FINAL PROJECT REPORT

Project Title: Identification of critical physiological targets in codling moth

PI:	Stephen F. Garczynski	**Co-PI:**	Laura Lavine
Organization:	USDA-ARS	**Organization:**	WSU Entomology
Telephone:	509-454-6572	**Telephone/email:**	509-335-7907
Email:	steve.garczynski@ars.usda.gov	**Email:**	lavine@wsu.edu
Address:	5230 Konnowac Pass Road	**Address:**	WSU Dept of Entomology
Address 2:		**Address 2:**	PO Box 646382
City:	Wapato	**City:**	Pullman
State/Zip	WA / 98951	**State/Zip:**	WA / 99164-6382

Cooperators: Dr. Amit Dhingra, WSU; Dr. Kevin Clark, University of Georgia; Dr. Kevin Wanner, Montana State University

Total Project Funding: $130,989

Budget History:

Item	Year 1:	Year 2:	Year 3:
Salaries	$14,000	$15,000	$16,000
Benefits	$ 1,019	$ 1,024	$ 1,204
Wages	$ 4,800	$ 4,992	
Benefits	$ 466	$ 484	
Equipment			
Supplies	$22,000	$22,000	$12,000
Travel	$ 500	$ 500	
Plot Fees			
Miscellaneous	$ 5,000	$ 5,000	$ 5,000
Total	$47,785	$49,000	$34,204

ORIGINAL OBJECTIVES

The goal of this project is to provide fundamental biological information on codling moth physiology that can be used to identify new targets for enhanced pest control. Identification of critical physiological targets in codling moth will provide information and methods that will allow us and other researchers to develop new strategies and tools for control of this major pest of apple.

1) Characterize pheromone biosynthesis activating neuropeptide (PBAN) and its receptor (PBANR) in late 5th instar larvae and adult females. Pheromone biosynthesis activating neuropeptide (PBAN) is a polypeptide (short protein) hormone that stimulates the production of pheromones in insects by interacting with its receptor (PBANR). Both PBAN and PBANR are potential protein targets that if blocked could inhibit pheromone biosynthesis (including codlemone), thus having great potential to enhance the effectiveness of mating disruption for codling moth control.

2) Characterize diapause hormone (DH) and its receptor (DHR) in eggs, neonate and 5th instar larvae. Diapause hormone (DH) is encoded by the same gene as PBAN. DH, along with other physiological factors, in some moth species regulates diapause through signals sent by its receptor (DHR). Both DH and DHR are potential targets that if altered may disrupt the codling moth's ability to enter or leave diapause, and allow researchers to take advantage of this physiological pathway for codling moth control.

3) Identify potential targets in eggs and neonate larvae by analyzing the transcriptome, and determine those that may be critical for insect survival. Because of the codling moth's life cycle, eggs and neonate larvae are accessible to control measures in the orchard. Sequencing the transcriptome (a compilation of genes that are being actively expressed) of eggs and neonate larvae will allow us to identify potentially critical protein targets in these codling moth life stages. After identification, we will characterize potential protein targets to gain a further understanding of the basic physiology of eggs and neonate larvae and assess their usefulness for codling moth control.

4) Identify potential targets in adult males and females by analyzing the transcriptome, and determine those that may be critical for insect survival. Adult males and females are also accessible to control measures in the orchard. Sequencing transcriptomes made from chemosensory organs (mouthparts, antennae and legs) will allow us to identify smell and taste receptors expressed in males and females. Further characterization of these receptors and their signal transduction pathways will help us to gain a further understanding of physiology as it is related to host and mate finding. Proteins important in these physiological pathways are potential targets for enhanced insect control measures.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

Year 1:

- A gene transcript encoding the codling moth receptors for three neuropeptide/peptide hormones (PBAN, Neuropeptide F and Insulin receptor) were cloned.
- Transcriptomes from codling moth eggs, neonate larvae, and adult male and female chemosensory organs were prepared.
- Full length transcripts of six heat shock proteins (HSP) were cloned and expression profiles for three HSPs in various codling moth stages were determined.

