

EVOLUTION OF THE MOLECULAR MECHANISMS OF PHEROMONE
RECEPTION IN EUROPEAN AND ASIAN CORN BORER MOTHS

by

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DEDICATION

For Michele

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ABSTRACT

The insect order Lepidoptera includes more than 180,000 species and some of the most well known pests of food and fiber crops. Ninety-eight percent of lepidopteran species belong to a taxonomic group called the Ditrysia. Modern Ditrysia use long distance sex pheromones to facilitate mating. The European corn borer, *Ostrinia nubilalis* (ECB) is a well known pest of agricultural crops throughout North America and Western Europe. The European corn borer species exists as two different pheromone races. Females of the species produce, and males are attracted to different blends of the isomers (Z)-11-tetradecenyl acetate and (E)-11-tetradecenyl acetate. The closely related Asian corn borer (*O. furnacalis*, ACB) has evolved to use a pheromone blend that is unique among all Lepidoptera, (Z)- and (E)-12-tetradecenyl acetate. *O. nubilalis* and *O. furnacalis* species can be used as models to study pheromone evolution. Pheromones are detected at the periphery of the olfactory system by olfactory sensilla located on the antennae. Proteins involved in pheromone detection at the periphery include: odorant receptors, pheromone binding proteins, and sensory neuron membrane proteins. In this study, the coding sequences of seven odorant receptors, five pheromone binding proteins, and two sensory neuron membrane proteins were cloned from Asian and European (E and Z race) corn borer antennae. Five odorant receptors and two pheromone binding proteins were expressed at high levels in male corn borer antennae based on quantitative real-time PCR assays. Several odorant receptors were heterologously expressed in *Xenopus laevis* oocytes, and odorant receptor 6 was found to respond specifically to (Z)-11-tetradecenyl acetate in electrophysiological studies. The coding sequences of all fourteen genes were analyzed by computational and statistical methods to identify candidate genes that may play a role in the detection of the ACB pheromone blend. Odorant receptor 3 and pheromone binding protein 3 may have evolved specificity to 12-tetradecenyl acetates. Future studies will clarify the role of these proteins in the evolution of pheromone detection at the molecular level. An improved understanding of the evolution of pheromone detection may lead to new pheromone based controls for these economically damaging species.

CHAPTER 1

A REVIEW OF SEX PHEROMONE
RECEPTION IN THE GENUS *OSTRINIA* (LEPIDOPTERA: CRAMBIDAE)

The insect order Lepidoptera includes more than 180,000 species including some of the most well known pests of food and fiber crops world-wide (Grimaldi & Engel, 2005). Most Lepidoptera are phytophagous and feed on a wide range of agricultural, stored product, forest, urban and nursery crops causing significant economic damage (Pedigo, 1989). These diverse insects encounter a broad spectrum of chemical signals in their search for food, mates, and oviposition sites. In order to detect these signals and respond appropriately these insects rely on their chemical senses. Ninety-eight percent of lepidopteran species belong to a taxonomic group called the Ditrysia (Grimaldi & Engel, 2005). Modern Ditrysia use long distance sex pheromones to facilitate mating (Löfstedt, 1993). Female moths emit blends of hydrophobic, volatile, long chain fatty acid derivatives that attract male moths from a distance. The use of a long distance sex attractant is thought to have contributed to speciation in the Lepidoptera (Carde & Haynes, 2004). Slight changes in the chemical structure of the sex pheromones such as carbon chain length, the number and location of double bonds, and different isomers of the same chemical, can all contribute to species specificity.

Basic and applied research into sex pheromones has been driven by the needs of agriculture, horticulture, forestry, and chemical industries (Witzgall, 2010). Broad spectrum insecticides are sometimes unacceptable for pest management due to insecticide resistance, negative impacts on health and the environment, and cost. In these cases, sex

pheromones can be used for pest management because they are species specific, effective in small quantities, and most are non-toxic to animals. Chemical ecologists have exploited the species specificity of sex pheromones to develop lures for pest management programs. Hundreds of lepidopteran sex pheromones representing more than 60 taxonomic families have been identified and many have been tested as lures (El Sayed, 2003). The most common use of sex pheromones is in detection and monitoring. Pheromone lures are sensitive enough to detect low density populations, and are useful in monitoring invasive species (Witzgall, 2010).

The European corn borer (ECB, *Ostrinia nubilalis*) is used as a model species to study the evolution of sex pheromone specificity because it has several attractive biological traits. Two different sex pheromone races exist in Europe and North America (Carde et al., 1978), termed the E and Z races, abbreviated ECB-E and ECB-Z. ECB-Z males are attracted to a 97:3 ratio of (Z)-11-tetradecenyl acetate (Z11-14:OAc) to (E)-11-tetradecenyl acetate (E11-14:OAc) while ECB-E males are attracted to a 1:99 ratio of the Z and E isomers. Most species in the genus *Ostrinia* use Z11 and E11-14:OAc as their major pheromone components, but a single species stands out as unusual among all *Ostrinia*. The Asian corn borer (ACB, *Ostrinia furnacalis*) is closely related to the ECB, but has evolved to use a pheromone consisting of variable ratios of (Z)-12-tetradecenyl acetate (Z12-14:OAc) and (E)-12-tetradecenyl acetate (E12-14:OAc) (Ishikawa et al., 1999; Roelofs & Rooney, 2003; Klun et al., 1980; Linn et al., 2007). Apparently, a major shift in pheromone production from the 11-tetradecenyl acetates to the 12-tetradecenyl acetates occurred in the lineage leading to the ACB (Baker, 2002). The shift in

pheromone production appears to have involved the activation of a desaturase gene used by ACB that is present but not active in ECB (Roelofs & Rooney, 2003). The gene encoding $\Delta 14$ desaturase is active in ACB but not in ECB, and ECB has an active $\Delta 11$ desaturase that is lacking in ACB (Roelofs & Rooney, 2003). These two species produce different sex pheromones from the same fatty acid precursor (Symonds 2010). The question remains how males were able to track the shift in pheromone production and evolve into the species we now know as the ACB.

In addition to the differences in pheromone production among races and species, corn borers of the genus *Ostrinia* are useful models because they have a negative impact on agriculture and the global economy. The ECB is one of the most damaging insect pests of corn with economic losses exceeding \$1 billion each year in the United States and Canada (Witkowski et al., 2008). Corn borer damage manifests as stunting and yield loss due to stalk tunneling and vascular damage, stalk lodging, and shank damage. Most yield loss can be attributed to the impaired ability of plants to produce grain due to the physiological effect of larval feeding (Rice, 2006). The ECB is native to Europe and was first found invading North America in 1917 in Massachusetts (Brindley, 1975). It is now distributed throughout North America east of the Rocky Mountains. The ACB is distributed from China to Australia and the Solomon Islands (Nafus & Schreiner, 1991). Populations of ACB and ECB in Eastern Europe and Asia do not appear to overlap, but this may be due to a lack of observation rather than true geographical isolation (Frolov, 2007). Information on the geographic distribution of *Ostrinia* species is incomplete.

The genus *Ostrinia* was originally classified into three groups based on differences in the uncus, a segment of the male genitalia (Mutuura & Munroe, 1970). Species group I was termed the *penitalis* group, group II, the simple uncus group, and group III, the trilobed uncus group. Group III is further subdivided into species with small medium and large tibia, with the small tibia group being the most primitive. ACB and ECB belong to the small-tibia subgroup of group III. The ten species of group III are confusingly similar in external appearance, except for the male mid-tibia morphology (Frolov, 2007). Mitochondrial DNA analysis does not support the small, medium, and large-tibia subdivisions of group III (Ishikawa et al., 1999; Marcon et al., 1999). Phylogenetic analysis of the cytochrome oxidase II (COII) gene indicates a close relationship between members of the small tibia subgroup, with the exception of ACB, which is more distant (Kim et al., 1999) (Fig.1). Still, the level of sequence divergence of COII in group III (0.15-2.38%) was similar to the level of sequence divergence within species (Kim et al., 1999).

Genetically similar ACB and ECB also have similar life cycles. Corn borer eggs are laid on leaves, and the early instars feed on the mesophyll. Eventually the larvae bore into the corn stalk where they develop into the fifth instar (sixth in ACB) and pupate or diapause as larvae. ECB is known to feed on about 250 different plants and is a pest of field crops such as peppers, beans, potato, and tomato. The ACB also feeds numerous crops other than corn including: peppers, ginger, sorghum, and cotton (Nafus & Schreiner, 1991). The ECB is polymorphic for the number of generations per year. The Z-race may have one, two, or several generations per year, while the E-race has two or

more generations per year. Both the E and Z-races are present in the United States, but the Z-race predominates west of Pennsylvania. The E-race rarely occurs alone and is not known to exist as a univoltine northern ecotype (Rice, 2006). The ACB has one or a few generations per year in northern regions, but in the tropics, generations are continuous and overlapping (Bell et al., 2006).

The E and Z pheromone races of ECB are well-known as models for reproductive isolation (reviewed in Dopman et al., 2010). The two races can be distinguished by at least two independent loci; one is responsible for differences in male electrophysiological response (*Olf*), and the other for differences in behavioral orientation (*Resp*) to female sex pheromones. Autosomal vs. sex-linked inheritance of these genes was determined using F1 and F2 and back-cross experiments (Roelofs et al., 1987). Different patterns resulted from autosomal vs. sex-linked inheritance (depending on the type of cross) because female Lepidoptera are heterogametic and males are homogametic. Male electrophysiological response is autosomal while behavioral orientation is sex-linked. The gene for female pheromone production is also autosomal and is not linked to male electrophysiological response (Roelofs et al., 1987). Variation in female pheromone production leading to race specific signals was recently found to be a result of allelic variation in a fatty-acyl reductase gene essential for pheromone biosynthesis (Lassance et al., 2010).

Pheromones are detected at the periphery of the olfactory system by pheromone sensitive sensilla located mostly on the antennae. Olfactory sensilla typically consist of three olfactory neurons with different diameters housed within a hair-like structure, the

trichoid sensillum (Hallberg et al., 1994) (Fig.2). The responses of pheromone sensitive olfactory neurons (ORNs) to pheromone components were characterized using single sensillum electrophysiology, and the spike-amplitude of the responses were found to be related to dendrite diameter (Hansson et al., 1994). The “large spiking” neuron in ECB-Z males responded to Z11 and Z12-14:OAc while the “small spiking” neuron responded to all ACB and ECB pheromone components (Domingue et al., 2010). In ECB-E males the “large spiking” neuron responded to all ACB and ECB components while the “small spiking” neuron responded to Z11 and E12-14:OAc. The “medium spiking” neuron responded to Z9-14:OAc (a behavioral antagonist) in both races of ECB (Domingue et al., 2007). ACB ORNs responded similarly to ECB-E ORNs except that the “small spiking” neuron responded to E12-14:OAc and the “medium spiking” neuron responded to Z11 in addition to Z9-14:OAc (Domingue et al., 2007, 2009) (Fig. 3). The broad responses of ORNs in these studies were unexpected because it had been thought that pheromone sensitive ORNs would need to be highly specific to detect small differences in pheromone blends or components (Carde & Haynes, 2004).

Rare ACB males are attracted to ECB pheromone blends, and rare ECB males are attracted to ACB pheromone blends (Löfstedt, 1993; Löfstedt et al., 1990, 1991; Phelan, 1997). Domingue et al. (2007, 2010) showed that the medium spiking antagonistic neuron was not responsive to Z11-14:OAc in rare ACB males that flew upwind to ECB females, and that the large spiking neuron was more responsive to E12-14:OAc in rare ECB-E males that flew upwind to ACB females. Therefore, the attraction of ACB males to ECB females can be attributed to an absence of behavioral antagonism to the Z11

pheromone component. In contrast, the attraction of ECB-E males to ACB females resulted from increased behavioral attraction to Z12-14:OAc. A change in one or more of the proteins involved in pheromone reception at the ORN is the most likely explanation for altered physiological responses of the ORNs.

Proteins involved in pheromone reception at the ORN include (but are not limited to): odorant receptors (ORs), pheromone binding proteins (PBPs), and sensory neuron membrane proteins (SNMPs). OR genes form one of the largest gene families in insects. Conserved intron/exon boundaries suggest that ORs arose from a common ancestor (Robertson et al. 2003). The comparison of OR genes within and/or between species shows that the OR gene family has undergone rapid evolution. Most ORNs express a single OR gene, but there have been several cases in *Drosophila* where two or three genes are expressed in a single ORN. These genes often arise from recent duplication events and may be functional or nonfunctional (Dobritsa et al. 2003, Goldman et al. 2005).

The coding regions of OR genes are not highly conserved across insect species, except in the OR83b gene family. *Drosophila* OR83b (known as OR2 in Lepidoptera) is an insect-specific receptor that has conserved functions across many insect species (Hill et al., 2002; Nakagawa et al., 2005; Robertson & Wanner, 2006 ; Bohbot et al., 2007; Engsontia et al., 2008) . OR2 functions as a chaperone and dimer partner for ligand binding ORs, but does not participate directly in odor detection (Neuhaus et al., 2005; Benton et al., 2006). Co-expression of OR2 is essential for odorant evoked activity of

ligand binding ORs in heterologous expression systems (Nakagawa et al., 2005; Wanner et al., 2007; Sato et al., 2008; Wicher et al., 2008).

ORs are seven transmembrane domain proteins expressed in the dendrite membrane of ORNs. Insect ORs were thought to be G-protein-coupled receptors (GPCRs) like mammalian ORs. However, insect ORs are oriented opposite of GPCRs in the membrane (the N-termini of insect ORs are intracellular while the C-termini are extracellular), and insect ORs lack sequence homology to GPCRs (Benton et al. 2006; Lundin et al. 2007; Wistrand et al. 2006). Recent evidence suggests that insect ORs are heteromeric ligand gated ion channels (Sato et al., 2008) or, that insect ORs are both GPCRs and ligand gated ion channels (Wicher et al., 2008). Wicher et al. (2008) suggested that rapid activation of insect ORs by ligands is followed by a G-protein dependent response. In both cases, the direct activation of ORs by ligands would explain the observed rapid activation kinetics of insect ORs (Sato et al., 2008; Wicher et al., 2008).

The study of lepidopteran ORs was advanced when technologies were developed to express ORs in heterologous systems. Dobritsa et al. (2003) developed a mutant strain of *Drosophila* with an “empty neuron”. The empty neuron was generated by deleting the gene for a receptor (OR22a/b) thereby eliminating electrophysiological responses without eliminating the ORN. Almost any OR can be expressed in the empty neuron system, and the properties of the OR can be studied *in-vivo*. Alternatively, ORs can be studied *in-vitro* by expression in human embryonic kidney 293 (HEK293) cells, HeLa cells, or

Xenopus laevis oocytes (Nakagawa et al., 2005; Neuhaus et al., 2004; Sato et al., 2008; Wetzel et al., 2001; Wicher et al., 2008).

Pheromone binding proteins (PBPs) are a subgroup of odorant binding proteins (OBPs) that may have arisen in the Lepidoptera (Vogt, 2003). PBPs are produced in the accessory cells surrounding pheromone sensitive ORNs, and are secreted into the sensillum lymph where they accumulate to high concentrations (Pophof, 2002). PBPs are small water soluble proteins that transport hydrophobic pheromone ligands through the watery sensillum lymph to the dendrite membrane. The ability to bind pheromone (and non-pheromone) ligands has been demonstrated for several PBPs (Damberger et al., 2007; Du & Prestwich, 1995; Grosse-Wilde et al., 2006; Horst et al., 2001; Leal et al., 2005; Maida et al., 2003). Evidence suggests that lepidopteran PBPs eject pheromone ligands close to the dendrite membrane as a result of conformational changes induced by low pH near the membrane (Damberger et al., 2007; Lautenschlager et al., 2005). The well studied *Bombyx mori* PBP1 (*BmPBP1*) binds to the pheromone component bombykol (Grosse-Wilde et al., 2006). When the ligand is bound at high pH the C-terminal tail of *BmPBP1* is a disordered loop, and at low pH the C-terminal tail forms a helix that occupies the same binding pocket that formerly housed the pheromone ligand (Lautenschlager et al., 2005). PBPs are not required for *in-vitro* assays of receptors, but they may affect the sensitivity and specificity of the receptor (Grosse-Wilde et al., 2007). PBPs may account for, or (in addition to ORs) may contribute to altered physiological responses of ECB and ACB *in vivo*.

Sensory neuron membrane proteins (SNMPs) are another class of proteins involved in pheromone reception at the ORN. SNMPs are members of the CD36 family of proteins (Rogers et al., 2001b). CD36 proteins may function in the binding and transport of hydrophobic ligands. SNMPs are two transmembrane domain proteins located in the dendrite membrane of pheromone sensitive ORNs. SNMP expression in ORN membranes suggests that these proteins play a role in odor detection. However, little is known about the specific functions of SNMPs in Lepidoptera. The *Drosophila* homolog of SNMP1 is essential for the detection of the volatile pheromone 11-cis-vaccenyl acetate (cVA) (Jin et al., 2008). When *HvCr13*, the receptor for the main pheromone component (Z11-16:Ald) of *Heliothis virescens*, was expressed in transgenic *Drosophila*, a SNMP protein was required for neuron responsiveness (Benton et al., 2007). However, *HvCr13* can respond to Z11-16:Ald in *in-vitro* assays without SNMPs (Grosse-Wilde et al., 2007). Therefore, it is unclear whether SNMPs are involved in pheromone detection in the Lepidoptera.

It is important to differentiate between peripheral and central responses to odors. The brain is responsible for transforming chemical signals into information that elicits a behavioral response. Is it possible that the wiring of the brain encodes the response to pheromone components or blends? Studies suggest that male response is caused by a factor upstream of the antennal lobe at the level of the ORN (Karpati et al., 2008). In the brain, the axon of an ORN forms a synapse with a projection neuron in the antennal lobe (AL). The AL is made up of a number of glomeruli where synaptic contacts are made between ORNs, projection neurons, and local interneurons. Male moths have a group of

enlarged glomeruli (the macroglomerular complex, MGC) dedicated to receiving information about female produced pheromones (Hansson & Anton, 2000; Karpati et al., 2008). ECB-E and ECB-Z males have identical MGC morphology (Karpati et al., 2008). ORNs specific to the major component arborize in the medial MGC, and ORNs specific to the minor pheromone component arborize in the lateral MGC. Therefore, the medial and lateral MGC have reversed functions in ECB-E and ECB-Z (Karpati et al., 2008). An explanation for this is an exchange of ORs between neurons in the same sensillum. The path of an ORN is determined separately from its response (De Bruyne & Baker, 2008); therefore changes in the peripheral response can change the behavioral response.

Despite differences in pheromone communication the E and Z races of ECB are otherwise difficult to distinguish. Genetic studies of allozymes, mitochondrial DNA, randomly amplified polymorphic DNA, and nuclear genes suggest that gene flow is ongoing (Cianchi et al., 1980; Harrison & Vawter, 1977; Marcon et al., 1999, Pornkulwat et al., 1998, Willett & Harrison, 1999). Of all the loci that have been tested, only the Triose phosphate isomerase (*Tpi*) locus showed significant differences in allele frequency between ECB pheromone races (Glover et al., 1990). *Tpi* was thought to be linked to *Resp* on the Z chromosome, but a map of the Z chromosome generated using AFLP (amplified fragment length polymorphism) and microsatellite markers showed that *Tpi* mapped to a region of the Z chromosome 28.1cM away from *Resp* (Dopman et al 2004) . Therefore, it is unlikely that differences in allele frequency at the *Tpi* locus are a result of linkage to *Resp* (Dopman et al., 2004). Field studies have shown that the proportion of ECB-E and Z hybrids is lower than would be expected given that they often occur

together and there are no significant post-zygotic barriers (Dopman et al., 2009). One explanation for this is that the differences in pheromone communication are contributing to speciation in E and Z-race ECB.

Differences in pheromone communication between E and Z-race ECBs contribute to speciation by promoting strong reproductive isolation in the field (Lassance, 2010). Comparison of the ACB and ECB pheromone communication systems provides an informative backdrop for understanding how shifts in pheromone composition can occur between closely related moth species (Roelofs et al., 2003; Baker, 2002). This may help to explain the diversity of pheromone communication systems used by moths in general (Domingue et al., 2007). The exact nature of the genetic changes affecting pheromone reception in male *Ostrinia* remains to be determined. Recently, Gould et al. (2010) identified a quantitative trait loci (QTL) containing at least four odorant receptor genes that determines differential male responses to female pheromone components in *Heliothis subflexa* and *Heliothis virescens*.

The following chapters describe how a functional genomics approach was used to identify and characterize seven sex pheromone ORs, five PBPs, and two SNMPs from male ECB-E, ECB-Z, and ACB moths. Specifically, the objectives of this study were to: clone all OR, PBP, and SNMP genes from ECB-E, ECB-Z, and ACB moths and measure their expression in male and female antennae, to analyze their sequences for evidence of positive selection, and to identify candidate genes that may have evolved specificity to the ACB sex pheromone. Examination of these genes will allow further exploration into

peripheral mechanisms contributing to the evolution of sex pheromone detection at the molecular level.

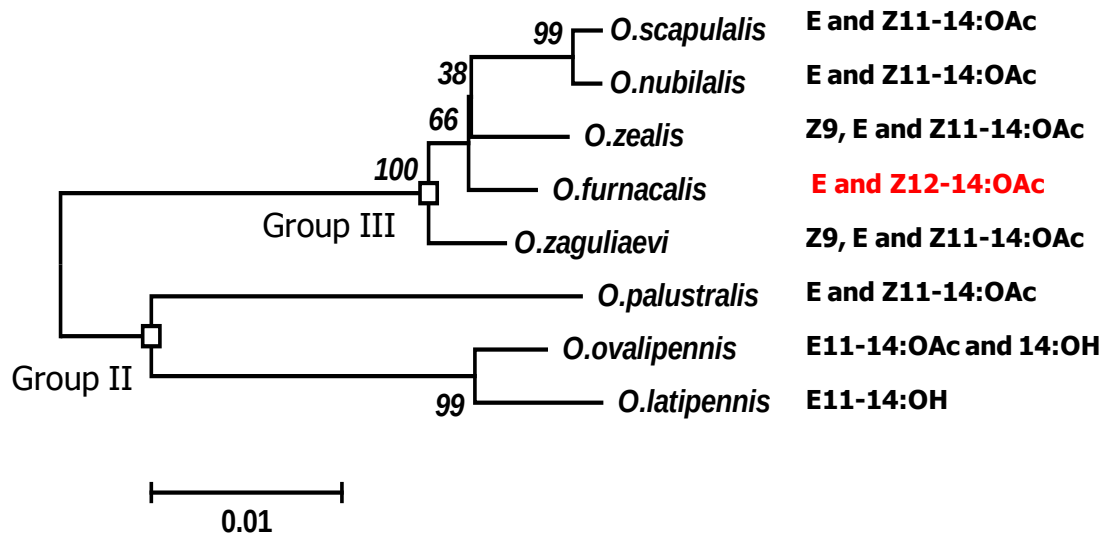


Figure 1. Phylogenetic relationship of *Ostrinia* cytochrome oxidase II (COII) sequences. The evolutionary history of *Ostrinia* COII nucleotide sequences was inferred using the neighbor-joining method in MEGA4 with 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths in units of the number of base substitutions per site. Three species from *Ostrinia* group II and five species from group III are represented. The major sex pheromone components used by each species are shown.

