Determining the Route of Detoxification of Insecticides Used to Control Navel Orangeworm (NOW)

Project No.: **12-ENTO1-Berenbaum**

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Objectives:
Our principal objective in this study is to use inhibitors of the three major detoxification enzyme systems (P450, COE, GST) to determine which systems contribute to metabolism of and potentially resistance to insecticides in six classes: organophosphates, pyrethriods, diamides, diacyl hydrazines, avermectins, and spinosyns.

A secondary objective is to continue the sequencing of the navel orangeworm genome using Illumina sequencing and manual annotation.

Interpretive Summary:
Representatives of six different chemical classes (organophosphates, pyrethroids, diamides, diacyl hydrazines, avermectins and spinosyns) have been registered for use in management of navel orangeworm (*Amyelois transitella*), yet little is known of the mechanisms by which this insect detoxifies or otherwise processes them. Although the assumption is often made that rotating insecticides with different modes of action delays resistance acquisition, applied individually in rotation or simultaneously in combination, insecticides can select for cross- or multiple-resistance, which presents an enormous challenge to sustainable management. Chemical control of navel orangeworm is carried out today without synergists and, in orchards with heavy infestations, via applications in rotation. Moreover, navel orangeworms may also be exposed to insecticides used to control other pest species in almond orchards or in nearby row crops; such exposure may amplify the selection pressure placed on detoxification systems used by navel orangeworm to metabolize these different classes of insecticides. To delay resistance evolution and preserve the efficacy of available control chemicals, it is important to understand the relative sensitivity of navel orangeworm to the insecticides used in almond orchards and neighboring row crops and to characterize the mechanisms of detoxification for the different classes of pesticide.

In this proposal, we have been using inhibitors of the three major detoxification enzyme systems—cytochrome P450 monooxygenases (P450), carboxylesterases (COE), and glutathione-S-transferases (GST)--to identify the detoxification systems principally responsible for metabolism and potential resistance to the six classes of control chemicals in use today for navel orangeworm management (Niu et al. 2012).

Materials and Methods:
Insecticides were tested against a laboratory colony of *A. transitella* (J. Siegel:USDA), which was maintained at conditions of 28 ± 4ºC with a photoperiod of 16:8 (L:D) hours. Insecticides were incorporated into standard insect diet at specific concentrations and fed to neonate larvae. A PROBIT analysis was conducted to generate the median lethal concentration that would kill 50% of the sample population at 48 hours (LC_{50}). Median lethal concentrations were determined for chlorpyrifos, azinphos-methyl, chlorantraniliprole, acetamiprid, and bifenthrin and were subsequently used to assay for synergism with piperonyl butoxide (PBO) and diethyl maleate (DEM). Data were analyzed with ANOVA and Tukey's HSD test, using Statistical Analysis Software Version 9.3.

Results and Discussion:
PROBIT analysis data for chlorpyrifos, azinphos-methyl, chlorantraniliprole, acetamiprid, and bifenthrin in neonate *A. transitella* from a laboratory strain revealed significant variation in toxicity (**Table1**). The extensive range of the median lethal concentration values indicates that neonates are more susceptible to certain classes of insecticides. Both representatives of the pyrethroids, bifenthrin and β-cyfluthrin, were more toxic to neonates than other insecticides tested. Neonates displayed greater tolerance toward the neonicotinoid acetamiprid relative to the other classes of insecticides.

