Lipid Interactions Involved with Pheromone Binding Protein Function in *Lymantria dispar*

by

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Abstract

The mechanism for insect olfaction is not fully understood. Pheromone-binding proteins (PBPs) are hypothesized to be critical for proper chemical signaling in insects. Using a pheromone-binding assay I have shown that endogenous fatty acids, which bind *Lymantria dispar* PBP1 and PBP2, cause increased affinity for pheromone. Using circular dichroism spectroscopy, I have shown that the structures of PBP1 and PBP2 are altered by the binding of endogenous ligands. I propose that the altered conformation and increased affinity are related.

The large concentration of fatty acids found in the sensillar lymph, where PBP1 and PBP2 have access to these ligands, is well above the critical micellar concentration. With various analytical techniques, most importantly of which was mass spectrometry, I identified many lipid components found in *L. dispar*'s lipidome. Analysis has provided insight into lipid bilayer preservation in the presence of endogenous fatty acid micelles. This thesis work provides evidence that lipids play an integral role in PBP function and proposes that they may be critical for olfaction.

Keywords: Insect olfaction; Pheromone-Binding Proteins; Fatty Acids; Membrane glycosphingolipids; disparlure; *Lymantria dispar*
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<th>Definition</th>
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<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>cVA</td>
<td>Cis-vaccenyl acetate</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
<tr>
<td>IP(1,4,5)P₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ORN</td>
<td>Olfactory receptor neuron</td>
</tr>
<tr>
<td>MOE</td>
<td>Molecular Operating Environment</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/Mass spectrometry</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>ESI-TOF</td>
<td>Electrospray Ionization Time-of-Flight</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N,O-Bis(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>PBP</td>
<td>Pheromone binding protein</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>SNMP</td>
<td>Sensory neuron membrane protein</td>
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1. General Introduction

1.1. Lipids

The word “Lipids” has traditionally referred to compounds that are soluble in non-polar solvents (Blanksby and Mitchell 2010). A more sophisticated definition has been proposed, whereby lipids are defined based on their route of biosynthesis (Figure 1.1) (Fahy, Subramaniam et al. 2005; Fahy, Subramaniam et al. 2009). As an example of the complexity and diversity of lipid structures in a single organism, the yeast lipidome was found to contain 342 distinct lipid species (Ejsing, Sampaio et al. 2009). Lipids encompass too broad a class of molecules to discuss in completeness. The simplest lipids are the fatty acids and most other lipids, including triacylglycerols, diacylglycerols, phospholipids, sphingolipids, wax esters and fatty alcohols are derived from fatty acids. Sterols and terpenes are normally derived from isopentenyl pyrophosphate (Figure 1.1).

Membrane lipids encompass mostly phospholipids, with some other minor but critical components such as sphingolipids, sterols, and various glycolipids (Figure 1.2). The general layout of the biological membrane is a lipid bilayer composed mostly of phospholipids, with a hydrophobic interior and polar headgroups oriented towards the exterior aqueous phase. Any glycosylated lipid will have its carbohydrates on the exterior of the extracellular half of the lipid bilayer (Luckey 2008). Lipid bilayers are highly dynamic environments with components able to diffuse laterally, rotationally or transversely where a lipid component will flip from one leaflet to the other (Luckey 2008). It is important to realize as well that inner and outer leaflet compositions do not mirror each other, a phenomenon referred to as lipid asymmetry (Luckey 2008). In fact even the same phospholipid may have different acyl chains attached between leaflets (Luckey 2008). The fluid mosaic model applies in general to lipid bilayers with the exception of
membrane microdomains, colloquially known as lipid rafts. Microdomains have properties that differ from the bulk membrane. For example, they often consist of clusters of cholesterol and sphingolipids and these regions are often several angstroms thicker than the bulk membrane. Biomembranes have evolved to contain hundreds of different lipid molecules which interact dynamically with the proteins embedded in the membrane and those located peripherally.

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**Figure 1.1**

Specific examples of the different lipid categories as proposed by Fahy et al. (Fahy, Subramaniam et al. 2009). 1 Fatty acid, 2 glycerolipid, 3 glycerophospholipid, 4 sphingolipid, 5 sterol, 6 prenol, 7 polyketide
1.1.1. The importance of lipids in biochemistry

Chemical signaling and biomembrane dynamics are two of the high-interest areas of research involving lipids. For example, the hydrolysis of phosphatidylinositol-4,5-bisphosphate \( \text{PI}(4,5)\text{P}_2 \) (Fig 1.3) produces the second messenger precursors diacylglycerol (DG), inositol-1,4,5-trisphosphate (IP\(_{1,4,5}\)P\(_3\)) and arachidonic acid (AA) (Wenk 2005). Interruption or interference with lipid metabolism has been linked to many metabolic and neurological diseases (Wenk 2005; Wenk 2010).

Figure 1.2

(A) Lactosylceramide, a common neutral glycolipid found in milk. (B) Fuca1-2Galβ1-3GalNAcβ1-4(NeuAca2-3) Galβ1-4Glcβ Core Ceramide; an example of an acidic glycosphingolipid belonging to the ganglioside family of glycosphingolipids.
1.2. Insect Olfaction

The first moth pheromone was characterized from the extract of 500 000 specimens in 1959 (Butenandt, Beckmann et al. 1959; Hansson 2002). At about the same point in time, electrophysiological experiments were first being used to monitor antennal activity in the presence of odorants (Schneider and Boeckh 1962). Since then, much of the olfactory receptor family of *Drosophila melanogaster* has been characterized (Clyne, Warr et al. 1999). Olfactory processing begins when the membrane surrounding the olfactory receptor dendrite becomes depolarized sending an electrical current down the dendrite to the body of the olfactory receptor neuron (ORN). The signal then proceeds to the antennal lobe of the central nervous system via the ORN’s axon (Hansson 2002). The antennal lobe is segregated into sections called glomeruli. Male insects have a large portion of the antennal lobe dedicated to glomeruli.
called the macroglomeruler complex (MGC), not present in females (Hansson 2002). Based on activity-dependant staining of neurons, it was determined that different glomeruli in the MGC respond to different components of the pheromone blend targeting different ORN’s (Hansson, Ljungberg et al. 1992). This led to the conclusion that all ORN’s expressing a specific receptor which is activated by specific odorant converge to the same glomerulus in the antennal lobe. The idea behind specific combinations of odorants producing a completely different “smell” than the sum of its parts is likely based on glomeruli activity pattern recognition by higher processing centres like the cortex (Hansson 2002). The cortex contains a separate neuropil region called mushroom bodies (MB) as well as the protocerebrum which have been shown to be involved in processing olfactory information in insects (Hansson, Ljungberg et al. 1992; Strausfeld, Hansen et al. 1998).

With insects, most of the research into understanding their exquisite olfactory system has been approached from a genetic and proteomics angle. With the marked importance lipids have shown with respect to chemical signaling, it was our goal to explore lipid interactions which may be integral to the signal transduction pathway in insects. The extreme sensitivity and selectivity of insect olfaction has led to interest in the mechanism. It has been proposed that with an understanding of the mechanism, drug design principles could be applied to develop more efficient and selective chemicals for pest management (Raffa and Raffa 2011). There is also work that suggests there are similarities between chemical signaling in insects and hormone/neurotransmitter signaling in mammals (Mullin, Chyb et al. 1994; Vosshall 2008). The similarity is believed to be in the neuroanatomy processing of odorants concluded by the observation that in Drosophila melanogaster, olfactory neurons linked to the same receptors converged to the same glomeruli. Specific patterns in glomeruli activity are related to odorant recognition in both the olfactory bulb of vertebrates and the antennal lobe of insects (Vosshall 2008).
1.2.1. **Current Insect Control Strategies**

There are many different pest control agents currently available. Most commercially available pesticides contain a mixture of several different chemicals acting by different mechanisms. They are effective at killing pests but are not without some serious side effects. Acetylcholinesterase inhibitors like the organophosphate malathion (Figure 1.4) binds the enzyme acetylcholinesterase preventing it from hydrolyzing the neurotransmitter acetylcholine causing it to build up and lead to paralysis and eventual death.

![Chemical structures]

**Figure 1.4**  
*Currently used pest control chemicals employing different modes of action. Allethrin is a synthetic pyrethroid; Malathion and Carbofuran are AcetylCholine Esterase inhibitors; Hexaflumuron is a chitin synthesis inhibitor.*

Carbamates like carbofuran also inhibit acetylcholinesterase, but do so reversibly where as organophosphates do so irreversibly (Figure 1.5). Both of these chemicals are non-selective and will cause death in any insect species in contact (Raffa and Raffa 2011). Sodium channel modifiers like allethrin prevent sodium channels from closing leading to hyperpolarization of the membranes and paralysis. Chitin synthesis inhibitors such as hexaflumuron prevent larvae from molting as they interfere with chitin biosynthesis, the N-acetylglucosamine (GlcNAc) polysaccharide which is the major component of the insect exoskeleton. Both these approaches are also non-selective (Raffa and Raffa 2011). There are also biological strategies such as juvenile hormones, microbial pesticides, and insecticidal viruses which are more selective than the other synthetic chemicals but take considerable cost and time to produce (Raffa and Raffa
2011). Because of this, studies into pheromone olfaction are the next step in discovering more selective pest control agents.