Year 2:

- Transcripts encoding chemosensory proteins (13 unique), odorant binding proteins (10 unique), general odorant binding proteins (3 unique), and pheromone binding proteins (5 unique) have been identified in transcriptomes of codling moth chemosensory organs.
- Transcripts encoding 25 unique heat shock proteins have been identified in the transcriptomes of codling moth eggs, larvae, and male and female chemosensory organs.
- A membrane receptor that interacts with codlemone has been tentatively identified via a cell based assay.
- A common odorant receptor with homology to the pheromone/kairomone receptor family has been detected in the genomes of codling moth, oblique-banded leafroller, *Pandemis* leafroller and oriental fruit moth.

Year 3:

- Completed characterization of codling moth transcriptomes and identified several classes of proteins that are critical for regulation of the codling moth's sense of smell. Gene transcripts encoding sensory neuron membrane proteins (SNMP) and odorant degrading enzymes (ODE) were identified.
- Characterized gene transcripts encoding three codling moth general odorant binding proteins and determined their expression profiles in chemosensory tissues of pupal and adult moths.

RESULTS & DISCUSSION

The chemosensory system is responsible for the detection of chemical compounds present in the insect's environment. The senses of smell (olfaction) and taste (gustation) play crucial roles in insect survival, often regulating feeding and reproductive behaviors. For the codling moth, the chemosensory system is critical for finding food sources, mates, and oviposition sites. Many codling moth control programs already include semiochemicals targeting the chemosensory system. Codlemone, a sex pheromone, is currently used in the orchard for mating disruption. A further understanding of the molecular pathways that are used by pheromones will allow for the potential discovery of new, more effective compounds that can be used in codling moth control programs.

Identification of major proteins involved in the detection of pheromones

To gain a better understanding of the codling moth sense of smell, the major research focus of this project has been to identify and characterize the main proteins involved in insect olfation. An overview of the components of the moth olfactory system is presented in Figure 1. Important proteins involved in the detection of an odorant, such as codlemone, include odorant binding proteins (OBP), odorant degrading enzymes (ODE), sensory neuron membrane proteins (SNMP), and odorant receptors (OR). As an odorant enters the antennal sensilla, it is bound by an OBP in the sensillar lymph. The odorant/OBP complex makes its way through the sensillar lymph until it reaches the olfactory neuron membrane, where interactions with its OR and SNMP occur. Odorant interaction with its OR causes the nerve to fire, sending a signal to the brain which can cause a behavioral response, such as the tracking response used by codling moth males when it is exposed to codlemone. To control nerve firing, ODEs degrade the odorants stopping the signals sent from the OR. Several studies indicate that each of the protein classes involved in the sense of smell are needed for the proper functioning of the moth's olfactory system.

We have analyzed transcriptomes generated from codling moth chemosensory tissues to identify gene transcripts encoding proteins that may be critical to the detection of codlemone and plant derived odorants. Last year we reported the identification of gene transcripts encoding 18 codling moth OBPs, of which 8 are predicted by homology to potentially bind pheromones and plant derived kairomones. In further analysis of the codling moth transcriptome data, we have now identified two transcripts encoding SNMPs and transcripts encoding several ODEs. It is interesting to

note that ODEs belong to the same family as insecticide degrading enzymes, and for codling moth we identified 10 glutathione *S*-transferases, 12 carboxylesterases, and 20 mixed function oxidases (Table 1). We are in the process of cloning the gene transcripts encoding codling moth OBPs, ODEs and SNMPs for future functional studies to determine their viability as targets for insect control.