Figure 2. Drawing of a pheromone sensitive trichoid sensillum. Three major classes of proteins involved in pheromone detection: odorant receptors, odorant/pheromone binding proteins (OBP/PBP), and sensory neuron membrane proteins (SNMPs) are shown.

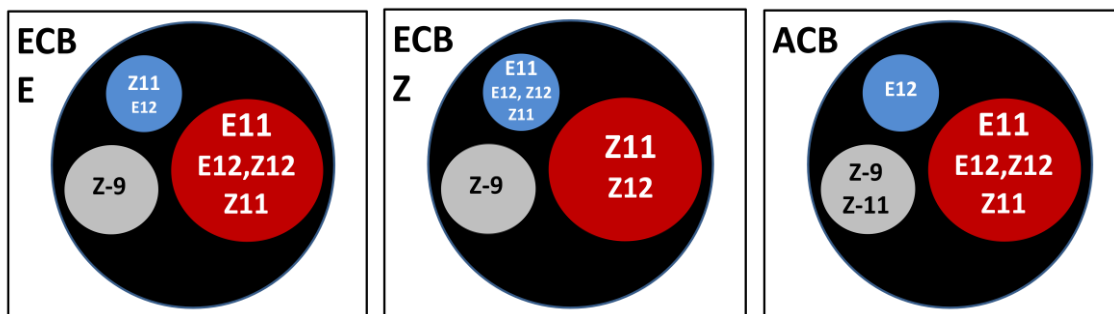


Figure 3. Representation of the small, medium, and large spiking neurons housed in type A sensilla. The differential responses of European corn borer E and Z-race, and Asian corn borer neurons to pheromone components: E11, Z11, and Z9-tetradecenyl acetate, and E and Z12-tetradecenyl acetate, are shown.

CONTRIBUTIONS OF AUTHORS AND CO-AUTHORS

Chapter 2: Sex Pheromone Receptor Specificity in the European Corn Borer Moth,
Ostrinia nubilalis

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Contributions: Jean E. Allen, co-author, performed quantitative real-time PCR and cloning. Kevin W. Wanner, co-author, constructed expressed sequence tag (EST) library. Andrew S. Nichols, co-author, expressed odorant receptors in *Xenopus* oocytes and performed electrophysiological recordings. Peggy L. Bunger performed rapid amplification of cDNA ends (RACE), PCR, and cloning. Stephen F. Garczynski provided degenerate primers. Charles E. Linn provided the insects used in the construction of the EST library. Hugh M. Robertson was involved with making the EST library. Charles W. Luetje provided the equipment used in electrophysiological recordings.

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CHAPTER 2

SEX PHEROMONE RECEPTOR
SPECIFICITY IN THE EUROPEAN CORN BORER MOTH, *OSTRINIA NUBILALIS*Abstract

Background: The European corn borer (ECB), *Ostrinia nubilalis* (Hubner), exists as two separate sex pheromone races. ECB(Z) females produce a 97:3 blend of Z11- and E11-tetradecenyl acetate whereas ECB(E) females produce an opposite 1:99 ratio of the Z and E isomers. Males of each race respond specifically to their conspecific female's blend. A closely related species, the Asian corn borer (ACB), *O. furnacalis*, uses a 3:2 blend of Z12- and E12-tetradecenyl acetate, and is believed to have evolved from an ECB-like ancestor. To further knowledge of the molecular mechanisms of pheromone detection and its evolution among closely related species we identified and characterized sex pheromone receptors from ECB(Z).

Methodology: Homology-dependent (degenerate PCR primers designed to conserved amino acid motifs) and homology-independent (pyrophosphate sequencing of antennal cDNA) approaches were used to identify candidate sex pheromone transcripts. Expression in male and female antennae was assayed by quantitative real-time PCR. Two-electrode voltage clamp electrophysiology was used to functionally characterize candidate receptors expressed in *Xenopus* oocytes.

Conclusion: We characterized five sex pheromone receptors, *OnORs*1 and 3-6. Their transcripts were 14-100 times more abundant in male compared to female antennae. *OnOR*6 was highly selective for Z11-tetradecenyl acetate ($EC_{50} = 0.86 \pm 0.27 \mu\text{M}$) and was at least three orders of magnitude less responsive to E11-tetradecenyl acetate. Surprisingly, *OnOR*1, 3 and 5 responded to all four pheromones tested (Z11- and E11-tetradecenyl acetate, and Z12- and E12-tetradecenyl acetate) and to Z9-tetradecenyl acetate, a behavioral antagonist. *OnOR*1 was selective for E12-tetradecenyl acetate based on an efficacy that was at least 5-fold greater compared to the other four components. This combination of specifically- and broadly-responsive pheromone receptors corresponds to published results of sensory neuron activity *in vivo*. Receptors broadly-responsive to a class of pheromone components may provide a mechanism for variation in the male moth response that enables population level shifts in pheromone blend use.

Introduction

Sex pheromone communication between male and female moths is believed to have contributed to their extensive speciation (1). More than 98% of the 150,000 described extant species of Lepidoptera belong to the Ditrysia, a monophyletic lineage that evolved during the last 110 million years (2). Female moths produce and release a mixture of related fatty acid derivatives from their pheromone gland to which males respond from long distances. In many cases, subtle changes in carbon chain length, double bond location and isomer blend differentiate the pheromones of closely related species (3). While a variety of mating systems have evolved in the Lepidoptera, female release of pheromone is a predominant ancestral trait (4). One long standing question has been the origin and mechanism of the variation in detection that enables the evolution of new pheromone blends.

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner), has provided a model system to study the evolution of sex pheromones among closely related races and species. Most of the 20 species in the genus *Ostrinia* use varying ratios of Z11- and E11-tetradecenyl acetate (Z11- and E11-14:OAc) as the two main components of their pheromone blend (5-7). An introduced pest from Europe, the ECB was first detected in North America in 1917 and exists as two different pheromone races (8). Males of the Z-race are attracted to a 97:3 blend of Z11- and E11-14:OAc whereas ECB(E) males are attracted to a 1:99 blend of the Z and E isomers (9-10). The closely related Asian corn borer (ACB), *O. furnacalis*, is unique in this genus, having evolved to use a pheromone blend with a shift in the location of the double bond, Z12- and E12-tetradecenyl acetate

(Z12- and E12-14:OAc) (11). Mating isolation between the Z- and E-races of ECB is controlled by a few major genetic loci, including *pher* and *resp*, controlling female blend production and male response, respectively (12-15). Desaturase enzymes in the female moth pheromone gland introduce double bonds at specific locations along the hydrocarbon chain. The recruitment of a novel $\Delta 14$ desaturase into the pheromone biosynthesis pathway of an ancestor of the ACB led to a novel pheromone blend (Z12- and E12-14:OAc) contributing to the divergence of this species from the ECB (16).

Male moths have evolved to detect female-produced sex pheromones with great sensitivity and specificity over a wide range of concentrations (17). A majority of the olfactory neurons on male antennae, housed within long trichoid sensilla, specifically respond to components of the female sex pheromone. The sex pheromones are detected by odorant receptors (ORs) expressed on the dendrites of the olfactory neurons (18-19). The trichoid sensilla on male ECB and ACB antennae typically house three different olfactory neurons that can be differentiated by the amplitude of their electrophysiological response spikes. For ECB(E) males, a large-spiking neuron responds to the main pheromone component, a small-spiking neuron responds to the minor component, and an intermediate-spiking neuron responds to Z9-tetradecenyl acetate (Z9-14:OAc) (20-24). The olfactory pathway responding to Z9-14:OAc antagonizes responses to the attractive pheromone pathway and prevents upwind flight to similar sex pheromone blends that include Z9-14:OAc (6).

Insect ORs are a family of chemoreceptors (Cr) that function as ligand-gated ion channels (25-27). A highly conserved OR termed 83b in *Drosophila melanogaster* and its

ortholog in other insect species acts as a chaperone and dimerization partner for other ORs that impart ligand specificity (28). Together OR83b+OR_x form a ligand-gated ion channel. Approximately 10% of the expected 60-70 OR genes encoded in moth genomes form a distinct phylogenetic subfamily that appears to be dedicated to sex pheromone detection (18-19). Seven silkworm (*Bombyx mori*) and six tobacco budworm (*Heliothis virescens*) ORs belong to this subfamily. All but two are expressed at higher levels in male antennae (29-30) and four respond to their respective sex pheromone components *in vitro* (18,31).

The behavioral response of male insects to sex pheromone can be closely linked to the activity of the peripheral olfactory neurons. Transgenic fruit flies expressing the silkworm pheromone receptor BmOR1 (18) in place of their sex and aggregation pheromone receptor DmOR67d (32) are attracted to the silkworm pheromone bombykol rather than their own pheromone vaccenyl acetate (33). Activation of the sex- and aggregation-specific olfactory pathway results in behavioral attraction independent of the actual signal. The neurological pathway of sex pheromone sensitive olfactory neurons and their projection to the antennal lobe was recently compared between ECB(Z) and ECB(E) males. In each case, the axons of the large-spiking neurons that respond to the main pheromone component, Z11-14:OAc for ECB(Z) and E11-14:OAc for ECB(E), projected to the same macroglomerulus in the male antennal lobe (34). The authors concluded that the major genetic locus that controls the altered olfactory response between the Z and E races did not result in a rewiring of the olfactory neurons, rather, the mechanisms must be located at the periphery. ORs belonging to the sex pheromone

receptor subfamily are excellent candidates because the activity of an olfactory neuron often parallels the response spectrum of the OR that it expresses (35). Here we employed a functional genomics approach to identify and characterize five sex pheromone receptors from ECB(Z) moths to further explore peripheral mechanisms contributing to the evolution of sex pheromone detection.

Results

Five candidate sex pheromone receptors identified from ECB(Z)

Two complementary approaches were used to identify the greatest possible number of candidate sex pheromone receptors in the absence of whole genome sequencing. First, degenerate PCR primers were designed to match a conserved amino acid motif in the carboxy(C)-terminus of known Lepidoptera sex pheromone receptors, (I/L/V)PW(E/D)(Y/F/C/H/A)M(D/N)(T/V/K/I/N). Using these degenerate primers, the C-terminus of five OR transcripts with amino acid homology to the Lepidoptera sex pheromone receptor subfamily were identified by 3' Rapid Amplification of cDNA Ends (RACE) reactions (GenBank accession numbers FJ385011 - FJ385015).

In a second approach, an EST library was created by high-throughput pyrophosphate sequencing of antennal cDNA. Seven partial cDNA sequences with amino acid homology to known Lepidoptera sex pheromone receptors were identified by tBLASTn searches of the assembled contigs (Text S1). The seven contigs varied from 178 to 1124 nucleotides (nt) in length, and were assembled from a minimum of 6 sequence reads to a maximum of 198 reads (Table S1). *OnOR2*, the ortholog of *DmOR83b* that acts as a chaperone and partner for most ORs, was represented by two

contigs (Table S1) of 1032 and 178 nt (62 and 6 reads, respectively). All cDNAs were partial sequences, 3' and 5' RACE was required to clone and sequence the complete open reading frames (ORFs).

As a result, the combined approaches yielded 5 unique cDNAs, *OnOr1* and 3-6 (GenBank Accession numbers GQ844876-GQ844881) that were cloned using primers designed from the RACE sequences. *OnOr1* and *OnOr 3-6* encode proteins ranging from 421 to 425 amino acids in length including motifs characteristic of the insect OR family (such as the conserved C-terminal serine and tyrosine residues,³⁶; Figure S1). All five ORs have BLASTp similarity to lepidopteran sex pheromone receptors that have been functionally characterized. *OnOR 1* and 6 are 36% and 41% identical to *Plutella xylostella* OR1 (37); *OnOR3* is 36% identical to *Diaphania indica* OR1 (37); and, *OnOR 4* and 5 are 63% and 99% identical to a sex pheromone receptor recently characterized from *O. nubilalis* (7).

ESTs representing *OnOr 4* and 5 were identified by 3' RACE with degenerate primers but were not represented by pyrosequencing contigs. Conversely, ESTs representing *OnOr6* were abundantly represented by pyrosequencing contigs but were not amplified using degenerate primers. These results illustrate the benefit of using two complementary approaches to identify candidate pheromone receptors, one dependent on sequence homology and the other independent of sequence homology, but dependent on adequate expression levels.

It was uncertain whether the pyrosequencing approach would provide sufficient sequence coverage of rare transcripts to assemble contigs that could be detected by

tBLASTn searches. The full length nucleotide sequences of *OnOrs 1-6* used as queries for BLASTn searches yielded only three new contigs (Figure S2). These contigs were not detected in our original tBLASTn searches because they contained intron or 3'UTR sequence and less than 120 nt of coding sequence.

OnOrs 1 and 3-6 are expressed at higher levels in male antennae

Expression levels of *OnOrs 1* and 3-6, averaged from four biological replications, were determined by quantitative real-time PCR (qPCR). The transcripts of all five candidate pheromone receptors were expressed at higher levels in male antennae, ranging from 14 to 100 times higher compared to female antennae (Figure 1). *OnOr2* was highly expressed at levels comparable to the reference gene *ribosomal protein S3 (OnRPS3)* and only 1.6 times higher in the male antennae. *OnOr 1* and 6 transcripts were detected at similarly high levels, whereas the transcripts of *OnOrs3-5* were approximately an order of magnitude less abundant (Figure 1). In general, *OnOrs1-6* were not expressed at significant levels in other tissues such as legs, abdomen and mouthparts (Figure S2). *OnOr1* expression in female but not male mouthparts, and *OnOr3* expression in male but not female abdomens, may be two interesting exceptions (Figure S2). Low level signal, more than two orders of magnitude below that of the reference gene *OnRPS3*, can result from non-specific PCR amplification that is detected by the SYBR green dye or by genomic DNA contaminating the RNA template. In addition to removing DNA from the RNA template by enzyme digestion, false expression signal from contaminating DNA was assessed by including RNA that was not reverse transcribed. These negative controls did not produce signals of expression confirming the purity of the RNA template. In

addition, several primer sets spanned an intron, and the absence of larger-sized amplicon that would result from genomic DNA template was confirmed by gel electrophoresis of the PCR product and by its melting point curve.

Specific and broad responses of different ECB sex pheromone receptors

Each of the five candidate ECB(Z) receptors was co-expressed in *Xenopus* oocytes with the obligatory functional partner *OnOR2*, and screened for responsiveness to a panel of ECB and ACB pheromone components (Z12-14:OAc, E12-14:OAc, Z11-14:OAc, and E11-14:OAc), and the antagonist Z9-14:OAc, at a 10 M concentration (Figure 2). *OnOR4/2* failed to be activated by any of the components tested, with the exception of a very slight response to the antagonist Z9-14:OAc (Figure 2C). Increasing the concentration of Z9-14:OAc to 300 M did not increase the response amplitude (unpublished results), suggesting that *OnOR4/2* may not be robustly expressed in our assay system, or the receptor responds to a ligand not tested here. *OnOR6/2* was specifically activated only by Z11-14:OAc (Figure 2E). Surprisingly, *OnOR1/2*, *OnOR3/2*, and *OnOR5/2* responded to all five components (Figure 2A, B and D). *OnOR3/2* and *OnOR5/2* exhibited only slight isomer selectivity, both favoring the E isomers over the Z isomers. *OnOR1/2* did not share this trend; it was more selective for E12-14:OAc over Z12-14:OAc, but surprisingly, was selective for Z11-14:OAc over E11-14:OAc.

OnOr6 is a highly specific receptor tuned to Z11-14:OAc

We next investigated the specificity of *OnOR6/2* through a range of pheromone concentrations. Dose-response analysis revealed *OnOR6/2* to be a sensitive receptor for

Z11-14:OAc, with an apparent EC_{50} of $0.86 \pm 0.27 \mu\text{M}$ (mean $\pm$ SEM, $n=4$) (Figure 3).

Although E11-14:OAc began to elicit a receptor response at higher concentrations, approximately half of this response can be attributed to the small amount of Z11-14:OAc present in our sample of E11-14:OAc (0.1%, personal communication, Pherobank, Wageningen, The Netherlands). If the remaining response is truly due to E11-14:OAc, then *OnOR6/2* is approximately 1000-fold selective for Z11-14:OAc over E11-14:OAc. These results demonstrate that *OnOR6/2* is highly specific for Z11-14:OAc, exhibiting a strong degree of isomer selectivity.

Based on relative efficacy, OnOr1 responds best to E12-14:OAc

Although *OnOR1/2* responded to all five components, this receptor exhibited unique preferences as compared to *OnOR3/2* and *OnOR5/2*. Therefore, dose-response analysis was performed for all five pheromones (Figure 4). While *OnOR1/2* was broadly activated by the various pheromones with similar potencies, we observed a wide range of relative efficacies that may provide a mechanism for *OnOR1/2* to differentiate among pheromone isomers (Table 1). Based on this analysis, we conclude that E12-14:OAc is the strongest activator of *OnOR1/2*.

Phylogenetic relationship of OnOrs1-6 within the pheromone receptor subfamily

The 28 published OR sequences from 8 different species that belong to the lepidopteran pheromone receptor subfamily group together generally at the superfamily level of taxonomy (Figure 5). *OnORs1, 3 and 6* are most related to each other and group together with two ORs from the diamondback moth, *Plutella xylostella*. *OnORs 4 and 5* group together on a separate lineage along with an OR from the light brown apple moth

Epiphyas postvittana and an OR from the cucumber moth *Diaphania indica*. With the current representation of published sequences there is no clear relationship between pheromone receptor phylogeny and their ligand response. For example, *HvCr14* and *PxOR1* both respond best to Z11-16:OAc and *HvCr13* and *MSepOR1* both respond best to Z11-16:Al (Figure 5)(31,37). The receptors do not appear to be orthologous in either case.

Discussion

Chemical communication in mating behavior is a prominent feature of moth biology that has contributed to their extensive divergence. To understand better how the molecular mechanisms of sex pheromone detection evolve we identified and characterized five sex pheromone receptors from the ECB(Z), a model example of an early stage of speciation (38). *OnOR6* was particularly interesting as it responded with high specificity and isomer selectivity to Z11-14:OAc, the main component of the ECB(Z) pheromone blend. Based on EC_{50} values, *OnOR6* is at least 1000 times more responsive to Z11-14:OAc compared to E11-14:OAc (Figure 3). Importantly, these *in vitro* results correspond to *in vivo* electrophysiological recordings that found a large-spiking neuron in ECB(Z) males, and a small-spiking neuron in ECB(E) males that responded specifically to Z11-14:OAc (22-23). Consequently, *OnOR6*(Z) should be expressed in the large-spiking neurons of ECB(Z). Its ortholog in ECB(E) males is likely expressed in the small-spiking neurons, but further research will be required to test this hypothesis.

We did not find a similar receptor that responded specifically to E11-14:OAc. An additional sex pheromone receptor that responds specifically to E11-14:OAc that was not identified by our approach might also exist. Traditionally it has been thought that male moth antennae possess olfactory neurons specifically tuned to each of the components of the female sex pheromone blend (39-41). Rather, we found that the remaining pheromone receptors responded generally and more broadly to the five compounds tested. *On*OR1 responded to all five compounds tested with EC50s ranging from 0.26 μ M to 2.73 μ M (Table 1). *Bm*OR1 and 3, the silkworm bombykol and bombykal receptors, responded with similar sensitivities to their pheromone ligands when co-expressed with *Bm*OR2 in *Xenopus* oocytes (EC50s 0.26 and 1.5 μ M, respectively)(18). These results are similar to recent electrophysiological data finding that the large-spiking neurons of ECB(E) males actually respond more broadly *in vivo* (22-23). This neuron responded best to E11-14:OAc but it also responded to the Z11-, E12- and Z12- 14:OAc components. However, co-expression of two or more pheromone receptors in the same olfactory neuron could also explain the more broad *in vivo* responses (23).

The existence of more broadly-responsive sex-pheromone receptors *in vitro*, and pheromone-sensitive olfactory neurons *in vivo*, suggests that not all components of a pheromone blend need to be detected with high specificity. Male moths respond to the ratios of the major and minor components in a pheromone blend (22). If behavioral attraction requires activity of both neuron types at specific ratios, behavioral specificity can be retained with one highly-specific neuron and one more generally-responding neuron. A combination of specific- and generally-responsive pheromone receptors may

provide the genetic variability for males to detect and track shifts in female pheromone blend production (42).

‘Rare’ ECB and ACB males, typically representing 3-5% of the population, are less specific in their behavioral response to related pheromone blends (6). Changes in the periphery that alter the strength or specificity of the olfactory neuron’s response to specific pheromone components could account for the rare responses (22-24). For example, a decrease in responsiveness of the small-spiking neuron of rare ECB(E) males to Z12-14:OAc may alter the firing ratio relative to the large-spiking neuron in a way that allows the ACB blend to mimic the ECB(E) blend (22). The antagonism-related olfactory neuron of normal ACB males responds to Z11-14:OAc in addition to Z9-14:OAc, preventing flight of ACB males to the ECB pheromone blend. However, this response to Z11-14:OAc in the antagonism pathway is lacking in rare ACB males that fly to the ECB pheromone (24). Amino acid polymorphisms between alleles of a more broadly tuned OR could account for subtle changes in olfactory neuron response. Such variation could also provide the genetic material for the evolution of altered detection and response to new pheromone blends (22). *OnOR1* in this study exhibited a more efficacious response to the E12-14:OAc ligand. Its ortholog in the ACB might be a candidate receptor for one of its main pheromone components, E12-14:OAc.

Alternatively, the broad *in vitro* responses measured in this study may not completely reflect their *in vivo* specificity. While the ORs are clearly one of the major determinants of olfactory neuron specificity, complexes of interacting proteins are involved in the signal transduction, including sensory neuron membrane protein 1

(SNMP1) and pheromone binding proteins (PBPs) (43). For example, PBPs can increase physiological sensitivity to pheromone ligands. *BmOR1* expressed in the empty neurons of *Drosophila* ab3 sensilla is activated by the silkworm sex pheromone bombykol. However, when co-expressed with *BmPBP1*, much lower concentrations of bombykol activate the *BmOR1*-expressing neuron (44). PBPs may also affect the specificity of the physiological response to sex pheromone. A PBP added to an *in vitro* assay altered the specificity of a moth pheromone receptor, making its response more specific (31). Similarly, the responses of *OnOR1* and 3 characterized in this study may be more specific *in vivo* in the presence of PBPs. Also, the responses of *OnOR1* and 3-6 to a larger panel of pheromone and general odors should be tested in future work. *OnOR5* in this study corresponds to an ECB OR that was recently reported to respond to E11-tetradecen-1-ol, a pheromone component used by ancestral species in the genus *Ostrinia* (7).