Figure 1.5
I. Mechanism for phosphorylation of an AChE serine hydroxyl resulting in inhibition of the enzyme (Fukuto 1990). II. Mechanism for carbamylation of AChE serine hydroxyl resulting in inhibition of the enzyme. Organophosphates are said to inhibit irreversibly compared to carbamates as the half-life of enzyme recovery from serine phosphorylation can range from hours to days, if at all, depending on substituents. The half-life for enzyme recovery from carbamylation is approximately 30 min (Fukuto 1990).

1.2.2. Proposed Models For Insect Olfaction

Despite the similarities in neuroanatomy there are several marked differences in the olfactory transduction process between insects and vertebrates. Insects have seven helix transmembrane receptors similar to G-protein coupled receptors, however the insect receptors have topology that is reversed in comparison to typical GPCR’s such as rhodopsin (Ha and Smith 2009). In addition, it was discovered that insect receptors are capable of forming ligand-gated ion channels which can depolarize the membrane in the absence of G-protein second messengers (Sato, Pellegrino et al. 2008; Ha and Smith 2009). This is not to say that GPCR’s should be disregarded completely with respect to
insect olfaction. Cyclic nucleotides and phosphoinositides, both trademarks of second messenger systems, have been detected concomitantly with odorant detection in insect olfactory neurons (Zufall and Hatt 1991; Wicher, Schafer et al. 2008). There is conflicting evidence on the involvement of cyclic nucleotides. In one study, the presence of cyclic nucleotides was shown to increase as a function of tuning receptor expression (Wicher, Schafer et al. 2008). Tuning receptors are one of the many membrane receptors hypothesized to be required for olfaction. They are referred to as tuning receptors because they are believed to tune co-receptor or83b, meaning that or83b by itself does not show odorant-dependant activation but requires its co-receptor (Sato, Pellegrino et al. 2008; Wicher, Schafer et al. 2008). The presence of these tuning receptors was hypothesized to activate a cyclase which leads to cyclic nucleotide production. This idea stemmed from the fact that cyclic nucleotides showed the ability to modulate or83b conductance. From these studies, it was proposed that olfaction may occur by both direct ligand-gated channels at high odorant concentrations and via a cyclic nucleotide second messenger system at lower concentrations (Ha and Smith 2009). However, work with or83b and its tuning receptor showed that inhibitors of G-proteins, adenylyl cyclase, guanylyl cyclase, phosphodiesterases, and phospholipase Cβ (all of which are part of the GPCR machinery) did not affect Ca²⁺ conductance (Smart, Kiely et al. 2008).

Both models, whether it be the G-protein model producing cAMP triggering or83b activation or direct ligand-gated activation of or83b by odorants, suggested that it was the odorant molecule which triggered the signal transduction cascade by binding one of the olfactory receptors. In 2008, Dean Smith and his group published results which strongly indicate that ligand bound pheromone binding proteins (PBP’s) cause activation of olfactory receptors (Jin, Ha et al. 2008).

### 1.2.3. Insect Pheromone Binding Proteins

Pheromone binding proteins were first identified in 1981 (Vogt and Riddiford 1981) as extracellular small proteins (~16kDa) having high affinity for pheromones. Pheromones are small molecules which are used for chemical communication within the same species. Their structure is highly specific to each species even though several
closely related species make use of pheromones which are very nearly identical (Figure 1.6). *Lymantria dispar* (gypsy moth) and *Lymantria monacha* (nun moth) have evolved different stereoisomers of the same molecule as their respective pheromones. The former uses (7R,8S)-7,8-epoxy-2-methyl-octadecane, commonly referred to as (+)-disparlure, while the latter uses (+)-disparlure, but adds a large amount of (-)-disparlure, which may be to fend off *L. dispar* males (Miller, Mori et al. 1977). *L. monacha* males do not respond well to (-)-disparlure, whereas *L. dispar* males do not fly upwind if (+)-disparlure is contaminated with more than 1% of (-)-disparlure. These insects are able to distinguish enantiomers while maintaining astonishing sensitivity which may be approaching single-molecule response (Kaissling and Priesner 1970). This extreme sensitivity and selectivity is believed to come from the interaction pheromone binding proteins, receptors and other proteins known to be necessary for olfaction, as described below for *D. melanogaster*.

![Figure 1.6](image)

*Figure 1.6*

Pheromones belonging to different insect species. (1) (+)-disparlure, (2) (-)-disparlure (3) Z-vaccenyl acetate (4) bombykol (5) (s)-olean
Dean Smith and his group have shown strong evidence which supports pheromone-bound PBP’s as the ligand for receptor activation and not pheromone by itself (Laughlin, Ha et al. 2008). They hypothesize that the conformational shift in LUSH (the PBP belonging to D. melanogaster) caused by cVA (Z-vaccenyl acetate; D. melanogaster’s native pheromone) should allow for single-molecule sensitivity (Ha and Smith 2009). When LUSH was knocked out, cVA sensitivity of the T1 neurons was removed (Xu, Atkinson et al. 2005). If cVA itself was the ligand for receptor activation then this should not have happened. In fact, after having determined the structure of cVA bound LUSH, Laughlin and his colleagues were able to create the mutant LUSH D118A which was able to activate the T1 neurons in the absence of any pheromone. Further, they showed that the co-receptors SNMP (sensory neuron membrane protein) and or67d are required for T1 activation as LUSH D118A had no effect in experiments where those receptors were knocked out (Laughlin, Ha et al. 2008). Clearly, they had shown that it was the pheromone binding protein with pheromone bound triggering membrane depolarization.

1.2.4. *Lymantria dispar*

The target species for this study has been *Lymantria dispar*, commonly referred to as the gypsy moth but will be referred to as *L. dispar* hence forth. The two main reasons for selecting this species are as follows: 1) the 5th instar larva of *L. dispar* is an infamous defoliator of hard wood trees and being non-native to North America it has few natural predators (Raffa and Raffa 2011). With a clear understanding of the pheromone olfaction process, the door to developing possible ligands which could out-compete (+)-disparlure would open a little wider. This would pave the way for insect control reagents which would be much more selective than current pest control strategies. 2) Although there has been a great deal of insect olfaction research carried out with *D. melanogaster*, lepidopterans have also been a focal point for much research with an emphasis on the pheromone binding proteins of *Bombyx mori*, *Manduca sexta* and in our group *L. dispar*. 
The life cycle of *L. dispar* includes 5 instars in larval development (the larvae will go through 4 molts before reaching the final instar). The 5th instar larvae are voracious feeders in preparation for pupation and metamorphosis. The adult female is flightless and must rely on attracting males by emitting plumes of pheromone down-wind (Charlton and Carde 1982). The adult male will follow the plume upwind to the source with the olfaction sensors located within the micron size hairs (*sensilla trichodea*) attached to his antennae (Plettner, Lazar et al. 2000). Each sensillum contains 2 or 3 distinct dendrites thought to respond to distinct pheromone plume components (Kaissling 1980).

The dynamic range of pheromone detection of male moth antennae spans six orders of magnitude from 1 μM (Kaissling 1977) to the believed limit of detection at 1 pM which translates to a single molecule per sensillum on 25 per cent of the 10 000 sensilla in 1 second (Schneider, Kasang et al. 1968). The sensilla are covered by a porous waxy cuticle through which pheromone can diffuse. At the centre of the sensilla is the dendrite which is shrouded with a biomembrane containing the necessary receptors for depolarization of the membrane (Figure 1.7). The biomembrane is surrounded by the sensillar lymph, an environment previously described as aqueous and rich in ions and PBP’s. PBP concentration in the lymph has been estimated to be in the millimolar range (Vogt 1980; Honson 2006).
1.2.5. *L. dispar* Pheromone Binding Proteins

The gypsy moth has two known PBP’s, *L. dispar* PBP1 (PBP1) and *L. dispar* PBP2 (PBP2). Both proteins have been sequenced and share homology with other insect OBP’s (Figure 1.8) (Du, Ng et al. 1994; Merritt, LaForest et al. 1998).

Although the crystal structure for *L. dispar* PBP1 and *L. dispar* PBP2 have not yet been solved, structures for other insect PBP’s such as that belonging to *Bombyx mori* have been elucidated (Sandler, Nikonova et al. 2000; Lee, Damberger et al. 2002). The structure of *Bombyx mori* PBP showed 6 alpha helices, four of which make up the
binding pocket. Therefore it can be hypothesized that since most insect odorant binding proteins (OBP’s) share high sequence homology, *L. dispar* PBP would have a similar protein structure, which includes 3 disulfide bridges and an unstructured C-terminus. PBP’s are small proteins typically ranging from 13 -17 kDa (110 – 150 residues) and have a pI~5 (Honson 2005). They are classified based on the number of amino acids constituting their C-terminus (Pesenti, Spinelli et al. 2008).