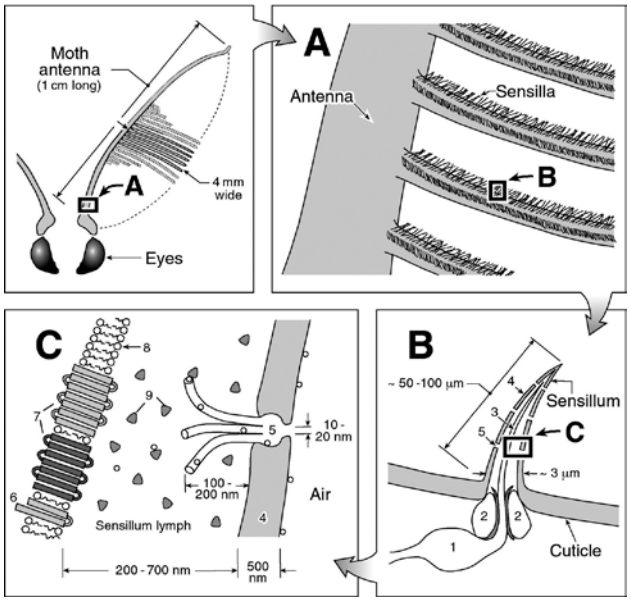

Figure 1. The route a pheromone takes through the sensillum lymph on its way to the olfactory nerve. A moth antenna (A) is highly branched, and the branches are covered with sensory hairs (sensilla). The sensilla are hollow cuticular structures that are innervated by a few olfactory neurons (1 in panel B). The cuticle of the hair (4) is traversed by pores (5). The dendrite of an olfactory neuron (3) protrudes into the space of the hair and is bathed by an electrolyte-rich sensillar lymph (panel C). The sensillar lymph contains odorant binding proteins (9 - triangles in panel C) and ions (Na+, K+, Cl–, phosphate). The dendritic (olfactory neuron) membrane (8 in panel C) houses the odorant receptors (7 in panel C) and the sensory neuron membrane proteins (6 in panel C).

Table 1. Potential odorant degrading enzymes annotated from codling moth transcriptome.

Tissue Source	Annotated Hits by Homology	GST	Esterase	Cyt P450
Male Antennae	**542**	**4**	**4**	**5**
Female Antennae	**475**	**4**	**3**	**3**
Male Legs and Mouthparts	**431**	**3**	**0**	**5**
Female Legs and Mouthparts	**350**	**1**	**4**	**6**
Eggs (Embryos)	**660**	**2**	**0**	**3**
Neonate Larvae	**617**	**4**	**1**	**4**
All Tissues	**2267**	**10**	**12**	**20**

Glutathione S-transferase (GST), Carboxylesterase (Esterase), mixed function oxidase (Cyt P450).

Characterization of gene transcripts encoding codling moth general odorant binding proteins

General odorant binding proteins (GOBPs) are members of a subfamily within the clade of OBPs. GOBPs are mainly expressed in antennae and have been shown to bind pheromones and plant volatiles, suggesting possible roles in mate and host finding. Because GOBPs bind both pheromones and plant compounds, we wanted to see where the gene transcripts are expressed for clues as to the types of compounds they may bind. First we cloned the full-length gene transcripts encoding the three GOBPs identified by transcriptome analysis. The transcript for antennal CpomGOBP1 is 698 nucleotides (nt), encoding for 163 amino acids, that for CpomGOBP2 is 1289 nt, encoding for 160 amino acids, while the transcript for CpomGOBP3 is 702 nt, encoding for 169 amino acids (data not shown).

To determine where the CpomGOBP transcripts are expressed in males and females, an expression profile was performed using RT-PCR to detect RNA transcripts in a variety of codling moth tissues (Fig. 2). CpomGOBP1 expression was limited to pupal heads, and adult antennae and mouth parts, with PCR products more strongly visualized in adult male and female antennae (Fig. 2, top panel). CpomGOBP2 transcripts were more broadly expressed, with PCR products detected in late pupae heads, and antennae, heads, mouthparts of adults from both sexes, and female abdomen tips (Fig. 2, second panel). Expression of CpomGOBP3 was similar to that of CpomGOBP1 with PCR products mainly detected in antennae and mouthparts, and also faintly detected in female heads (Fig. 2, third panel). CpomActin, which should be expressed in all tissues, was used as a control (Fig. 2, bottom panel).