The male moth olfactory system that responds to the female-produced sex pheromone is believed to be subject to stabilizing selection. Duplication of desaturase enzyme genes and their differential activation in the pheromone glands of female ECB and ACB moths provides a mechanism for sudden changes in the pheromone blend (45-46). The origins of variation in male detection and response that enable the evolution of new sex pheromone blends has been a long-standing question (16). To address this, the asymmetric tracking hypothesis proposed that male responses were broad enough to track changes in female production (42). Physiological studies of the pheromone-sensitive ORNs of rare ECB males that respond to ACB pheromone provided support for this

hypothesis (22). The existence of both specifically- and broadly-responsive sex pheromone receptors may represent a molecular mechanism; however, further *in vitro* and *in vivo* experiments will be required to test this hypothesis.

Ethics Statement

The care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health.

Materials and Methods

Insects and RNA extraction

ECB(Z) pupae were purchased from Benzon Research (Carlisle, Pennsylvania) and provided from a colony maintained at the New York State Agricultural Experiment Station. Antennae were dissected from male and female adults within 3 days of emergence. Mouthparts, legs, and abdomens were dissected separately. All tissues were stored at -80° C. For gene expression studies antennae were collected from four batches each consisting of 35-50 male and 35-50 female moths. RNA was extracted from frozen tissue using a Dounce homogenizer and an RNeasy Mini kit (Qiagen, Valencia, CA). RNA was quantified and assayed for purity by absorbance at 260nm, 280nm, and 230nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

Pyrosequencing and OR EST identification

cDNA was prepared by the University of Illinois Urbana-Champaign W.M. Keck Center for Comparative and Functional Genomics from 200 µg of pooled antennal total

RNA (100 µg from male and female antennae). The cDNA was pyrosequenced using a Roche 454 GS-FLX system and the sequence reads assembled into contigs. FASTA files of the non-redundant contigs were formatted as BLAST databases and searched using a PC version of standalone BLAST. Silkworm OR sequences were used as queries in tBLASTn searches to identify EST contigs with homology to known lepidopteran sex pheromone receptors. Detailed methods and results of the EST library will be presented elsewhere.

OR cloning

3' and 5' RACE-ready cDNA was generated from male ECB antennal total RNA using the SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA). Forward and reverse gene-specific primers designed from ESTs with homology to lepidopteran sex-pheromone receptors were combined with the SMART RACE primers (Invitrogen, Carlsbad, CA) to amplify PCR products. PCR reactions used the Advantage 2 Polymerase Mix (Clontech) under the following conditions: 94°C for 3 minutes, 24 cycles of 94° for 20 seconds, 68° for 6 minutes, followed by 1 cycle of 72° for 5 minutes. In some cases a second internal gene-specific reverse primer was used for nested 5'RACE. 3' and 5' RACE products were gel purified (Qiagen MinElute Gel Extraction Kit), cloned into the TOPO pCR2.1 vector (Invitrogen TOPO TA cloning kit) and sequenced in both directions. The resulting sequences were used to design forward and reverse primers (with restriction enzyme sites for pGEMHE) to amplify the complete ORFs of five unique ORs (*OnOrs1* & 3-6) and the *DmOr83b* ortholog. Each TOPO clone was sequenced in both directions and the inserts subcloned into the pGEMHE vector

which was subsequently sequenced in both directions. The relationships of translated OR sequences were analyzed by constructing a neighbor-joining phylogenetic tree using PAUP software (47). Corrected distances were used to construct the tree and uncorrected distances to perform bootstrap analysis (n = 1000 replicates) as described in (48).

Gene expression

Genomic DNA was digested from Total RNA used for gene-expression with the TURBO DNA-free kit (Applied Biosystems, Foster City, CA). cDNA was synthesized from 300-600 ng of Total RNA using SuperScript III Reverse Transcriptase (Invitrogen) and 50µM Oligo(dT)₁₂₋₁₈ primer and incubated at 52°C for one hour followed by inactivation at 70°C for 15 minutes. qPCR primers were designed using Primer3 software (49) with the following criteria: primers 15-30 base pairs in length, annealing temperature 58-60°C and a 75-100 nt amplicon. *OnRPS3-F*, TGGTAGTGTCTGGCAAGCTC, *OnRPS3-R*, CGTAGTCATTGCATGGGTCT; *OnOr1-F*, CGGCGTCAGCACCATGA, *OnOr1-R*, TCTCCCATTGTTTGCAGAATG; *OnOr2-F*, GCTCTGAAGAAGCCAAGACC, *OnOr2-R*, CAAGTCCAGTGAAACCGTGA; *OnOr3-F*, GGCGCACCGCTCATATC, *OnOr3-R*, CCCAACGCTTTGATGGTGAT; *OnOr4-F*, CTGGTGACCCTGGAGATGAT, *OnOr4-R*, CAAATGCCTCGGATGTTTTAG; *OnOr5-F*, TCACGGTCGGCGTCACTA, *OnOr5-R*, TTCGCAAGAACATGAAGTAAGAAAA, *OnOr6-F*, AGAGACGGAAAAGCTGAAGG, and *OnOr6-R*, TATCCCCAACATGGTGTTCA. Each primer set was validated by calculating standard curves with 10x serial dilutions of template (three replicated wells for each template dose). The threshold cycle (CT) was plotted against the log of the template dilution and primers with slopes ranging from 3.1 to 3.5 were used (a slope of 3.3 represents 100% efficiency).

qPCR experiments were performed using 96 well plates (Bio-Rad, Hercules, CA), the IQ5 Real Time PCR Detection System (Bio-Rad) and IQ SYBR Green Supermix (Bio-Rad). Each 15µl reaction was replicated in triplicate. Cycling conditions were as follows: 95°C for 1 minute, 40 cycles of 95°C for 10 seconds and 58°C for 1 minute, followed by melting temperature analysis: 95°C for 1 minute, 58°C for 1 minute and 67 cycles of 55-88°C for 10 seconds. Baseline cycle and threshold values were calculated automatically using default settings. No-template and no-reverse transcriptase controls were included in each experiment. As a final validation, qPCR products were cloned into TOPO pCR-4 and sequenced to ensure that the expected product was amplified. Expression levels of *OnOrs* 1-6 were calculated relative to the control gene, *OnRpS3*, using the $2^{-\Delta CT}$ method (50).

Preparation of oocytes

Xenopus laevis frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. Frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were surgically removed. Oocytes were separated from follicle cells by treatment with collagenase B (Roche, Indianapolis, IN) for 2 h at room temperature.

cRNA injections

Capped cRNA encoding each candidate pheromone receptor was synthesized from linearized template DNA cloned in pGEMHE using mMessage mMachine kits (Ambion, Austin, TX). cRNAs were then injected into Stage V-VI *Xenopus* oocytes at a

concentration of 25 ng/cRNA species/oocyte. Oocytes were incubated at 18°C in Barth's saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 CaNO₃, 0.41 CaCl₂, 0.82 MgSO₄, 15 HEPES, pH 7.6, and 100 µg/ml amikacin) for 2-5 days prior to electrophysiological recording.

Electrophysiology and data analysis

Pheromone-induced currents were measured under two-electrode voltage clamp using an automated parallel electrophysiology system (OpusExpress 6000A; Molecular Devices, Union City, CA). Micropipettes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. The holding potential was -70 mV. Pheromones were perfused with ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.5). Pheromone stock solutions (1 M) were prepared in DMSO and stored at -20°C. On the day of each experiment, fresh dilutions were prepared in ND96. Unless otherwise noted, pheromones were diluted in ND96 and applied for 20 sec at a flow rate of 1.65 ml/min with extensive washing in ND96 (10 min at 4.6 ml/min) between applications. Pheromone compounds typically greater than 99% purity were purchased from Pherobank, Plant Research International B.V., Wageningen, The Netherlands. Current responses, filtered (4-pole, Bessel, low pass) at 20 Hz (-3 db) and sampled at 100 Hz, were captured and stored using OpusXpress 1.1 software (Molecular Devices). Initial analysis was done using Clampfit 9.1 software (Molecular Devices). Dose-response analysis was done using PRISM 4 software (GraphPad, San Diego, CA). Dose-response curves were fit according to the equation: $I = I_{max} / (1 + (EC_{50}/X)^n)$, where I represents the current response at a given pheromone concentration, X ; I_{max} is the maximal response; EC_{50} is the concentration of

pheromone yielding a half-maximal response; and n is the apparent Hill coefficient.

Relative efficacies of pheromones were normalized to the maximal response elicited by Z11-14:OAc.

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Table 1. Summary data of the activation of *On*OR1/2 and *On*OR6/2 by ECB and ACB pheromones and the antagonist Z9-14:OAc.

	EC ₅₀		Relative Efficacy
<i>On</i> Or1/2	($\mu\text{M} \pm \text{SEM}$)	Hill slope	(% response to Z11-14:OAc)
Z9-14:OAc	1.54 ± 0.33	1.85	36.1 ± 2.8
Z12-14:OAc	2.73 ± 0.24	1.68	87.4 ± 2.3
E12-14:OAc	0.26 ± 0.05	1.30	515 ± 30.9
Z11-14:OAc	0.34 ± 0.05	1.10	100.7 ± 3.9
E11-14:OAc	1.92 ± 0.20	1.31	55.0 ± 2.0
<i>On</i> Or6/2			
Z11-14:OAc	0.86 ± 0.27	0.91	100

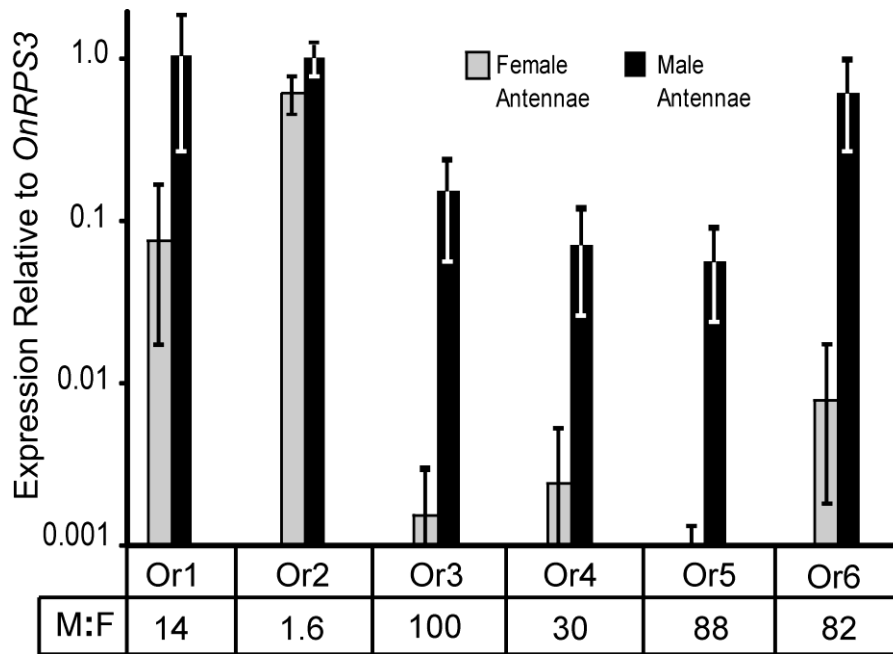


Figure 1. Male-biased expression of five ECB(Z) sex pheromone receptor genes. Ratios of male to female expression (M:F) are presented below each bar. Gene expression, determined by real-time quantitative PCR with SYBR green, is reported relative to the reference gene *OnRPS3* on a logarithmic scale. Expression values are presented as averages (with standard error bars) of four biological replicates and three nested technical replicates. Sex-biased expression is supported by nested ANOVA analyses of the normalized CT values, $P=0.03$, 0.04 , 0.001 , 0.06 , 0.001 and 0.003 , *OnORs1-6* respectively.

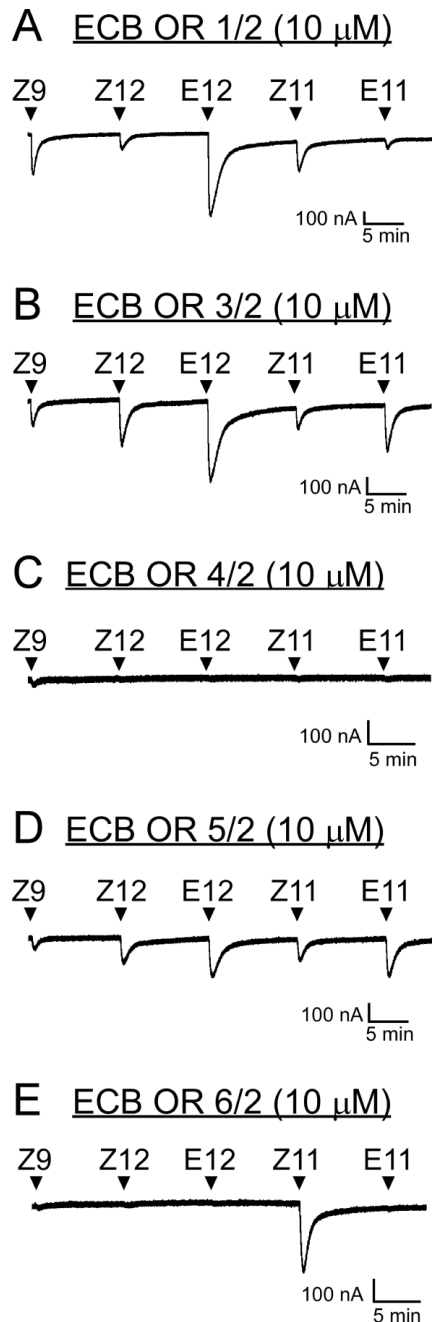


Figure 2. Functional screen of candidate ECB(Z) pheromone receptors. Oocytes expressing *OnOR2* and either *OnOR1* (A), *OnOR3* (B), *OnOR4* (C), *OnOR5*, (D) or *OnOR6* (E) were challenged with 20 sec applications (arrowheads) of various ECB and ACB pheromones (at 10 μ M): Z9-14:OAc (Z9), Z12-14:OAc (Z12), E12-14:OAc (E12), Z11-14:OAc (Z11), and E11-14:OAc (E11). Each application was separated by 10 min washing in ND96 (4.6 ml/min). Pheromone-induced currents were measured by two-electrode voltage clamp electrophysiology.

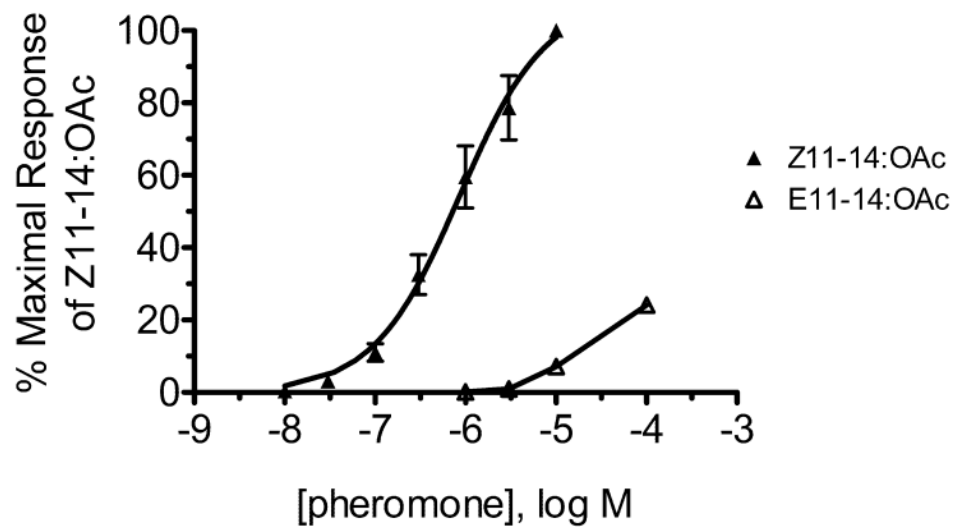


Figure 3. Dose-response relationships for Z11-14:OAc and E11-14:OAc activation of *OnOR6/2*. Pheromone-induced currents were measured by two-electrode voltage clamp electrophysiology. Refer to Table 1 for EC_{50} , Hill slope, and relative efficacy values. Data is presented as means $\pm$ SEM (Z11-14:OAc, $n=4$; E11-14:OAc, $n=5$).

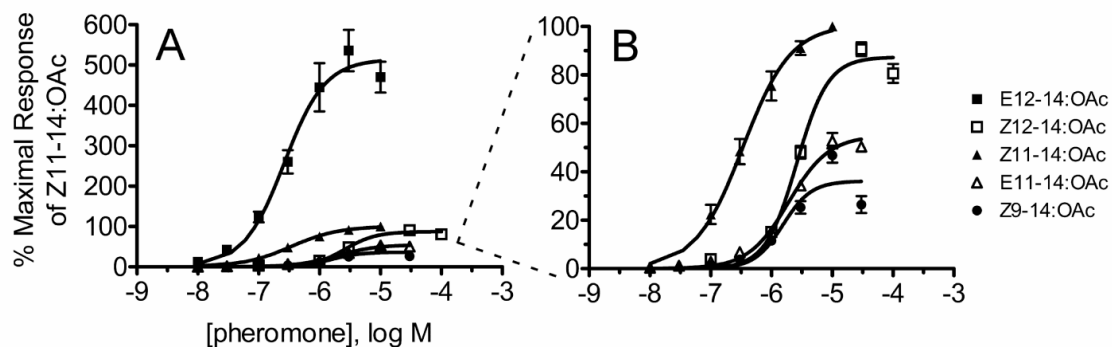


Figure 4. Dose-response relationships for E12-14:OAc, Z12-14:OAc, Z11-14:OAc, E11-14:OAc and Z9-14:OAc activation of *OnOR1/2*. Left and right graphs have different y-axis scales of the same data points. Pheromone-induced currents were measured by two-electrode voltage clamp electrophysiology. Refer to Table 1 for EC₅₀, Hill slope, and relative efficacy values. Data is presented as means $\pm$ SEM (E12-14:OAc, n=5; Z12-14:OAc, n=6; Z11-14:OAc, n=7; E11-14:OAc, n=5; and Z9-14:OAc, n=5).

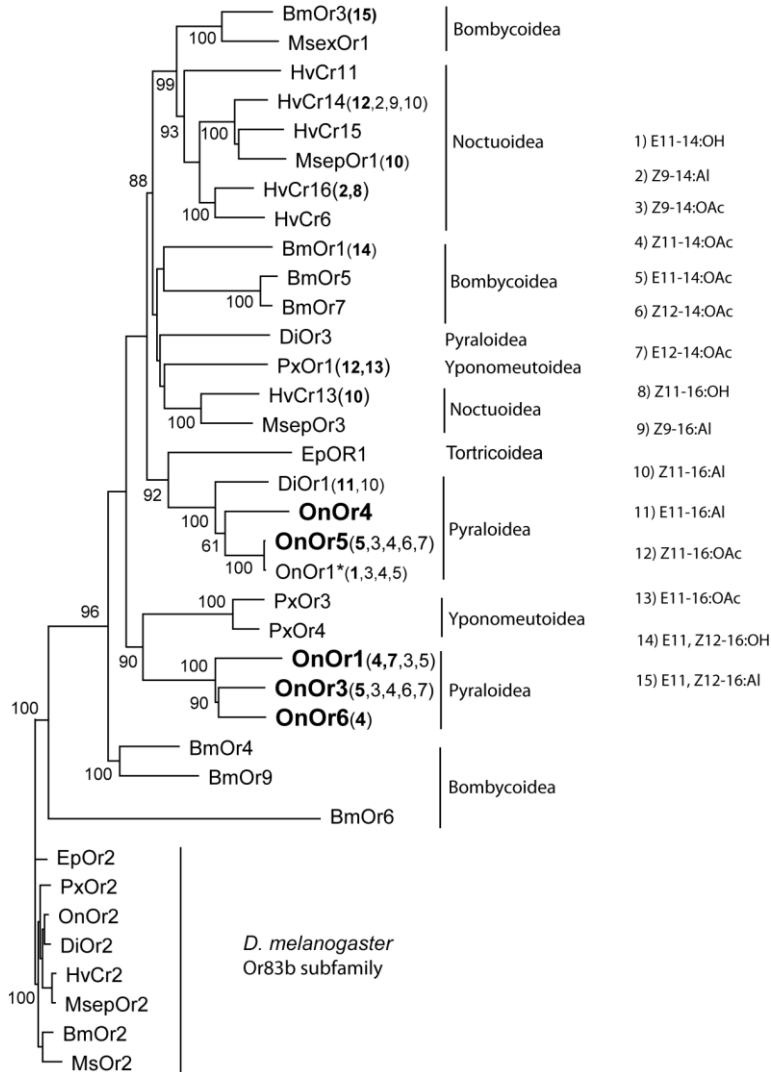


Figure 5. Phylogenetic relatedness of *OnORs*1-6 to the Lepidoptera sex pheromone receptor subfamily, neighbor-joining (corrected distance) tree. Bootstrap values are presented as a percentage of $n = 1000$ replicates at significant branch points. The tree is rooted with lepidopteran orthologs of *DmOR83b*. The responses of receptors that have been functionally characterized are indicated by numbers corresponding to the 15 pheromone compounds listed, bolded numbers indicate the strongest response. Bm, *Bombyx mori*; Di, *Diaphania indica*; Ep, *Epiphyas postvittana*; Hv, *Heliothis virescens*; Msex, *Manduca sexta*; On, *Ostrinia nubilalis*; Px, *Plutella xylostella*; Msep, *Mythimna separata*. Superfamily taxonomies are delineated by vertical bars. ECB receptors reported in this study are bolded; *OnOR1** was reported in (7) and is identical to *OnOR5* in this study. Pheromone ligands: 1) E11-14:OH; E11-tetradecen-1-ol, 2) Z9-14:Al; Z9-tetradecenal, 3) Z9-14:OAc; Z9-tetradecenyl acetate, 4) Z11-14:OAc; Z11-tetradecenyl acetate, 5) E11-14:OAc; E11-tetradecenyl acetate, 6) Z12-14:OAc; Z12-tetradecenyl acetate, 7) E12-14:OAc; E12-tetradecenyl acetate, 8) Z11-16:OH; Z11-hexadecen-1-ol, 9) Z9-16:Al; Z9-hexadecenal, 10) Z11-16:Al; Z11-hexadecenal, 11) E11-16:Al; E11-hexadecenal, 12) Z11-16:OAc; Z11-hexadecenyl acetate, 13) E11-16:OAc; E11-hexadecenyl acetate, 14) E10, Z12-16:OH; E10,Z12-hexadecadien-1-ol, and 15) E10, Z12-16:Al; E10,Z12-hexadecadienal.

CONTRIBUTIONS OF AUTHORS AND CO-AUTHORS

Chapter 3: Asian Corn Borer Pheromone Binding Protein 3, a Candidate for Evolving Specificity to the 12-Tetradecenyl Acetate Sex Pheromone

Co-authors: Jean E. Allen and Kevin W. Wanner.

Contributions: Jean E. Allen, co-author, performed rapid amplification of cDNA ends (RACE), PCR, cloning, quantitative real-time PCR, statistics, phylogenetic analysis, sequence analysis, and structural modeling. Kevin W. Wanner, co-author, constructed expressed sequence tag (EST) library.