The C-terminus of lepidopteran PBP’s are among the longer C-termini and are comprised of greater than 12 amino acids in an unstructured motif (Sandler, Nikonova et al. 2000; Lee, Damberger et al. 2002). It has been observed that the C-terminus undergoes conformational changes under certain conditions. With LUSH and ASP1, two medium length PBP’s, the C-terminus alters its conformation upon pheromone binding (Laughlin, Ha et al. 2008; Pesenti, Spinelli et al. 2008). It has also been proposed on the basis of NMR structural data that at acidic pH, *Bombyx mori* apoPBP forms a 7th helix with the C-terminus (Horst, Damberger et al. 2001). As PBP approaches the membrane, the pH has been proposed to become acidic, causing the C-terminus to form an alpha helix and insert itself into the binding cavity forcing out any ligand present (Lautenschlager, Leal et al. 2005). In support of this idea, *Bombyx mori* and *Antheraea polyphemus* are not capable of binding their native pheromones at acidic pH (Prestwich 1993; Leal, Chen et al. 2005; Damberger, Ishida et al. 2007). However, these experiments focused strongly on the C-terminus and did not consider structural changes to the rest of the protein under such acidic conditions. In an environment where the pH is dramatically below the PBP’s pI, the protein will take on an overall positive charge. The electrostatic interactions holding the secondary and tertiary structure together may be altered enough to cause decreased pheromone affinity. Further, the work of Smith and coworkers showed that the olfactory receptor ligand was a specific conformation of pheromone-bound PBP and not pheromone by itself (Laughlin, Ha et al. 2008), so although the acidic pH may cause a 7th alpha helix to form, it is not clear at this point that the helix ejects ligand from the binding cavity.
### Table 1.8

<table>
<thead>
<tr>
<th>Species</th>
<th>C-terminus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmorPBP</td>
<td>WAPSMDVAVGELLAEV</td>
</tr>
<tr>
<td>ApolPBP</td>
<td>WVPNMDLVIGEVLAEV</td>
</tr>
<tr>
<td>LdisPBP1</td>
<td>WAPTLDVAVGELLADT</td>
</tr>
<tr>
<td>LdisPBP2</td>
<td>WAPDVELLVADFLAESQ</td>
</tr>
</tbody>
</table>

**Figure 1.8**

Comparison of C-terminus sequence of various Lepidopteran PBP’s. Hydrophobic residues are highlighted in yellow and charged residues are highlighted in purple.

### 1.2.6. Endogenous Ligands of *L. dispar* PBP’s

The hemolymph of insects has been identified as containing an abundance of fatty acids. For example, the hemolymph fatty acid composition of *Periplanata americana* (Oguri and Steele 2003), *Pycroscelus striitus* (Tan 1973), *Tenebrio mulitor* and *Schistocercu greguriu* (Thompson and Barlow 1970) have all been elucidated. Although the sensillar lymph, the PBP-containing matrix of the sensilla, is quite a different system than hemolymph it was hypothesized that it too may contain several ligands which could associate with PBP1 or PBP2. This idea was further developed from the observation of the large number of hydrophobic residues making up both PBP’s (Figure 1.9). In the proposed aqueous environment of the sensillar lymph (Krieger and Breer 1999) these ligands could potentially associate with these two proteins.

Fatty acids and cholesterol were identified in the sensilla of *Lymantria dispar* (Honson 2006). These fatty acids included palmitic acid (0.053 M), linoleic acid (0.006 M), oleic acid (0.092 M) and stearic acid (0.059 M). Further, Honson was able to isolate PBP1 and PBP2 from the antennal aqueous homogenate by FPLC and extract any endogenous ligands bound (Honson 2006). PBP1 was found to have oleic acid (127 μM) and stearic acid (43.4 μM) bound, while PBP2 bound endogenous ligands included
palmitic acid (6.8 mM), linoleic acid (7.3 mM), oleic acid (1.5 mM) and stearic acid (2.2 mM). It was impressive that these ligands bound so strongly that they were not separated even after passing through a strong anion exchange column for FPLC. To quantify the strength of this interaction, isothermal calorimetry experiments were used to calculate dissociation constants ($K_d$) for the endogenous ligands (Honson 2006). Dissociation constants were calculated for PBP1-bound oleic acid (2.04 μM), PBP2-bound oleic acid (1.41 μM) and PBP2-bound linoleic acid (5.07 μM). In comparison by the same experiment, the $K_d$ of (+)-disparlure was calculated to be 11.10 μM for PBP1 and 2.35 μM for PBP2. The endogenous ligands were shown to bind the PBP’s as strongly as (+)-disparlure.

<table>
<thead>
<tr>
<th>Percent Breakdown of hydrophobic residues</th>
<th>PBP2</th>
<th>CPBP2</th>
<th>PBP1</th>
<th>CPBP1</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>12.1</td>
<td>17.6</td>
<td>11.1</td>
<td>18.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Ile</td>
<td>4.8</td>
<td>0</td>
<td>4.3</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>Leu</td>
<td>9.7</td>
<td>17.6</td>
<td>12.3</td>
<td>18.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Met</td>
<td>6.7</td>
<td>0</td>
<td>7.4</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>Phe</td>
<td>4.2</td>
<td>5.9</td>
<td>3.7</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>Pro</td>
<td>3.6</td>
<td>5.9</td>
<td>2.5</td>
<td>6.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Trp</td>
<td>1.8</td>
<td>5.9</td>
<td>1.2</td>
<td>6.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Val</td>
<td>8.5</td>
<td>11.8</td>
<td>7.4</td>
<td>12.5</td>
<td>6.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>51.4</strong></td>
<td><strong>64.7</strong></td>
<td><strong>49.9</strong></td>
<td><strong>62.5</strong></td>
<td><strong>38.2</strong></td>
</tr>
</tbody>
</table>

Figure 1.9

*Lymantria dispar* PBP2 model created with Molecular Operating Environment® based on sequence homology with *BmorIGOBP2* PDB ID 2WCJ (Left). Hydrophobic residues are highlighted in yellow. Breakdown of hydrophobic residues in *Lymantria dispar* PBP’s and their C-terminus in comparison to the vertebrate fatty acid-binding protein Bovine Serum Albumin (BSA)
1.3. Lipidomics

1.3.1. Fatty Acids

Fatty acids are long chain carboxylic acids found ubiquitously in nature. The length of their carbon chain bestows properties not found among short chain carboxylic acids. To begin with, where the pK\(_a\) of most carboxylic acids is approximately 5, the pK\(_a\) of fatty acids is significantly higher in the area of 8 or 9. There are studies which have shown a correlation between chain length, unsaturation and isomerism and pK\(_a\) in fatty acids (Kanicky and Shah 2002). Secondly, the extended carbon chain makes fatty acids amphipatic molecules, containing both a hydrophobic and hydrophilic part. This property is important as it allows fatty acids to form aggregates. Fatty acid short-hand normally includes the carbon chain length, degree of unsaturation and position of unsaturation. For example 9Z-octadecenoic acid, commonly referred to oleic acid, is an 18 carbon monosaturated fatty acid. It is commonly abbreviated as (Δ9)18:1, where Δ9 refers to the position of the unsaturation relative to the α-carbon. Fatty acids may exist as free fatty acids or esterfied to a parent head group such as a phospho-head group or a sphingosine base as is the case with sphingolipids (Figure 1.1).

1.3.2. Insect Fatty Acids

Insects contain a variety of different fatty acids in their fat body, hemolymph and cuticle, some of which are species specific. For example, *Acrithosiphon pisum* biosynthesizes primarily 18:0, while the *Ceratitis capitata* and *Drosophila melanogaster* produce mostly 12:0 and 14:0 respectively. *Trichoplusia ni* and the *Musca domestica* do not synthesize fatty acids shorter than 16 carbons (Stanley-Samuelson, Jurenka et al. 1988). Other examples include *Galleria mellonella* of the order Lepidoptera which has high levels of esterfied 20:1 in males but not females (Stanley-Samuelson 1984). While
the composition may vary between insect species on a quantitative level, it is believed that qualitatively there is a conserved fatty acid pattern among all insect orders which includes mostly saturated and monounsaturated fatty acids and possibly 18:2 and 18:3 (Stanley-Samuelson, Jurenka et al. 1988). These observed fatty acid composition patterns between species may not be just a result of diet as insects have the ability to produce many variations of fatty acids, therefore the patterns may be an example of evolutionary adaptation (Stanley-Samuelson, Jurenka et al. 1988). Stanley-Samuelson also argues that although insects have the ability to produce many fatty acid types, diet, environment as well as developmental stage also may influence fatty acid biosynthesis. Certain insect species have the ability to synthesize polyunsaturated fatty acids (PUFA’s) such as 18:2 with Δ12 desaturases. This has proven to be true in at least 15 insect species (Blomquist, Dwyer et al. 1982).

