**Project Summary: Chemical Perception Genes from the Lepidoptera, Odorant and Gustatory Receptors Mediating Host Selection and Feeding Behavior**

## 1.0 Introduction

A substantial foundation of insect chemical ecology and electrophysiology research has been built over the last 100 years, but it is only in the last decade that tremendous scientific progress in our molecular and neurological understanding of the chemical senses has occurred. The scientific community is currently witnessing the integration of these disciplines, which promises to provide new insights into insect pest behaviors mediated by the chemical senses, leading to new and improved strategies for their integrated management. The watershed event stimulating the molecular progress was the discovery of the receptor genes that bind and recognize chemical stimuli – a highly sought goal worldwide, and one which earned Buck and Axel the 2004 Nobel price in Physiology or Medicine for their identification of the first odorant receptors from rat olfactory neurons in 1991 (Buck and Axel, 1991). The first insect chemoreceptor genes were discovered from *Drosophila melanogaster* only after its genome was sequenced in 1999-2000 (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Robertson et al 2003), and now in five short years, most of these have been functionally characterized (Hallem et al., 2006). Several large research efforts are underway to transform this progress into new olfactory based tactics for managing mosquito pests. Similarly, identifying the odorant and gustatory receptors from several important pest species is the first critical step required to extend this progress to the Lepidoptera. With the completion of the *Anopheles gambiae* (Diptera) and *Apis mellifera* (Hymenoptera) genomes, we know that each encodes approximately 100-200 chemorecptors (odorant and gustatory receptors, Ors & Grs) that typically share only 20-30% amino acid identity (Hill et al., 2002; Robertson et al., 2003; Robertson and Wanner, 2006). Greater similarity is required for homology based discovery approaches, and for this reason, the recently completed silkmoth (*Bombyx mori*) genome (Xia et al., 2004) will be a gateway to the lepidopteran chemoreceptors (as taxonomic similarity increases, so does receptor homology). Our goal is to identify odorant and gustatory receptor genes from several economically important lepidopteran pest species, focusing on receptors that mediate important pest behaviors such as host plant selection and feeding. We will actively disseminate our results for the immediate benefit of groups working on these species, thus facilitating the development of new pest management techniques. By representing five different taxonomic families within the Lepidoptera, we will stimulate the discovery of receptor genes from many other pest species.

**Objectives:**

1. Identify the odorant and gustatory receptor genes from the silkmoth (*Bombyx mori*) genome sequence.
2. Profile silkmoth receptor gene expression patterns in adult and larval sensory organs to identify candidates that mediate important pest behaviors.
3. Use the silkmoth genes as probes to identify odorant and gustatory receptors from important lepidopteran pest species, representing five different taxonomic families. Construct cDNA libraries from sensory organs and screen them for homologous receptors, focusing on candidates that mediate important pest behaviors (utilizing taxonomic similarity for the homology based strategy).
4. Profile the expression patterns of the new receptors in the pest species. Publish and disseminate the results to the broad research community to facilitate the development of new pest control techniques based on exploiting the chemoreceptors.

*1.1 The Lepidoptera are a significant pest group*

The Lymantridae, Noctuidae, Plutellidae, Pyralidae and Tortricidae are five families that contain some of the most important pests of agriculture and forestry. These taxonomic families include a vast range of pests of agricultural (pre and post harvest), urban, forest, and nursery crops, resulting in tremendous food, fiber and monetary loss in the US and worldwide, and they account for a large portion of pesticide use (for example, Pedigo, 1989).

*1.2 Host selection and feeding are key pest behaviors*

In the Lepidoptera, host plant selection primarily results when females lay eggs, and damage results from feeding by the larval stage, two eminent pest behaviors. However, there is considerable variability in these behaviors – some female moths are flightless, and the larvae are capable of significant dispersal, either by wind at early stages, or by crawling at later stages. Similarly, there is a wide range in the selectivity of oviposition behavior. These critical pest behaviors involve sequential steps that are mediated by the chemical senses of olfaction (volatile stimuli) and gustation (contact stimuli) (Bernays and Chapman, 1994). Olfactory and gustatory neurons, typically housed within hollow hair-like sensilla, can be located on many different parts of the insect body, including the adult antennae, proboscis, labial palps, tarsi and ovipositor, and the larval antenna and maxilla.

The behavioral sequence leading to oviposition begins with searching, orientation and encounter (first phase), leading to a second phase that includes landing, contact evaluation and acceptance or rejection (reviewed by Ramaswamy, 1988, Renwick, 1989 and Renwick and Chew, 1994). Visual cues such as shape and color are important while searching for a host, but plant volatiles and the olfactory system (mainly the antennae) are critical to female moths for orienting to their host plant. Moths can exhibit significant discriminatory abilities when orientating, even to the degree of selecting susceptible varieties of a host species based upon the volatiles they emit (Khan et al., 1987). Physiological and developmental states can be important, mated female moths will fly upwind towards host volatiles in wind tunnels (Renwick and Chew, 1994). The last step in the orientation phase occurs when the mated moth lands on a plant surface.

After landing on a plant surface, a balance of stimulatory and inhibitory signals from contact gustatory receptors mediates the decision to accept or reject the plant as a host for oviposition (Chapman, 2003). These contact chemosensilla can be located on the antennae, proboscis, front tarsi and ovipositor of the female moths. Again, the behaviors may occur sequentially and incorporate the use of various sensory organs; female moths may tap the plant surface with their antennae and front tarsi, fan their wings, extend their proboscis to the surface, or drag their ovipositor (Renwick and Chew, 1994). A variety of plant chemicals and cuticular waxes can act as oviposition stimulants and deterrents, or so called sign stimuli (Renwick, 1989; Chapman, 2003). Some moth species will also use oviposition-deterring pheromones to space egg deposition.

Larval feeding behavior also involves a sequence of events, including orientation and attraction to a food source, test biting and sustained feeding, all mediated by olfaction and gustation (Schoonhoven, 1968; 1987). Olfactory cues can attract or repel caterpillars, and induce or inhibit test biting, while contact taste sensilla regulate continued feeding and host discrimination. For example, caterpillars whose antennae have been ablated will proceed to the test bite stage before rejecting a non-host plant, intact caterpillars will not take the test bite (Schoonhoven, 1987).