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CHAPTER 3

ASIAN CORN BORER
PHEROMONE BINDING PROTEIN 3, A CANDIDATE FOR EVOLVING
SPECIFICITY TO THE 12-TETRADECENYL ACETATE SEX PHEROMONE

Abstract

Most moth species in the genus *Ostrinia* use varying ratios of Z11- and E11-tetradecenyl acetate as their main sex pheromone components. The Asian corn borer is unique within the genus having evolved to use pheromone components with a shift in the location of the double bond, Z12- and E12-tetradecenyl acetate. We identified cDNAs representing five pheromone binding proteins (PBPs) and two sensory neuron membrane protein genes from an antennal transcriptome. The coding regions of the orthologous genes were cloned from the Asian corn borer and the E and Z sex pheromone races of the European corn borer. Their nucleotide sequences and transcript expression levels were analyzed to identify candidate genes from the Asian corn borer that may have evolved specificity to the 12-tetradecenyl acetate ligand. PBP2 and PBP3 transcripts were expressed at high male-biased levels. PBP3 had the most nonsynonymous nucleotide substitutions resulting in ten amino acid changes. Based on the predicted three-dimensional structure of PBP3, six of these ten amino acid changes occur in domains that may interact with the pheromone ligand. Future studies will determine whether PBP3 has evolved specificity to the Asian corn borer sex pheromone.

Key words: Pheromone binding protein; sensory neuron membrane protein; selection; *Ostrinia furnacalis*; *Ostrinia nubilalis*

1. Introduction

The sex pheromone detection system of male moths (Order Lepidoptera) is sensitive enough to detect a few molecules, and specific enough to differentiate between similar compounds (Hansson, 1995). Female moths emit species-specific pheromones that cause conspecific males to fly upwind to locate potential mates (Baker, 2008; Hansson et al., 1987). Sex pheromones in the Lepidoptera are believed to contribute to mating isolation and speciation (Carde & Haynes, 2004). Male attraction to specific female-produced pheromone blends is thought to subject the genes responsible for pheromone detection to strong selective pressures. A better understanding of the genes involved in male response to female-produced sex pheromone blends among closely related species may help determine the molecular mechanisms contributing to reproductive isolation in the Lepidoptera.

Female-produced pheromones are detected at the periphery of the male moth olfactory system by odorant receptor neurons (ORNs) housed within trichoid sensilla on the antennae. Pheromones are detected by a combination of proteins encoded by several different gene families which are expressed in and around the ORN. These include (but are not limited to): odorant receptors (ORs), odorant binding proteins (OBPs, including pheromone binding proteins, PBPs), and sensory neuron membrane proteins (SNMPs) (reviewed in Hallem & Carlson, 2004; Rutzler & Zwiebel, 2005). OR genes encode transmembrane proteins that are expressed on the surface of the ORN dendrite. Typically, a single ligand-responsive OR is expressed on each neuron along with a highly conserved partner OR, termed OR2 in the Lepidoptera and OR83b in *Drosophila*. OR2 acts as a

chaperone and dimer partner for most other ORs (Benton et al., 2007). Together the OR2+OR_x complex forms a ligand-gated ion channel (Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008). Therefore, ORs detect and discriminate volatile odors and initiate the signal transduction cascade that leads to olfactory nerve impulses ultimately processed and interpreted by the brain (Pelosi et al., 2006).

Lepidopteran SNMPs form a unique clade of genes homologous to the human fatty-acid transporter CD36 (Nichols & Vogt, 2008; Rogers et al., 1997; Rogers et al., 2001b). Two differentially expressed SNMP lineages have been identified in Lepidoptera suggesting each subtype has a distinct function. SNMP1 associates with pheromone sensitive ORNs in the antennae suggesting it has a role in pheromone detection (Rogers et al., 1997, 2001a). SNMP2 also associates with pheromone sensitive sensilla, but is expressed within sensillar support cells rather than within neurons (Forstner et al., 2008; Rogers et al., 2001b). SNMPs are 519-525 amino acids in length and contain two transmembrane domains near the N- and C-termini. In *Drosophila*, SNMP is required for pheromone sensitivity in trichoid sensilla (Jin et al., 2008).

PBPs are a subgroup of OBP specific to the Lepidoptera that are not found in other insect orders (Vogt, 2003). Synthesized by accessory cells, PBPs accumulate to high concentrations in the lymph of pheromone-sensitive sensilla. They are thought to act as transporters, making hydrophobic pheromones soluble and possibly participating in their protection and/or inactivation (Du & Prestwich, 1995). PBPs are small (15-17kDa), soluble, hydrophilic proteins 120-150 amino acids in length. The signal peptide consisting of about 20 amino acids is cleaved off of the mature protein. Six conserved

cysteines and several conserved amino acid motifs are characteristic of the PBPs (Xu et al., 2009). Whether PBPs contribute to pheromone ligand discrimination is a long-standing question.

Moths in the genus *Ostrinia* are used as models to study the evolution of sex pheromones among closely related species. Most *Ostrinia* species use varying ratios of E11- and/or Z11-tetradecenyl acetate (E11- and Z11-14:OAc) as sex pheromone components. Therefore, a recognition system for these two components may have existed prior to the diversification of this genus (Ishikawa et al., 1999; Linn et al., 2007; Miura et al., 2009). The European corn borer (*Ostrinia nubilalis*, ECB) exists as two different pheromone races (Brindley et al., 1975). Males of the Z-race (ECB-Z) are attracted to a 97:3 blend of Z11- and E11-14:OAc while E-race males (ECB-E) are attracted to an opposite 1:99 blend of the Z and E isomers (Carde et al., 1978; Roelofs et al., 1985). The adzuki bean borer, (*O.scapulalis*) displays a similar polymorphism in pheromone blend usage (Huang et al., 2001). The Asian corn borer (*O.furnacalis*, ACB) is unique within the genus *Ostrinia*, having evolved to use a pheromone blend with a shift in the location of the double bond, E12- and Z12-tetradecenyl acetate (Klun et al., 1980).

Ten years ago Willett and Harrison (1999a) used degenerate PCR primers and genomic DNA to identify a PBP from both ECB races and the ACB, all with identical amino acid sequences. Using high-throughput massively parallel sequencing of ECB-Z antennal complementary DNA (cDNA) we reexamine the potential role of PBPs and SNMPs in sex pheromone discrimination between closely related species in the genus *Ostrinia*. We report a total of five PBPs and two SNMPs expressed in ACB, ECB-E, and

ECB-Z antennae. PBP2 and PBP3 are differentially expressed at high levels in male corn borer antennae. We also show that PBP3 sequences exhibit 10 amino acid differences between ACB and ECB, raising the possibility that PBP3 has evolved specificity for one or both of the ACB pheromone components.

2. Materials and Methods

2.1 Antennal transcriptome sequencing

cDNA was prepared by the University of Illinois Urbana-Champaign W.M. Keck Center for Comparative and Functional Genomics from 200ug of pooled antennal total RNA (100ug from male and female antennae). ECB-Z pupae were purchased from Benzon Research (Carlisle, PA). The cDNA was pyrosequenced using a Roche 454 GS-FLX system and the sequence reads assembled into contigs. FASTA files of the non-redundant contigs were formatted as BLAST databases and searched using a PC version of standalone BLAST. Lepidopteran PBP and SNMP sequences were used as queries in tBLASTn searches to identify expressed sequence tag (EST) contigs with homology to known lepidopteran sex pheromone receptors (Fig. S1). Detailed methods and results of the EST library will be presented elsewhere.

2.2 RNA extraction

Univoltine Z-race (ECB-Z) and Bivoltine E-race (ECB-E) European corn borer pupae were provided by the New York State Agricultural Experiment Station (NYSAES)(Geneva, NY) during September and October 2009. The European corn borers were from the same colonies used by Willett & Harrison (1999a, 199b). Adults were dissected within three days of emergence. Antennae, mouthparts, legs, and abdomens of male and

female moths were dissected separately and stored at -80°C. For gene expression studies antennae were collected from three batches each consisting of: 87, 102, and 81 female ECB-E moths, 92, 94, and 86 male ECB-E moths, 95, 59, and 111 ECB-Z female moths, and 96, 67, and 120 male ECB-Z moths. Mouthparts, legs, and abdomens were dissected from a single batch of ECB-Z race moths. Asian corn borer antennae preserved in RNAlater (Ambion, Austin, TX) were provided by NYSAES in three batches each consisting of antennae from 50-75 male and 50-75 female moths. RNA was extracted from frozen or preserved tissue using a Dounce homogenizer and an RNeasy Mini kit (Qiagen, Valencia, CA). DNA was digested from total RNA preparations using on-column DNase (Qiagen). RNA was quantified and assayed for purity by absorbance at 260nm, 280nm, and 230nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

2.3 Rapid Amplification of cDNA Ends (RACE) and open reading frame (ORF) cloning

RACE was performed to obtain the 5' ends and UTR regions of SNMP1 and PBP2, and the 3' ends and UTR regions of PBP2, 3, and 5. Sequences of PBP1 and SNMP2 were complete. RACE was performed using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA). RACE ready cDNA was used in 3' and 5' RACE reactions with gene specific primers (Table S1) designed from the contig sequences (Fig. S1). Cycling conditions were as described for program one in the SMART RACE manual. RACE products were gel purified (Qiagen MinEluteGel Extraction kit), cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Full length PBP and SNMP ORFs were amplified from male ACB, ECB-E, and ECB-Z antennal cDNA using primers (Table S1) designed to the 3' and 5' untranslated regions, with two exceptions. The 5' end primers for amplifying PBP 5 and the 3' end primers for SMNP2 were designed within the reading frame. Amplification reactions were performed using Phusion High-Fidelity DNA polymerase (Finnzymes Inc., Woburn, Massachusetts). Cycling conditions were: 98°C for 30 sec, 28 cycles of 98°C for 10 sec, 50°C for 30 sec, 72°C for 60-90 sec, followed by a final extension at 72°C for 5 min. PCR products were gel purified, cloned into the pCR2.1 vector (Invitrogen), and sequenced. Plasmid inserts were sequenced by Molecular Cloning Laboratories (South San Francisco, CA) using 10µm M13 forward (GTAAAACGACGGCCAG) and M13 reverse (CAGGAAACAGCTATGA) primers. At least three clones were sequenced for each cDNA.

2.4 Quantitative real-time PCR (qPCR)

150-300ng of total RNA was used in cDNA synthesis reactions with SuperScript III Reverse Transcriptase (Invitrogen) and 50µM Oligo(dT)₁₂₋₁₈ primer at 50°C for one h, and inactivated at 70°C for 15 min. Quantitative PCR primers (Table S1) unique to each set of three orthologous corn borer genes were selected using Primer3 software (Rozen & Skaletsky, 2000). Each primer set was validated by calculating standard curves with 10x serial dilutions of template (three replicated wells for each template dose). Threshold cycle (CT) was plotted against the log of the template dilution and only primers with efficiencies of 90-105% were used. qPCR experiments were performed using 96 well plates and IQ SYBR green Supermix on an IQ5 Real Time PCR Detection System (Bio-

Rad, Hercules, CA). Each 15µl reaction was replicated in triplicate. Cycling conditions were as follows: 95°C for 1 min, 40 cycles of 95°C for 10 sec, 58°C for 1 min, followed by melting temperature analysis at: 95°C for 1 min, 58°C for 1 min, 67 cycles of 55-88°C for 10 sec. No-template and no-reverse transcriptase controls were included in each experiment. Expression levels of PBP and SNMP genes were calculated relative to the control gene RpS3 using the $2^{-\Delta CT}$ method described by Livak and Schmittgen (2001). qPCR results were analyzed by fully nested ANOVA using SAS 9.2 software (SAS institute Inc, Cary, NC). Tukey's HSD (Honestly Significant Difference) test was used to compare means. Genes were assigned different letters (Fig. 3) when the means of the normalized expression levels were statically different between sexes ($p=0.05$, Tukey's HSD).

2.5 Sequence analysis

Multiple alignments of PBP and SNMP amino acid sequences obtained from GenBank were generated using MUSCLE ((Edgar, 2004). These alignments were used to generate multiple codon alignments using PAL2NAL (Suyama et al., 2006). Multiple codon alignments were used to generate phylogenetic trees, and these were used in tests of selection. Phylogenetic trees based on codon alignments were constructed in MEGA4 using the maximum composite likelihood method with the pairwise deletion option and 1000 bootstrap replications (Tamura et al., 2007). Phylogenetic trees based on amino acid sequence alignments (Fig. 2A, 2B) were also constructed in MEGA4 using the JTT matrix-based method with the pairwise deletion option and 1000 bootstrap replications (Jones et al., 1992).

Amino acid sequence alignments were used to reconstruct the phylogenetic relationships of 63 Lepidopteran PBPs. In this reconstruction the PBP sequences clearly formed three clades (Fig. 2A). Consequently, individual trees were constructed for each clade (A, B, and C) based on nucleotide sequences, and these were used in tests of selection. There were 42 sequences and 378 nucleotide positions in clade A, 11 sequences and 309 nucleotide positions in clade B, and 10 sequences and 432 nucleotide positions in clade C. Similarly, the SNMP sequences formed two clades (Fig. 2B), and these were tested independently. There were seven sequences and 1505 nucleotide positions in the SNMP1 clade, and six sequences and 1398 nucleotide positions in the SNMP2 clade. All clades had a mean synonymous distance of 0.5-0.7.

Tests of selection were performed using the codeml procedure implemented in the PAML 4.4 package (Yang, 1997), which estimates ω ratios of the normalized nonsynonymous (dN) to synonymous (dS) substitution rate by the maximum likelihood method (where $\omega > 1$ is considered evidence of positive selection and $\omega < 1$ evidence of purifying selection). Assumptions of variable selective pressures acting on lineages or codons were tested using branch-specific or site-specific models, respectively. Likelihood-Ratio Tests (LRTs) were used to compare models, and significant results were determined using chi-squared tests (Anisimova et al., 2001).

In addition to branch-specific and site specific analysis, pairwise comparisons were made between nucleotide sequences of ACB and ECB-E, ACB and ECB-Z, and ECB-E and ECB-Z PBPs and SNMPs. The unweighted method described in Nei & Gojobori (1986) was used to make pairwise comparisons of synonymous and

nonsynonymous differences. The total numbers of synonymous differences (S_d) and nonsynonymous differences (N_d), as well as the total numbers of amino acid differences, for ACB/ECB-E and ACB/ECB-Z sequence comparisons were averaged (Table 1). Ratios of nonsynonymous to synonymous substitutions per site (d_N/d_S ratios) were calculated as an average of ACB/ECB-E and ACB/ECB-Z sequence comparisons (Table 1).

2.6 Structural modeling

For structural modeling, PBPs were edited so that the signal peptide sequence was removed. Signal peptides were predicted using the SignalP 3.0 Server (Bendsten et al., 2004). Structures were predicted using a HMM-HMM comparison approach with HHPred (Soding et al., 2005). The top three hits (all with an E value of 0) from the RCSB Protein Data Bank (<http://ftp.wwpdb.org>) were: PDB ID: 2wcj, *Bombyx mori* general odorant binding protein (Zhou et al., 2009), PDB ID: 1qwv, *Antheraea polyphemus* pheromone binding protein (Mohanty et al., 2004), PDB ID: 1dqe, *Bombyx mori* pheromone binding protein (Sandler et al., 2000). These were used as templates to create a model using Modeller 9v7 (<http://www.salilab.org/modeller>). Atomic coordinates provided by Modeller were modified using MolProbity 3.17 (Davis et al., 2007). Structural quality was assessed using the Verify3D Structure Evaluation Server (http://nihserver.mbi.ucla.edu/Verify_3D). Motifs were analyzed using MEME 4.1.1 and MAST 4.3 (Bailey et al., 2009). Transmembrane regions of SNMPs were predicted using TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>).

3. Results

3.1 Five PBPs and two SNMPs identified from ACB, ECB-E, and ECB-Z

An EST library was created by high throughput pyrophosphate sequencing of cDNA from ECB-Z antennae. Five partial and two complete cDNA sequences with amino acid homology to known Lepidoptera PBPs and SNMPs were identified by tBLASTn searches of the assembled contigs (Fig. S1). 3' and 5' RACE were used to clone and sequence the complete ORFs of the five partial sequences from ECB-Z antennal cDNA. The complete ORFs of the five PBPs and two SNMPs from ACB, ECB-E, and ECB-Z antennal cDNA were cloned using PCR primers flanking the coding region designed from ECB-Z 5' and 3' UTR sequences (GenBank Accession numbers: GU826166-GU826170, GU828019-GU828028, HM044388-HM044393).

The five PBP ORFs encode proteins of 163-169 amino acids with predicted signal peptides of 20-24 amino acids. Consistent with the PBP family, they have six conserved cysteine residues, a calculated isoelectric point of 4.9-5.4, and predicted molecular weights of 15.9-16.5kDa. All five PBPs have BLASTp similarity (and group phylogenetically) with lepidopteran PBPs that have been functionally and structurally characterized (Fig. 2A). ECB-Z PBPs 1 to 5 are 61%, 55%, 50%, 41%, and 34% identical to *Bombyx mori* PBP1 respectively (Sandler et al., 2000). The amino acid sequences of ACB PBP1 and ECB-Z PBP1 are 100% identical to previously published sequences, (GenBank Accession) AF133632 and AF133637, while ECB-E PBP1 is 99% identical to AF133635 (Willett and Harrison, 1999a).

The six conserved cysteine residues of ECB and ACB PBPs are spaced at intervals C1-X₃₀₋₃₁-C2-X₄-C3-X₄₃-C4-X₁₀₋₁₁-C5-X₉-C6 (X is any amino acid). All PBPs contain motifs typical of the OBP/PBP family (data not shown) (Xu et al., 2009). Phylogenetic analysis indicates that ACB and ECB PBPs 1-3 cluster within a large lineage that includes many well-characterized Lepidopteran PBPs (Fig. 2A, clade A). ACB and ECB PBPs 4 and 5 fall into separate lineages within the PBP subfamily (Fig. 2A, clades B and C). Of the five corn borer PBPs, PBP2 and PBP3 are the most closely related. All three PBP clades in Figure 2A include sequences representing moth families from the modern macrolepidoptera as well as older basal moth families such as the Tortricidae and Yponomeutidae.

The SNMP ORFs encode proteins 525-527 amino acids in length and are predicted to have two transmembrane helices flanking a large extracellular loop (Fig. 1B). Corn borer SNMP1 and 2 separate into two distinct lepidopteran lineages (Fig. 2B). The amino acid sequence of ECB-Z SNMP1 is 68% identical to *Manduca Sexta* SNMP1, and ECB-Z SNMP2 is 69% identical to *M. sexta* SNMP2 (Rogers et al., 2001b).

3.2 PBPs 2 and 3 are expressed at high male-biased levels in corn borer antennae

PBP and SNMP expression levels in male and female corn borer antennae were determined by qPCR. Expression levels are reported relative to the reference gene *Ostrinia* ribosomal protein S3 (RpS3) that was expressed consistently in all tissues. PBPs 2 and 3 were expressed at significantly higher levels in male compared to female ACB and ECB antennae (Fig. 3). Expression levels of PBP2 were 98, 67, and 14 fold higher in male compared to female ACB, ECB-E, and ECB-Z antennae, respectively. The

expression level of PBP3 was 372 fold higher in male compared to female ACB antennae, and 67 and 34 fold higher in ECB-E and ECB-Z male antennae. Transcripts of PBPs 1, 4, and 5 were less abundant overall and on average, expression levels were two to five fold higher in female compared to male corn borer antennae. SNMP1 and 2 were expressed at levels comparable to RpS3 overall and on average, were expressed at levels two to three fold higher in male compared to female corn borer antennae (Fig. 3).

PBPs 2 and 3 were detected at low to moderate levels in male heads (Table S2). Residual antennal stubs remaining on the head after dissection and high expression of PBP 2 and 3 in male antennae may explain these results. SNMP2 was detected at low levels in all body tissues except the female abdomen. All other genes were detected at negligible levels in tissues other than the antennae (Table S2). By including RNA that was not reverse transcribed as a negative control, false signals of expression were eliminated as a concern. In addition, SNMP primer sets spanned an intron and the absence of PCR products resulting from contaminating genomic DNA was confirmed by gel electrophoresis and melting temperature analysis.

3.4 ACB and ECB PBPs may have been subjected to variable selective pressures

To investigate selective pressures acting on PBP genes, we tested the uniformity of selection along lineages using branch and site-specific models using the PAML 4.4 package (Yang, 1997). First, the branch models M0 and M1 were compared. In codeml, M1 calculates ω for each branch while M0 calculates a single average ω for all branches. Likelihood ratio tests indicated that the variation in ω in PBP clade A and C, and the SNMP1 clade, was unlikely to be due to chance alone ($p < 0.01$). Next, ω was calculated

for branches containing ACB and ECB PBPs and SNMPs. None of the branches tested had $\omega > 1$. Because adaptive evolution is often restricted to a fraction of sites, site-specific models were used to investigate variable selective pressure among sites. There was no evidence for variable selective pressure among sites except when the ACB, ECB-E, and ECB-Z PBP3 sequences were compared ($p=0.05$). Although ACB and ECB PBP3 exhibited evidence for variable selection, the data set was considered too small to reliably determine which sites were under selection (Anisimova et al., 2001).

3.3 ACB and ECB PBP3 have diverged

Differences in amino sequences of ACB and ECB PBP1 are encompassed by the Willett and Harrison (1999) sequences (GenBank Accession: AF133630-AF133643) with one exception, a conservative substitution of aspartic acid at position 133 in ECB-E PBP1 (Fig. 1A, Fig. S2). The d_N/d_S ratios for ECB-E sequences compared with ECB-Z sequences were generally very small (0-0.2, data not shown). In the case of PBP3, there are two synonymous and no nonsynonymous substitutions between the ECB races and therefore, the d_N/d_S ratio is equal to zero. Although the d_N/d_S ratio is less than one, we see an increased rate of nonsynonymous substitutions in ACB/ECB PBP3 sequence comparisons (Table 1). Synonymous substitutions exceed nonsynonymous substitutions in all other ACB/ECB sequence comparisons. SNMP sequences are approximately three times longer than PBP sequences, and still have very few nonsynonymous substitutions (Table 1). Fractional numbers for S_d , N_d , and amino acid differences in Table 1 result from averaging, or in the case of PBP3, from the method of counting synonymous and

nonsynonymous differences when there are two substitutions in one codon (Nei & Gojobori, 1986).

3.5 Amino acid changes may affect PBP3 function

ACB and ECB PBPs fold into small globular proteins with six alpha helices stabilized by three disulfide bonds between conserved cysteine residues (Fig. S3). The model of PBP3 was generated using a consensus based approach and was of good quality (Verify 3D quality 58.2). A Ramachandran plot indicated that 96.5% of all residues were in favored regions and 98.6% of all residues were in allowed regions. There were two outliers, Met 133, and Glu 136 (data not shown). The divergent amino acid positions of PBP3 were mapped onto the model. There were two changes in $\alpha 1a$, one between $\alpha 1b$ and $\alpha 2$, three in $\alpha 2$, two in $\alpha 3$, and two in the disordered C-terminal region (Fig. 1A). Amino acid changes in $\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, and the C-terminus may be of interest as these regions participate in ligand binding in a homologous PBP, *B.mori* PBP1 (Horst et al., 2001; Lautenschlager et al., 2005). Although the three-dimensional structure of the *B. mori* PBP with bound bombykol has been determined by X-ray diffraction, we consider its phylogenetic distance and ligand specificity to be too distant from ACB and ECB to specifically inform PBP3 residues involved in ligand binding (Sandler et al., 2000). The Gln in ACB PBP3 at position 54, which lies in $\alpha 2$, was unusual as 94% of PBP sequences in Figure 2A had Glu or Asp in this position. The lysine in position 85 ($\alpha 3$) in ECB PBP3 is highly conserved, and ACB PBP3 was the only sequence, out of 63 sequences, with Glu in this position.