1.3.3. **Glycosphingolipids**

Glycosphingolipids are among the most complex lipids. Aside from variation in chain length, unsaturation and isomerism of the N-linked fatty acyl, there can be variation in the length, unsaturation, isomerism and hydroxylation degree and position of the sphingosine base. A further complicating part is the carbohydrate tree, which can include different sugars linked at different positions and can even include different anomers. Glycosphingolipids are named with the carbohydrates prefixing the ceramide (Figure 1.10). Glycosphingolipids are classified based on carbohydrate composition (Merrill, A.H. 2002). Acidic carbohydrates like sialic acids or glucuronic acids belong to acidic glycosphingolipids and those containing non-charged carbohydrates like glucose, galactose and N-acetylglucosamine are neutral glycosphingolipids.
Glycosphingolipid structures consist of the carbohydrate part and the ceramide part which is everything excluding the sugars. The ceramide is made of the sphingosine base, sphing-4-enine or (2S,3R)-2-amino-4-octadecene-1,3-diol by its systematic name. The sphingosine base is N-linked with a fatty acyl chain.

1.3.4. Biological Significance of glycosphingolipids

The biochemical roles traditionally associated with glycosphingolipids include cell-cell recognition, cell adhesion and signal transduction (Kasahara, Hayashi et al. 1996). The majority of glycosphingolipids have the a high tendency to cluster together with cholesterol to form highly ordered microdomains in biological membranes termed lipid rafts (Degroote, Wolthoorn et al. 2004). Glycosphingolipids have been heavily researched because of their ability to form special biomembrane environments which house membrane proteins. Glycosphingolipids have been hypothesized to help regulate transmembrane ionic gradients (Curatolo 1987). An important physical property of glycosphingolipids is that, unlike most other membrane lipids (i.e. phosphatidylycholines), they have the ability to act as hydrogen bond donors and acceptors (Pascher 1976). Because of this, their $T_m$ (melting temperature) is higher than phospholipids. This coupled with their reduced overall surface area compared to other membrane lipids in the liquid-disordered phase leads to the hypothesis that glycosphingolipids stabilize biomembranes.
1.3.5. **Glycosphingolipids in Insects**

There has been very limited work done on isolating and analyzing insect GSL’s (Wiegandt 1992). Unlike vertebrate GSL’s which mostly have lactose as the core disaccharide (Galβ(1-4)Glcβ(1-1)Cer), insect GSL’s have mactose (Manβ(1-4)Glcβ(1-1)Cer) as their core (Abeytunga, Oland et al. 2008). This insect disaccharide core has been identified in *Aedes aegypti* (Sugita, Nishida et al. 1982), *Lucilia caesar* (Dennis, Geyer et al. 1985) and *Calliphora vicina* (Seppo, Moreland et al. 2000). The sphingosine base chain lengths have also been found to be shorter in insects compared with those in vertebrates (Abeytunga, Oland et al. 2008). Insect GSL analysis by tissue has been undertaken by Thusitha and coworkers on *Manduca sexta* (Abeytunga, Oland et al. 2008) and by Sickmann and coworkers on Calliphora vicina in 1992 (Sickmann, Weske et al. 1992). *Calliphora vicina* GSL analysis was also published by Dennis and coworkers in 1985 (Dennis, Geyer et al. 1985). More recently, GSL analysis was carried out on the embryo of *D. melanogaster* (Seppo, Moreland et al. 2000). Not too surprisingly there were variations found between *C. vicina* and *D. melanogaster* glycosphingolipid profiles. Among the differences were GlcNAc substitution and extent of GlcNAc glycosylation. Phosphoethanolamine derivitization of GlcNAc is something that has been observed in multiple insect species but not in vertebrates (Dabrowski, Dabrowski et al. 1990; Helling, Dennis et al. 1991). In comparison to *Lucilla calliphora*, there were a greater number of GSL’s found in the embryo of *D. melanogaster* (Seppo, Moreland et al. 2000). Developmental stage likely plays a role in the differences seen in Dipteran GSL’s. It is also likely that variations exist between species. Studies on the interactions between these GSL’s and surrounding proteins are therefore of interest.
1.4. Aim and Scope of this Work

The PBP’s of *L. dispar* have been studied in our group for years. *L. dispar* PBP’s have a variety of endogenous ligands available for binding and they bind these ligands with affinities on the same order of magnitude as (+)-disparlure (Honson 2006). Pheromone binding kinetics of PBP-disparlure binding were studied by Gong, Y. (Gong, Tang et al. 2010). Using stopped-flow fluorescence she developed a 2-step model for pheromone binding for *L. dispar* PBP’s.

My goal was to investigate the endogenous lipid-PBP interaction shown to exist by Honson. By stripping recombinantly expressed PBP’s of the majority of lipids which may have adsorbed during expression, I was able to study the pheromone binding affinity of PBP1 and PBP2 at equilibrium with and without endogenous fatty acids bound. I was also able to probe the secondary structures of these proteins, using circular dichroism spectroscopy, under those conditions. I found that both PBP’s show an increased affinity for disparlure when endogenous fatty acids are bound. The PBP’s also displayed a global increase in $\alpha$-helix structure when bound with endogenous fatty acids. An attempt to correlate the two experiments was made by preparing a homology model of PBP1 and PBP2 and docking the endogenous ligands from the experiments to determine sites of preferred binding. Close attention was paid to the residue-fatty acid interactions at the docking sites of highest stability. Both protein models showed docking sites of highest stability for the endogenous ligands adjacent to both the C-terminus and the entrance to the internal binding cavity.

The second goal of this project was to study the lipidome of *L. dispar*. The high concentration of fatty acids found in the sensillar lymph (Honson 2006) was shown by dynamic light scattering experiments to form vesicles. Because micelles and vesicles are able to intercalate and dissolve lipid bilayers, I paid particular attention to the polar lipids found in the biomembrane during the analysis. Lipid-bound fatty acids and other lipids
were identified from lipid extracts of the antennal sensilla and other tissues. Because of the observed influence on pheromone binding and protein structure, a quantitative free fatty acid analysis was completed to learn whether a distinct composition of fatty acids are specific to the antennal sensilla where PBP's are primarily found. Finally, several of the polar membrane lipids were isolated by chromatographic techniques in order to characterize their structure by a variety of techniques including NMR and mass spectrometry.

The lipidome of *L. dispar* influences the function of the insect’s olfactory system. While this has not been completely confirmed by this thesis project, several interesting observations including PBP-fatty acid interactions and lipids found to be specific to the olfactory tissues have, at minimum, called for further investigation.
2. Endogenous Ligands of *L. dispar* Pheromone Binding Proteins

2.1. Abstract

The gypsy moth, *Lymantria dispar*, and other insects use pheromones for communication with members of their own species. Pheromones are detected by sensory hairs (sensilla), mainly on the antennae of the adult males. The sensory hairs contain pheromone binding proteins (PBPs) and endogenous ligands for PBPs. The endogenous fatty acids associate with *Ldis*PBP1 and *Ldis*PBP2 with low micromolar. The results of a PBP-disparlure binding assay suggest that these endogenous fatty acids affect the binding affinity of these proteins for pheromones. Circular Dichroism spectroscopy was used to probe conformational changes the secondary structure brought about by the association of fatty acids. The marked increase in overall $\alpha$-helical content seen in *Ldis*PBP1 and *Ldis*PBP2 was investigated further by probing the unstructured C-terminus for conformational changes. Docking endogenous fatty acids on potential binding sites of homology modelled PBP’s was carried out in an attempt to correlate the increased pheromone affinity and change in structural conformation. We propose here that the change in protein conformation and the increase in binding affinity, brought on by association with these endogenous fatty acids, are related.
2.2. Introduction

We have observed ligand binding selectivity for the gypsy moth, *Lymantria dispar*, PBP1 and PBP2 towards various ligands (Plettner, Gong et al. 2009; Gong, Tang et al. 2010). Although PBPs and OBPs are essential for olfaction and show ligand binding selectivity, their biological role and mode of action are still unclear. They are thought to bind pheromones at the cuticle pore and diffuse through the sensillar lymph until they approach an olfactory receptor. The mechanism of interaction between PBP and receptor leading to membrane depolarization are still unclear.

A question central to PBP-ligand dynamics is whether PBPs have an empty binding site, in the absence of pheromone in the lymph. Given that oleamide has been identified as an endogenous ligand for a locust chemosensory specific protein (CSP) (Pelosi, Ban et al. 2003) it is likely that the antennal lymph of other insect species contain a variety of potential ligands for PBPs, none of which have been identified. In addition, the presence of endogenous ligands may result in the PBP adopting a certain conformation, perhaps exposing residues involved in pheromone-specific interactions (Gong, Tang et al. 2010). Lastly, endogenously bound ligands may act to synergize or antagonize pheromone binding, as has been seen with mixtures of odorants (Plettner, Honson et al. 2003; Plettner, Chen et al. 2011).

The two known gypsy moth PBPs (*Ldis*PBP1 and *Ldis*PBP2) belong to the class of “long” OBPs, with a hydrophobic, disordered C-terminal peptide of 10-18 amino acids in length (Tegoni, Pesenti et al. 2008). The function of the C-termini of long insect OBPs and the reason why they have a large proportion of hydrophobic residues is poorly
understood. The goal of this work is to explore the binding of endogenous ligands to 
$LdisPBP1$ and $LdisPBP2$ and their possible contribution to olfaction, if any. As the crystal 
structure for $LdisPBP1$ and $LdisPBP2$ has not yet been obtained, based on sequence 
homology to other OBPs of similar size to $LdisPBPs$, it is likely that $LdisPBPs$ are mostly 
alpha-helical with flexible loops, particularly between helices 2 and 3, and a non-
structured, flexible C-terminus. Previous modeling and kinetic studies with gypsy moth 
PBPs suggest that the loop and C-terminus delineate an external site where ligands, 
such as the pheromone (+)-disparlure, can be captured as the first step of a two-phase 
binding mechanism (Plettner, Gong et al. 2009).