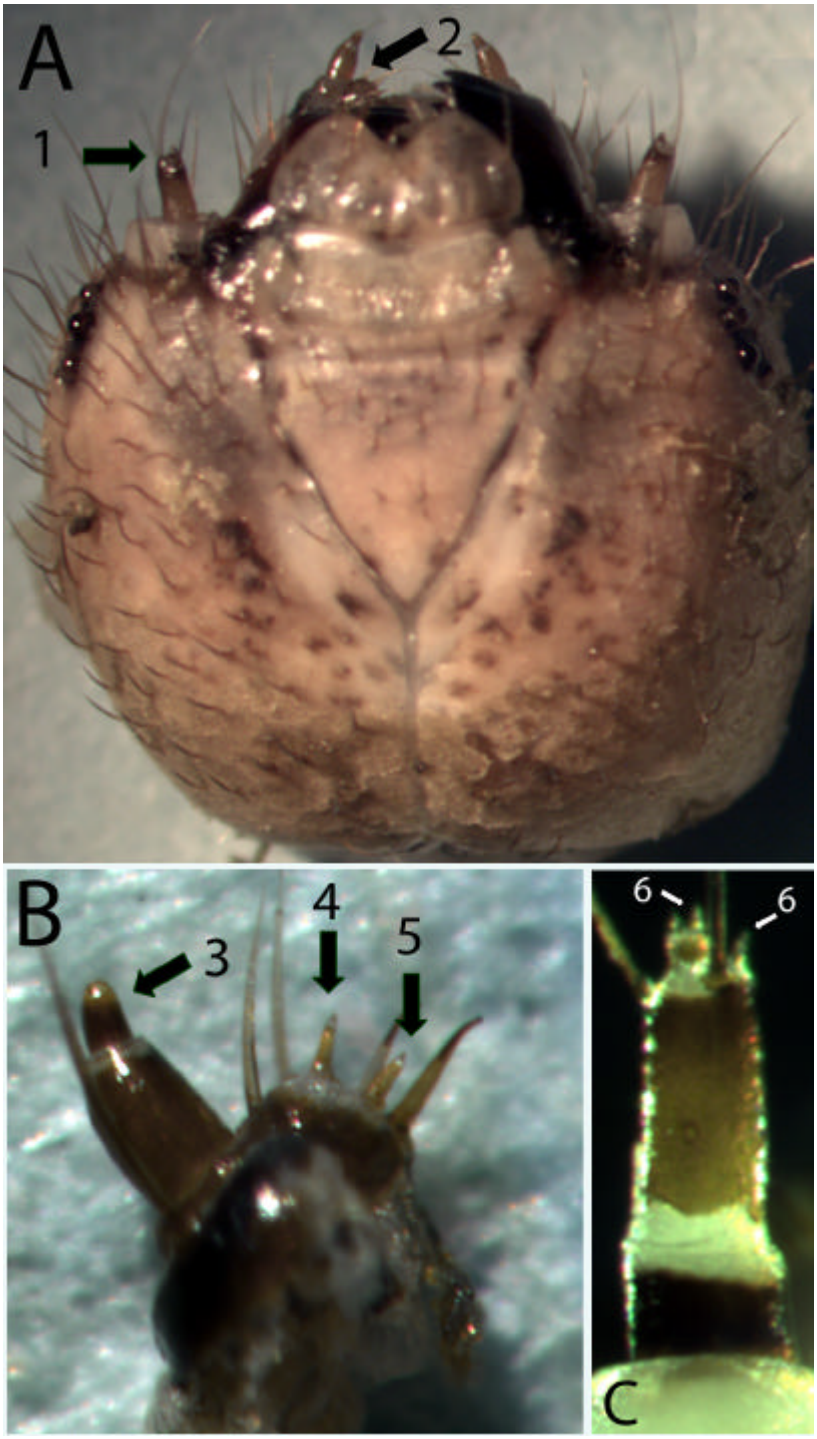



**Figure 2. A)** Dorsal view of the head of a 5th instar silkworm larva illustrating the antenna (1) and the maxilla (2). **B)** Maxilla, with the maxillary palp (3) and the lateral (4) and medial (5) styloconic taste sensilla on the maxillary lobe. **C)** Antenna with three olfactory basiconic sensilla (6).

Each caterpillar antenna has three basiconic sensilla that house 16 olfactory neurons that function in short range olfactory attraction (Schoonhoven, 1987; Bernays and Chapman, 1994). A maxilla located on each side of the mouth is further divided into the maxillary palp and the maxillary lobe (Figure 1). The palp holds 3 olfactory and 5 gustatory basiconic sensilla (housing 14-19 sensory neurons) that mediate feeding behavior; its removal impairs the ability of the caterpillar to distinguish between host species. Two styloconic sensilla (lateral and medial) on the tip of the maxillary lobe house 4 gustatory neurons each; remarkably, these 8 gustatory neurons primarily control the feeding behavior of caterpillars. Their removal abolishes host discriminatory ability. Typically, one of these 8 gustatory neurons is tuned to feeding deterrents, while the remaining 7 are tuned to nutrients (such as sugars and salts) and specific feeding stimulants (Schoonhoven, 1987; Bernays and Chapman, 1994).

*1.3 Chemoreceptors are the key components of the peripheral sensory system*

The transduction of chemical signals into nerve impulses occurs at the tips of sensory neuron dendrites, a region often referred to as the peripheral sensory system (reviewed in Rutzler and Zwiebel, 2005 and Hallem et al., 2006). The dendrites are housed within specialized hollow hair-like chemosensilla, and the axons project to either the subesophogeal ganglion (gustatory neurons) or to spherical glomeruli that compose the antennal lobe (olfactory neurons). A viscous fluid termed the sensillum lymph surrounds the sensory neurons, and provides the only barrier to the external environment. Chemical ligands enter through pores in the sensillum wall; olfactory sensilla are typically multiporous, contact (taste) sensilla typically have a single pore located at the tip. The sensillum lymph contains abundant levels of odorant binding proteins (OBPs) which transport hydrophobic chemicals to the neuron membrane, and odor degrading enzymes (ODEs), both of which were discovered more than 20 years ago (Vogt and Riddiford, 1981). While OBPs and ODEs have signicant functions, and may modulate the sensillum environment, it is the neuron bound chemoreceptors (first discovered from *D. melanogaster* only five years ago) that confer specificity to the chemical stimuli. Upon activation by their cognate ligands, chemoreceptors (members of the G-protein coupled receptor superfamily) interact with secondary messenger systems on the neuron membrane to trigger the nerve impulses.

The critical role of chemoreceptors is poignantly illustrated by the work of John Carlson, who has developed a mutant fruit fly with a specific olfactory neuron devoid of chemoreceptors, but otherwise functional (reviewed in Hallem et al., 2006). Various different *Drosophila* chemoreceptors can be expressed in this “empty neuron” and their ligand binding characteristics determined by single sensillum electrophysiology. Using this method, the electrophysiologial characteristics of a “wild type” sensillum can be replicated in the empty neuron, simply by juxtaposing its chemoreceptor into the empty neuron (Hallem et al., 2004). Therefore, in many (but not all) cases, insect sensory neurons may function as blank modules, their signaling mode largely determined by the chemoreceptor itself. For example, chemical stimuli can inhibit or stimulate the firing rate, and the temporal response dynamics to discrete stimuli can vary – electrophysiologists have documented these phenomenon for more then 30 years (for example, Kaisling, 1970). Remarkably, all of these electrophysiological traits can be replicated in the *Drosophila* empty neuron simply by changing the chemoreceptor (Hallem et al., 2004).

