.1) and *Bombyx mori* (Bm; NP_001093316.1) adiponectin receptors. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.

```
Ll (1) -----------------------------------------------------------------------------------------GKKAPQMGFSA
Rp (1) -MPVGSLLVMSCVLAACLAQERRAMGFVGMRGKKDTPDMEEYKRAPSTMGFQGVRGKKDDLIGEPDDTFLEEFKRAPAAMG---FQGMRGKKTPAMGFMG
Pa(101)MRGKKDPITQQEFLQEFLDKRAPNMGFMGMRGKKDPTDFDYFDKRAPSLGFQGMRGKKDQWEEDPDMYKRAPSAGFHGMRGKKDFDDGDFMAEKRMGFMG
Pc(10) MVMVVVTGALAEEQDTADRERRAPSGFLGMRGKKDVSTPLENIVPAVN----------DYTLQDSFPASLYGLRDDNGPVV---LAVPWRVKKAPSGFLG
Pi(10) LVVLALAACVSQAQEASDRERRAPSGFLGMRGKKDAAAPLNEVDEASAN--------DYPILPDPIAARLYAFRNGNAPVG---LAVPLRGKKAPSGFLG

Ll(12) MRGK------------------------KEDYGFWAGEEDHPG--FYDPRGR-RPPSGFFGMRGKKVP--------------SAFFGMRGKKGP-SGFMG
Rp(97) MRGKK-----------------------DSDYGWWEEDKRAPASGFFGMRGKKAPASGFFGMRGKKGPSS------------SAFFGMRGKKGP-SGFMG
Pa(201)MRGKKESDFEGDDYPEGLADDDVWGDQDEEFTGGEDVNKRAPASGFFGMRGKKVPASGFFGMRGKKGPSVGFFAMRGKKAPSAGFMGMRGKKAPGSGFMG
Pc(97) MRGKKS---------------------GEEAFGEAGMDSELET-------LLKRAPSGFLGMRGKKAP--------------SGFLGMRGKKAP-SGFLG
Pi(99) MRGKKS---------------------DEEIFGDASDDSDLET-------LLKRAPSGFLGMRGKKAP--------------SGFLGMRGKKAP-SGFLG

Ll(70) VRGK----KDGADDLDALMQI---
Rp(161)VRGK----KDSPDDLNHLLQLLRE
Pa(301)MRGKKDSEMEGAEDLDSLLQYLGA
Pc(154)MRGKK--HFDDESEIDAYIQALTA
Pi(156)MRGKK--YYDDDGEMDALIQAFTA
```

Figure 6. Alignment of *L lineolaris* putative tachykinin precursor (Ll) with *R. prolixus* (Rp; ACS45389.1), *Periplaneta Americana* (Pa; AAX11212.1), *Procambarus clarkii* (Pc; BAC82426.1) and *Panulirus interruptus* (Pi; BAD06363.1) tachykinin precursor. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.

```
Ll (1)  ---YQGGWSVHAG-PNRAYLHYVILIVIYSALIFLGVLSNVVVSFVVARRPQMHTARNLY
Ph(40)  AKILQGFWSSGKD-RNLSSQAEAALITIYTFLIIGGLITNFLVCFVVARRKQMHTARNLY
Ag(40)  --IRQGLIEQYSNNRKVADPWYHILIIMYGTLIVFGATGNSLVVLAVARKPQMRTARNMF
Ap(40)  EEVYSIMLEHKRDSRNLDKSTETLLIIVYFGLMIVGLSANLTVIYVVARRAQMHTSRNLY

Ll (57) IVNLTVSDLTLCYMICMPFTLVHIINRHWTLG--VALCKLVPFVQGTNIMVSVGTITVI---------
Ph(100) IVNLTVSDISLC-LICMPFTLVMILRRQWTLG--PVLCKLVSFLQATNIMVSVGTITVIALDRYFTIC
Ag (80) IVNLAVSDLLLC-LVTMPLTLVEILTKYWPMGRLPFLCKSIGTLQATSIFVSTISITAIALDRYQVIV
Ap(101) IVNLAVSDMTLC-LVCMPFTLTSILRHQWTMG--TVLCKLVPLLQGTNIMVSVGTITVIAIDRYWVIV
```

Figure 7. Alignment of *L lineolaris* putative neuropeptide Y receptor (Ll) with *P. humanus* (Ph, XP_002427965.1), *Anopheles gambiae* (Ag, XP_313000.2) and *A. pisum* (Ap, XP_001943708.1) neuropeptide Y receptors. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues. The 7tm_1 motif domain is indicated by boxed residues.