Do gypsy moth PBP’s have any endogenous ligands and is there any reason for 
these ligands to interact with PBP? Here we have identified several free fatty acids in the 
sensillar lymph which are available for interaction with PBP’s. Further, we show that 
$LdisPBP1$ and $LdisPBP2$ associate with endogenous lipids present in the antennal 
sensillum lymph of $L. dispar$ males. The impact of PBP-endogenous ligand interaction on 
protein secondary structure was also investigated as well as the influence on disparlure 
binding affinity.
2.3. Materials and Methods

2.3.1. Materials

All reagents used were of the highest purity available. Ethyl acetate and hexane were distilled in glass prior to use. Palmitic, linoleic, oleic acid, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), (+)-disparlure, and dimethyldichlorosilane were from Sigma-Aldrich (St. Louis, MO). Stearic acid was from Matheson Coleman & Bell (Cincinnati, OH), cholesterol was obtained from Fisher Scientific (Fair Lawn, NJ), potassium hydroxide from BDH Inc. (Toronto, ON), and sodium chloride and Tris from Caledon (Georgetown, ON).

2.3.2. PBP Expression, Purification and Isolation

PBPs were expressed in Escherichia coli (with the pHN1+ vector, supplied by L. Chen and G.L. Verdine, Harvard, via G.D. Prestwich) and isolated as inclusion bodies. These were then subjected to a denaturation/renaturation scheme and purified as described elsewhere (Prestwich 1993; Plettner, Lazar et al. 2000). The PBPs had the correct mass by matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF MS) and were positively identified by Western blots. PBP2 was delipidated using either
charcoal or hydrophobic polymer coated beads (BioRad HIC-methyl macro Prep 50μm, (BioRad, USA), and confirmed by solvent extraction followed by N,O-
Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivitization and GC-MS analysis of the extract.

2.3.3. tPBP Expression, Purification and Isolation

Truncated PBP’s (tPBPs) lack the C-terminus of the full protein (~ 17 amino acids). The construct for expression was designed by Dr. Erika Plettner (in 2007). Expression was carried out by Dr. Yong-Mei Gong and Brian So in 2009. Briefly, a forward primer with restriction enzyme (Nco I) site and a reverse primer with restriction enzyme site (Hind III) were used in a polymerase chain reaction (PCR) to amplify the PBP gene lacking the nucleotides responsible for coding the C-terminus amino acids.

The amplicon created by PCR was ligated into pET-22b(+) vector (Novagen), in phase with the signal sequence on the vector that directs the expressed protein into the periplasmic space. The vector was transformed into JM109 competent cells, and the plasmid encoding the truncated PBP was isolated. The isolated plasmids containing tPBP1 or tPBP2 were transformed into E. coli BL21(DE3) and cultured in LB medium containing 50 μM ampicillin. Isopropyl thiogalactoside (IPTG) was used to induce
expression of tPBPs. Cells were harvested, and the protein was released from the periplasm, using an osmotic shock procedure. Proteolysis was avoided by adding PMSF and ABSF during periplasmic extraction. Truncated PBPs were purified by 12% native PAGE as described for full-length PBPs. The purified proteins were characterized by MALDI-TOF MS and SDS-PAGE.

PBPs were delipidated (Appendix Figure A2.1) using hydrophobic interaction chromatography (HIC) polymer coated beads BioRad HIC-methyl macro Prep 50μm (BioRad, USA). Delipidation was confirmed by solvent extraction followed by BSTFA derivitization and gas chromatography/mass spectrometry (GC-MS) analysis of the extract.

2.3.4. Circular Dichroism Spectroscopy

A fatty acid stock aqueous solution (pH > 10) containing palmitic acid (1135 μM), linoleic acid (246 μM), oleic acid (1944 μM), and stearic acid (1009 μM) was prepared with MilliQ water (18 MΩ)(Szostak, Hanczyc et al. 2003). The pH of this solution is adjusted to greater than 10 with 1M NaOH. The solution was then vortexed and agitated overnight (shaker) under argon. The C-terminal peptide (Biomatik Corporation) standards (CPBP’s) were prepared for each 2-propanol (IPA) titration so that the final concentration of the peptide would be 50μM. Both PBP’s as well as the truncated PBP’s were prepared at 10 μM, while CPBP’s were prepared at 50 μM, with varying concentrations of total fatty acids.
Measurements were made on a JASCO-810 CD spectropolarimeter. The cell path length was 1 mm, and spectra were recorded from 190 nm to 250 nm in 0.1 nm increments accumulating 6 scans per sample. Analysis of secondary structure content was performed with the analysis software SELCON3 (the self-consistent method for protein CD analysis, version 3) (Sreerama, Venyaminov et al. 2000). The reference set SMP56 was used, as it contained the largest representation of proteins (43 soluble proteins and 13 membrane proteins).

2.3.5. **PBP-Pheromone Binding Assay**

All PBP’s used in the binding assay were delipidated for at least 3 days with Macro- Prep Methyl HIC Support beads (BioRad, Ca.) at 5°C. All Eppendorf tubes were coated with 1-tetradecanol as described elsewhere (Plettner, Lazar et al. 2000). PBP1 and PBP2 were diluted with phosphate buffer (100 μM, pH 8.1) appropriately so that the final assay concentration would be 5 μM. A fatty acid stock aqueous solution (pH > 10) was prepared with palmitic acid (1135 μM), linoleic acid (246 μM), oleic acid (1944 μM), and stearic acid (1009 μM). The fatty acid mixture (10 μM) was titrated to the assay samples which would have a final volume of 400 μL and shaken at 350 rpm for 1 hour. All disparlure stereoisomers were synthetically prepared in our lab (Yu, unpublished) and diluted in distilled ethanol before being titrated in with PBP assay samples to a final concentration of 20 μM. PBP-disparlure mixtures were incubated for 2 hours on ice.
Subsequently, all assay samples were passed through a Bio-Gel P2 45-90 μm (BioRad, Ca.) column to separate unbound disparlure. Samples were acidified with 6M HCl and disparity and fatty acids were solvent extracted with distilled hexane/ethyl acetate (1:1) containing internal standard methyl palmielaidate (119 ng/μL) as an internal standard. The extract was evaporated to dryness and silylated with BSTFA and allowed to react for 1 hour at room temperature. The assay samples were analyzed by gas chromatography under the following conditions: Inj. Temp. 250˚C; Det. Temp. 300˚C; Split mode 20 mL/min total flow; Initial column temp. 100˚C held for 5 min, 20˚C/min to 205˚C and held 10 min, 5˚C/min to 250˚C. The GC (Perkin Elmer Clarus 500) was fitted with a Supelco SPB-5 capillary column and a flame-ionization detector (FID).

2.3.6. **PBP-Endogenous Ligand Docking Simulations**

Homology modeling has been shown to be an effective tool for constructing 3-dimensional protein models when the crystal structure is not available (Tramontano and Morea 2003). This is so because, unlike the almost limitless number of possible sequences in nature, the number of possible peptide folds is limited. Therefore sequences sharing sequence homology can be used to template each other if the structure of one is not known (Arnold, Bordoli et al. 2006). Swiss-Model is a web-based homology modeling server which includes the databases and applications required for modeling (Arnold, Bordoli et al. 2006). There are four steps required with any homology modeling experiment (Arnold, Bordoli et al. 2006; Kiefer, Arnold et al. 2009): selection of a homology template, sequence alignment of the target and template proteins, model
construction and model validation. PBP1 and PBP2 were modeled on *B. mori* GOBP2 (general odorant binding protein) with (10E,12Z)-tetradecadien-1-ol bound (PDB ID 2WCJ).

Fatty acid docking simulations with PBP1 and PBP2 were carried out with Molecular Operating Environment® (MOE). All ligands were constructed with MOE and imported into a MOE ligand database as '.pdb' files. All ligands in the database were computationally washed to remove any disruption by solvent or salt molecules surrounding the structure. Partial charges were subsequently adjusted on all ligands in order to be able to calculate electrostatic potential maps later on. Finally, the ligands were energy minimized to place them in an energetically favorable conformer.