*1.4 The Insect Chemoreceptor Super Family; Odorant and Gustatory Receptors*

Until recently, the field of chemical ecology concerned with olfaction and gustation in insects was severely limited in the area of sensory perception because no receptors had been identified despite extensive efforts. We now know that chemoreceptors form large, ***diverse*** gene families, in the order of 100-200 genes within each of the insect genomes sequenced to date, a property thought to reflect their ability to recognize an array of diverse chemical stimuli (Table 1). The coding region of each gene averages about 1200 nucleotides translating into a protein with about 400 amino acid residues, that includes seven membrane spanning domains. This

**Table 1.** Odorant and Gustatory genes annotated from insect genomes.

| **Species** | **Glomeruli #** | | **Or #** | **Gr #** | **References** |
|---|---|---|---|---|---|
| *D. melanogaster* | 43 | (Laissue et al. 1999) | 61 | 66 | (Roberson et al. 2003; Voshall et al. 2000) |
| *A. gambiae* | 61 | (R. Ignell) | 79 | 76 | (Robertson et al. 2003; Fox et al. 2001) |
| *B. mori* | 60 | (Ai&Kanzaki 2004) | 52+? | ? | (Our results herein; Sakurai et al. 2004) |
| *Heliothine sp.* | 62-66 | (Berg et al., 2002) | 17+? | ? | (Krieger et al. 2004) |
| *A. mellifera* | 160 | (Joerges et al. 1997) | 170 | 10 | (Robertson and Wanner, 2006) |
| *T. castaneum* | ? | ? | | >140 | (Robertson, unpublished) |

sequence diversity has meant that all insect chemoreceptor gene sequences that have been published to date originate from genome sequences; using phylogenetic techniques, they resolve into two main subgroups, the odorant receptors (Ors), which are expressed in olfactory neurons, and the gustatory receptors (Grs) which are expressed in taste neurons (Robertson et al., 2003, Robertson and Wanner, 2006). Olfactory neurons express a single type of Or gene (or sometimes two), and all olfactory neurons expressing the same Or project to the same glomerulus in the antennal lobe (reviewed in Hallem et al., 2006). Therefore, the number of Ors in an insect genome tends to approximate the number of glomeruli (Table 1), and we can predict that the silkmoth and *Heliothine* genomes should encode a total of about 60-70 Ors. While the odorant receptor genes are highly divergent, there are some conserved features such as the intron splice sites (Figure 2). Specifically, introns e-h are in the same alignment position and phase as the four ancestral commonly shared introns in the Drosophila and Anopheles Ors (Robertson et al., 2003). In particular, the final phase 0 intron h is a conserved position shared by most members of the chemoreceptor superfamily and this characteristic is used to support the monophyly of the superfamily (Scott et al. 2001). This last exon also represents a conserved amino acid region.

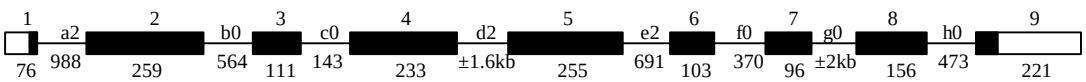



**Figure 2.** Gene structure of *Manduca sexta* Or1 (unpublished results). Exons are shown as boxes to scale and introns as thin lines not to scale, with their exact or estimated lengths below them. Coding regions are in black. The naming letter and phase of the introns are shown above them.

*1.5 The first insect chemoreceptors from the Drosophila melanogaster genome sequence*

The laboratories of John Carlson, Richard Axel, and Andrew Chess broke open this field by identifying the first candidate Or sequences from the fledgling *Drosophila melanogaster* genome project (Clyne et al. 1999; Vosshall et al. 1999; Gao and Chess 1999), and with its completion, a total of 62 Or genes are now recognized (Vosshall et al. 2000; Robertson et al 2003). Their assignment as odorant receptors was confirmed by demonstrating their expression in small subsets of olfactory sensory neurons in the antennae and/or maxillary palps (Vosshall et al. 2000). The *D. melanogaster* gustatory receptors were identified soon after; Clyne et al. (2000) identified 19 candidates and Scott et al. (2001) and Dunipace et al. (2001) subsequently expanded the total to 56 gustatory receptors (specific expression in gustatory sensilla was also demonstrated). Dr. Robertson has added to this total, now bringing it to 69 gustatory receptors encoded by 61 genes (Robertson et al., 2003).

*1.6 Using the D.melanogaster sequences to identify chemoreceptors from the recently sequenced genomes of A. gambiae, A. mellifera, and Tribolium castaneum*

Although the sequence homology of chemoreceptors between different insect orders is low, it is sufficient to identity candidate genes from genome sequences using BLAST search tools. A tBLASTn search compares the protein query with all translations of the genome sequence of interest. Contiguous genome sequences that encode peptides that share amino acid similarity with the query sequence are identified. At this point, it becomes challenging. The candidate genomic sequences are downloaded from GenBank, scrutinized to ensure that they are not spurious sequences, and the genes constructed manually in a text editor. Or and Gr genes typically have six or more introns, ranging in size from 100 nucleotides to many thousands, making their manual assembly difficult. While some software programs, such as intron splice site predictors, are helpful, I have realized from working with Dr. Robertson that building the complete gene families requires an intimate knowledge of the subtle characteristics of Or and Gr genes that comes with experience. As new sequences are identified, they can in turn be used as BLAST queries in an iterative process, and as sequence representation increases, the annotation of insect chemoreceptors becomes somewhat easier.

In this way, Dr. Robertson used the *D. melanogaster* Or and Gr protein sequences to identify and manually build the chemosensory receptor families from the *A. gambiae* and *A. mellifera* genome sequences. In late 1999 he identified the first few mosquito Ors in the 5% of the *A. gambiae* genome then available as 17,500 BAC end sequences, and he collaborated with Larry Zwiebel at Vanderbilt, Nashville, TN to publish the complete repertoire of 79 Or and 76 Gr genes (Fox et al. 2001; Hill et al. 2002). Larry Zwiebel is currently leading an international effort to functionally characterize the mosquito Ors and translate this information into new methods to control this important pest (http://www.gcgh.org/subcontent.aspx?SecID=389). Lauren Kent, a graduate student in Dr. Robertson's lab, is currently working to functionally characterize *A. gambiae* gustatory receptors. Dr. Robertson is also a member of the honey bee genome consortia, and has identified and built 170 Ors, but only 10 Grs, from the honey bee genome (Robertson & Wanner, 2006). The large number of Ors is thought to reflect the importance of olfaction to foraging honey bees, while the reduction in the number of Grs may reflect a reduced dependence of the honey bee on gustatory discrimination, since they have a symbiotic relationship with plants, and are raised in a nurturing hive environment. As a Post Doctoral Research Associate, I have assisted Dr. Robertson's technician, Kim Walden, to characterize the expression patterns of the honey chemoreceptors using an olfactory specific

microarray chip and quantitative PCR. We have already identified three candidate female queen pheromone blend receptors that are expressed at higher levels in male drone antennae. Finally, Dr. Robertson is also responsible for annotating the flour beetle, *Tribolium castaneum*, chemoreceptors from its recently sequenced genome. To date, he has identified approximately 140 gustatory receptors (unpublished results). Having the sequences from several different insect orders (Diptera, Hymenoptera and Coleoptera) an important point has become clear: The majority of the receptors appear to originate from gene expansion within each insect order, and there are few clear examples of orthologous lineages ***between*** different insect orders.