```
Ll (1)  -------NFKMNKNPS------DSQLPRVRDFSREEFIRDRLG--SQQLPFALPVTLVYAAIFITGVVGNLAICRVITKNQSMQTSTNYYLFSLAVSDLS
Ph (1)  -MTNNNNNFDVTSNSSEITTTRDDEDSVNFNISLEQYLLRTLGPKHLALTTVIPLTVIYIFIFVTGIFGNISVCVVIIKNPSLHTATNYYLFSLAVSDLT
Am (1)  METSDDDSYDFWKNWDLKN------------LTEAEYLAKVLGPKYLSMRMVIPLTIIYMIIFVTGIFGNITTCTVIIKNPAMQTATNYYLFSLAISDLI
Tc (1)  ---MDNDTKVMCMNNSSS---------------LDTYVQCYRGAQQQPLEMAIPLTVVNVLIFVSGFFGNVAVCIVIIKHRSLHTATNYYLFNLAISDLT
Ap (1)  ----------------------------------------------------------------------------------------------------

Ll(86)  LLILGLPNEVSLYWKQYPWQFGTPVCKLRALVSEMTSYTSVLTIVAFSMERYLAICHPLHNYSMSGLHRAVKIIGALWVVSLLAASPFAIYTKINYLQYP
Ph(100) LLTLGLPNDLSVYWQQYPWLFGKGLCKVRALVSEMVSYTSVLTIVAFSMERYLAICHPLHHYAMSGLKRAVKIIGILWIVSLVGATPFAVYTTVNYLDYP
Am(89)  LLVLGLPNELSLFWQQYPWVLGVSLCKIRAYVSEMSSYVSVLTIVAFSMERYLAICHPLRVYTISGLKRPIRFILAAWLIALISAIPFAIYTKVNLVEYP
Tc(83)  LLIFGLPNDVMLYWHQYPWPFGVEFCKLRALLSEMASYVSVLTIVAFSTERYLAICYPLYLHTMSGLQRAVRIIACLWLCAFVSALPFCIYTRVHYLFYP
Ap (1)  FSVSGLPNDLSVYWQQYPWPLGEVLCKFRALVSEMTSYTSVLTIVAFSMERYLAICHPLHSYAMSGLKRAVRIIAVVWVISFFAALPFAMFTTVDYVDFP
                            *

Ll(186) EGSGNDLPETAFCAMLKENIPKGFPIYELSFLLFFLVPMKIIIVLYILIGRKIQSRGNE-LSANMDGSVHRDARHLKSRRNIIRMLSKFD----------
Ph(200) PGSGQKVLESGFCAMLSTNIPSNWPIYEISSFLFFLLPMLVILILYIRMGIAIRESGAD-DSMKRLQGLVHKPRHSNSRKSIIRMLAAVVITFFLCWAPF
Am(189) PESGNYSADSAMCAMLTI--YADFPLYELSTIIFFLIPMLIILVVYTRMGLKIRNSTKDTLNSVVQGAIHGDSRQIQSRKSVIKMLSAVVILFFICWAPF
Tc(183) PNSTNILPDSAFCGMLYQ--PEGIPLTELSTLIFFIIPMLAIAVQYTKMGLEIAKTTRKTLGHGLRGSVHRDSRRTQSNRSVIKMLSAVVIAFFLCWAPF
Ap(101) PGNGP-ILESAFCAMLDKNVPTGVPVYELSSLLFFLVPMMIIIVLYVLIGLQIRQSSRHSLGKQMQGNVHGETKQIQSKKSIVRMLAAVVIAFFLCWAPF
```

Figure 8. Alignment of *L lineolaris* putative CAPA receptor (Ll) with *P. humanus* (Ph, XP_002426611.1), *Apis mellifera* (Ap, NP_001091702.1), *Tribolium castaneum* (Tc, XP_973937.1) and *A. pisum* (Ap, XP_001950333.1). Two *L. lineolaris* contigs were combined for the alignment as they may be part of the same gene. Asterisk indicates the junction between these contigs, CAPA 2 and 1. The 7tm_1 motif domain is indicated by boxed residues. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.