PBP1 and PBP2 models were prepared in a similar fashion to the ligands. Prior to docking, potential docking sites were identified on the protein (protein will be referred to as the receptor here). Using Site Finder (a MOE tool), six potential sites suitable for ligand docking were identified for PBP2 and seven sites for PBP1. Dummy atoms were placed at each site targeted for docking. The dummy atoms are markers used by the docking algorithm to know where to place the ligand. Ligands were docked using an induced fit protocol with GBVI/WSA and London rescoring. Triangle matcher was used for placement and Forcefield for refinement. 20 poses were retained for every ligand excluding duplicates. Ligands docked included palmitic acid, linoleic acid, oleic acid and stearic acid.
The GBVI/WSA DG estimates the free energy of binding of the ligand from a given pose (Corbeil, Williams et al. 2012). The function is the sum of terms (Corbeil, Williams et al. 2012) where

\[ \Delta G \approx c + \alpha \left[ \frac{2}{3} (\Delta E_{coul} + \Delta E_{sol}) \Delta E_{vdw} + \beta \Delta S A_{weighted} \right] \]

\( c \) represents average gain of rotational and translational entropy, \( E_{coul} \) is a coulombic electrostatic term calculated using a constant dielectric of 1, \( E_{sol} \) is the solvation electrostatic term, \( E_{vdw} \) is the contribution of van der waals interactions to binding and \( S A_{weighted} \) is the term taking into account surface area of the ligand. \( \alpha \) and \( \beta \) are constants which are forcefield-dependant.
2.4. Results

2.4.1. CDS of PBP C-terminus peptides in a hydrophobic environment

The reason for PBP1 and PBP2 binding endogenous fatty acids with such high affinity is not understood. To determine if the interaction with endogenous ligands affects the secondary structure of these proteins, I probed the secondary structure of both PBPs, the C-terminally truncated PBPs and the C-terminus peptide with circular dichroism spectroscopy, a technique which is highly sensitive to changes in secondary structure. (Sreerama and Woody 2000) PBP1 and PBP2 have long unstructured C-termini. The C-terminus for both proteins contains a high number of hydrophobic residues which may be sites for interaction with non-polar molecules. As the v/v fraction of isopropyl alcohol increased in the presence of CPBPs, the overall alpha helix content of the peptides increased (Appendix Figure A2.2) (Figure 2.1).
Figure 2.1

*Overall secondary structure of 50 \( \mu M \) CPBP1 (left) and CPBP2 (right) as a function of %v/v isopropyl alcohol. Data points in white are \( \alpha \)-helix fractions and points in black are unstructured fractions. Points represent the average \( \pm \)S.E. of at least 3 replicates.*

The inflection point is statistically significant as calculated by a JMP version 8 t-test with ANOVA \( P < 0.0001 \). The results for the same experiment with CPBP2 also showed an increase in the overall alpha helix content of the peptide with increase in the v/v fraction of isopropyl alcohol titrated (Figure 2.1). The inflection point is statistically significant as calculated by a JMP version 8 t-test with ANOVA \( P < 0.0001 \). Bars are the mean \( \pm \)S. E. of 3 replicates. A significant difference between the results of the two peptides is the maximal overall alpha helix content for CPBP2 of 95% compared to 56% for CPBP1. The overall unstructured content of the CPBP1, as estimated by SELCON3 analysis, decreased with increasing isopropyl alcohol content (Figure 2.1).
With a clear indication that the C-terminus of PBPs alters its conformation as a result of hydrophobicity, the complete proteins were titrated with the same fatty acids shown to bind PBP’s in the lymph. The concentrations of the fatty acids in the experiment were well below the concentrations present in the lymph. This is due to the spectroscopic limitations of the experiment. The larger concentrations of fatty acids lead to greater scattering of light and poor signal to noise ratio. As the concentration of fatty acid increases with both PBP1 and PBP2, the overall alpha helix content increases (Figure 2.2). In both cases, the pH of the samples was kept at 8 and the concentration of the protein at 10 μM. The basis for circular dichroism in molecules is that they must be chiral and since fatty acids are not chiral molecules any absorbance difference between left and right polarized light must come from the protein. SELCON3, the program used to estimate the alpha helix content in the protein, makes its approximations based on a reference set. The same reference set (SMP56) is used for all approximations so, although the absolute values are estimates, the relative values for each protein are of interest. The structure of CPBPs appears to be unaffected by fatty acids in the experiment carried out (Figure 2.2). With the full proteins, a significant increase in overall α-helix content was seen with PBP2 and to a much lesser extent with PBP1. The increase appears to have a limit, after which any further amount of fatty acids will not affect structure. Finally, both tPBPs show increase in α-helix content with increasing fatty acid concentration and seem to have a larger threshold for reaching an α-helix maximum,
**Figure 2.2**

Left: overall $\alpha$-helix content of CPBP1 (50 $\mu$M) and CPBP2 (50 $\mu$M) with increasing fatty acid mixture concentration. Right: Overall $\alpha$-helix content of recombinently expressed full-length PBP’s (10 $\mu$M) and truncated PBP’s (10 $\mu$M) with increasing fatty acid mixture concentration. Bars represent the average ± S.E. of at least 3 replicates. Bars sharing common letters are not significantly different (Student’s t-test, $P < 0.05$)

2.4.2. Binding Assay

A significant increase in binding affinity for all isomeric forms of disparlure ((+),(-), (±)) is seen for both PBP1 and PBP2 when the delipidated proteins are allowed to adsorb endogenous ligands prior to disparlure binding (Figure 2.3) (Appendix Figure A2.3). It is important to note that all samples were extracted after passing through the P2 gel column in order to remove any unbound disparlure (Appendix Figure A2.4). One exception is PBP$_2$$_{endo}$, which does not appear to show stronger affinity for (+)-disparlure than delipidated PBP2. Finally, the C-terminally truncated PBP’s also show a stronger affinity for (+)-disparlure with endogenous ligands adsorbed.
Figure 2.3

Mean dissociation constants for various recombinantly expressed *L. dispar* PBP’s binding disparlure enantiomers. tPBP’s are C-terminally truncated proteins. The subscript ‘endo’ refers to those proteins which were allowed to adsorb endogenous ligands prior to pheromone binding. Points represent the average ± SE of at least 4 replicates. Bars sharing common letters are not significantly different (Student’s t-test, *P* < 0.05).

### 2.4.3. *L. dispar* PBP homology SWISS models

We have used two different sets of information to determine the quality of the SWISS MODEL PBP’s. The first is the QMEAN Z-score which describes the quality of the homology model (Arnold, Bordoli et al. 2006). The second is the Ramachandran plot which describes protein structure based on torsion angles of
Φ (phi) and Ψ (psi). Φ and Ψ (Figure 2.4) describe the orientation of the carbonyl-carbon relative to the alpha-carbon (Ψ) and of the alpha-carbon relative to the amide nitrogen of the next residue (Φ).

![Figure 2.4](image-url)

Peptide torsion angles phi and psi. Carbon atoms presented in grey, oxygen atoms presented in red, nitrogen presented in blue, side chain groups presented in green. Subscript ‘i’ denotes separate residue.

The data points of a Ramachandran plot represent the phi and psi torsion angles for each residue in the protein. Residues which are part of α-helices tend to have clusters in the region of -47˚ for Φ and -57˚ for Ψ. The top-left quadrant centered around 120 for psi and -120 for phi represents the region for residues belonging to β-sheets and the region centered at 60 phi and 30 psi represents left-handed or irregular α-helices.

Ramachandran plots were built for homology models of PBP1 and PBP2 (Figure 2.5). Data points given in green represent residues having torsion angles ideal for a given motif whereas yellow represent residues which have less ideal but still acceptable torsion angles. The red crosses represent residues with forbidden torsion angles. These residues likely have side chains which are sterically hindered.
Figure 2.5

Ramachandran plots for (A) PBP1 2WCJ homology model and (B) PBP2 2WCJ homology model.

The QMEAN scoring function was developed by Benkert et al. (Benkert, Biasini et al. 2011) and is used to assess acceptability of a SWISS MODEL. It is a collection of individual descriptors describing protein interactions such as distance-dependant interaction potentials between atoms of individual residues and long-distance potentials related to secondary structure. The QMEAN also takes into account factors like torsion angle potential and solvation potential. In total there are 6 terms which are combined linearly to calculate the QMEAN which is then converted into a Z-score (Benkert, Biasini et al. 2011). The QMEAN Z-score is a comparison of the QMEAN and its individual terms for the prepared model to those for structures of similar size, prepared by X-ray crystallography or NMR (Benkert, Biasini et al. 2011). The PBP1 model has 28.06% sequence homology with 2WCJ and the PBP2 model has 30% sequence homology. A SWISS Model algorithm calculates QMEAN scores for the models to provide a measure of quality (Table 2.1) (Benkert, Biasini et al. 2011). The QMEAN Z-scores for PBP1 and PBP2 are -3.14 kcal/mol and -3.56 kcal/mol respectively. Both are more than 3 standard deviations from QMEAN Z-score which would result from the structure of the actual
proteins. The major contribution to the large Z-score is the torsion angle energy which is calculated to be -3.30 kcal/mol for PBP1 and -3.75 kcal/mol for PBP2.

<table>
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<th>Z-score</th>
<th>Raw score</th>
<th>Z-score</th>
</tr>
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<td>C-(\beta) interaction energy</td>
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<td>-0.70</td>
<td>-79.71</td>
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<tr>
<td>All-atom pairwise energy</td>
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<tr>
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<tr>
<td>QMEAN4 score</td>
<td>0.51</td>
<td>-3.14</td>
<td>0.47</td>
<td>-3.56</td>
</tr>
</tbody>
</table>

Table 2.1
SWISS MODEL interaction energy Z-scores for L. dispar PBP1 and PBP2 homology models from template protein 2WCJ.