*1.7 The first lepidopteran chemoreceptors*

In the Lepidoptera, research has been directed towards identifying the receptors in the male antennae that perceive the female produced sex pheromone. This was a highly sought goal, since Lepidopteran sex pheromone research has more than 100 years of history and a large research community. Progress towards this objective has been shaped by three approaches: 1) antenna specific EST (expressed sequence tag) sequencing projects, 2) the partial (but privately owned) genome sequence of *Heliothis virescens*, generated from the collaboration between Bayer and Exelixis, and 3) the first publicly released Lepidopteran genome sequence, that of the silkmoth *B. mori*.

Dr. Robertson began an antennal EST sequencing project in 1997, when he made a cDNA library from mRNA isolated from male and female tobacco hawkmoth (*Manduca sexta*, F. Sphingidae) antennae. Many novel proteins, not previously identified in moths, were discovered including four new odorant binding proteins (OBPs) and two new pheromone binding protein (PBPs) (Robertson et al. 1999). After further efforts to reduce the redundancy of the library (by screening for and subtracting common clones), and sequencing a total of about 1,500 ESTs, two putative odorant receptors were discovered, MsOr 1 & 3. Other researchers taking the same approach have achieved similar results, such as Richard Newcomb from New Zealand, who has identified three Ors from the antennae of the apple pest, *Epiphyas postvittana* (personal communication, collaborative letter of support attached). Dr. Robertson's former Ph.D. student, Harland Patch, has characterized MsOr1 and his results indicate that it is a putative pheromone receptor (a manuscript is in preparation). An examination of the sequence of MsOr1 indicated that it encoded 7 transmembrane domains, a feature that all Ors share. Furthermore, quantitative PCR experiments indicated that MsOr1 is expressed more then 1000-fold greater in male, as compared to female, antennae, and in situ hybridization experiments were able to pinpoint expression in the neurons of pheromone sensitive trichoid sensilla on male antanne, but not on female antennae (which do not possess pheromone sensitive sensilla).

Most recently, a German group that has access to the privately owned genome sequence of *H. virescens*, and a Japanese group with access to the silkmoth genome sequence, identified putative lepidopteran pheromone receptors (Kreiger et al., 2004; Sakurai et al., 2004; Kreiger et al., 2005; Nakagawa et al., 2005). Krieger et al. (2004) has published the full or partial sequences of 17 Or and 3 Gr genes from the *H. virescens* genome; several candidate pheromone receptor genes were identified based upon selective expression in male antennae. Using in situ hybridizations, they were able to localize expression of HvCr13 specifically to the pheromone sensitive trichoid sensilla of male *H. virescens* antennae. Six silkmoth Ors that were expressed at higher levels in adult male as compared to female antennae were identified in a similar approach. Two receptors, BmOr1 & 3, were localized to the pheromone sensitive male trichoid sensilla and further were demonstrated to bind the two main pheromone components, bombykol and

bombykal, respectively, using an in vitro assay (Sakurai et al., 2004; Nakagawa et al., 2005).

*1.8 We have identified > 45 new Ors from the silkmoth, and characterized their expression*

While lepidopteran sex pheromones are a very important field, I believe that resources need to be allocated towards general Ors that mediate host plant selection and feeding behaviors that are critical to pest speices. Prior to joining Dr. Robertson's lab, I began a methodical effort to identify the complete Or family from the recently sequenced silkmoth genome. I have continued this work in Dr. Robertson's lab, where we have identified approximately 55 partial silkmoth sequences with homology to known Ors. We designed primers to these sequences, and performed quantitative real-time PCR (qPCR) to test for enrichment in the adult moth antennae, and for sex biased expression (Table 2). We used SYBR green dye, the relative method of qPCR, and we normalized expression relative to levels in the abdomen, a tissue that should exhibit only background levels. Using this technique, we confirmed the expression of at least 33 new Ors in the adult antennae, at levels ranging from 60 to 15,000 times greater then that in the abdomen (Table 2). We were able to confirm (and more accurately quantify) the male enriched expression of BmOr1 and 3-7 (red font, Table 2) that had been previously published. More significantly, we have identified the first female specific Ors from the Lepidoptera (BmOr19 and 30, in blue font). At least 11 other Or genes are expressed at very high levels, > 1000 times that of the abdomen (green font). Several of these may be important in host selection behavior, such as BmOr12, 48, 56 and 57, which are all highly expressed and moderately female biased. These results have been consistent over two replicated experiments.

BmOr19 and 30 are particularly noteworthy for their apparent complete absence from male antennae, which indicates that they mediate chemoreception specific only to females. The very high expression of BmOR19 indicates that it will be represented in a large number of sensilla on the female antenna, which provides a clue towards its cognate ligand. Female silkworm possess about 6000 trichoid sensilla on each antenna, each of which houses two olfactory neurons, the majority of which respond either to linalool (and other terpenoids) or benzoic acid (Heinbockel and Kaissling, 1996). Terpenoids are commonly used by insects to discriminate between host plants, while the function of a benzoic acid receptor is not clear.

Several of the Ors that were not detected in the adult antennae (such as BmOr20, 24 and 26) were expressed in the larval antennae (Figure 3). BmOr2, a member of the Drosophila 83b subfamily, a group of Ors that actually act as a chaperone for the other Ors, was highly expressed in the laval antennae as was expected. Interestingly, BmOr10 was highly expressed in the maxilla, a sensory organ located alongside the caterpillar mouth (Figure 1), and that has three olfactory basoconic sensilla that mediate feeding behavior.

*1.9 Lepidoteran Or sequences are conserved at the family level of taxonomy*

We have identified the greatest number of lepidopteran Ors to date, 45 full length (or close to full length) new silkmoth Ors. Along with the 17 HvOr sequences published by Krieger et al. (2004), we have constructed a phylogenetic tree to analyze their evolution within the Lepidoptera (Figure 5A), yielding two critcal insights: 1) The Ors form subfamilies that are represented by both *B. mori* (Family Bomboycidae) and *H. virescens* (Family Noctuidae) (Figure 5A). Sequences within each subfamily have greater similarity, and therefore can be used to identify homologous genes from other taxonomic families. In some cases, the subfamilies may have conserved functions – almost all of the Ors that group into the same subfamily with the

**Table 2.** Expression levels of silkmoth Or genes in male and female adult moth antennae relative to abdomens (fold increase), determined by quantitative real-time PCR (normalized with a ribosomal control gene). Only genes with significant levels above background are included. Enriched in male antennae(**red**); specific to female antennae (**blue**); generally high (**green**).