4. Discussion

Lepidopteran OBPs show affinities for specific chemical groups or structures (Vogt et al., 1991). It has been proposed that each insect pheromone component could have a unique high affinity PBP (Du & Prestwich, 1995). While it appears that this is not the case, selectivity of PBP-ligand binding may still play a role in discrimination by acting as a filter prior to binding at the receptor (Lautenschlager et al., 2007) or by interacting with the receptor (Grosse-Wilde et al., 2007). Proteins that confer a level of binding specificity to the pheromone detection system in moths should show fixed amino acid differences between races or species that use structurally different pheromones (Willett & Harrison, 1999a; Willett, 2000). Only PBP1 had been reported in the corn borer model system and the amino acid sequences of the ACB and ECB PBPs were identical (Willett & Harrison, 1999a).

In this study we have identified five genes expressed in ACB and ECB antennae with all the hallmarks of the PBP family. Consistent with Willett and Harrison (1999a) PBP1 sequences had the fewest amino acid changes between species, and its expression was not male biased. Rather, we found that PBP2 and 3 are expressed at high levels in male compared to female antennae. PBP3 nucleotide sequences are almost identical in ECB-E and ECB-Z, but PBP3 sequences have the greatest number of nonsynonymous changes amongst all PBPs when sequences from ACB and ECB are compared. *Bombyx mori* PBP1 can bind very different ligands with only minor side chain adjustments (Lautenschlager et al., 2007). It is possible that PBP3 has evolved to bind the ACB pheromone components, Z12- and E-12-tetradecenyl acetate.

PBPs 2 and 3 appear to be the result of an ancestral gene duplication event (Fig. 2A) and both are significantly male-biased in their expression (Fig. 3). The fact that PBP3 sequences appear to have accumulated differences in ACB versus ECB may be consistent with its neo- and/or subfunctionalization as a result of selective pressure caused by the positional change of the double bond in the ACB sex pheromone. However, our analysis could not determine whether the changes were the result of positive selection or if the increased rate of nonsynonymous substitutions was due to a relaxation of purifying selection in the ACB. With the exception of PBP3, our PAML analysis supports the idea that purifying selection is the predominant force acting on the evolution of the OBP gene family (Vieira et al., 2007).

Alternatively, the divergent sequences of ACB and ECB PBP3 could be the result of a high degree of natural variation in the PBP sequences. Three sequences for *Agrotis ipsilon* PBP2 were found to differ by 2 or 10 amino acids (Abraham et al., 2005). Diversity of PBPs in *Agrotis* species depends not only on the number of genes within the genome but also the number of allelic variations (Abraham et al., 2005). We may have cloned a PBP3 allele in the lab reared ACB colony that is very different from other PBP3 alleles in wild ACB and ECB populations. Willett and Harrison (1999b) found that there was a substantial amount of variation within the PBP1 nucleotide sequence for the E- and Z-race, but that the variation was apportioned among alleles rather than among races. The lack of sequence variation in ACB and ECB SNMPs, and the comparable expression levels in male female antennae suggest that SNMPs are under strong functional constraints, and serve similar functions in both sexes and species. SNMPs may function

to stabilize OBP-bound molecules at the surface of the ORN, and therefore, facilitate the delivery of odor molecules to nearby OR proteins (Rogers et al., 1997).

Though not statistically significant using Tukey's HSD, female-biased expression of PBPs 1, 4, and 5 in this study raises the question of female-biased functions for PBPs. *Manduca sexta* PBP2 and 3 are expressed equally in males and females (Robertson et al., 1999). *M. sexta* PBPs 2 and 3 group with ACB and ECB PBPs 4 and 5, respectively (Fig. 3). These PBPs may be related to ancestral OBP lineages that detect general odors. Alternatively, PBPs expressed in female antenna may play a role in the autodetection of pheromone compounds. Autodetection has been demonstrated by electroantennography in some Lepidoptera species (Gokce et al., 2007; Groot et al., 2005; Stelinski et al., 2006, Yang et al., 2009). Interestingly, the male-produced aphrodisiac pheromones of ECB are structurally similar to the female-produced sex pheromones, and therefore genes involved their detection may be shared by both sexes (Lassance & Löfstedt, 2009).

Similar to *B.mori* PBP1, helices $\alpha 1$, $\alpha 3$, $\alpha 5$, and $\alpha 6$ appear to form the binding pocket of ACB PBP3, and the loop between $\alpha 3$ and $\alpha 4$ covers the entrance (Horst et al., 2001). Divergent sites that may be of interest in future studies include two on $\alpha 1$, two on $\alpha 3$, and two on the C-terminal region that has been predicted to fold into the binding pocket and expel the pheromone ligand in response to low pH in the vicinity of the neuron membrane (Horst et al., 2001; Lautenschlager et al., 2005). However, the regions of the PBP3 protein that participate in ligand binding in ACB and ECB are unknown, so it is difficult to determine which amino acid changes are most significant to function. Future research is needed to determine whether any of these amino acid substitutions are

fixed and specific to the ACB, making them candidates for mutagenesis experiments. Functional analysis will be required to determine whether any fixed polymorphic amino acids contribute to ligand specificity and discrimination between the 11-14:OAc and 12-14:OAc pheromone ligands used by ECB and ACB.

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Table 1. Pairwise comparisons of synonymous and nonsynonymous differences in pheromone binding protein (PBP) and sensory neuron membrane protein (SNMP) nucleotide sequences between European and Asian corn borers. The total number of synonymous differences (S_d) and nonsynonymous differences (N_d) for ACB/ECB-E and ACB/ECB-Z sequence comparisons were averaged, as were ratios of nonsynonymous to synonymous substitutions per site (d_N/d_S ratios). The average number of amino acid (AA) changes between ACB/ECB-E and ACB/ECB-Z are shown.

	S_d	N_d	d_N/d_S	AA
PBP1	6.5	2	0.08	2
PBP2	14	4.5	0.08	4.5
PBP3	7.5	11.5	0.4	10
PBP4	8.5	6.5	0.2	6.5
PBP5	12.5	2.5	0.05	2.5
SNMP1	37.5	6	0.04	5.5
SNMP2	34	3.5	0.03	2.5

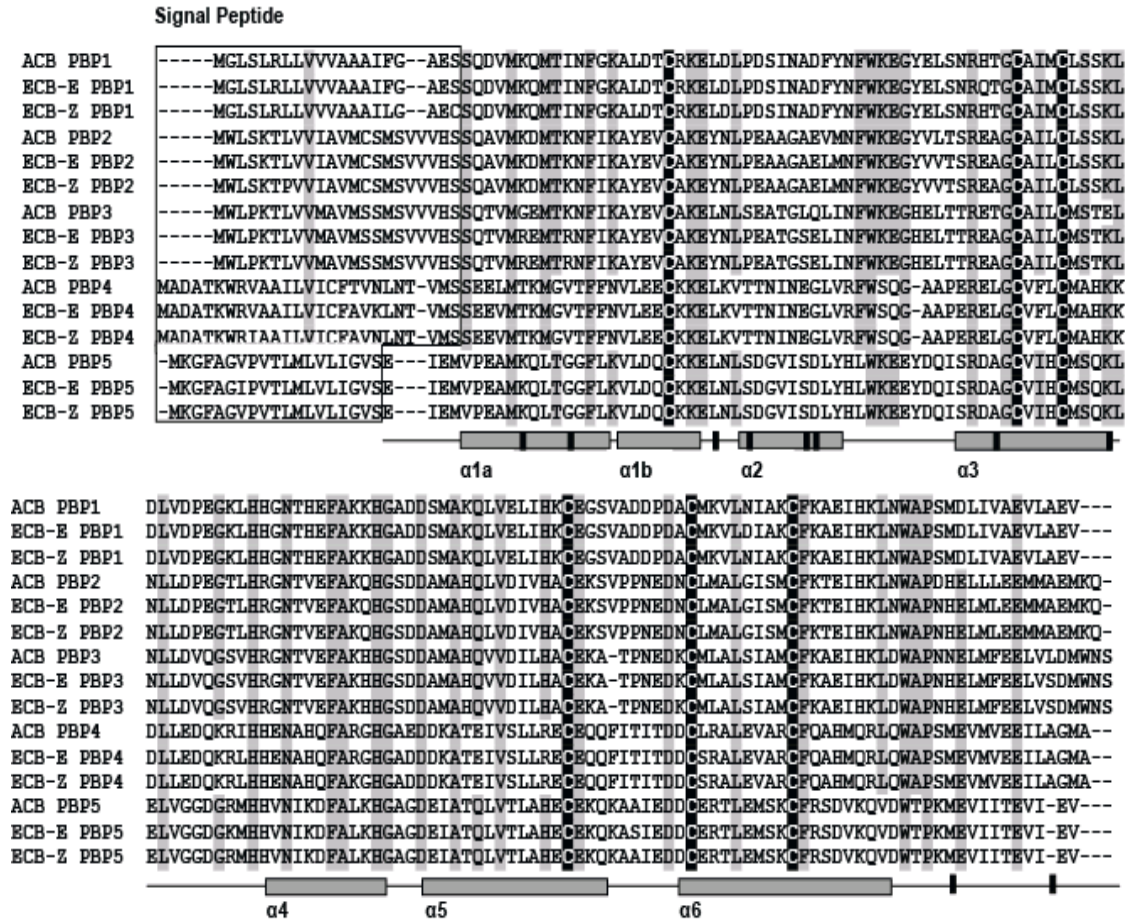


Figure 1A. Amino acid alignment of five pheromone binding proteins from *Ostrinia furnacalis* (ACB), *Ostrinia nubilalis* E-race (ECB-E), and *Ostrinia nubilalis* Z-race (ECB-Z). The signal peptide sequences are boxed. Amino acids conserved in at least 80% of the sequences are shaded in grey, and conserved cysteines appear in white on a black background. Shaded boxes below the alignment indicate approximate positions of six alpha (α) helices. Vertical bars below the alignment indicate the position of amino acid differences in ACB PBP3.



Figure 1B. Amino acid alignment of two sensory neuron membrane proteins (SNMPs) from *Ostrinia furnacalis* (ACB), *Ostrinia nubilalis* E-race (ECB-E), and *Ostrinia nubilalis* Z-race (ECB-Z). Amino acids conserved in at least 80% of the sequences are shaded in grey. Shaded boxes below the alignment indicate approximate positions of two transmembrane spanning helices (Tm α). The region flanked by the transmembrane helices is predicted to form a large extracellular loop.

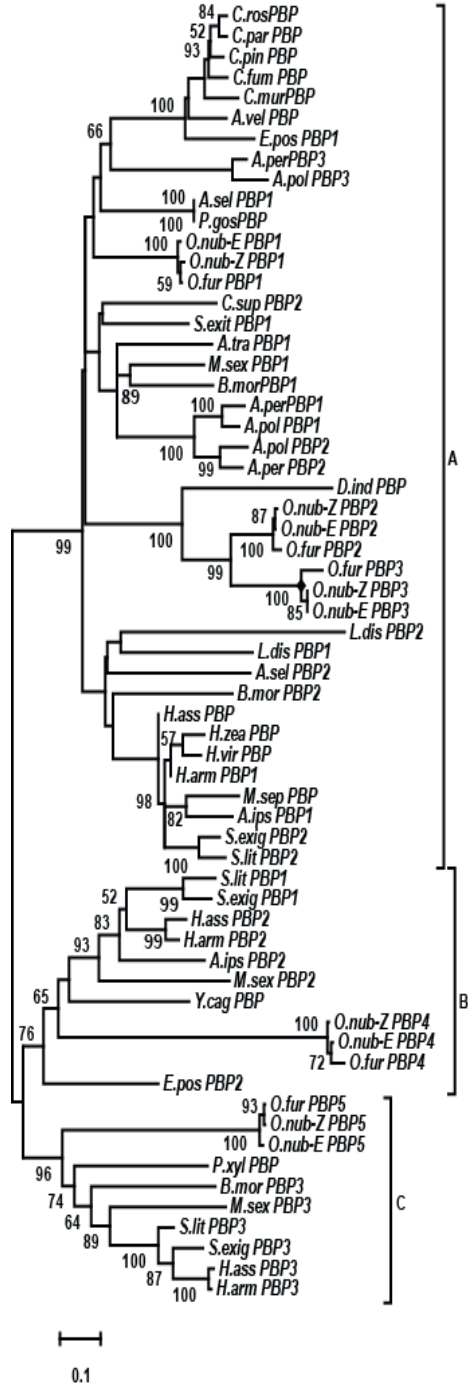


Figure 2A. Evolutionary history of Lepidopteran pheromone binding proteins inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 11.6 is shown. Distances were computed using the JTT matrix-based method and the scale is in units of the number of amino acid substitutions per site. Bootstrap values are presented as a percentage of $n=1000$ replicates at branch points. Branches reproduced in less than 50% of bootstrap replications are shown but not labeled. For analysis of positive selection, nucleotide sequences were divided into 3 clades; A, B, and C. The branch leading to *Ostrinia furnacalis* (*Ofur*, ACB), *Ostrinia nubilalis* E-race (*Onub-E*, ECB-E), and *Ostrinia nubilalis* Z-race (*Onub-Z*, ECB-Z) PBP3 is marked with a diamond.

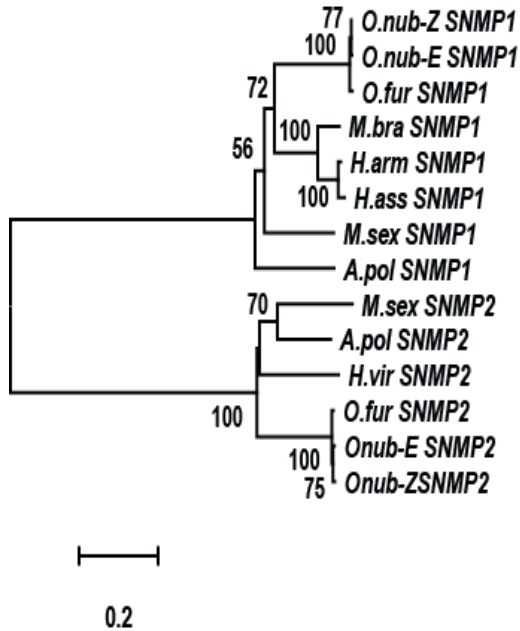


Figure 2B. Evolutionary history of Lepidopteran sensory neuron membrane (SNMP) proteins inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 2.9 is shown. Distances were computed using the JTT matrix-based method and the scale is in units of the number of amino acid substitutions per site. Bootstrap values are presented as a percentage of $n=1000$ replicates at branch points. Branches reproduced in less than 50% of bootstrap replications are shown but not labeled. *Ostrinia furnacalis* (*Ofur*, ACB), *Ostrinia nubilalis* E-race (*Onub-E*, ECB-E), *Ostrinia nubilalis* Z-race (*Onub-Z*, ECB-Z). GenBank protein accession numbers: *Antheraea polyphemus* SNMP1, AAC47540; *Mamestra brassicae* SNMP1, AAO15603; *Helicoverpa armigera* SNMP1, AAO15604; *Helicoverpa assulta* SNMP1, ACC61201; *Manduca sexta* SNMP1, AAG49366; *Plutella xylostella* SNMP1, ADK66278; *Heliothis virescens* SNMP2, CAP19028; *Antheraea polyphemus* SNMP2, CAP19029; *Manduca sexta* SNMP2, AAG49365.

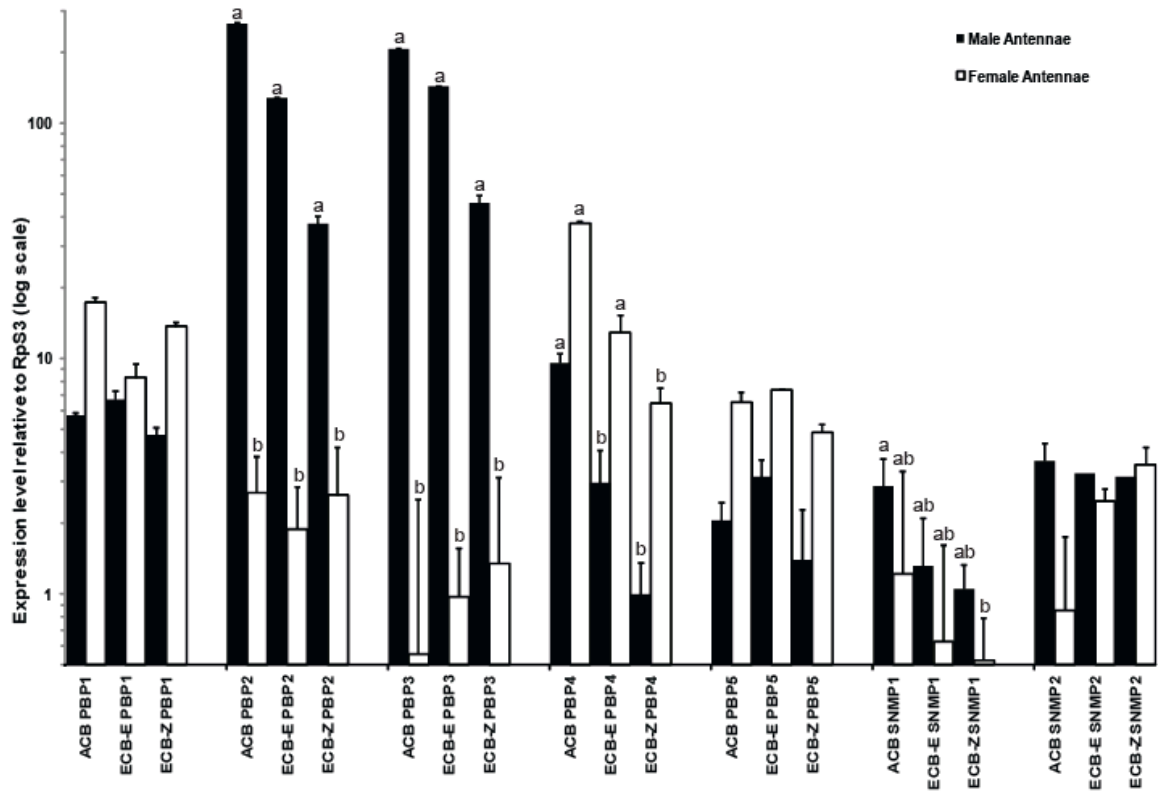


Figure 3. Expression levels of five pheromone binding protein (PBP) and two sensory neuron membrane protein (SNMP) genes relative to ribosomal protein S3 (RpS3) in male and female *O. furancalis* (ACB), *O. nubilalis* E-race (ECB-E), and *O. nubilalis* Z-race (ECB-Z) antennae (log scale). Expression levels were measured using quantitative real-time PCR with SYBR green, and relative expression was calculated using the $2^{-\Delta CT}$ method. Error bars represent the 95% confidence interval of three biological replications. Tukey's Honestly Significant Difference test was used to compare means. Genes were assigned different letters when the means of the normalized expression levels were statically different between sexes ($p=0.05$).

CHAPTER 4

ODORANT RECEPTOR 3, A CANDIDATE FOR EVOLVING
SPECIFICITY TO THE 12-TETRADECENYL ACETATE SEX PHEROMONEIntroduction

The sex pheromone communication system of moths (Order Lepidoptera) has been the subject of multidisciplinary studies for more than a century (Schneider, 1992). Female moths emit blends of pheromones that attract males from long distances. Female sex pheromones are blends of volatile fatty-acid derivatives. Subtle changes in the chemistry of the blends differentiate closely related species and promote reproductive isolation (Ando et al., 2004). Female sex pheromone production and male response are under the control of different genes (Dopman et al., 2004; Roelofs et al., 1987). The genes underlying male response to female pheromones are thought to be under strong selective pressures (Carde & Haynes, 2004). Indeed, a recent study has identified a genetic region associated with pheromone detection that is the basis for sexual isolation of two closely related moth species (Gould et al., 2010).

Twenty species of moths belonging to the genus *Ostrinia* (Crambidae) have been classified as three different groups based on male genital morphology (Mutuura & Munroe, 1970). The sex pheromones of nine *Ostrinia* species have been characterized, and six of these species are very closely related members of group III (Ishikawa et al. 1999). The sequence divergence of mitochondrial COII gene sequences in these six species was estimated at 0.15-2.38% (Kim et al., 1999). Five of these species

(*O.nubilalis*, *O.scapularis*, *O.zea*, *O.zaguliaevi*, *O.orientalis*) use varying blends of E11 and Z11-tetradecenyl acetate (E11-14:OAc, Z11-14:OAc) as their primary sex pheromone components. One species, the Asian corn borer (*Ostrinia furnacalis*, ACB), uses blends of E and Z12-14:OAc as its primary sex pheromone. The three characterized species of *Ostrinia* group II (*O. palustralis*, *O.latipennis*, *O. ovalipennis*) use different blends of E and Z11-14:OAc, and a fourteen carbon alcohol, E11-14:OH (Ishikawa et al., 1999). E11-14:OH is the main sex pheromone component used by *O. latipennis*. These *Ostrinia* species, which are morphologically and genetically similar but use different pheromones, are ideal models for studying the evolution of sex pheromone communication.

Sex pheromone communication in the Lepidoptera is mediated by odorant receptor (OR) proteins expressed in the dendrite membrane of pheromone sensitive olfactory receptor neurons (ORNs). Insect ORs are unrelated to the mammalian G protein-coupled receptor family of mammalian ORs. In insects, ORs form a large family of seven transmembrane domain receptor proteins. The N-terminus of an insect OR is located intracellularly while the C-terminus is located extracellularly, which is opposite of the orientation of mammalian ORs (Benton et al., 2006).

Seven pheromone receptors have recently been identified from two *Ostrinia* species, *O.nubilalis* (ECB) and *O.scapularis* (Miura et al., 2010; Wanner et al., 2010). Several ORs from *O. nubilalis* and *O. scapularis*, and one from *O.latipennis* have been functionally characterized (Miura et al., 2010; Wanner et al., 2010). *O.nubilalis* OR6 was expressed at high levels in male antennae and was specifically tuned to Z11-14:OAc

(Wanner et al., 2010). The *O.scapulalis* and *O.latipennis* homologs of *OnubOR5* responded best to E11-14:OH (Miura et al. 2009). Other receptors from *O. nubilalis* and *O. scapulalis* responded more broadly to sex pheromone components in general (Miura et al., 2010; Wanner et al., 2010). Additionally, Muira et al. (2010) reported genes homologous to *OscOR1* and 3-8 from eight species (*O. nubilalis*, *O.furnacalis*, *O. scapulalis*, *O.zea*, *O.zaguliaevi*, *O. palustralis*, *O. latipennis*, and *O. ovalipennis*).