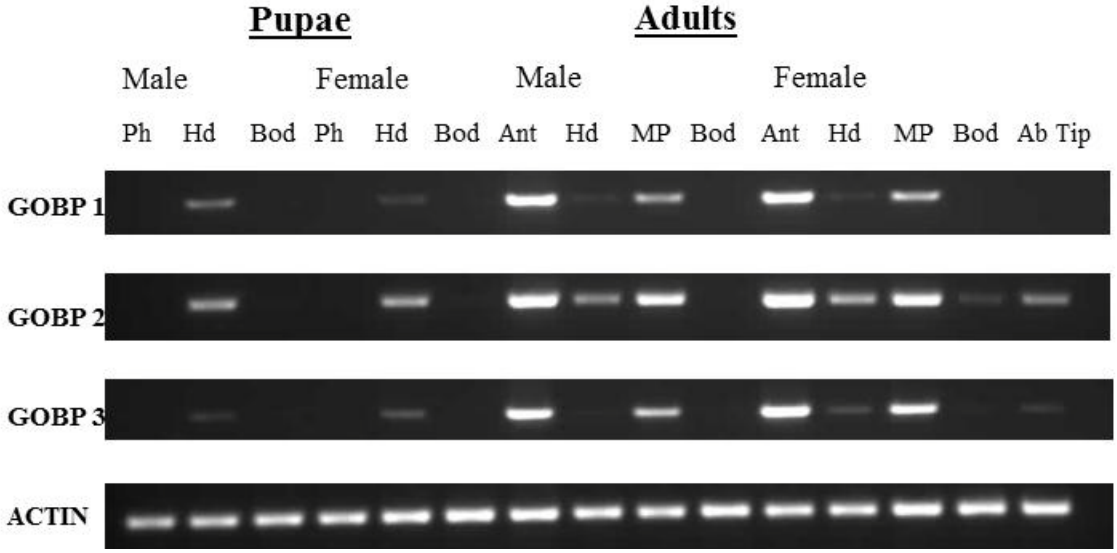

Figure 2. CpomGOBP1, CpomGOBP2, and CpomGOBP3 expression profile in cDNA prepared from total RNA extracted from pharate pupae (Ph), male and female pupae heads (Hd) and bodies (Bod), adult male and female antennae (Ant), heads (Hd), mouthparts (MP) and bodies (Bod), and adult female abdomen tips (Ab Tip). Top Panel: Detection of CpomGOBP1 using transcript specific primers in RT-PCR reactions. Second Panel: Detection of CpomGOBP2 using specific primers in RT-PCR reactions. Third Panel: Detection of CpomGOBP3 using specific primers in RT-PCR reactions. Bottom Panel: Detection of CpomActin using specific primers in RT-PCR reactions. For CpomGOBP1, CpomGOBP2, CpomGOBP3 and CpomActin, PCR products were visualized on 1.5% agarose gels by ethidium bromide staining and UV illumination. Bands produced were of the size predicted for the transcript specific primer pair.

Last year, we reported detecting abnormalities in messenger RNAs (mRNA - gene transcripts that encode proteins), that code for pheromone receptors, and we have detected similar abnormalities in mRNAs that code for GOBPs. Figure 3 shows a diagram representing the structural features of mRNA molecules. Of major interest is the 3'untranslated region (3'UTR) and the Poly-A tail. What we have found is that several gene transcripts that code for pheromone receptors and the three GOBPs alter the length of their 3'UTRs by alternating the places where the poly-A tail is added. By altering the length of the 3'UTRs of these gene transcripts, the insect can regulate how much protein gets made and localize where the protein is inserted into the membrane. This type of mechanism would be important in codling moth detection of sex pheromone and would allow for males to increase protein amounts as they detect increasing sex pheromone concentrations as they approach females. Having transcripts with a variety of 3'UTR lengths can potentially regulate the number of receptors on a nerve membrane, or increase the number of OBPs in response to pheromone detection. This rare mechanism has been previously cited as a means for translational control of gene transcripts in mammals, but has yet to be reported for insects. Collaborative research projects to explore this mechanism for detection of pheromones have been initiated with Richard Newcomb at Hort Research in New Zealand.