| BmOr no. | ? | ? | ? :? | BmOr no. | ? | ? | ? :? |
|---|---|---|---|---|---|---|---|
| ***1*** | ***0.25*** | ***2701*** | ***0.000093*** | 33 | 52 | 223 | 0.23 |
| 2 | 16384 | 17560 | 0.93 | 35 | 1.8 | 3.7 | 0.49 |
| ***3*** | ***37*** | ***198668*** | ***0.00019*** | 36 | 169 | 97 | 1.7 |
| ***4***[a] | ***1911*** | ***30574*** | ***0.063*** | **37A** | **4390** | **2896** | **1.5** |
| ***5*** | ***39*** | ***1783*** | ***0.022*** | 37B | 84 | 74 | 1.1 |
| ***6*** | ***169*** | ***10086*** | ***0.017*** | 38 | 1.9 | 2.3 | 0.83 |
| ***7***[a] | ***1911*** | ***30574*** | ***0.063*** | 39 | 64 | 36.8 | 1.7 |
| 8 | 74 | 79 | 0.94 | 40 | 60 | 84 | 0.71 |
| 9 | 338 | 239 | 1.4 | 41 | 84 | 60 | 1.4 |
| 10 | 832 | 478 | 1.7 | 42 | 4.9 | 4.9 | 1.0 |
| **11** | **1218** | **1261** | **0.97** | 43A | 239 | 128 | 1.9 |
| **12** | **15287** | **4705** | **3.2** | 43B | 588 | 388 | 1.5 |
| **13** | **3566** | **10086** | **0.35** | 44 | 7 | 8 | 0.88 |
| **14** | **891** | **1024** | **0.87** | 45 | 294 | 111 | 2.6 |
| 15 | 338 | 97 | 3.5 | 46 | 9 | 8 | 1.1 |
| **16** | **1552** | **4390** | **0.35** | 47 | 5.7 | 5.3 | 1.1 |
| 17 | 12 | 5.3 | 2.3 | **48** | **7643** | **2353** | **3.2** |
| **18** | **5793** | **5404** | **1.1** | 49A | 362 | 128 | 2.8 |
| **19** | **7132** | **9** | **792** | 49B | 274 | 104 | 2.6 |
| 24 | 20 | 60 | 0.33 | 54 | 512 | 549 | 0.93 |
| 25 | 13 | 6 | 2.2 | **55** | **1097** | **891** | **1.2** |
| 27 | 119 | 111 | 1.1 | **56** | **10809** | **1552** | **7.0** |
| 28 | 169 | 169 | 1.0 | **57** | **1552** | **256** | **6.0** |
| **30** | **776** | **8.6** | **90** | 59 | 388 | 223 | 1.7 |

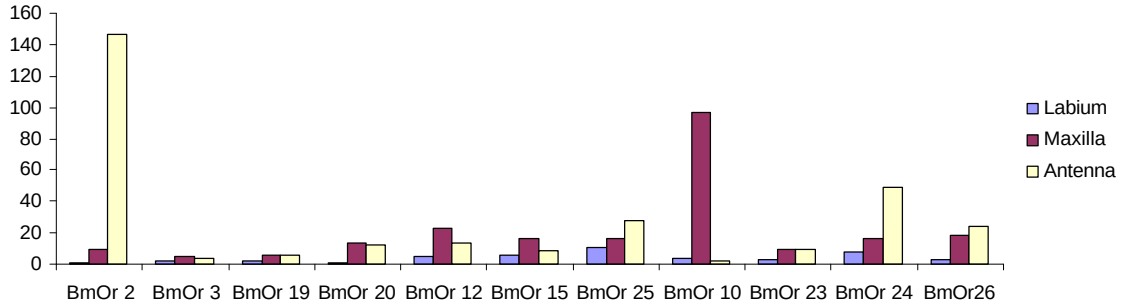



**Figure 3**. Expression level of silkmoth Or genes in 5th instar larval mouth parts, the labium (lower lip), maxilla and antenna (see Figure 1). Fold increase over levels in the larval abdomen, determined by quantitative real-time PCR.

putative pheromone receptors are expressed at higher levels in male antennae. The *H.virescens* pheromone blend has six components, and there are six HvOrs in the pheromone subfamily.
2) *B. mori* and *H. virescens* Ors that are paired together in subfamilies have nucleotide identities at the carboxy-terminus that commonly exceed 50% over a 120 bp region (Figure 4B), and commonly exceed 80% over shorter stretches (Figure 4). Therefore, the conserved carboxy-terminus region can be used as probes to successfully screen cDNA libraries from other taxonomic families.