2.4.4. Docking Endogenous Fatty Acids with PBP1 and PBP2

Each of the endogenous fatty acids found to bind PBP1 and PBP2 were docked at all the possible sites on the proteins. The sites were identified by MOE as possible docking sites for ligands and indicated numerically in Figure 2.6. The free energy of binding for each ligand was calculated at each site (Figure 2.6). The internal binding cavity is labeled as site 1 in both proteins. The remaining docking sites are all on the proteins' exterior.
Figure 2.6  
Fatty acid docking on SWISSModel homology modeled PBP using MOE. Bar graphs are free energy of binding estimations (GBVI/WSA score) for lowest energy pose of each ligand. Top-right is PBP1 and bottom-right is PBP2 models with docking sites numbered. Palmitic acid is displayed at each external site with its solvent exclusion (Connolly Surface) surface.

The C-termini are colored red and the N-termini are colored blue. Stearic acid was used to illustrate the docking sites in PBP1 and palmitic acid was used for PBP2, as these fatty acids showed the greatest decrease in free energy of binding compared to all ligands at all sites. The ligand surfaces shown represent the solvent exclusion volume of the ligand. Solvent molecules are not able to breach this surface because of steric hinderence under the assumption that atoms are taken to be spherical with van der waals radii (Connolly 1983). The ligand surface is colored by atom. For both proteins,
Figure 2.7

Homology model of PBP2 with palmitic acid docked at site 3. The blue ribbon is the N-terminus and the red ribbon is the C-terminus. Hydrogen bonds as low as 0.2 kcal/mol in energy are shown. Palmitic acid interactions are diagrammed on the bottom right.

the internal binding cavity (site 1) showed the lowest free energy of binding by a factor of about 2 compared to all other sites. Of the external sites identified on PBP1, site 2 showed the lowest free energy of binding for all ligands (-5.85 kcal/mol for stearic acid) compared to other sites. Of the external sites identified on PBP2, site 3 showed the lowest free energy of binding (-5.61 kcal/mol for palmitic acid) for all ligands compared to all other sites.
Figure 2.8  
*Homology model of PBP1 with stearic acid docked at site 2. The blue ribbon is the N-terminus and the red ribbon is the C-terminus. Hydrogen bonds as low as 0.2 kcal/mol in energy are shown. Palmitic acid interactions are diagrammed on the bottom right.*

Both site 2 on PBP1 and site 3 on PBP2 are in the vicinity of the C-terminus (Figure 2.7 and Figure 2.8). Figure 2.7 and Figure 2.8 show PBP2 and PBP1 respectively, docked with the ligand calculated to have the lowest free energy of binding. The figures show a cross-section of the 3D docked proteins. The C-terminus is colored red and the N-terminus is colored blue. The docked ligands are shown in green with possible hydrogen bonding as low as 0.2 kcal/mol shown with hashed lines. On the right-hand side of both figures is a diagram of calculated ligand interactions at the docking site. The dashed line surrounding the ligand shows the surface of solvent exclusion. The diagram shows the possible interactions with solvent and adjacent residues for the docked ligand.
2.5. Discussion

Extraction of PBP1 and PBP2 revealed that these proteins associate with endogenous fatty acids. The \textit{in vivo} concentrations of fatty acids associated with each protein were estimated to be in the micromolar range for PBP1 and in the millimolar range for PBP2. These concentrations are above the critical micellar concentration suggesting that these acids likely aggregate \textit{in vivo} into micelles, more complex multi-lamellar structures or a combination of two. Single chain amphiphiles, such as fatty acids begin to spontaneously aggregate in aqueous solutions when the pH approaches the pK\textsubscript{a} of the fatty acid (Hargreaves and Deamer 1978). The pK\textsubscript{a} values of palmitic, linoleic, oleic, and stearic acid in a spread monolayer have been determined to be 8.7 (Shah, Kanicky et al. 2000), 9.2, 9.9, and 10.2, respectively (Kanicky and Shah 2002). The pK\textsubscript{a} value of oleic acid vesicles in water has been estimated to be between 8.0-8.5 (Cistola, Hamilton et al. 1988). While the pH of the sensillar lymph is not known, it may be higher than neutral, because of a vacuolar ATPase and a H\textsuperscript{+}/K\textsuperscript{+} antiporter expressed on the support cell apical membrane(Klein and Zimmermann 1991). This ion pumping system removes H\textsuperscript{+} from the lymph and pumps K\textsuperscript{+} into the lymph, thereby raising the pH of lymph as high as 9-10, much like the midgut of lepidoptera, where a similar K\textsuperscript{+} secretion system operates (Wieczorek, Huss et al. 2003). This is the reason why we tested binding at a higher pH value (8.0) than the neutral pH usually used.

Our experiments suggest that PBPs naturally associate with fatty acid aggregates. Structural characterization of homologous PBPs reveal an unstructured C-
terminal tail (Sandler, Nikonova et al. 2000; Wieczorek, Huss et al. 2003). Homology modeled PBP2 contains an 18-residue C-terminal tail, with 10 hydrophobic residues. CPBP2 also contains 5 residues which are polar neutral or acidic. The MOE energy minimized CPBP2 model (Appendix Figure A2.5) illustrates how the peptide can form an amphipatic helix. The increase in alpha helix content with increase in hydrophobicity may be attributed to the lack of hydrogen bonding allowed for the charged residues of the peptide. In an attempt to regain the hydrogen bonds lost due to the reduced number of donors and acceptors available in a hydrophobic environment, the peptide might dimerize by hydrogen bonding with the charged side of another amphipathic helix.

Binding of ligands to OBPs has been found to induce conformational changes in these proteins (Lee, Damberger et al. 2002; Laughlin, Ha et al. 2008). Here we have shown that endogenous ligands associate with PBP’s to also induce conformational changes. From early work with PBPs, in which delipidation was not done, we know that fatty-acid laden PBPs are capable of binding odorants (Du, Ng et al. 1994; Plettner, Lazar et al. 2000). *L. dispar* PBP’s have shown the ability to bind ligands other than disparlure, such as various dialkoxybenzenes which resemble aromatic plant volatiles (Paduraru, Popoff et al. 2008). Endogenous ligands associating with an insect binding protein have previously been shown: e.g., in *Locusta migratoria*, the endogenous ligand of a chemosensory specific protein (CSP) isoleoamide (Ban, Scaloni et al. 2003), whereas in two *Helicoverpa* species (*armigera* and *assulta*) 1-dodecene is the endogenous ligand of odorant-binding protein 10 (OBP10) (Sun, Huang et al. 2012). PBPs may have neuron-activating conformations that are accessed only when the cognate pheromone is bound (Laughlin, Ha et al. 2008; Gong and Plettner 2011). It is
known that isolated insect odorant receptors can be stimulated with pheromone by itself (not bound to PBPs) (Nakagawa, Sakurai et al. 2005; Wicher, Schafer et al. 2008), but distinct responses have been observed when pheromone bound to various PBPs is delivered to the dendrite (Vandenberg and Ziegelberger 1991). The *Drosophila melanogaster* OBP LUSH is known to adopt a particular conformation in one of the loops when the pheromone is bound, and this conformation has been shown to be activating (Laughlin, Ha et al. 2008).

Circular dichroism of the C-terminal peptides in the presence of fatty acids (Figure 2.2) showed no change in the secondary structure. This is in stark contrast to the dramatic change seen for the peptides in the presence of IPA. This lack of similarity may be a consequence of the overwhelming ratio of unstructured to alpha helical peptides in solution. The IPA mixture is a much more hydrophobic environment allowing a greater fraction of the peptide molecules to adopt an alpha helix structure. The full protein as well as the truncated analogs showed increase in alpha helical content as the total fatty acid concentration increased. The larger surface area of the protein may have led to fatty acid adsorption having a more discernible impact on secondary structure.
The binding affinity of PBP1 and PBP2 is directly affected by the presence of endogenous ligands (Figure 2.3). Adsorption of these fatty acids clearly leads to a change in protein secondary structure in the form of increased alpha helical content. We propose that these PBP’s are potentially being ‘tuned’ or held in a favorable conformation by the endogenous ligands in order to bind disparlure. Further, we also propose that the C-terminus of PBP1 and PBP2 is not integral to pheromone binding, as the truncated proteins displayed analogous binding affinity in the presence of...
endogenous ligands as well as increased alpha helical content. The C-terminus undoubtedly serves some alternative purpose given its ability to undergo dramatic structural change in a hydrophobic environment and the amphipatic nature of the resulting alpha helix (Fig. 2.9). This alternative purpose may be to anchor into the lipid bilayer which is represented much more effectively by a 2-propanol environment than by an aqueous environment containing fatty acid micelles.