E and Z11-14:OAc are used by many lepidopteran species as sex pheromones (El-Sayed, 2010). Sex pheromone receptors with similar specificities may have evolved several times within the Lepidoptera. However, the fact that other species within the same family and superfamily as *Ostrinia* (Crambidae and Pyraloidea) use Z11-14:OAc as a sex pheromone component suggests that a receptor for Z11-14:OAc may have evolved prior to the divergence of the *Ostrinia* (Davis et al. 1991; Wakamura et al. 1999). Interestingly, to date only the ACB has been found to use E and Z12-14:OAc as sex pheromone components (El-Sayed, 2010). Production of E and Z12-14:OAc in ACB females results from a gene encoding a desaturase enzyme with a unique function in ACB (Roelofs & Rooney, 2003). Similarly, the odorant receptor genes involved in the male response to E and Z12-14:OAc may have been subjected to selection for specificity to this unique pheromone blend.

Mechanisms that could alter sex pheromone detection between closely related species include changes in the coding regions of sex pheromone receptors that alter receptor specificity and changes in noncoding regions that alter receptor expression. To test these hypotheses we have cloned the open reading frames (ORFs) of all known

Ostrinia sex pheromone receptors from the ACB and two pheromone races of the ECB (ECB-E and ECB-Z). Expression levels of the seven genes were analyzed to test for global changes in gene regulation between the closely related species and races. The coding sequences were analyzed for evidence of selection. These experiments are expected to provide important insights to inform future functional studies of ACB and ECB pheromone receptors.

Material and Methods

RNA Extraction

ECB and ACB antennae were prepared for RNA extraction as described in Wanner et al. (2010) and Allen & Wanner (accepted for publication). Briefly, ECB-Z pupae were purchased from Benzon Research (Carlisle, Pennsylvania) and provided from a colony maintained at the New York State Agricultural Experiment Station (NYAES). ECB-E pupae and ACB antennae were also provided by NYAES. RNA was extracted from frozen tissue using a Dounce homogenizer and an RNeasy Mini kit (Qiagen, Valencia, CA). RNA was quantified and assayed for purity by absorbance at 260nm, 280nm, and 230nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

Cloning Full Length ORs

Full length ORs were amplified from male ACB, ECB-E, and ECB-Z antennal cDNA using primers designed to the 3' and 5' untranslated regions (Table S1). Amplification reactions were performed using Phusion High-Fidelity DNA polymerase

(Finnzymes Inc., Woburn, Massachusetts). Cycling conditions were: 98°C for 30 sec, 28 cycles of 98°C for 25 sec, 55°C for 30 sec, 72°C for 90 sec, followed by a final extension at 72°C for 10 min. PCR products were gel purified, cloned into the pCR2.1 vector (Invitrogen), and sequenced. Plasmid inserts were sequenced by Molecular Cloning Laboratories (South San Francisco, CA). At least three clones were sequenced for each cDNA.

Phylogenetic Analysis

cDNA and amino acid sequences from *O.zaguliaevi*, *O.zealis*, *O.scapulalis*, *O.nubilalis*, *O.furnacalis*, *O.palustralis*, *O.ovalipennis*, and *O.latipennis* ORs 1 and 3-8 reported in Muira et al. (2009 & 2010) were obtained from GenBank. ORs 5 and 6, 4, 3, and 1 in Muira et al. (2010) correspond to ORs 1 group A and B, 3, 4, and 5 reported in Wanner et al. (2010), respectively. The sequence corresponding to OR3 (Muira et al., 2010) was excluded from the data set, as it was only one nucleotide different from Wanner et al. (2010) OR3. Muira et al. (2010) did not find an OR corresponding to OR6 (Wanner et al., 2010), and therefore, no additional *Ostrinia* sequences were available for OR6. ORs 7 and 8 refer to the same gene in Muira et al. (2010) and this report. E-race *O.scapulalis* and Z-race *O.nubilalis* were used in Muira et al. (2009, 2010).

The final data set contained seventy five OR sequences. Amino acid sequences were aligned using MUSCLE, and PAL2NAL was used to generate a multiple codon alignment (Edgar, 2004; Suyama et al., 2006). The PAL2NAL web server generates multiple codon alignments from amino acid alignments and their corresponding nucleotide sequences. A phylogenetic tree was created in MEGA 4 based on the multiple

codon alignment (Tamura et al., 2007). The neighbor joining method was used to estimate evolutionary distances in units of base substitutions per sites (Tamura & Nei, 1993). Alignment gaps were eliminated only in pairwise sequence comparisons.

Sequence Analysis

Tests of selection were performed using the codeml procedure implemented in the PAML 4.4 package (Yang, 1997), which estimates ω ratios of the normalized nonsynonymous (dN) to synonymous (dS) substitution rate by the maximum likelihood method (where $\omega > 1$ is considered evidence of positive selection and $\omega < 1$ evidence of purifying selection). Assumptions of variable selective pressures acting on specific lineages and or codons were tested using branch-specific and site-specific models, respectively. We tested patterns of selection in seven gene clusters (Table 1). For each gene cluster, a phylogenetic tree was reconstructed from a codon alignment as described previously. The OR1 gene cluster was unusual in that it contained two subgroups (groups A&B), which may be recently duplicated genes or two alleles of a single gene. For our purposes OR1 groups A and B were analyzed together.

First, the branch specific models M0 and M1 were compared for each gene cluster. In codeml, M0 computes a single ω for all branches, and M1 computes an individual ω for each branch. Likelihood-Ratio Tests (LRTs) were used to compare models, and significant results were determined using chi-squared tests (Anisimova et al., 2001). Degrees of freedom (*df*) for chi-squared tests were equal to the difference in the number of parameters estimated. For each analysis, correction for multiple testing (Bonferroni correction) was applied. For gene clusters where the free ratio model (M1) fit

the data significantly better than M0, branches with ratios significantly different from one were identified by running M2 under two conditions: in condition A the d_N/d_S ratio was calculated, and in condition B the d_N/d_S ratio was set to one. Again, LRTs were compared, and significant results were determined using chi-squared tests.

Next, the site-specific models M1a (nearly neutral) and M2a (selection), and M7 (beta) and M8 (beta and ω) were compared for each gene cluster except OR6. For OR6, d_N/d_S ratios were determined for pairwise comparisons of three protein coding sequences using the maximum likelihood method implemented in codeml (runmode -2). When selection models M2a and M8 fit the data significantly better than the neutral models (M1a and M7) the Bayes empirical Bayes (BEB) procedure was used to calculate the posterior probabilities (PPs) of codons under positive selection (Yang et al. 2005). We then examined the distribution of the predicted positively selected codons by mapping the corresponding amino acids onto the odorant receptor topology using TOPCONS and TOPO2 (Bernsel et al. 2009; <http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl>). A consensus sequence was generated from an alignment of full length ACB, ECB-E, and ECB-Z sequences and the consensus sequence was used to predict the transmembrane regions.

Quantitative Real-Time PCR (qPCR)

qPCR was performed as described in Allen & Wanner (accepted for publication). Briefly, 150-300ng of total RNA was used in cDNA synthesis reactions. Quantitative PCR primers unique to each (except OR7 & 8) set of three orthologous corn borer genes were selected using Primer3 software (Rozen & Skaletsky, 2000). ORs 7 and 8 were not

included in qPCR experiments as the sequences were unknown at the time the qPCR experiments were done. qPCR experiments were performed using 96 well plates and IQ EvaGreen Supermix on an IQ5 Real Time PCR Detection System (Bio-Rad, Hercules, CA). Each 15µl reaction was replicated in triplicate. No-template and no-reverse transcriptase controls were included in each experiment. Expression levels of OR genes were calculated relative to the control gene RpS3 using the $2^{-\Delta CT}$ method described by Livak and Schmittgen (2001). qPCR results were analyzed by fully nested ANOVA using SAS 9.2 software (SAS institute Inc, Cary, NC). Tukey's HSD (Honestly Significant Difference) test was used to compare means. Genes were assigned different letters when the means of the normalized expression levels were statically different between sexes ($p=0.05$, Tukey's HSD).

Results

OR Genes Form Distinct Phylogenetic Clusters

A total of twenty three ORFs were cloned from ECB-Z, ECB-E and ACB antennae, representing seven unique pheromone receptor genes (or possibly 8, if OR1A and 1B are distinct genes rather than different alleles of one gene). ORs 1 and 3-8 have been demonstrated to belong to the lepidopteran pheromone receptor lineage (Mira et al. 2010; Wanner et al. 2010). The pheromone receptors cloned in this study group into distinct gene clusters with orthologous *Ostrinia* receptors reported in Mira et al. (2010) (Fig. 1). Within this phylogenetic relationship, ORs 1A, 1B, and 8 form a highly

homologous lineage related to ORs 6 and 3. ORs 4 and 5 are closely related while OR7 is more distantly related (Fig. 1).

OR Genes Are under Variable Selective Pressures

To investigate the evolutionary pressures affecting OR genes, we estimated the rates of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions in the seven OR gene clusters using branch-specific models (Yang 1998; Yang and Nielsen 1998). The one ratio model (M0) was an adequate fit for OR gene clusters 1, 4, 5, and 8 (Table 2). The individual d_N/d_S ratio for each of these gene clusters was calculated, and was determined to be less than one in each cluster (Fig. 1), consistent with purifying selection. For OR gene clusters 3, 6, and 7 the free ratio model fit the data significantly better than the one ratio model, which suggests that selective pressure varies among lineages (Table 2). In the OR3 gene cluster there was strong evidence for positive selection on the lineage leading to ACB OR3 ($\omega=2.9$, $p=0.008$). There was strong evidence for purifying selection acting on the lineages leading to *Ostrinia* group II species (*O.palustralis*, *O.ovalipennis*, *O.latipennis*) OR3 ($\omega=0.2$, $p<0.001$). The free ratio model best fit the OR7 sequence data, and no clear pattern of selection could be discerned. The d_N/d_S ratio for the lineage leading to ACB OR6 was elevated relative to the ECB OR6 lineage, but was not significantly different from one.

Branch models are useful for detecting selection along lineages but they average selective pressures over all codon sites within the sequences. In most functional genes the majority of sites are subject to purifying selection, and only a fraction of sites are

subject to positive selection (Nielsen, 2005). To detect positive selection acting on specific codons we estimated the rates of synonymous (d_s) and nonsynonymous (d_N) nucleotide substitutions in seven OR gene clusters using site-specific models (Yang 1998; Yang and Nielsen 1998). Significant LRTs (M1a/M2a and M7/M8 comparisons) indicated that positive selection was occurring on a fraction of sites in ORs 3, 7, and 8 (Table 3). In OR3, significant LRTs were obtained when the *Ostrinia* group II sequences were removed from the analysis and the remaining eight sequences were analyzed. To determine which codons were subject to positive selection, we used the BEB procedure implemented in models M2 and M8. Only codons with PPs greater than 0.80 were considered, and because M2a has been shown to be an extremely conservative model of selection (Wong et al. 2004; Yang et al. 2005), codons with PPs greater than 80% in both M2a and M8 were emphasized (Table 3). However, the codon models implemented in PAML ignore the physiochemical properties of the amino acid being substituted, and therefore, some codons with $\omega > 1$ may not be advantageous or biologically relevant substitutions.

Positively Selected Sites May Affect Receptor Function

Under a relaxation of selective pressure a random distribution of amino acid substitutions would be expected, whereas an overrepresentation of amino acid changes in functional domains would be expected under positive selection. To investigate the distribution of amino acid substitutions in OR proteins we mapped predicted positively selected sites onto topology models of ORs 3, 7, and 8. Interestingly, the distribution of

positively selected sites appears to be quite different in OR3 compared to ORs7 and 8. In OR3, two positively selected sites are located on the intracellular amino terminus, and single sites are located on the second and third transmembrane regions (Fig. 2). In contrast, all but one of the positively selected sites of ORs7 and 8 are located beyond the third transmembrane segment within the second extracellular loop, the second intracellular loop, and the fifth and sixth transmembrane segments (Fig. 2).

Prediction of positively selected sites is not practical from a small number of similar sequences (Nielsen, 2005); therefore we conducted pairwise comparisons of OR6 sequences from ACB, ECB-E, and ECB-Z using the maximum likelihood method. Pairwise comparisons are unlikely to detect positive selection, as selective pressure is averaged over all lineages and codon sites. However, it is clear that the d_N/d_S ratios of ACB/ECB OR6 pairwise comparisons are elevated relative to the ECB-E/ECB-Z comparison. There is more than twice the number of amino acid substitutions in ACB/ECB OR6 comparisons relative to the ECB-E/ECB-Z comparison (Table 4).

No Differences in OR Expression Levels between ECB and ACB

OR expression levels in male and female corn borer antennae were determined by qPCR. Expression levels are reported relative to the reference gene *Ostrinia* ribosomal protein S3 (RpS3) that was expressed consistently in all tissues. ORs 1 and 3-6 were expressed at significantly higher levels in male compared to female ACB and ECB antennae (Fig. 3). There were no significant differences in OR expression level between species or strains of the same sex. On average, the expression levels of ORs 1, 3, 4, 5, and

6 were 165, 1076, 119, 347, and 307 fold higher in male compared to female corn borer antennae, respectively. ORs 1, 2, 3, and 6 were expressed at levels 24-175% of RpS3 while ORs 4 and 5 were expressed at 3-12% of RpS3 (Fig. 3).

Expression of ORs 1-6 were detected at negligible levels in tissues other than the antennae (reported in Wanner et al., 2010). By including RNA that was not reverse transcribed as a negative control, false signals of expression were eliminated as a concern. In addition, ORs 1, 3, 4, and 5 primer sets spanned an intron and the absence of PCR products resulting from contaminating genomic DNA was confirmed by gel electrophoresis and melting temperature analysis.

Discussion

Ostrinia species that are morphologically and genetically similar but use different pheromones are ideal models for studying the evolution of sex pheromone communication. The ACB is closely related to the ECB, but the ACB uses a sex pheromone, 12-tetradecenyl acetate, that is unique in the Lepidoptera. To identify candidate ORs that may have evolved specificity to 12-14:OAc, orthologous OR sequences from ACB, ECB-E, ECB-Z and were cloned and examined for evidence of positive selection. In addition to the twenty three OR sequences reported here, Miura et al. (2010) reported a total of fifty two OR sequences from eight *Ostrinia* species. Analyzed together, these sequences form seven distinct phylogenetic branches representing ORs1 and 3-8.

Insect ORs are postulated to have evolved by a birth and death process where genes arise by duplication and diverge in sequence and function over time (Sanchez-Gracia et al., 2009). Analysis of nonsynonymous and synonymous substitutions has been used to detect functional diversification following gene duplication in phylogenetically related sequences (Guo & Kim, 2007; Vieira et al., 2007; Smadja et al., 2009). In this study, the normalized nonsynonymous (d_N) to synonymous (d_S) substitution rate (ω) was calculated for specific lineages and codon positions within seven *Ostrinia* OR gene clusters. There were uniform normalized d_N/d_S ratios for all lineages within ORs gene clusters 1, 4, 5 and 8 (Table 2, Fig. 1). In all cases ω was significantly less than one, suggesting that purifying selection is acting on ORs 1, 4, 5, and 8.

Most functional genes are subject to strong purifying selection (Nielsen, 2005; Sanchez-Gracia et al., 2009). With a normalized d_N/d_S ratio of 0.13, OR5 is a clear example of strong purifying selection that can be supported by functional studies. Miura et al. (2009) characterized *OscA*OR5 and *Olat*OR5 as receptors for E11-14:OH. E11-14:OH is the main sex pheromone of *O. latipennis*, a member of the more basal *Ostrinia* group II species complex. Interestingly, there is no known function for E11-14:OH in *O. scapulalis* or other members of the more modern group III species complex. Regardless, the function of OR5 as a receptor for E11-14:OH is conserved in *O. scapulalis*. The functional conservation of OR5 throughout the evolution of *Ostrinia* species would provide an explanation for the strong purifying selection acting on this OR gene cluster.

New functions for duplicated genes evolve intermittently (Nielsen, 2005). For example, functional divergence of duplicated OR genes could occur when new pheromones such as 12-14:OAc arise. There was evidence for variable selective pressures acting on the lineages within OR gene clusters 3, 6, and 7. The lack of a consistent pattern of selection in the OR7 gene cluster may be the result of relaxed purifying selection. Relaxation of purifying selection also appears to be acting on the lineage leading to ACB OR6 (Fig. 2). This result is consistent with the hypothesis that OR6 is a critical receptor for *Ostrinia* species that use Z11-14:OAc as their main sex pheromone component, and the orthologous receptor in ACB has been released from purifying selection because it has evolved to use a sex pheromone that OR6 does not detect (Wanner et al., 2010).

Perhaps the most significant results of this study are those of the selection analysis of the OR3 gene cluster. Within the OR3 cluster, sequences from the basal *Ostrinia* group II species are under strong purifying selection while purifying selection seems to be relaxed in the group III species (Fig 1). The lineage leading to ACB OR3 is the only lineage where there is evidence to suggest that positive selection is occurring (Fig. 1). These results suggest that OR3 sequences have been released from a conserved ancestral function in group III species, and the accumulation of nonsynonymous substitutions in ACB OR3 may affect receptor specificity. Therefore, ACB OR3 is the best candidate for evolving specificity for the 12-14:OAc pheromone.

Positive selection is often restricted to a small number of sites (Nielsen, 2005). For this reason, site-specific analyses were used to identify specific codons under positive

selection. These analyses are considered liberal given that a correction was not applied for multiple testing, in favor of reducing false negatives at the expense of elevating false positives. ORs 3, 7 and 8 yielded significant sites that may be subject to positive selection (Table 3). Positively selected amino acid sites were located in the N- and C- termini, the intra- and inter-cellular loops, and transmembrane domains.

Insect ORs are a family of ligand-gated ion channels whose function has only recently been elucidated (Sato et al., 2008; Wicher et al., 2008). There are no three-dimensional structures available for insect ORs and little is known about how their structure affects their function. Due to the lack of structural information it is difficult to speculate on the functional nature of specific amino acid residues. However, it is interesting that two of the positively selected residues of OR3 are located in the second and third transmembrane domains. These regions have been implicated in the activation of *Drosophila* OR85b by the odorant, 2-heptanone (Nichols & Luetje, 2010). In contrast, the majority of positively selected residues in ORs 7 and 8 reside in the third intracellular loop (OR8), and the fifth and sixth transmembrane domains. The third intracellular loop is postulated to interact with the obligatory co-receptor, OR83b in *Drosophila* (OR2 in Lepidoptera) (Benton et al., 2006). Transmembrane segments six and seven are conserved among insect ORs, and may have a common function (Benton et al., 2006). Nonsynonymous substitutions in these regions may lead to non-functionalization of ORs 7 and 8.

Differential expression of ORs in closely related species may also contribute to difference in sex pheromone detection. Therefore, the expression levels of the seven OR

genes of ACB, ECB-E, and ECB-Z were analyzed. There were no global changes in gene regulation between the closely related species and races. Although we did not make statistical comparisons between OR genes, it is interesting that OR3 was expressed at higher levels in the ACB, while OR6 was expressed at higher levels in the ECB. This difference in expression would be consistent with the theory that OR3 has evolved specificity for 12-14:OAc. Functional studies will be required to determine if ACB OR3 is specific for 12-tetradecenyl acetate. The results presented here lay the foundation for future mutational studies that will determine which amino acid substitutions in OR3 (if any) are critical to receptor specificity.

Table 1. Seven odorant receptor gene clusters. The number of odorant receptor (OR) sequences in each gene cluster, the number of codons in the nucleotide alignment, and the number of species represented in tests of selection are shown. The *Ostrinia palustralis* OR1 and OR7 sequences were not included in our analysis, as they were available in GenBank but were not included in Muira et al. (2010). Only the European and Asian corn borers (*O. nubilalis* and *O. furnacalis*) were represented in the OR6 gene cluster.

Gene	Sequences	Codons	Species
OR1	16	424	7
OR3	11	422	8
OR4	13	410	8
OR5	12	424	8
OR6	3	421	2
OR7	10	397	7
OR8	11	427	8

Table 2. Evidence of variable selective pressures acting on odorant receptor (OR) lineages. The branch-specific models implemented in codeml (PAML 4.4 package) were used to test for variable selective pressures acting on seven OR lineages. The log likelihoods (lnL) for branch models M0 (uniform nonsynonymous to synonymous substitution rate) and M1 (variable nonsynonymous to synonymous substitution rate) were compared in likelihood ratio tests, and the p values were determined from the chi-squared distributions. The Bonferroni correction for multiple testing was used. The uniform rate branch model (M0) is a better fit for OR gene clusters 1, 4, 5, and 8; and the variable rate branch model (M1) is a better fit for OR gene clusters 3, 6, and 7.

	n	lnL M0	lnL M1	df	P value
OR1	16	-3215.6	-3198.8	28	0.21
OR3	11	-3005.5	-2976.1	19	6.1E-6**
OR4	13	-3381.9	-3365.1	22	0.05
OR5	12	-2791.1	-2777.6	20	0.13
OR6	3	-1983.6	-1979.7	2	0.02*
OR7	10	-2706.7	-2686.9	16	8.8E-4**
OR8	11	-2269.8	-2260.2	18	0.59

n =number of sequences, df is degrees of freedom

** Significant at the 5% level after Bonferroni correction

* Significant at the 10% level after Bonferroni correction

Table 3. Predicted positively selected codon positions in odorant receptors (OR) 3, 7, and 8. The site (codon) specific models implemented in codeml (PAML 4.4 package) were used to test for positive selection acting on specific codons in three OR gene clusters. The log likelihoods for branch models M1a (neutral) and M2a (selection) and M7 (beta) and M8 (beta & ω) were compared in likelihood ratio tests, and the p values were determined from the chi-squared distributions. Specific codons under section were predicted using Bayes Empirical Bayes (BEB) analysis. Predicted positively selected sites with posterior probabilities (PPs) greater than 80% in both M2 and M8 BEB analysis are underlined.

N, n			p value	p value	Positively selected sites
			M2a>M1a	M8>M7	(PP>80%)
OR3	8	422	0.02	6E-3	<u>20, 33, 45, 78, 144</u>
OR7	10	397	5.0E-3	3.8E-3	75, <u>173</u> ,176,284, <u>314</u> , <u>316</u>
OR8	11	427	0.03	0.03	182, 270, <u>275</u> , 278, 311, <u>312</u>

N is the number of sequences

n is the number of codon positions in the alignment.

Table 4. Pairwise comparisons of odorant receptor (OR) 6 from the Asian corn borer (ACB) and two pheromone races of the European corn borer (ECB-E and ECB-Z). The d_N/d_S were estimated using the maximum likelihood method implemented in codeml (PAML 4.4 package). The d_N/d_S ratio represents the nonsynonymous to synonymous substitution rate between two sequences. N_d and S_d are numbers of nonsynonymous and substitutions per sequence pair, respectively, and AA is amino acid substitutions per sequence pair.