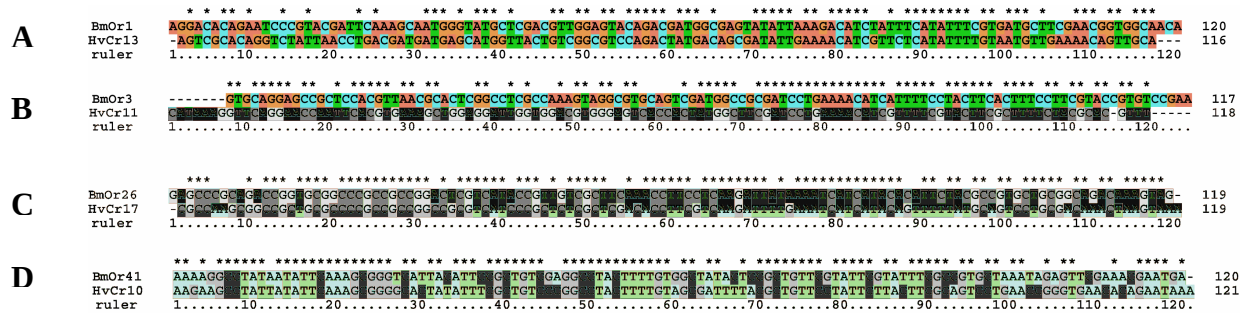
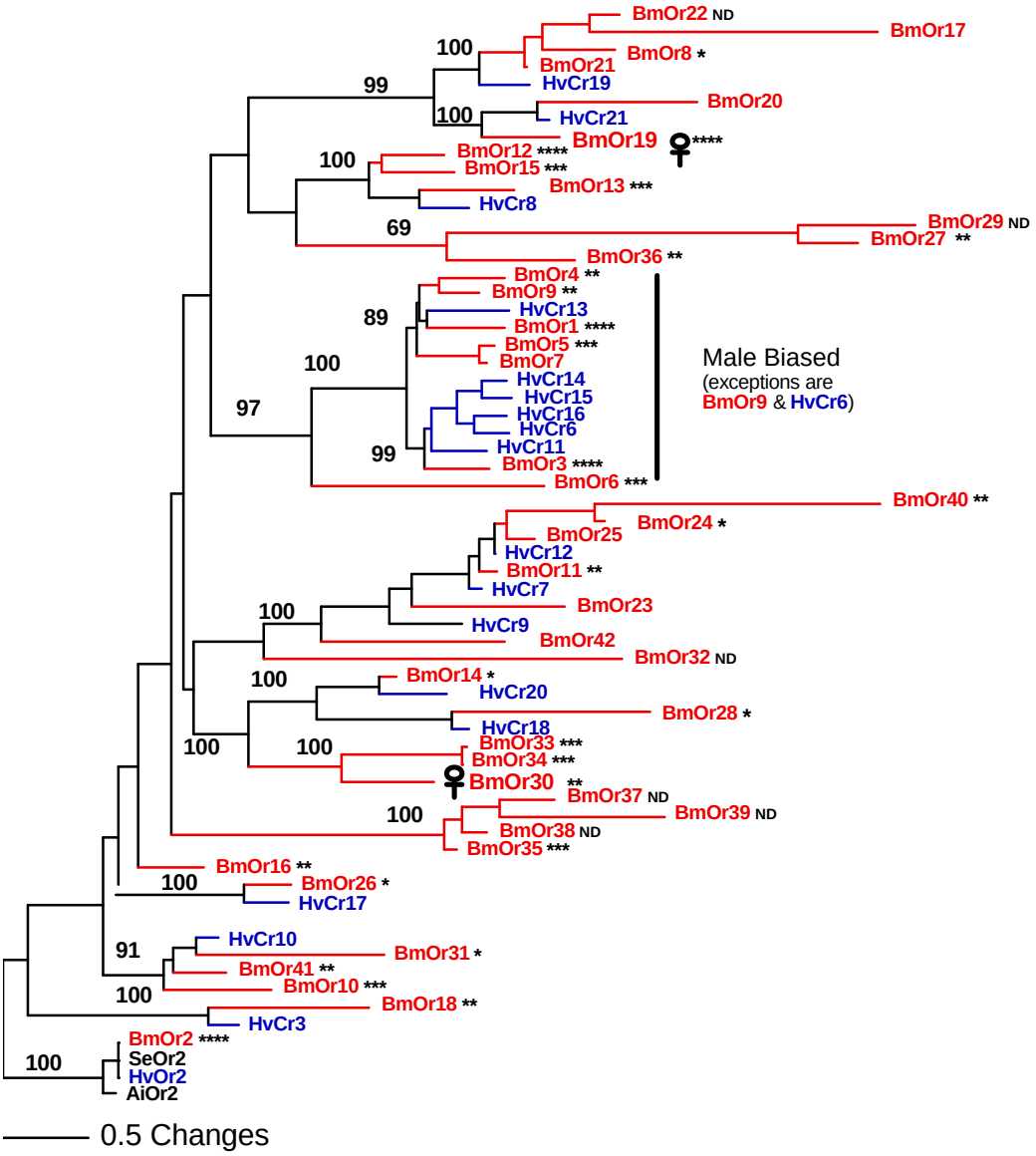



**Figure 4.** Pairwise alignment of the last 120 nt of BmOr1 & HvCr13 (A); BmOr3 & HvCr11 (B); BmOr26 & HvCr17 (C); and BmOr41 & HvCr10 (D). The * above the alignment indicates nucleotide identity. Note that the *H. virescens* genes were simply named Crs, although they belong to the Or subfamily.

*1.10 Five pest species that represent five important taxonomic families*

The apple codling moth, *Cydia pomonella* (F. Tortricidae ), diamond back moth, *Plutella xylostella* (Plutellidae), European corn borer, *Ostrinia nubilalis* (Pyralidae ), gypsy moth, *Lymantria dispar* (Lymantridae) and the corn earworm, *Helicoverpa zea* (Noctuidae) are five significant pest species that represent five important taxonomic families. The codling moth is the most economically important insect pest of pome fruits in North America and the western world (Barns, 1991), contributing to high amounts of insecticide use in these crop systems. The gypsy moth is notorious for its introduction to (and continual spread within) the US in 1869, and it defoliates millions of acres of forest each year. In 1999 the USDA Forest Service implemented the National Gypsy Moth Slow the Spread (STS) Project whose goal it the use novel integrated pest management (IPM) strategies to reduce the rate of gypsy moth spread (http://www.exosect.com/solutions/pests/gypsy_moth.asp#significance). The corn earworm and the European corn borer are two of the most serious pests of corn, a major cash grain crop in the US (Pedigo, 1989). The diamondback moth is a significant pest of Brassica crops such as canola and vegetable crops such as cabbage, cauliflower, and broccoli, and it has developed insecticide resistance from the prophylactic use of insecticides over many years (Talekar and Shelton, 1993). All of these pest species use their chemical senses to locate and select their host plants, and in many cases, their chemical ecology has been studied (Renwick and Chew, 1994) for the development of pest management techniques such as trap cropping (Shelton and Badenes-Perez, 2006). Many scientists study these pest species, reflected by more than 1100 publications listed on pubmed alone for these five species. The identification of their chemoreceptor genes will benefit this scientific community, and lead to the development of new and alternative pest management techniques.

**Figure 5A.** Neighbor-joining (corrected distance) phylogenetic tree of the *B. mori* and *Heliothis virescens* (F. Noctuidae) Ors identified to date, rooted using lepidopteran orthologs of DmOr83b. The *H. virescens* Ors are reported in Krieger et al. (2004). Bootstrap support is reported as a % of 1000 replicates. Gene expression levels relative to *BmOr2* are depicted using asterisks: expression levels approximately equal to BmOr2, ****; 10-1 X lower, ***; 10-2 X lower, **; and 10-3 X lower, * . Lack of an asterisk indicates that expression was not detected. ND = no data.



**2.0 Progress Reprort** N/A

**3.0 Rationale and Significance**

This proposal is directly aligned with FY 2006 research priority number 1: "Chemical perception and signaling genes to elucidate interactions between pests …with agricultural commodities …" Without dispute, insect chemoreceptors are the preeminent gene family responsible for mediating the perception and signaling of olfactory and gustatory stimuli in their environment. Therefore, they also mediate critical pest behaviors such as host crop selection and feeding, particularly in the Lepidoptera, an important pest group. Our proposal is focused on identifying these genes from five important pest species that represent five important pest families, and transferring this information to the broad research community. We will not focus on function, an important, but difficult task at this time, one which would consume the entire resources of a grant simply for a few receptors from a single species. Rather, as observed with the fruit fly and the mosquito, much greater progress will be facilitated by identifying the sequences for use by the research community at large, and it will open the "receptor window" to many important species within the Lymantridae, Noctuidae, Plutellidae, Pyralidae and Tortricidae whose pest behaviors rely on the chemical senses.