Figure 9. G protein-coupled receptor 155 (GPR 155) is conserved among wide variety of animal taxa. Alignment of *L lineolaris* putative GPR 155 (Ll) with *T. castaneum* unkown protein (Tc; XP_972562.1), *A. mellifera* (Am; XP_001120777.1), *Macaca mullata* (rhesus monkey) (Mm; XP_001089835.2), *Taeniopygia guttata* (zebra finch) (Tg; XP_002196451.1), *Takifugu rubripes* (a puffer fish) (Tr; NP_001098703.1), *Ciona intestinalis* (a sea squirt) (Ci; XP_002121506.1) and *Xenopus laevis* (African clawed frog) (Xl; NP_001085895.1) GPRs155. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.

```
Am (1) -----------------------------------MKVEPIDNLYLALIQCFAIILCGYIAGRFDVITKIEANGLNTFVGTFALPSLIFMSLAKLNFTLV
Ci (1) -----------------------------------MSAAGFTNLFPALVNCFVIIIFGYLAGWFNVVTPSQGKGISNFVATFALPATIFKSMVELNFATV
Tr (1) MDAASRFVLIHGKNIS--HNTLAG-----SAAVPP---MSIDKLFPALLECFGIILCGYIAGRADLITESQAKGLGNFVSKFALPALLFKNMVLLDFGDV
Mm (1) MNSFSNLPAENLTIAVNMTKTLPTALTHGFNSTNDPPSMSITRLFPALLECFGIVLCGYIAGRANVITSTQAKGLGNFVSRFALPALLFKNMVVLNFSNV
Tg (1) MDSYSDFSAKNLSSSDNMSLSVPG--QAGLNTTGSPPSMSISRLFPALLECFGIILCGYIAGRANIITSTQGKGLGNFVSRFALPALLFKNMVVLNFSNV
Xl (1) MDIASGIAWKNFSSSVNMNNVLGDG-ANPANATGESTSMSINRLFPALLECFGIIMCGYIAGRANVISATQAKGLGNYVSRFALPALLFKNMVVLDFSNV

Am(66) NWKFLFAVLLAKSCVFFVVLSISLIIKR-RSNPGCAALFAIFTTQSNDFAIGYPMIHALYGKTHPEYAAYLYLMAPISLVILN-----------------
Ci(66) NWSFMLACMFGKATVFILVIVLTLIMLR-RNGVGRSALYAIFATQSNDFALGYPIVKVLYAKTHPELLQYIYLAAPVSLLILNPIGFIMLEIDKQWRKED
Tr(91) IWAFLWSVLVAKVAVFVLVCVLTLMVASPDSRYSKAGLYAIFATQSNDFALGYPIVDALYRSTYPEYLQYIYLVAPVSLMLLNPIGFALCEVQKWRQSDR
Mm(101)DWSFLYSILIAKASVFFIVCVLTLLVASPDSRFSKAGLFPIFATQSNDFALGYPIVEALYQTTYPEYLQYIYLVAPISLMMLNPIGFIFCEIQKWKDTQN
Tg(99) NWSFLYSVLIAKAAVFFLVCVLTLLVASPENRFSKAGLFPIFATQSNDFALGYPIVEALYQATYPEYLQYIYLVAPISLMMLNPLGFIFCEIQKWRNNRT
Xl(100)NWSFLYSILIAKASVFIVVCVLTLLVADPQSRFSKAGLFPIFATQSNDFALGYPIVEALYQTTYPKYLQYIYLVAPVSLMMLNPLGFVLCEIQKWRDNPN

Ll (1) ------------GNFIFAHEVPDALEALLKVFGSAFTASALFLLGLRMVGSVRNFKGEALMVPICLIAVKELVLPLVTERFQVWS------------LG
Am(148)-----------PIGFVLLEIVPPVLATVLNIFGNAFSASALFLLGLMMVGKVHKLKGTALVIPGILISIKLLVLPLVIRESIILLN------------PG
Ci(215)NPIVLMVFLGLAFHFICGGKLPYLPKQILTTLANSFSATALFYLGLSMVGKLSKQKGVNLLVPCVLIVAKILLLPILIRLFMYLFSSVLPVHVPQITNTT
Tr(209)NPVVFMVMIGILGHFALGQQIPAVLSEFIDGLANSFGGAALFYLGLTMVGQLRKLTRDTGVALILLITAKLLVMPLVCKDMVDILD------------VG
Mm(219)NPIVFMVFIGIAFNFILDRKVPVYVENFLDGLGNSFSGSALFYLGLTMVGKIKRLKKSAFVVLILLITAKLLVLPLLCREMVELLD------------KG