OR6	N_d	S_d	d_N/d_S	AA
ACB vs. ECB-E	30	12	0.65	29
ACB vs. ECB-Z	25	8	0.82	24
ECB-E vs ECB-Z	11	12	0.23	11

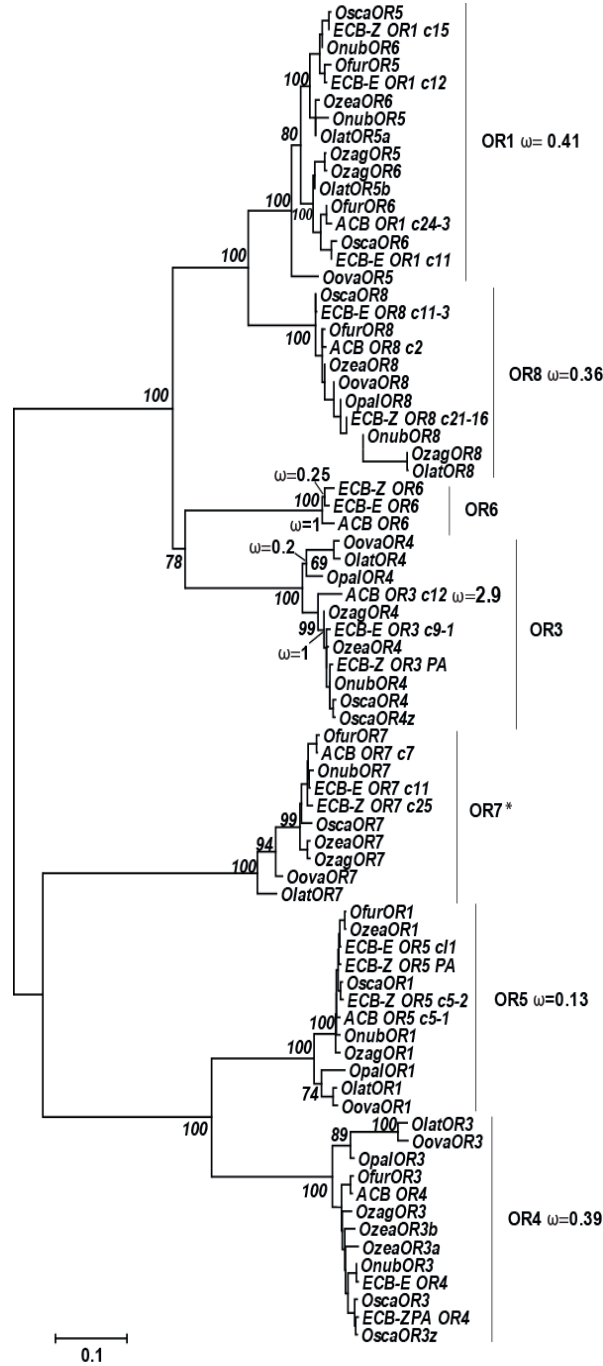
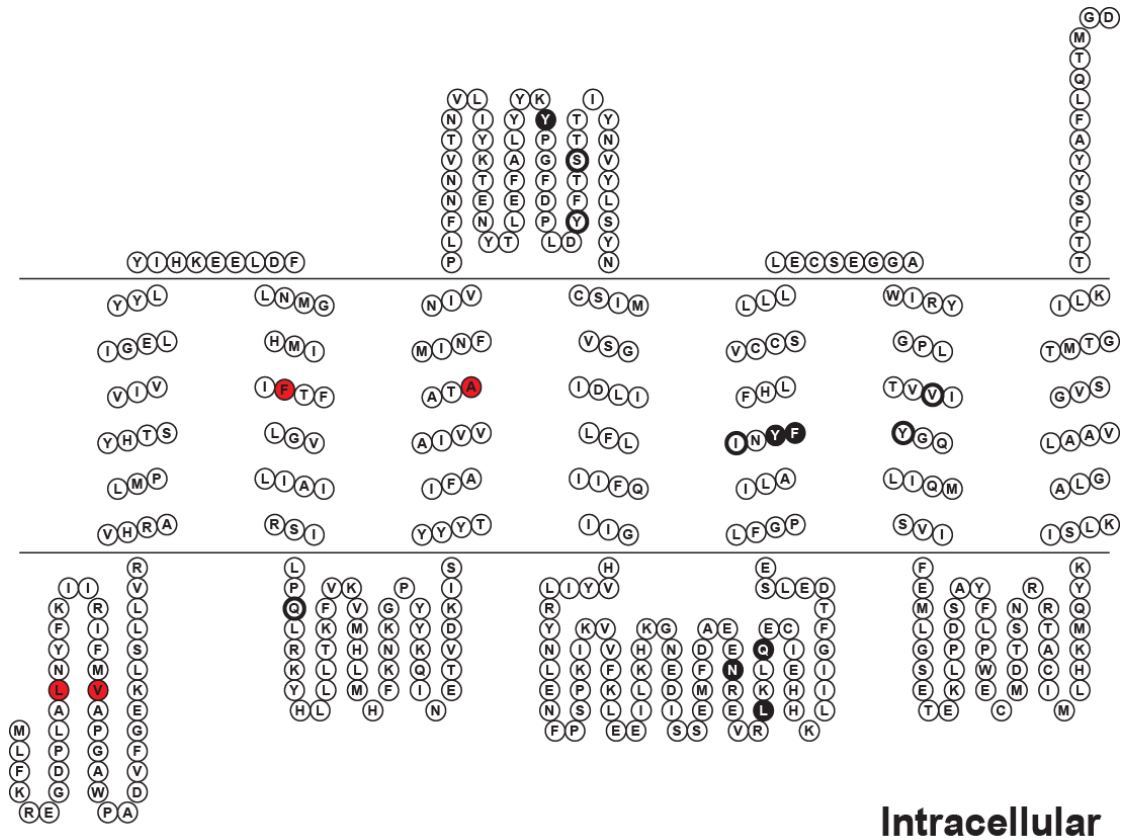


Figure 1. Phylogenetic relationship of seventy five odorant receptor (OR) nucleotide sequences representing seven OR genes. The phylogenetic relationship was inferred using the neighbor-joining method in MEGA4 with 1000 bootstrap replicates. Bootstrap values for major branches are shown. The tree is drawn to scale, with branch lengths in units of the number of base substitutions per site. The normalized nonsynonymous to synonymous substitution rate (ω) is shown. ORs 1, 4, 5, and 8 have one uniform ω for all lineages in the gene cluster, while ORs 3 and 6 have two or more rates (ω) for lineages within the gene cluster. ω is greater than one on the lineage leading to ACB OR3. *The OR7 gene cluster has individual rates (ω) for each lineage (not shown).

Extracellular



Intracellular

Figure 2. Location of predicted positively selected sites in Asian and European corn borer odorant receptors. Predicted positively selected sites from Bayes Empirical Bayes analysis with posterior probabilities (PPs) greater than 80% are mapped onto an odorant receptor seven transmembrane topology. The topology was predicted using a consensus sequence of Asian and European corn borer ORs, the transmembrane prediction web server, TOPCONS, and the drawing program, TOPO2. Residues with PPs greater than 80% in codeml site model M2 or M8 are shaded in red (OR3), black outlines (OR7) and, solid black (OR8).

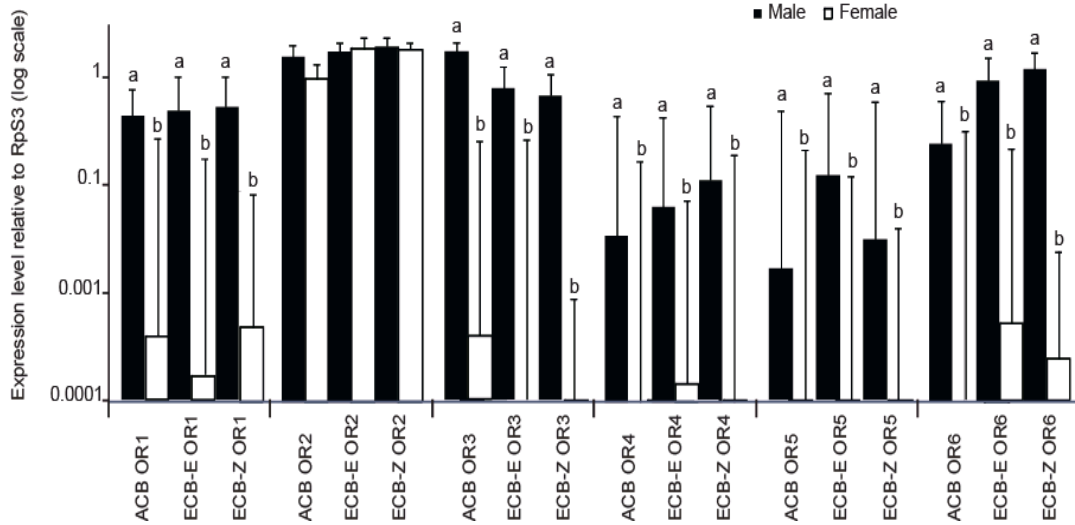


Figure 3. Male biased expression of odorant receptors (ORs) Asian and European corn borer antennae. Expression level of six OR genes relative to ribosomal protein S3 (RpS3) in male and female *O. furancalis* (ACB), *O. nubilalis* E-race (ECB-E), and *O. nubilalis* Z-race (ECB-Z) antennae (log scale). Expression levels were measured using quantitative real-time PCR with EvaGreen, and relative expression was calculated using the $2^{-\Delta CT}$ method. Error bars represent the 95% confidence interval of three biological replications. Tukey's Honestly Significant Difference test was used to compare means. Genes were assigned different letters when the means of the normalized expression levels were statically different between sexes ($p=0.05$).

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The goal of this research was to identify genes expressed in the peripheral olfactory system that may contribute to male response to female sex pheromone components, and to identify candidate genes that have evolved specificity to the unique ACB sex pheromone. In this study, seven sex pheromone receptors from ACB and ECB were identified. In collaboration with Andrew Nichols and Charles Luetje (University of Miami), we found that the OR6 pheromone receptor is specific for the Z isomer of 11-14:OAc in ECB-Z. In addition, four previously unknown pheromone binding proteins were identified. For over a decade, it was supposed that ACB and ECB had one PBP (PBP1), and that there were no fixed differences in the amino acid sequence between ACB and ECB. PBPs 2 and 3 are likely carriers for pheromone components of ACB and ECB, as they are expressed at high levels in male antennae. ACB PBP3 has several amino acid changes compared to ECB that may affect its ligand binding specificity. Similarly, OR3 is divergent between ACB and ECB, and may also have amino changes that affect ligand binding specificity. Two previously unknown SNMPs were identified, and it was determined that these genes are not involved in the detection of specific pheromone ligands based on their comparable expression levels in male and female antennae, and their conserved amino acid sequences.

The discovery of these seven ORs, five PBPs, and two SNMPs in ACB and ECB generates new questions. PBPs may not have an effect on the ligand binding specificity

of odorant receptors that are already highly specific for a particular pheromone component (such as OR6 in ECB-Z), but they may affect the sensitivity. The abundance of PBPs in pheromone sensitive sensilla may help to explain why olfactory neurons in male antennae can be activated by as little as one molecule of pheromone ligand (Kaissling & Priesner, 1970). On the other hand, PBPs may affect the ligand binding specificity of ORs that respond more broadly to sex pheromone components, such as ORs 1, 3, and 5 in ECB-Z. In this scenario, combinatorial interactions between PBPs and generally responsive ORs could contribute to altered ligand binding specificity. Future studies will determine if ACB OR3 has evolved specificity for 12-14:OAc, and if the addition of PBP3 affects the ligand binding specificity.

This research provides the basis for several approaches to determine the specific amino acids involved in binding 12-tetradecenyl acetate sex pheromone components: Gene genealogies can be used to determine which amino acid polymorphisms are fixed between the two species. This will be accomplished by cloning and sequencing the ORs from different populations of ACB, ECB-E, and ECB-Z. Next, specific codons that are fixed between the species, and appear to be under positive selection, can be mutated. The mutant receptors can be expressed in *Xenopus laevis* oocytes and the electrophysiological responses measured to determine if the ligand binding specificity has been affected. Ultimately, receptors can be “back mutated” to ancestral specificities. *In vitro* functional assays incorporating ORs, PBPs, and SNMPs will be important, as proteins often function in complexes, and changes in sex pheromone detection may involve changes in more than one gene family.

The gene responsible for differences in female pheromone production has been characterized in ACB and ECB, but the many of the genes involved in male electrophysiological and behavioral responses are still unidentified. There is still some disagreement over whether these responses are encoded at the periphery of the olfactory system or further upstream, in the central nervous system. The results presented here suggest some possibilities for control at the peripheral level. While ACB and ECB are interesting from an evolutionary standpoint, understanding how sex pheromone reception contributes to speciation is also critical for effective control of these damaging pest species.

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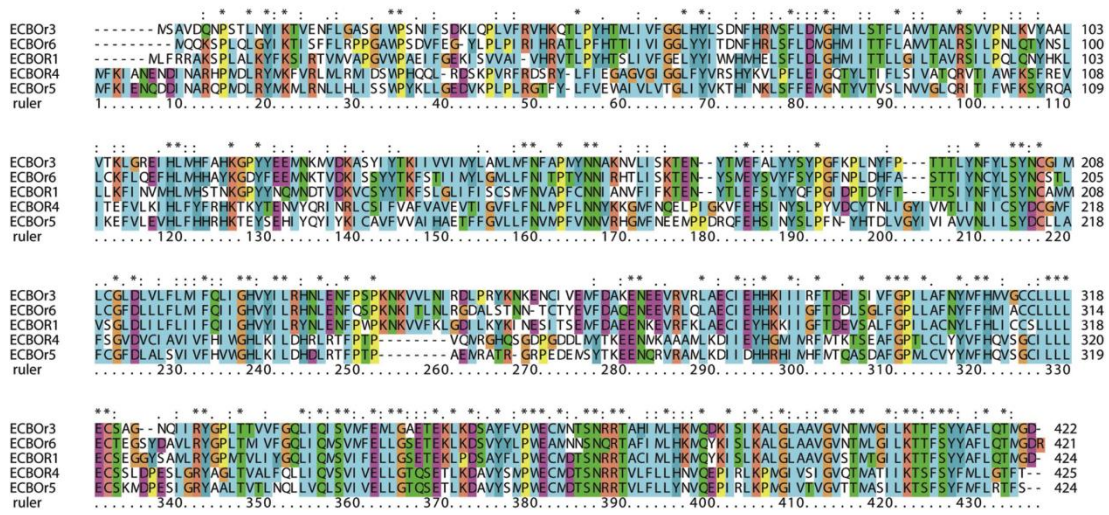
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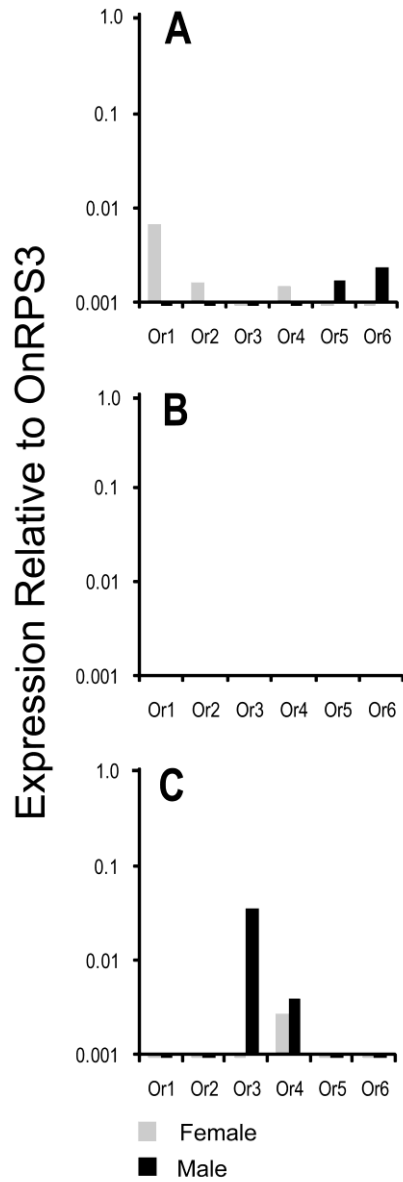
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APPENDICES

APPENDIX A

CHAPTER 2 SUPPLEMENTARY MATERIAL

Supplementary Figure 1. ClustalX alignment of *OnORs*1 and 3-6.



Supplementary Figure 2. Expression of *OnORs* 1-6 in three different tissues of adult male and female moths: A) heads (with mouthparts); B) legs; and, C) abdomens. Gene expression, determined by real-time quantitative PCR with SYBR green, is reported relative to the reference gene *OnRPS3*.

APPENDIX B

CHAPTER 3 SUPPLEMENTARY MATERIAL

Table S1. Primer sequences for rapid amplification of cDNA ends (RACE), quantitative real-time PCR (qPCR), and full-length cloning of open reading frames from Asian and European corn borers

PBP1	RACE	<i>CB-SNMP1</i> RACE R, GGGCATTGTGTCAGGGCTGTCACAGTAG
	qPCR	<i>CB-PBP1</i> F, GCATGAAGGTGCTGAACATC <i>CB-PBP1</i> R, CTAACACCTCGGCAACAATC
	Cloning	<i>CB-PBP1</i> UTR F, TCAACTGTGCCCCGA <i>CB-PBP1</i> UTR R, AAGCCTCGGTCCATTA
PBP2	RACE	<i>CB-PBP2</i> RACE F, AGGCTACGTGGTGACGAGTCGTGAGG <i>CB-PBP2</i> RACE R, ACCAGTTGGTGAGCCATAGCGTCGT
	qPCR	<i>CB-PBP2</i> F, CATGTGCTTCAAGACCGAGA <i>CB-PBP2</i> R, CTTCAATTCGGCCATCATCT
	Cloning	<i>CB-PBP2</i> UTR F, GTTAGATAAAATAACCGAAG <i>CB-PBP2</i> UTR R, CTCAGTCCTAAGTCCATAGCGAT
PBP3	RACE	<i>CB-PBP3</i> RACE F, ACCGAAGATGTGGCTGCCGAAGAC
	qPCR	<i>CB-PBP3</i> F, GGCTACACCCAATGAGGACA <i>CB-PBP3</i> R, AAACATCAGCTCGTGTTGG
	Cloning	<i>CB-PBP3</i> UTR F, TCTAGATGATGTGACCGAAG <i>CB-PBP3</i> UTR R, TGGATAAAAGAATAAACTACCTCA
PBP4	qPCR	<i>CB-PBP4</i> F, CATCACCATAACCGACGACT <i>CB-PBP4</i> R, GATCTCCTCCACCATGACCT
	Cloning	<i>CB-PBP4</i> UTR F, GGATTCAAAATCTGAAACAAGATG <i>CB-PBP4</i> UTR R, CAAGTTTCAAAGCCGACTC
PBP5	RACE	<i>CB-PBP5</i> RACE F, GCGAGATAGAAATGGTGCCC-GAAGC
	qPCR	<i>CB-PBP5</i> F, ACGAGTGCGAGAAGCAGAAG <i>CB-PBP5</i> R, GTCCAGTCGACCTGCTTGA
	Cloning	<i>CB-PBP5</i> F, ATGAAAGGGTTTGCG <i>CB-PBP5</i> UTR R, ATCCAGTTTATTGAAAAGATTTAAC
SNMP1	RACE	<i>CB-SNMP1</i> RACE F3, TCTTCATCGTGGTCCTCGATGTC
	qPCR	<i>CB-SNMP1</i> F, CTGGATAGCGACCCACAGTT <i>CB-SNMP1</i> R, GTTTGGCAACCATTGGAGTT
	Cloning	<i>CB-SNMP1</i> UTR F, ACTGCTGCGCTCACATGTAAC <i>CB-SNMP1</i> UTR R, AACGTCAGAAGTCGCCTGA
SNMP2	qPCR	<i>CB-SNMP2</i> F, GTACTGCGGACAGCTGAATG <i>CB-SNMP2</i> R, CAGTTTTTCCGGCACGTTAT
	Cloning	<i>CB-SNMP2</i> UTR F, TGGGATTTTGGTGTGTTTGTAG <i>CB-SNMP2</i> R, CTAATGCACTTTATTAACGTCAGAATTGGG
RpS3	qPCR	<i>CB-RpS3</i> F, TGGTAGTGTCTGGCAAGCTC <i>CB-RpS3</i> R, CGTAGTCATTGCATGGGTCT

CB (corn borer)

Table S2. Expression level of five pheromone binding protein (PBP) and two sensory neuron membrane protein (SNMP) genes in body parts of male and female *Ostrinia nubilalis* (ECB) Z-race relative to the reference gene ribosomal protein S3. Expression levels were measured using quantitative real-time PCR with SYBR green, and relative expression was calculated using the $2^{-\Delta CT}$ method.

	Head		Leg		Body	
	Male	Female	Male	Female	Male	Female
PBP1	0.01	0.00	0.00	0.00	0.00	0.00
PBP2	0.34	0.01	0.00	0.01	0.00	0.00
PBP3	0.27	0.03	0.00	0.02	0.00	0.00
PBP4	0.02	0.00	0.00	0.02	0.00	0.00
PBP5	0.00	0.01	0.00	0.00	0.00	0.00
SNMP 1	0.01	0.01	0.00	0.00	0.00	0.00
SNMP 2	0.13	0.02	0.02	0.04	0.01	0.00

PBP1 Hits

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Figure. S1. Nucleotide sequences identified by tBLASTn searches of assembled expressed sequence tag (EST) contigs with homology to known Lepidoptera pheromone binding proteins.

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ACB	PBP2	CAACATGGCTCTGACGACGCTATGGCTCACCAACTGGTTGACATTGTCCATGCTTGCAGAGAAGTCCGTCCCCGCCCAATGAAGACAAC	GCCTGATGGCGT								
ECB-E	PBP2	Q H G S D D A M A H Q L V D I V H A C E K S V P P N E D N C L M A	..G.....C.....								
ECB-Z	PBP2	Q H G S D D A M A H Q L V D I V H A C E K S V P P N E D N C L M A	..G.....C.....								
		410	420	430	440	450	460	470	480	490	
ACB	PBP2	TGGGCATCTCCATGTGCTTCAAGACCGAGATCCACAAGCTGAAC	TGGGCGCCGACACGAGCTGTGCTAGAGGAGATGATGGCCGAAATGAAGCAA								
ECB-E	PBP2	L G I S M C F K T E I H K L N W A P D H E L L L E E M M A E M K Q	A.....A.....A.....								
ECB-Z	PBP2	L G I S M C F K T E I H K L N W A P N H E L M L E E M M A E M K Q	A.....A.....A.....								
		10	20	30	40	50	60	70	80	90	100
ACB	PBP3	ATGTGGCTGCCGAAGACGCTTGTGGTGATGGCAGTGATGAGCTCAATGAGCGTGGTGGTGCAATTCATCACAACAGTGATGGGAGAAATGACAAAGAATT									
ECB-E	PBP3	M W L P K T L V V M A V M S S M S V V V H S S Q T V M G E M T K N	A.....G.....								
ECB-Z	PBP3	M W L P K T L V V M A V M S S M S V V V H S S Q T V M R E M T R N	A.....G.....								
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ECB-Z	PBP3	F I K A Y E V C A K E Y N L P E A T G S E L I N F W K E G H E L T T	TA.....C.....T.....C.G.....T.....								