This proposal directly meets the long term goal of reducing pesticide use through the development of sustainable, integrated pest management techniques. By identifying the genes that directly mediate the chemical senses of these important pest species, we will open the door to new approaches in chemical ecology, a field which has a long history of developing new insect management techniques, such as pest monitoring, trapping (the use of crop trapping appears to be on the rise, Shelton and Badenes-Perez, 2006) and disruption techniques based upon attracting or repelling insects. The fact that olfactory and gustatory receptors represent a new class of "target" genes is reflected by a large initiative in mosquito control (whose receptors have been identified). The Grand Challenges in Global Health (Bill and Melinda Gates Foundation, http://www.gcgh.org/subcontent.aspx?SecID=389) has funded two significant projects: "Disruption of Malaria Transmission by Chemical Manipulation of Anopheline Olfactory Responses" ($8.5 million US), and "Molecular Approaches to Alter Olfactory-Driven Behaviors of Insect Disease Vectors" ($5 million), a clear recognition of the significance of this gene family to the development of future insect management tactics. To quote from this source: "Molecular technology will be used to identify compounds that interfere with the host-seeking behavior of malaria-transmitting mosquitoes by stimulating or blocking the responses of specific odor receptors." We feel that the timing of our proposal is excellent. As previously mentioned, functional assays are currently difficult, but as we identify chemoreceptors from the Lepidoptera, new functional tools will be emerging from the mosquito and fruit fly research. This will likely include refined in vitro ligand binding assays able to screen large chemical libraries.

A wealth of chemical ecology and electrophysiology knowledge has been accumulated for many important lepidopteran pest species. Many scientists eagerly await the opportunity to incorporate the chemoreceptors into their research programs, which will facilitate the transfer of these new and exciting developments to important lepidopteran pest species. Finally, we have demonstrated that our proposal is viable, and that we possess the expertise to successfully complete it.

**4.0 Approach**

*4.1 Objectives 1 & 2. Identify the odorant and gustatory receptor genes from the Bombyx mori genome sequence and identify candidates that mediate important pest behaviors*

Technically, identifying and constructing the silkmoth chemoreceptors is straightforward, however it is laborious and time consuming. I have constructed 45 new silkmoth Ors (in addition to the 7 published sequences) and I have the fragments of about 10 others, bringing the known total to about 62. Based upon the roughly one to one ratio of Ors to glomeruli (Table 1), this may be close to the complete complement. I will continue with the same approach to identify and build the silkmoth gustatory receptors. I will use the published fly and mosquito Grs that have been entered onto GenBank (National Center for Biotechnology Information), as well as the unpublished beetle Grs that Dr. Robertson has identified, to search for homolgous genes in the silkmoth genome sequence. Protein sequences will be used to perform tBLASTn (Altschul *et al.*, 1997) searches of assembled scaffolds available through two internet websites: http://kaikoblast.dna.affrc.go.jp/ (Silkmoth Genome Research Program, National Institute of Agrobiological Sciences, Japan) and http://silkworm.genomics.org.cn/ (Beijing Genomics Institute, China). As an alternative, the complete silkmoth genome can be down loaded and queried using a stand alone PC version of the BLAST software. Genomic scaffold sequences will be down loaded and used to construct Gr genes manually in the PAUP text editor (Swofford, 1998), using homology with known Or exons and an online program to predict exon/intron splice sites (for example, Softberry, www.softberry.com/berry.phtml). In some cases I have amplified and sequenced PCR products from the Or genes, including 3' RACE, to resolve problems such as missing exons – I will continue to use this method.

Using quantitative PCR to profile gene expression, I have identified two Or genes specifically expressed in adult female silkmoth antennae (BmOr19 and 30, Table 2), and one expressed highly in the caterpillar mouthparts (BmOr10)(Figure 3). Based upon this specific expression, they likely mediate important pest behaviors such as host selection and feeding – I will apply this successful approach to the silkmoth Gr genes, sex specific expression in organs with contact taste sensilla will be of particular interest, due to the role they play in host selection.

**Figure 6.** Containers for rearing larger numbers of silkmoth on artificial diet.

Adult organs such as the antennae, front tarsi, proboscis, labial palps and ovipositor are easily collected from reared insects. Over the last year we have reared large numbers of silkmoth to the adult stage (Figure 6). High quality eggs and larvae can be purchased from Mulberry Farms (http://www.mulberryfarms.com/) at reasonable prices due to their use as feeder insects for the reptile pet business. The larval sensory organs are much smaller, and therefore require large numbers to be dissected, and the ability to rear in large numbers will be important.

Gr expression in the caterpillar mouthparts, particularly in the lateral and medial styloconic sensilla found on the maxillary lobe, will be of particular interest due to their direct role in mediating caterpillar feeding. Here, very detailed dissections will be required to separate the sensory organs, and we have refined a technique precisely for this. By first freezing the heads on dry ice, the specific sensory organs cleanly break off when contacted by a minutia probe under a dissecting microscope. This work promises to identify the highly sought "biter" receptors expressed in the deterrent sensory neuron found only in the medial styloconic sensilla (Ishikawa, 1965). In fact, stimulation of this single deterrent neuron on the caterpillar mouthparts is sufficient to prevent feeding behavior.

We have used RNeasy columns (Qiagen, Valencia, CA USA) to isolate total RNA from abundant tissues, and acid phenol chloroform extraction with a carrier to capture RNA from small quantities of tissue. PCR is very sensitive, therefore all traces of genomic DNA must be removed by DNAse I digestion prior to synthesizing 1$^{st}$ cDNA for qPCR.

We use an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and SYBR Green dye (SYBR Green PCR Master Mix, Applied Biosystems) for real-time quantitative PCR (qPCR). Primer design is critical for qPCR, since the amplification efficiency needs to approximate 100%, so that different primer sets (representing different genes) can be compared. We have used ABI Primer Express 2.0 software (Applied Biosystems), set to select for an optimal primer annealing temperature of 59°C, amplicon sizes of 50-150 bp, and a minimum and maximum GC content of 30 and 80%, respectively, to design more than 50 primer sets for the silkmoth Or genes. Each of these primer sets was first validated by constructing a standard curve using 10x serial dilutions of template, if the efficiency is 100%, the slope of the regression line (CT vs the log[template dilution]) will equal 3.3 . Our Or primer sets typically produced slopes within 5% of this value, with $r^2$ values typically above 98%. With this criteria satisfied, relative expression levels of each Or gene was calculated relative to a reference tissue using the ? ? CT equation**.** Transcript levels were first normalized to a control gene, such as *B. mori* ribosomal protein S3, and the purity of the amplicon verified by a dissociation curve. Using this methodology, we will profile silkmoth Gr gene expression in adult and larval sensory organs to identify candidates that mediate host plant selection and feeding behavior.