Tg(217)NPIVFMVFIGIASNFILGQKIPEYLENFLDGLASSFSGSALFYLGLTMVGQTKKLTKGMVVSLILLITAKLLMMPFLCREMVELLD------------KS
Xl(218)NPIVFMVFIGIAFNFVLGQKIPIYLENFLDGLASSFSGAALFYLGLTMVGQTGKLEKSSFVALLLLITAKLLVLPLICRELVELLD------------KT

Ll(76) HKS--QRMKAHSALRVPVGTIPSAPGVFVYATNYGLEVKLIATAMVACSFVSAPLMFISAKMVSISH--LSPLKTLSSLAAFELDLSIVGIFACVILLSV
Am(225)ENA--TDTQDLSTYGFLYGTIPTAPALFIFTLRYNLEIDLIASAMVACTFLSAPLMFVSAKLIDAVASGISPENYEHQLNIFSFDVSIASATVCIWLIIC
Ci(315)TNTSTSYLTTLSNFGFLYGTFPTAPSVIIYATKYGMEVDRLASGMVLCTTLSAPIMYVSAWTLSIPS--MQYNFYKSQVILVDRDVSIVGIMCALWCIAV
Tr(297)VNSTSANHTSLSNFAFLYGVFPTAPSVAIYAGHYNMELEVVTSGMVISTFLSAPIMYVSAWLLTIPL--MDPAPLVAELENVSFNISIVSLIALVWTIVV
Mm(307)DN--VVNHTSLSNYAFLYGVFPVAPGVAIFATQFNMEVEIITSGMVISTFVSAPIMYVSAWLLTFPT--MDPKPLAYAIQNVSFDISIVSLISLVWSLAI
Tg(305)DS--TVNHTSLSNYAFLYGVFPAAPGVAIFASQFNMEVGIITSGMVISTFVSAPIMYVSAWLLTIPS--MDPNPLAAALQNVSFDISIVSLISLTWSLTV
Xl(306)SD--MANHTSLTNYAFLYGVFPVAPGVAIFASQFNMELEIITPGMVISTFVSAPIMYVSAWLLTIPS--MDVKPLVSALQNVSFDISILSILSLIWSLAV

Ll(172)FT--------------------------------------------------------------------------------------------------
Am(323)FIGLEKRKYKCITHKCTFCIIIAQLATAIGVVIWTKLESHNSDSVLWYIQFILITMGVYASRIWTMAIAATLLYLSSRSLDFVYNIQKWFYPIG--WGIP
Ci(413)FMGT--KKFRRVPHIITLFLTCSMFFMCVVAVVAMSS--KSYSNTAKLICYALLFFGVKSTRLLSVSLAISLLVIKIYGEAKLWQLRWFFIFVPGLLSAG
Tr(395)MLLS--RKFKRLPHLFALNLFLAQFLVCISMILWNSL-VKYDNLLGRTVTFTLLYSSLYSTYIWTGLIPLCLALINRDDL--LRLRPGVFMALG--WGVP
Mm(403)LLLS--KKYKQLPHMLTTNLLIAQSIVCAGMMIWNF--VKEKNFVGQILVFVLLYSSLYSTYLWTGLLAISLFLLKKRER--VQIPVGIIIISG--WGIP
Tg(401)VVLS--KKYKQIPHMITTNLLVAQFIACIGMVAWNFT-VKEKDITIQILVFIFLYSSLYSTYLWTGFLSFSLFLLKKRET--VKIPIGFIIIAG--WGIP
Xl(402)LLLS--KKYKRLPHLLTCNLLLSQLIACVGMMMWNFI-IQERNVVGQILVFVILYSALYSSYFWTGLLSLSLILLKKRD---GKVPVAFLVLVG--WGVP
```

Figure 10. Proprotein convertase (furin) presents a high degree of relatedness among insect orders. Alignment of *L lineolaris* putative furin (Ll) with *P. humanus* (putative) (Ph; XP_002422846.1), *Spodoptera frugiperda* (Sf; CAA93116.1), *N. vitrippenis* (predicted) (Nv; XP_001601381.1) and *T. castaneum* (predicted) (Tc; XP_969307.1) furins. Two *L. lineolaris* contigs were combined for the alignment as they may be part of the same gene. Sequences are at least 94% identical in the region of overlap. The proprotein convertase P-domain is indicated by boxed residues. Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.