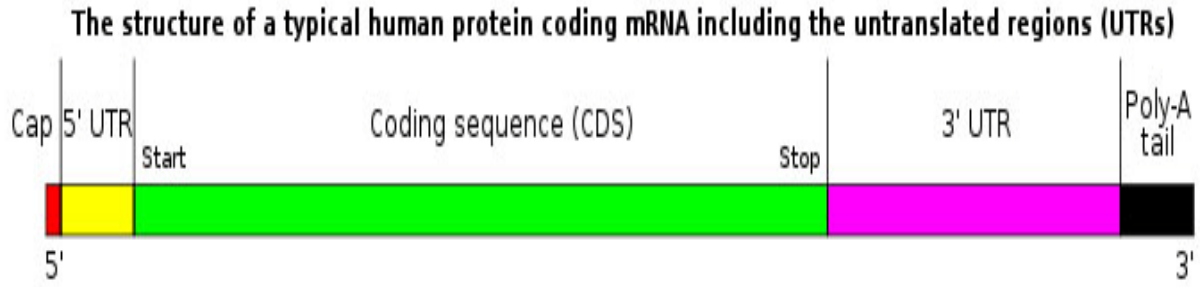

Figure 3. The structure of a typical mRNA molecule (also referred to as a gene transcript). At the extreme 5' end of the mRNA is a Cap structure which helps protect the molecule from degradation. The Cap is followed by a 5' untranslated region (5'UTR) which is a variable stretch of nucleotides that contains regulatory elements. Following the 5'UTR is the Coding sequence (CDS) which has a specific nucleotide sequence that encodes for a specific amino acid (protein) sequence. After the CDS is the 3'untranslated region (3'UTR) which is a stretch of nucleotides that contain regulatory elements and sequences that signal the addition of a Poly-A tail.

EXECUTIVE SUMMARY

The chemosensory system, which includes the senses of taste and smell, is responsible for the codling moth's ability to detect chemical cues in its environment. The codling moth, as well as other pest insects, use the senses of smell and taste to detect food sources and to locate mates or oviposition sites. The goal of this project was to determine protein components expressed in chemosensory organs and other stages of codling moth by analyzing transcriptomes prepared from key tissues housing the machinery that the insect used to detect food and odorant sources. Transcriptome analysis, coupled with cloning and characterization of key olfactory proteins, has provided fundamental biological information on codling moth physiology that can be used to identify new targets for enhanced pest control by disruption of the insect's sense of smell.

To gain a better understanding of the codling moth sense of smell, proteins encoding the major constituents of the molecular machinery used to detect odors in antennae were identified. Gene transcripts encoding pheromone and other odorant receptors (membrane proteins which are involved in nerve signaling in response to a pheromone or other odors), odorant binding proteins (small soluble proteins that are thought to shuttle pheromones and other odors to the membrane receptors), odorant degrading enzymes (proteins that degrade pheromones and other odorants) and sensory neuron membrane proteins (membrane proteins which are involved in the interaction of pheromones and pheromone receptors) were identified, cloned and characterized. A major finding was that gene transcripts encoding pheromone receptors and general odorant binding proteins use a mechanism in which they produce variant 3'untranslated regions of their mRNA. This is potentially important in explaining how a moth can detect concentration gradients of sex pheromones and other attractive odorant sources.

The results of the research performed in this project has led to new ideas on how moth's detect pheromones and other odorants, and has led to the initiation of several new collaborations with research groups in California, New Zealand and France. Research on the interaction of pheromones with their membrane receptors is already providing insights on the design of parapheromones that interact more strongly with pheromone receptors, and are more attractive to moths. These same design strategies can be used to synthesize novel codlemone derivatives which may provide better codling moth control in the orchard. Research involving altering odorant degrading enzyme function is also underway. Several enzyme inhibitors are available and can be used to determine the role of odorant degrading enzymes in the molecular detection of pheromones. It is hypothesized that alteration of odorant degrading enzymes will cause an habituation-like affect and perhaps be a useful supplement in mating disruption strategies. Lastly, several researchers are now interested in studying the regulation of the production and localization of pheromone receptor proteins by alteration of the 3'untranslated region of the mRNAs that encode them. If we can understand how a moth produces proteins in response to detecting pheromone concentration gradients it will help us understand the basis of how mating disruption is working and provide clues on how to enhance its effectiveness.