Table 1. Toxicity of Six Pesticides to Neonate Navel Orangeworms (NOW)

Insecticide	*n*	Slope (SE)	LC_{50} ± 95% CL (µg/g)	x^2	*P*	LC_{50} chosen (µg/g)
Chlorpyrifos	555	4.20 (0.330)	0.41 (0.38-0.45)	7.49	0.19	0.41
Azinphos-methyl	636	2.73 (0.194)	1.50 (1.35-1.65)	6.22	0.4	1.5
Chlorantraniliprole	320	1.10 (0.325)	3.2 (0.92-4.83)	0.76	0.68	4
Acetamiprid	274	2.49 (0.253)	26.56 (21.04-32.40)	1.98	0.58	25.00
Bifenthrin	239	1.72 (0.226)	0.12 (0.09-0.17)	4.56	0.34	0.15
β-cyfluthrin	355	1.33 (0.216	0.031 (0.014-0.048)	5.65	0.23	0.04

In terms of interactions between synergists and pesticides, an increase in survivorship with the application of PBO to the organophosphates indicates that P450s bioactivate members of this chemical class rather than detoxify them. PBO synergized the toxicity of acetamiprid and β-cyfluthrin but had no significant effect on the toxicity of bifenthrin and chlorantraniliprole. PBO and DEM did not synergize the toxicity of chlorantraniliprole. Only azinphos-methyl is a substrate for GST-mediated detoxification; this enzyme system does not appear to be directly involved in metabolism of the other insecticides.

If navel orangeworms detoxify other classes of insecticides through enhanced cytochrome P450 enzyme activity and resistance begins to develop by this route, then the rotation of organophosphate insecticides, which are bioactivated and not detoxified by P450s, may provide an effective alternative for chemical control of resistant navel orangeworm populations. The difference in the effects of PBO toward the pyrethroids from this research suggests

contributions from enzyme systems other than the P450s. Initial assays involving the esterase inhibitor s,s,s-tributyl phosphorotrithioate (DEF) suggest that this compound does not synergize the toxicity of either bifenthrin or β-cyfluthrin. The ineffectiveness of DEF toward β-cyfluthrin may be attributed to the presence of a cyano group protecting the ester linkage in the compound. The mechanism for bifenthrin detoxification has yet to be determined. Future research will require bioassays that examine the effects of DEF against each member selected from the registered insecticide classes.

Our experiment provides insights into the susceptibility of navel orangeworms to the different classes of insecticides and the enzyme systems involved in their detoxification. If insecticides from different classes that share a common mode of detoxification are used on a rotational basis, then cross-resistance may arise at an increasing rate. To generate optimal control strategies and minimize resistance evolution, it is essential to understand how detoxification enzymes respond to selective pressures exerted by different insecticides.

Research Effort Recent Publications:

No publications have yet been produced from this project but a poster describing these findings, presented by graduate students Catherine Dana and Mark Demkovich, won the Best MS Student Poster Prize at the Entomological Society of America North Central Branch meeting in June in Rapid City, SD. The poster may be found at this address: http://www.life.illinois.edu/cdana/files/Demkovich_Dana_NCB_ESA_2013.pptx. Figures depicting the results of the PBO synergism studies can be found in the Powerpoint; they are also available upon request.

References Cited:

Niu, G., Pollock, H., Lawrance, A., Siegel, J., and Berenbaum, M. 2012. Effects of a naturally occurring and a synthetic synergist on toxicity of three insecticides and a phytochemical to navel orangeworm (Lepidoptera: Pyralidae). *Journal of Economic Entomology* 105 (2): 410-417.

Genome Sequencing of Navel Orangeworm

Materials and Methods:
Our *A. transitella* draft genome sequence is complete, with extremely deep sequencing coverage with short reads from ILLUMINA HiSeq2000 machines. Our effort was greatly assisted by lack of heterozygosity in the sample; fortuitously, SPIRL-1966 is a long-standing laboratory strain of *A. transitella* that appears to be largely monomorphic (as judged by mapping reads back to our genome assembly), so we have been able to apply this approach to this ca. 400 Mbp genome. Specifically, the UIUC Roy Carver Biotechnology Center generated a 500-bp shotgun fragment library from a single female, thereby reducing genomic heterogeneity even further, and sequenced it deeply to approximately 40X coverage with paired-end 100-base reads. These reads were assembled in SOAPdenovo, the Short Oligonucleotide Assembly Program for *de novo* genome assembly from the BGI, resulting in essentially all unique regions of the genome, including presumably all protein-coding exons and most introns, being assembled into contigs (regions of contiguous known sequence). We then generated an additional 1-kb fragment size shotgun library, as well as 5-, 10-, and 20-kb mate-pair libraries (in which most of the sequence between the ends has been removed), from additional pooled male and female individuals. We sequenced these libraries in combination to an additional 40X coverage of paired-end 100-base reads. This information was employed primarily to arrange the unique contigs into scaffolds of ordered contigs with sequence gaps of roughly known size between them containing repetitive regions such as transposons. Finally, a subprogram within SOAPdenovo called GAPCLOSER was employed to utilize the mate-pair information to place paired-end reads with repetitive sequence within these sequence gaps and attempt to fill them in.