```
Ll (1) ----------------------------VNDAVEARALSLNPDHIDIYSASWGPEDDGKTVDGPGPLARRAFINGVTTGRKGKGSIFVWASGNGGRHTDS
Sf(227)AAVAYNQYCGVGIAYNASIGGVRMLDGVVNDAVEARALGLNPDHIDIYSASWGPEDDGKTVDGPGPLARRAFIYGVTSGRRGKGSIFVWASGNGGRHTDS
Nv(204)AAVAFNRFCGVGVAYNSSIGGVRMLDGPVNDAVEARALGLNPDHIDIYSASWGPEDDGKTVDGPGPLARRAFIYGVTSGRQGKGSIFVWASGNGGRHTDS
Ph (70)AAVAFNDFCGIGVAYNASIGGVRMLDGTVNDAVEAKALGLNPDIIDIYSASWGPEDDGKTVDGPGPLARRAFIYGVTNGRKGKGSIFVWASGNGGRHTDS
Tc(248)AAVAYNQFCGIGVAYNSSIGGVRMLDGVVNDAVEARALGLNPEHIDIYSASWGPEDDGKTVDGPGPLARRAFIYGVTSGRKGKGSIFVWASGNGGRHTDS

Ll(73) CNCDGYTNSIFTLSIS------------------------------------------------------------------------------------
Sf(327)CNCDGYTNSIFTLSISSATQGGYKPWYLEECSSTLATTYSSGTPGHDKSVATVDMDGRLRSDHICTVEHTGTSASAPLAAGICALALEANPELTWRDMQY
Nv(304)CNCDGYTNSIFTLSISSATQGGYKPWYLEECSSTLASTYSSGTPGNDKSVTTVDMDARLREDYICTVEHTGTSASAPLAAGIAALALEANPTLTWRDMQY
Ph(170)CNCDGYTNSIFTLSISSATQGGFKPWYLEECSSTLATTYSSGTPGHDKSVATVDMDGKLRPDHICTVEHTGTSASAPLAAGICALALEANPHLTWRDMQY
Tc(348)CNCDGYTNSIFTLSISSATQGGYKPWYLEECSSTLATTYSSGTPGHDKSVATVDMDARLRPDHICTVEHTGTSASAPLAAGICALALEANPSLTWRDMQY

Ll(89) ----------------------------------------------------------------------------------------------------
Sf(427)LVVMTSRPQPLEKEGGWIINGVKRKVSHKFGYGLMDASEMVSLAEQWVSVPPQHICKSQEINEDKQIESTFGYTLSAHMDVNGCSSTVNEVRYLEHVQCK
Nv(404)LVVLTSRSQPLEKESGWILNGVKRKVSHKFGYGLMDAGAMVSLAEQWTNVPTQRICKSQEINEDRQIDSSYGYTLSVSMDVNGCAGSLNEVRFLEHVQCK
Ph(270)LVLLSSRPGPLEKESGWAVNGVKRKVSHKFGYGLMDAAAMVNLAEQWTTVPPQHICKSQEINEERKIEPIHGKTLQLHMDVNGCSDTVNEVRFLEHVQCK
Tc(448)LVVLTSRSAPLEKESGWITNGVGRKVSHKFGYGLMDAGALVSLAEKWTTVPPQHICKSQELVEDRQIDAAYGTTLDFHMDVDACSNSLNEVHFLEHVQCK

Ll(90) ---------------------------ERPRDVVSSNFDDWPFLSVHYWGENPKGRWTLQVINAGNRHVNQAGILRKWQLIFYGTVSDPVRLKPPGSSRP
Sf(527)ISLRFFPRGNLRILLTSPMGTVSTLLFERPRDVVSSNFDDWPFLSVHFWGEHAEGRWTLQIVNAGNRHVNQAGILKKWQLIFYGTSVDPVRLRTK---RP