*4.2 Objectives 3 & 4. Identify odorant and gustatory receptors from the apple codling moth, corn earworm, diamond back moth, European corn borer and gypsy moth*

We will screen cDNA libraries constructed from sensory organs, using the silkmoth Ors and Grs as probes in a homology screen. This is the method of choice, since large numbers of clones can be screened, and it is much less expensive compared to sequencing thousands of ESTs in a random approach. We demonstrated that 8 of 10 *B. mori* and *H. virescens* paired Or genes share as much as 70-85% nucleotide identity near the ends of their ORFs (Figures 4 & 5B). Therefore, we will begin with species that have even greater taxonomic similarity to the silkmoth, so that we can expect even greater levels of nucleotide identity that will benefit the homology screening. Towards this, we will screen a *Manduca sexta* antennal cDNA library since we have one available (Robertson et al., 1999) and the Sphingidae moths are one of the most closely related families to the Bombycidae (silkmoth) (The tree of life web project, http://tolweb.org/tree?group=Ditrysia&contgroup=Neolepidoptera). These additional sequences can be used as probes as we continue to work outwards to related families, such as the Lymantridae (gypsy moth) and Noctuidae (corn earworm), all members of the Macrolepidoptera. Rates of gene diversion will vary in different taxonomic lineages. Therefore, greater representation from the Macrolepidoptera will benefit the homology based approach as we

extend it to the more distantly related families, the Pyralidae (European corn borer), Tortricidae (apple codling moth) and the Plutellidae (diamond back moth) (The tree of life web project, http://tolweb.org/tree?group=Ditrysia&contgroup=Neolepidoptera). We will include the published *H. virescens* sequences as probes.

Or genes from *B. mori* and *H. viresens* group together into homologous subfamilies which may exhibit conserved functions, such as the putative pheromone receptors (Figures 5A). We will prioritize our efforts towards subfamilies identified in objective 1 that may mediate important pest behaviors. However, we will not *limit* ourselves to these subfamilies – we feel that it will be important to identify a broad representation of receptors, and to further characterize their expression levels in each pest species. Also, while we will focus on receptors that mediate female moth and larval host selection and feeding behaviors, we will certainly include the candidate pheromone receptors in our screens, due to their importance and utility to the more broad research community.

Furthermore, our efforts to identify chemoreceptors from the Tortricidae are strengthened by collaboration, Dr. Thomas Unruh, USDA-ARS, Wapato WA has offered to make apple codling moth cDNA libararies, and Dr. Richard Newcomb, Science Leader - Molecular Olfaction Group, HortResearch, Auckland, New Zealand, has offered the sequences of several Tortricid Ors that he has discovered from the light brown apple moth (*Epiphyas postvittana)* as probes for screening. Both collaborators are also interested in using the silkmoth sequences to screen their own in house codling moth libraries.

Our first preference is to purchase the insects that we require. Corn earworm and diamond back moth eggs and larvae can be purchased from Bio-serve (http://www.insectrearing.com/products/eggslarv.html) and reared on artificial diet. The gypsy moth is reared at the Forest Pest Management Institute in Sault Ste. Marie, Ontario; alternatively, insects can be collected from infestations in neighboring states. If required, we can rear insects such as the European corn borer in house.

The construction and screening of cDNA libraries has become a routine molecular biology protocol described in standard manuals such as Molecular Cloning by Sambrook and Russell (2001). Dr. Robertson has worked with more than 10 cDNA libraries, including those described in Robertson et al. (1999), and I constructed a cDNA library during my Ph.D. studies. The first critical step is to isolate large amounts of high quality, poly A selected RNA. We will focus on sensory rich adult organs such as the antenna and proboscis which are readily available. We will also consider specific female tarsal leg segments that are enriched in contact sensilla. Molecular kits can be purchased for all stages of the process: poly A selection, $1^{st}$ and $2^{nd}$ strand cDNA synthesis, ligation into the vector and probe synthesis and library screening. The Stratagene cDNA library kit which uses the lamda ZAP phagemid vector is one standard kit. It has the option of mass excising the pBluescript phagemid so that screening can be conducted using E. coli bacteria as well as viral plaques. Clonetech sells a kit designed to select for full length cDNA clones. An important feature, phagemid libraries typically yield libraries with $10^6$ inserts, allowing the detection of rarely expressed genes. After plating the library and transferring the DNA to nylon membranes, the colonies are screened with labeled probes ($^{32}$P or digoxigenin, for example). During screening, we may consider a combinatorial approach, where several Ors representing a subfamily can be synthesized and combined to screen a library.

A PCR strategy using degenerate PCR primers designed to conserved regions of the receptor proteins can also be used. This will become more reliable as our sequence representation increases (homology will be evident in the phylogenetic trees that we construct),

particularly within specific subfamilies. While this approach can be unpredictable, it is relatively inexpensive and easy, and therefore is worth attempting since both PIs have extensive experience with this method.

*4.3 A discussion of risk factors*

The first objective, annotating the silkmoth chemoreceptor genes and characterizing their gene expression pattern, is risk free (and very valuable to the research community at large). The second objective, using these as probes in homology based screens entails some risk, particularly as the taxonomic distance between the species increases. Using the *Heliothis virescens* chemorecptors to screen a *Helicoverpa zea* library should entail almost no risk, since they both belong to the same subfamily (Heliothinae) within the Noctuidae. Similarly, we feel that the risk of using the silkmoth receptors to screen species in the families Sphingidae, Lymantridae and Noctuidae will be low, since we expect nucleotide identity to exceed 80% for certain conserved regions. However, the degree of conservation in the more distantly related families (Pyralidae, Plutellidae and Tortricidae ) is an uncertainty. Here, the availability of tortricid Ors from collaborators will be beneficial. The Gr sequences are more risky as compared to the Or sequences. Only three have been identified from the Lepidopera so far. Until we annotate the silkmoth Grs, and identify their homologous counterparts in other lepidopoteran families, we cannot assess their relatedness.

In some cases, function may be conserved in some receptor subfamilies (such as the pheromone receptors) – therefore identifying silkmoth receptors that mediate important behaviors may provide similar leads in pest species. However, function will not be conserved in all of the subfamilies. For this reason, we emphasize the importance of taking a comprehensive approach to identifying the greatest number of receptors possible, and characterizing their expression in the pest species.

*4.4 Timeline for milestones*

**Year 1.** We currently have most of the silkmoth Ors, and we have an antennal cDNA library from *M. sexta* (most closely related to the silkmoth), so we will begin screening this immediately. The silkmoth Grs will be built and their expression patterns profiled. We will make cDNA libraries from gypsy moth antennae, and from corn earworm antennae, proboscis and tarsi. Our collaborator Tom Unruh will make a cDNA library the apple codling moth, and we will send silkmoth Ors to Richard Newcomb to screen his tortricid libraries.

**Year 2.** The gypsy moth and corn earworm cDNA libraries will be screened for Grs and Ors, Tom Unruh will screen the apple codling moth library. We will construct antennal cDNA libraries from the European corn borer and the diamond back moth. Clones will be sequenced as they are identified. New homology trees will be constructed to identify conserved subfamilies as candidates for degenerate PCR primers. Results will be presented at the annual ESA and Achems meetings. A publication will be written, and the receptor sequences entered onto a public data base.

**Year 3.** The European corn borer and diamond back moth libraries will be screened for Grs and Ors. The expression pattern of receptor genes that we have discovered will be profiled in each pest species. Results will be presented at the annual ESA and Achems meetings. Two publications will be written, and the receptor sequences entered onto a public data base.

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