		210	220	230	240	250	260	270	280	290	300
											
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		R E T G C A I L C M S T E L N L L D V Q G S V H R G N T V E F A K									
ECB-E	PBP3	AG.....A.....T.....									
		R E A G C A I L C M S T K L N L L D V Q G S V H R G N T V E F A K									
ECB-Z	PBP3	AG.....A.A.....T.....									
		R E A G C A I L C M S T K L N L L D V Q G S V H R G N T V E F A K									
		310	320	330	340	350	360	370	380	390	400
											
ACB	PBP3	CACCATGGCTCTGACGACGCAATGGCTACCAAGTGGTAGACATTCTCCATGCTTGCGAAAAGGCTACACCCAACGAGGACAAGTGCATGTTGGCGTTGA									
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ECB-E	PBP3	T.....A.									
		H H G S D D A M A H Q V V D I L H A C E K A T P N E D K C M L A L									
ECB-Z	PBP3	T.....A.									
		H H G S D D A M A H Q V V D I L H A C E K A T P N E D K C M L A L									
		410	420	430	440	450	460	470	480	490	
											
ACB	PBP3	GCATCGCCATGTGCTTCAAGGCCGAGATACACAAGCTGGACTGGGCACCCAACAACGAGCTGATGTTTGAGGAGTTGGTGTTAGATATGTGGAATTCA									
		S I A M C F K A E I H K L D W A P N N E L M F E E L V L D M W N S									
ECB-E	PBP3	C.....CC.....G									
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ECB-Z	PBP3	C.....CC.....G									
		S I A M C F K A E I H K L D W A P N H E L M F E E L V S D M W N S									
		10	20	30	40	50	60	70	80	90	100
											
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ECB-E	PBP4	A.....G.....A.....T.....G.....									
		M A D A T K W R V A A I L V I C F A V K L N T V M S S E E V M T K									
ECB-Z	PBP4	A.....G.....T.....G.....									
		M A D A T K W R I A A I L V I C F A V N L N T V M S S E E V M T K									


```

      ....
ACB  PBP4  GGCT
      A
ECB-E  PBP4  ....
      A
ECB-Z  PBP4  ....
      A

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ECB-E  PBP5  .....A.....G..C..A.....
      M K G F A G I P V T L M L V L I G V S E I E M V P E A M K Q L T G
ECB-Z  PBP5  .....G..C..A.....
      M K G F A G V P V T L M L V L I G V S E I E M V P E A M K Q L T G

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ECB-E  PBP5  .....G.....C.....
      G F L K V L D Q C K K E L N L S D G V I S D L Y H L W K E E Y D Q I
ECB-Z  PBP5  .....C.....
      G F L K V L D Q C K K E L N L S D G V I S D L Y H L W K E E Y D Q I

      210     220     230     240     250     260     270     280     290     300
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ECB-Z  PBP5  .....G.A..T.....A.....G
      S R D A G C V I H C M S Q K L E L V G G D G R M H H V N I K D F A

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		310	320	330	340	350	360	370	380	390	400	
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ECB-E	PBP5	L K H G A G D E I A T Q L V T L A H E C E K Q K A A I E D D C E R										
		C.....T.....T.....T.....										
ECB-Z	PBP5	L K H G A G D E I A T Q L V T L A H E C E K Q K A S I E D D C E R										
		G.....T.....A.....										
		L K H G A G D E I A T Q L V T L A H E C E K Q K A A I E D D C E R										
		410	420	430	440	450	460	470	480	490		
ACB	PBP5											
		CCCTGGAGATGTCCAAGTGCTTCGCGAGCGACGTCAAGCAGGTCGACTGGACGCCCAAGATGGAGGTCATCATCACCGAAGTTATAGAAGTT										
ECB-E	PBP5	T L E M S K C F R S D V K Q V D W T P K M E V I I T E V I E V										
		T.....										
ECB-Z	PBP5	T L E M S K C F R S D V K Q V D W T P K M E V I I T E V I E V										
												
		T L E M S K C F R S D V K Q V D W T P K M E V I I T E V I E V										
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ECB-Z	SNMP1	M Q L Q K P L K I G L G M M G A G L F G I I F G W V L F P V I L K										
												
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		GCCAGTTGAAGAAGGAAATGGCGTTGTGCGAAAAAACCGACGTGCGAGCCATGTGGGAGAAGATTCCCTTCGCCCTGGACTTCAAAGTGTACATGTTCAA										
ECB-E	SNMP1	S Q L K K E M A L S K K T D V R A M W E K I P F A L D F K V Y M F N										
												
ECB-Z	SNMP1	S Q L K K E M A L S K K T D V R A M W E K I P F A L D F K V Y M F N										
												
		S Q L K K E M A L S K K T D V R A M W E K I P F A L D F K V Y M F N										

	610	620	630	640	650	660	670	680	690	700
ACB SNMP1	 TGTACTGCTTTGAAGAAAGAAGGCGCTACTGGGATGACTTTTGAACCTAACAATCAGTTTAGGTTCTCGTTGTTTGGGAATGCGAAACGGCACCATAGATC C T A L K K E G A T G M T F E P N N Q F R F S L F G M R N G T I D									
ECB-E SNMP1	..C.....A.....C..... C T A L K K E G A T G M T F E P N N Q F R F S L F G M R N G T I D									
ECB-Z SNMP1	A..T.....C..T..... C T A L K K E G A T G M T F E P N N Q F R F S L F G M R N G T I D									
	710	720	730	740	750	760	770	780	790	800
ACB SNMP1	 CCCACGTGGTCACCGTGCGCAGAGGCATCAAGAACGTCATGGATGTGGGAAAAGTCATCGCCATAGATGGGAAGACAGAGCAGGACGCTCTGGAGGGACAA P H V V T V R R G I K N V M D V G K V I A I D G K T E Q D V W R D K									
ECB-E SNMP1	A..A..A.....C.....G..... P H V V T V R R G I K N V M D V G K V I A I D G K T E Q D V W R D K									
ECB-Z SNMP1	T.....C.....G..... P H V V T V R R G I K N V M D V G K V I A I D G K T E Q D V W R D K									
	810	820	830	840	850	860	870	880	890	900
ACB SNMP1	 ATGCAACGAGTTCTGAAGGAACGGACGGCAGGCTCTCCACCTTTCTCTCACTGAGAAGGACAACCTTGAGTCGTTCTCTGATGACCTTTGCAGGTCTTTTC C N E F E G T D G T V F P P F L T E K D N L E S F S D D L C R S F									
ECB-E SNMP1	A..T.....G.....A... C N E F E G T D G T V F P P F L T E K D N L E S F S G D L C R S F									
ECB-Z SNMP1	T.....G.....A... C N E F E G T D G T V F P P F L T E K D N L E S F S G D L C R S F									
	910	920	930	940	950	960	970	980	990	1000
ACB SNMP1	 AAGCCCTGGTACCAAAAGAAGACCTCCTACAGAGGGATCAAGACAAACCGATACGTAGCCAACATTGGGGACTTTGCCAATGATCCTGAACTGCAGTGCCT K P W Y Q K K T S Y R G I K T N R Y V A N I G D F A N D P E L Q C									
ECB-E SNMP1	G.....C.....C..... K P W Y Q K K T S Y R G I K T N R Y V A N I G D F A N D P E L Q C									
ECB-Z SNMP1	A..A.....G.....C.....C..... K P W Y Q K K T S Y R G I K T N R Y V A N I G D F A N D P E L Q C									

	1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
ACB SNMP1										
	CGTTGGGTTTCGTGGGCCTTGTTGGCTCTCTGGTGTACCACTTCAAGGGTAAAATGATCAACTTCGCACCTTTCCCCGAGCTCCGCCAGGTGACGAAGGTC									
ECB-E SNMP1	V G F V G L V G S L V Y Q F K G K M I N F A L S P S S A Q V T K V									
	...C.....C.....									
	V G F V G L V G S L V Y Q F K G K M I N F A L S P S S A P V T K V									
ECB-Z SNMP1	...C..A....A..G.....G.....C.....C.....									
	V G F V G L V G S V V Y Q F K G K M I N F A L S P S S A P V T K V									
	1510	1520	1530	1540	1550	1560	1570	1580		
ACB SNMP1										
	AATCCGGAGATTAAACCAGCAAAACCAGCCGAAGGACATAAGCATTATCGGGGAGTCCCAGAACCCCCCAAAGTGGACATGTAA									
ECB-E SNMP1	N P E I N Q Q N Q P K D I S I I G E S Q N P P K V D M *									
	G.....									
ECB-Z SNMP1	N P E I N Q Q N Q P K D I S I I G E S Q N P P K V D M *									
										
	N P E I N Q Q N Q P K D I S I I G E S Q N P P K V D M *									
	10	20	30	40	50	60	70	80	90	100
ACB SNMP2										
	ATGCTCGGGAAACACACGAAGTTGTTTTTCGGCGTATCGCTCGTTGCTTTGATTGTGTCTGTGATTTTGGCGGCCTGGGGCTTTCCTAAGATTGTCAGCA									
ECB-E SNMP2	M L G K H T K L F F G V S L V A L I V S V I L A A W G F P K I V S									
	A.....G.....A.....A.....									
ECB-Z SNMP2	M L G K H T K L F F G V S L V A L I V S V I L A A W G F P K I V S									
	G.....A.....A.....									
	M L G K H T K L F F G V S L V A L I V S V I L A A W G F P K I V S									
	110	120	130	140	150	160	170	180	190	200
ACB SNMP2										
	AACAAATTCAAAGAACATCCAGATCGACAACCTCGTCGGTGATGTTTCGAGAAGTGGCGGAAGATACCGATGCGGCTCACGTTCAACGTATACGTGTTCAA									
ECB-E SNMP2	K Q I Q K N I Q I D N S S V M F E K W R K I P M P L T F N V Y V F N									
	A.....									
ECB-Z SNMP2	K Q I Q K N I Q I D N S S V M F E K W R K I P M P L T F N V Y V F N									
	A.....A.....									
	K Q I Q K N I Q I D N S S V M F E K W R K I P M P L T F N V Y V F N									

	210	220	230	240	250	260	270	280	290	300
ACB SNMP2										
	CGTGACCAACGTGGAAGACGTGAACAACGGCGCCAAAGCCCCGACTGCAGCAGATAGGGCCATACGCTTACAAAGAATACCGCGAGCGAACAGTACTCGGG									
ECB-E SNMP2	V T N V E D V N N G A K P R L Q Q I G P Y A Y K E Y R E R T V L G									
										
	V T N V E D V N N G A K P R L Q Q I G P Y A Y K E Y R E R T V L G									
ECB-Z SNMP2										
	V T N V E D V N N G A K P R L Q Q I G P Y A Y K E Y R E R T V L G									
	310	320	330	340	350	360	370	380	390	400
ACB SNMP2										
	TACGGGGACAACGACACAGTATCCTATACCTGAAGAAGACCTTCATCTTCGACCAGGAGGCGTCAGGCTTACTAAGCGAGGACGACGAAGTCACTGTCA									
ECB-E SNMP2	Y G D N D T V S Y T L K K T F I F D Q E A S G L L S E D D E V T V									
										
	Y G D N D T V S Y T L K K T F I F D Q E A S G S L S E D D E V T V									
ECB-Z SNMP2										
	Y G D N D T V S Y T L K K T F I F D Q E A S G S L S E D D E V T V									
	410	420	430	440	450	460	470	480	490	500
ACB SNMP2										
	TCCACTTCTCTATATGGCTGCTATCCTGACAGTGAACGACATGATGCCAGTATAACAGGCGTGGTCAACGGAGCTTTGGAGCAGTTCTTCACCAACCT									
ECB-E SNMP2	I H F S Y M A A I L T V N D M M P S I T G V V N G A L E Q F F T N L									
										
	I H F S Y M A A I L T V N D M M P S I T G V V N G A L E Q F F T N L									
ECB-Z SNMP2										
	I H F S Y M A A I L T V N D M M P S I T G V V N G A L E Q F F T N L									
	510	520	530	540	550	560	570	580	590	600
ACB SNMP2										
	GACTGACCCTTTCTTAAGGGTCAAGGTCAAGGATCTCTTCTTCGATGGCGTCTACGTCAACTGTGCGGGAAACCATTCTGCTTTGGGTCTCGTCTGCGGC									
ECB-E SNMP2	T D P F L R V K V K D L F F D G V Y V N C A G N H S A L G L V C G									
										
	T D P F L R V K V K D L F F D G V Y V N C A G N H S A L G L V C G									
ECB-Z SNMP2	...C..T.....									
	T D P F L R V K V K D L F F D G V Y V N C A G N H S A L G L V C G									

	610	620	630	640	650	660	670	680	690	700
ACB SNMP2	AAACTGAAGGCTGACGCTCCCCAGACAATGCGCCCTGCTGGAGATGGCAATGGGTTTACTTCTCGATGTTTCTCATATGAACAGAACAGAATCAGGAC									
ECB-E SNMP2	K L K A D A P Q T M R P A G D G N G F Y F S M F S H M N R T E S G									
ECB-Z SNMP2	K L K A D A P Q T M R P A G D G N G F Y F S M F S H M N R T E S G									
	710	720	730	740	750	760	770	780	790	800
ACB SNMP2	CATACGAGATGATCCGAGGGCGCGAGAATATTAAAGAGCTCGGTCACATCATATCATACAAAGGCAAGAGCTTCATGAAGAACTGGGGCAACGATATGTA									
ECB-E SNMP2	P Y E M I R G R E N I K E L G H I I S Y K G K S F M K N W G N D M Y									
ECB-Z SNMP2	P Y E M V R G R E N I K E L G H I I S Y K G K S F M K N W G N D M Y									
	810	820	830	840	850	860	870	880	890	900
ACB SNMP2	CTGCGGACAGCTGAATGGGTGAGACGCGTCCATCTTCCCGCCGATTGATGAAAATAACGTGCCGGAACCTGTACACCTTTGAGCCGAGGTTTGCAGA									
ECB-E SNMP2	C G Q L N G S D A S I F P P I D E N N V P E K L Y T F E P E V C R									
ECB-Z SNMP2	C G Q L N G S D A S I F P P I D E N N V P G K L Y T F E P E V C R									
	910	920	930	940	950	960	970	980	990	1000
ACB SNMP2	TCACTGTACGCAAGCCTTGTTGGAAAGAGTTTCGATTTTCAACATGAGCGCGTACTACTACGAGATTTCAGCGACGCGTTGGCATCTAAGAGCGCCAATC									
ECB-E SNMP2	S L Y A S L V G K S S I F N M S A Y Y Y E I S S D A L A S K S A N									
ECB-Z SNMP2	S L Y A S L V G K S S M F N M S A Y Y Y E I S S D A L A S K S A N									

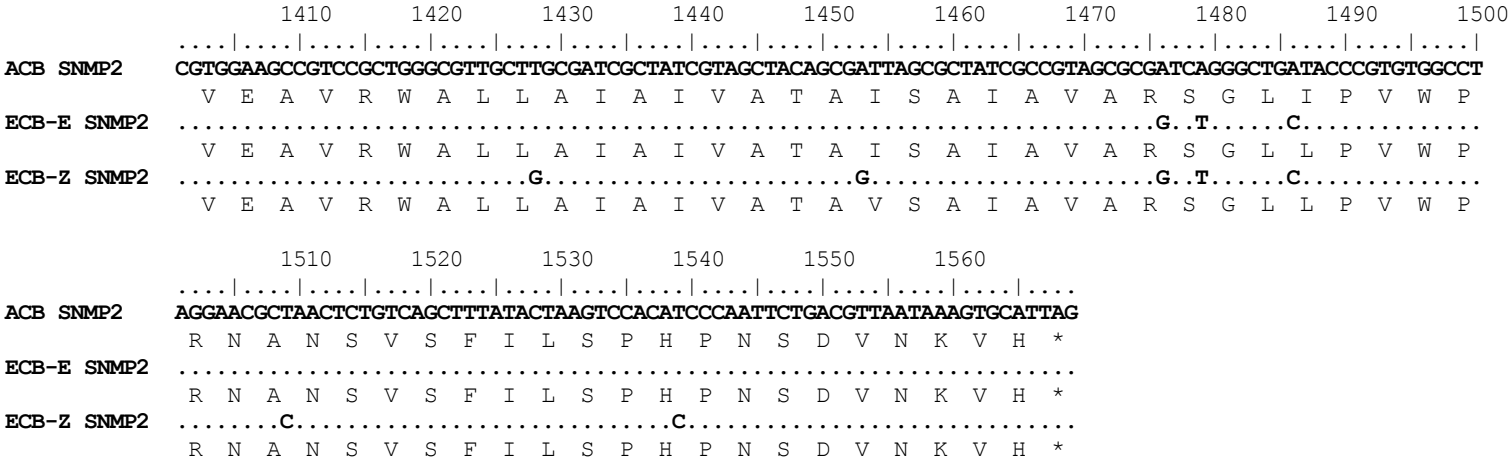


Figure S2. Nucleotide and amino acid sequence comparisons of *Ostrinia furnacalis* (ACB) and *Ostrinia nubilalis* (ECB) pheromone binding protein (PBP) and sensory neuron membrane protein (SNMP) genes. Exact nucleotide matches are indicated by dots.

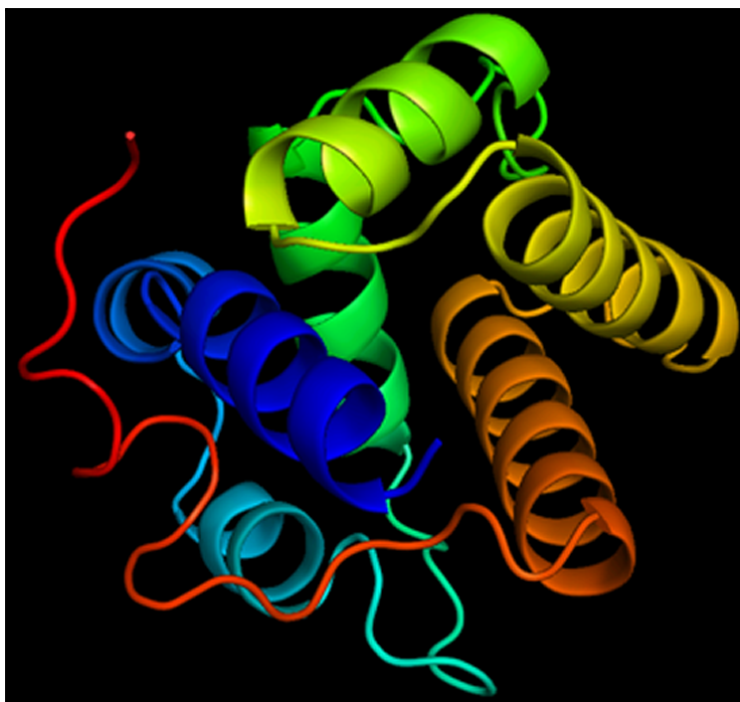


Figure S3. Three dimensional structure of *Ostrinia furnacalis* (ACB) pheromone binding protein 3. PBP3 was modeled using a consensus based approach and visualized using PyMol.

APPENDIX C

CHAPTER 4 SUPPLEMENTARY MATERIAL

Table S1. Forward and reverse primers for amplifying full length odorant receptors (OR) from Asian and European corn borers.

OR1 Fl F	CATGTCTAGATTTGCTAATCTCCCATTGTT
OR1 Fl R	GATCGGAATTCTTTGTAATGTTATTCAGAA
OR2 Fl F	CTACGAATTCGGATGACCAAAGTGAAAGCTCAGG
OR2 Fl R	ACTGTCTAGACTACTTCAGTTGTACCAAAACC
OR3 Fl F	TAGAATTCATGTCCGCCGTTGACCAAAA
OR3 Fl R	TCAAGCTTCTAATCTCCCATTGTTTGCA
OR4 Fl F	GAAACAAAAAGAATTAATAAAAATG
OR4 Fl R	TTAAGTGAATGTTCCAGTAGCATG
OR5 Fl F	TGTGAACGAATGTGAGAAATGTTTA
OR5 Fl R	CGAAGCTTTTAGCTGAACGTTGCAAGA
OR6 Fl F	TAGGATCCCATCAAACAAAATGCAACAGAAATC
OR6 Fl R	TATCTAGATCATTATCTATCTCCCATTGTTTG
OR7 Fl F	CGGGGAGCGCAAAGAATATAC
OR7 Fl R	GCTGCGGACATTTGCTACTGATGATTAA
OR8 UTR F1	ACTTCTTTTCTAGTTGCTACTTTCCGG
OR8 UTR R1	TTAAGTGTCGTTGAACTTCAAAAGCTA

OR1 Group A

OnubOR5d, AB508314; OovaOR5, AB508320; OscaOR5, AB508296; OlatOR5a, AB508308; OfurOR5, AB508302

>ECBZ_OR1_Clone15

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CACACGAGTCTCATTGTCTTCCGGGAGCTTTACTACATATGGATGCACAT
GCATGAGTTAAGTTTTCTCGATTTGGGACATATGATTATAACAACCCTTT
TAGGAATACTAACAGCGGTCCGTAGTATACTACCACAATTGCAGAAC
TATCATAAGTTGCTGCTAAAATTTATAAATGTGATGCATTTGATGCATTC
CACAAATAAAGGACCGTATTACAATCAGATGAACGATACTGTGACAAAAG
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CCCATTTTAGCTTGTA ACTATTTGTTTCATTTGATTTGCTGTAGCTTGTT
ACTACTGGAATGTTCCGAAGGTGGATATAGTGCAATGTTACGTTATGGGC
CTATGACTGTACTCATATACGGTCAACTGATTCAAATGTCAGTTATTTTT
GAGTTGTTGGGTTCCAGAGACTGAAAAGTTGCCAGATT CAGCTTACTTCCT
GCCGTGGGAGTGCATGGACACCAGCAACCGGCGGACGGCGTGCATCATGC
TGCACAAGATGCAGTACAAGATCAGCCTCAAGGCGCTGGGGCTGGCGGCC
GTCGGCGTCAGCACCATGACCGGGATATTGAAGACAACATTTTCATACTA
CGCATTTCTGCAAACAATGGGAGAT

>ECBE_OR1_Clone1-12

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 CGCATTTCTGCAAACAATGGGAGAT

OR Group B

OlatOR5b, AB508309; OzagOR5, AB508329; OzagOR6, AB508330; OfurOR6,
 AB594451; OzeaOR6, AB508336; OnubOR6, AB508315; OseaOR6, AB594449

>ACB_OR1_clone24-3

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OR3 Group

OscaOR4, AB508294; OscaOR4z, AB508295, OpalOR4, AB508324; OovaOR4,
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 OzagOR4, AB508328; OnubOR4, AB508313

>ACB_OR3_Clone12

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OR4 Group

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OR5 Group

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OR7 Group

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OR8 Group

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Figure S2. Nucleotide sequences used in phylogenetic and sequence analyses. The nucleotide sequences of twenty three odorant receptor (OR) clones from Asian (ACB) and European (ECB-E, ECB-Z) corn borer cDNA are given along with the GenBank accession numbers of the nucleotide sequences published in Miura et al. (2010).