Nv(504)VSLRFFPRGNLRLLLTSPMGTTSTLLFERPRDVLNSNFDDWPFLSVHFWGEKAEGRWTLQVINTGSRHVNSPGILKKWQLVFYGTASNPIRIRTK---QY
Ph(370)ISLRFLPRGNLRILLTSPMGTTSTLLFERPRDVAASNFDDWPFLSVHYWGEKAEGRWTLQIINTGNRHLNQPGILKKWQLIFYGTVTNPIRLRSP---AS
Tc(548)ISLRFFPRGNLRIILISPMGTTSTLLFERPRDVVSSNFDDWPFLSVHFWGERAEGRWTLRIVNAGNRHVNQPGVLKKWQLIFYGTSVNPIRVRPAGRPQS

Ll(163)NHFVYP--------------------------SQQPALAPFF--------
Sf(624)SPVAPP--------------------------FAFPTAADGYETIGDSFY
Nv(601)NPVQAPSEAQLSSIHYPAHYERAQAQPVRASDYDYPNADFFSTSGFQAYQ
Ph(467)KPFAFP--------R----------------PQEQPYYLSSGFNHFQNYP
Tc(648)SPWKSP----------------------------LNTFTFPTVSESVTQV
```

```
Ll (1) ------------------------------------------TSKRNSLFDAKGRFHWTMNGVGLEFNHLFGFGVLDAGAMVALAKQWKTVPARYHCEAG
Ap(451)GKCTKSHSGTSAAAPEAAGVFALALEANPDLTWRDIQHLTVLTSKRNSLFDAKRRFQWTMNGVGLEFNHLFGFGVLDAGGMVSLARQWHTVPARYHCQGG
Fa(52) GKCTTTHSGTSAAAPEAAGVFALALEANPGLTWRDIQHLTVLTSKRNSLFDAKGRFHWTMNGVGLEFNHLFGFGVLDAGAMVALSKLWRTVPARYHCEGG
Nv(387)GQCTTTHSGTSAAAPEAAGVFALALEANPQLTWRDIQHLTVLTSKRNSLFDAKGRFHWTMNGVGLEFNHLFGYGVLDAGAMVALASKWKTVPPRYHCMAG
Ph(322)GKCTTTHSGTSAAAPEAAGVFALALEANPALTWRDIQHLTVLTSKRNSLFDAKGRFHWTMNGVGLEFNHLFGFGVLDAGAMVALAKQWQMVPARYHCEAG

Ll(59) TIKKMQKITNLEPIYMKIDTNACLNSDTQVN-----------------
Ap(551)SHTNIRKFTTKSGIILKLNTDACRDTDTHVKYLEHVQAVITLNASRRGDV
Fa(152)TVKEQREIRNGKSIQLQITTDACRGQDTQVTYL-----------------
Nv(487)TVQQVQEVPSHRSILLKIETDACAGTDLAVNYLEHVQAVISVNATRRGDL
Ph(422)NLIGAYEIHSSRSLRLKIKTTACLGSDTQVSYLEHVQAVITLNASRRGDV
```

Figure 11. Alignment of *L lineolaris* (Ll) putative proprotein convertase (type-2) with *Forficula auricularia* (putative) (Fa; ABV81592.1), *N. vitripennis* (predicted) (Nv; XP_001600872.1), *P. humanus* (putative) (Ph; XP_002427932.1) *A. pisum* (Ap; XP_001951256.1) proprotein convertase 2 (type 2). Light grey shading represents identical residues, black/white text are conserved residues, dark grey/black text are similar residues.