Results and Discussion:
The result is our fourth draft genome assembly, which is a total of 423 Mbp long in approximately 80 thousand scaffolds. While this may sound like a highly fragmented assembly, like most ILLUMINA-based draft assemblies, the vast majority of these scaffolds are short and probably from repetitive and heterochromatic regions of the genome. The average scaffold size is 5,288 bp, but the standard measure to assess such an assembly is the scaffold N50, which indicates the scaffold size above which half the genome sequence is represented, a kind of median measure. The scaffold N50 is 1.529 Mbp, which is excellent for a medium-sized genome such as this one. The largest scaffold is 9.750 Mbp, so most of the genome assembly is in very large scaffolds. We therefore anticipate that most genes will be fully represented within single scaffolds, allowing the genome assembly to be submitted for automated genome-wide gene modeling. Initial tests confirm this to be the case. Specifically for the P450s, 62 of the 63 full-length transcripts that Master's student Katherine Noble identified from our midgut transcriptome align full length to exons in single scaffolds, with only one broken between scaffolds. Preliminary examination of this TBLASTN search output suggests that an additional ~30 P450 genes are present in the genome, bringing the total to a level similar to the 85-95 genes reported in the published silk moth *Bombyx mori*, monarch butterfly *Danaus plexippus*, and diamondback moth *Plutella xylostella* genomes (http://drnelson.uthsc.edu/CytochromeP450.html).

Our next step is to submit this draft assembly to NCBI for automated genome-wide gene modeling using GNOMON. Because our focus is specifically on the P450s, we will manually check, and if necessary improve, all P450 gene models, and additionally make sure none have been missed. The result will be a high-quality CYPome for *Am. transitella*.

At present, we have a tentative inventory of the 18 most abundant P450 transcripts (>10,000 reads/kb) expressed in midguts of *Am. transitella* larvae fed xanthotoxin, a furanocoumarin found in several hostplants, including figs (**Table 2**). Of the total 63 P450 transcripts expressed in midguts, many of the 18 are likely to contribute to detoxification, based on characterizations of their P450 subfamilies in other lepidopteran genomes and their very high expression level in this xanthotoxin-induced transcriptome**.**

Research Effort Recent Publication

Noble, K., 2013. Xenobiotic detoxification in the navel orangeworm *Amyelois transitella* (Lepidoptera: Pyralidae). Master's thesis, University of Illinois at Urbana-Champaign, IL

Table 2. Relative expression levels of P450s in Navel Orangeworm (NOW) Gut Transciptome

		(Noble 2013
P450	**Clan**	**#Read/kb**
CYP6AB43	3	228,391
CYP333B19	Mito	163,115
CYP6B54	3	108,907
CYP9A66	3	108,146
CYP6B55	3	76,050
CYP6B44v2	3	65,235
CYP6AB11v2	3	56,948
CYP321C1v2	3	49,418
CYP321C3	3	47,267
CYP6AE55	3	47,057
CYP6AN17	3	41,318
CYP9A67	3	35,480
CYP6AE54	3	31,633
CYP6AB42	3	30,781
CYP6AB41	3	19,400
CYP333A10	Mito	14,237
CYP6AB40	3	10,697
CYP306A1	2	10,635