The binding site with the lowest free energy of binding for both proteins and all ligands was site 1 (the internal binding cavity), and each protein had a particular external site that was calculated to have a greater affinity for fatty acids than the rest (Fig. 2.6). For example, stearic acid bound at site 2 on PBP1 was ~ 0.5 kcal/mol lower than any of the other external sites for PBP1. It was also interesting that both site 2 on PBP1 and site 3 on PBP2 were in close proximity to the C-terminus of their respective proteins (Fig. 2.6). Further, the C-terminus has several acidic residues which align on the same side of the barrel when the peptide becomes $\alpha$-helical. Almost complimentarily, the N-terminus, which appears adjacent to the C-terminus in the homology models, has basic residues all on the same side of its own $\alpha$-helix. While endogenous fatty acid binding seems to alter the PBP core structure, but not the C-terminus, a hydrophobic environment such as a lipid bilayer may still alter the conformation of the C-terminus for alternative reasons. The amphipatic helix of the C-terminus is very close to the amphipatic N-terminus. It may be possible that the C-terminus and N-terminus interact with the bilayer in a way which may improve docking with a membrane receptor. The N-terminus can interact electrostatically with the phosphate groups of the bilayer while the C-terminus may insert into the bilayer, forming an amphipatic helix which could possibly interact with a membrane receptor such as SNMP. This could explain how the pheromone-bound PBP
complex is required as a ligand for olfactory membrane receptors (Laughlin, Ha et al. 2008).

2.6. Conclusion

The C-terminus is able to form an α-helix in a hydrophobic environment but not in a fatty acid emulsion, in which the core of PBPs showed an increase in alpha helical content. We hypothesize that this structural change in the core is directly related to the increase in pheromone affinity observed for L. dispar PBPs. Therefore, while the C-terminus may not be directly involved in pheromone binding, it might play alternative roles. In kinetics experiments, it has been proposed to capture hydrophobic ligands during collisional encounters between the PBP and the ligand (Gong, Pace et al. 2009). Secondly, the C-terminus may help PBPs associate with bilayer membranes and possibly with other transmembrane proteins, such as the sensory-neuron membrane protein (SNMP) (Jin, Ha et al. 2008) (Figure 2.9). Interestingly, the C-terminal α-helix is estimated to be ~17 Å long (Appendix Figure A2.5) and could, therefore, span half the lipid bilayer. Such an interaction with the bilayer could be strengthened further by electrostatic attraction between the positively charged N-terminal α-helix and the negative charges on the exterior of the phospholipid bilayer. Consistent with this proposal, it has been demonstrated that the PBP of D. melanogaster (LUSH) regulates the ion permeability of the dendritic membrane in conjunction with the SNMP: deletion of
the PBP causes the neuron in the respective sensilla to be silent and unresponsive to odorants (Xu, Atkinson et al. 2005), whereas deletion of SNMP causes the neuron to be permanently activated and also unresponsive to odorants (Jin, Ha et al. 2008). Third, the C-terminus and the core of the PBP form external binding sites where fatty acids can bind. These fatty acids have been shown here to occur in sensillar lymph in high concentration and to modulate the interactions between the PBP and the pheromone.

Our observations suggest that PBP’s are naturally able to bind ligands other than pheromone, and here we have demonstrated that the endogenous fatty acids strengthen the PBP-pheromone interaction, in comparison to fatty acid-free PBPs. This strengthened interaction likely comes from an increased α-helix content of the core of the protein upon interaction of the PBP with fatty acids.
3. Analysis of the *L. dispar* Lipidome

3.1. Abstract

Lipids make up the major part of biological membranes and regulate many biological processes. Insect olfaction occurs through depolarization of the membranes containing olfactory receptors. The lipid environment of these receptors is likely critical to their function. We have carried out a partial lipid analysis of *Lymantria dispar*, with particular attention paid to fatty acids and polar membrane lipids. Using GC/MS, we have identified lipid-bound fatty acids found in the antennal sensory hairs. We have quantified the free fatty acids palmitic acid, linoleic acid, oleic acid and stearic acid in different parts of *L. dispar* finding that oleic acid is the most abundant throughout the insect. Finally, we isolated two glycosphingolipids from the antennal extract and proposed structures based on mass spectrometry and NMR analysis.
3.2. Introduction

The importance of lipids and lipid metabolism to insect proliferation has been fundamental. The perpetuation of many species has been attributed in large part to their ability to implicate lipid metabolism with vital life processes such as reproduction, embryogenesis, flight, metamorphosis (Beenakkers, Vanderhorst et al. 1985), and communication. The *L. dispar* sensilla contain concentrations of free fatty acids well above the critical micellar concentration (Honson 2006). Because the environment surrounding the olfactory neuron is aqueous, these free fatty acids are likely to assemble into fatty acid vesicles (Honson 2006). It is well established that fatty acid micelles and other detergents have a destabilizing influence on biological membranes (Henriksen, Andresen et al. 2010). It is for this reason that the sensillar biomembrane in *L. dispar* should have a composition which is able to counter the destabilization of the free fatty acids. It is primarily based on these two observations that the total lipid analysis of *L. dispar* is undertaken in a better attempt to understand the role lipids play in the olfaction process.

A variety of lipid classes are expected in the total lipid analysis of *L. dispar*. Cuticular lipids may include wax esters, alcohols, aldehydes and free fatty acids (Golebiowski, Bogus et al. 2011). For example, long chain wax esters are prevalent in the waxy cuticle of adult *Bemisia tabaci*, the cuticular lipids of *Acanthoscelides obtectus* include hydrocarbons, aldehydes, methyl and ethyl esters of free fatty acids, alcohols, sterols, and triacylglycerols (Golebiowski, Malinski et al. 2008), and the cuticular lipids of *Calliphora vicina* contains only hydrocarbons, free fatty acids and triacylglycerols.
Fatty acids can exist as free acids or as lipid bound esters. Insects are able to biosynthesize saturated and monounsaturated C16 and C18 fatty acids (Beenakkers, Vanderhorst et al. 1985). However, metabolic studies using radiolabeled precursors have shown that many insects are unable to produce polyunsaturated fatty acids and therefore must rely on diet to acquire them (Beenakkers, Vanderhorst et al. 1985). De novo biosynthesis of linoleic acid from $^{14}$C-acetate has put forth the conclusion that certain species may be able to produce polyunsaturated fatty acids (Blomquist, Dwyer et al. 1982). Interestingly, it has been shown that Drosophila melanogaster is able to shorten long chain fatty acids from the carboxyl end (Beenakkers, Vanderhorst et al. 1985). The diversity of possible fatty acids makes characterizing the lipidome of any insect a complex problem.

The biological membrane components include the receptor proteins involved in signal transduction. Phospholipids make up the majority of biomolecules in the membrane. Other membrane lipids can include sphingolipids which may be glycosylated in some cases, such as is the case with Lucilia caesar, Calliphora vicina, and Drosophila melanogaster, all of which contain glycosphingolipids with N-acetylglucosamine and N-acetylgalactosamine (Abeytunga, Oland et al. 2008). The composition may lend some insight into the way in which PBP1 and PBP2 may interact with the receptors, whether through an electrostatic interaction of an amphipatic helix or through hydrophobic insertion. Finally sterols are important membrane components, particularly cholesterol which is critical for the formation of liquid ordered domains in biomembranes (Luckey
2008). Cholesterol is also a precursor for ecdysteroids including 20-hydroxyecdysone, an important moulting hormone (Lagueux, Harry et al. 1981; Beenakkers, Vanderhorst et al. 1985).

3.3. Materials and Methods

3.3.1. Materials

18 MΩ water was used in all cases except for ESI-MS where JT-Baker HPLC Water was used. Chloroform and methanol were spectro grade (Caledon). Hexane (EMD) and ethyl acetate (Sigma-Aldrich) were distilled prior to use. Benzene (Caledon) and diethyl ether (Caledon) were reagent grade. BSTFA (Supelco) were used as received and stored under dessicant. Sulfuric acid, acetic and phosphoric acid were from Anachemia. 1-naphthol was from Anachemia and cupric acetate was from Matheson, Coleman & Bell. The TLC plates were Macherey-Nagel DC-Fertigfolien ALUGRAM Xtra SIL G/UV254 silica plates. Palmitic (Fisher), linoleic (Sigma), oleic (Mallinckrodt) and stearic acid (Matheson, Coleman and Bell) were all reagent grade. Mass spectrometry acetonitrile (JT-Baker) was LC/MS purity. Formic acid was from Acros organics.
3.3.2. Extraction of Lipids

The whole insects were dissected prior to extraction with a dissection kit. Approximately 150 antennae were placed in a test tube with glass beads (3 mm diameter, ~ 5 mL). The test tube was flash frozen in liquid nitrogen and shaken. The sensilla hairs were sheared from the main branches by the glass beads and clung electrostatically to the test tube while the larger antennal backbone and branches could be removed. Fewer insects were needed for tissues containing larger concentrations of lipids per milligram of tissue weight. For example, only 2 or 3 insects were needed for lipid extraction of the abdomen.

The tissues were extracted with 2:1 chloroform (Caledon, spectro grade) / Methanol (Caledon, spectro grade) overnight at 5˚C (Plettner 1995). The extracts were then filtered with a disposable pipet and cotton filter to remove solid matter. The extracts were subsequently concentrated under vacuum and stored at -20˚C or colder.

3.3.3. Qualitative 2D-TLC Analysis

Separation of lipids from the extracts was achieved with a modified existing method (Blomquist, G.D. 1987). Pre-coated TLC-sheets Alugram® Xtra SIL G/UV_{254} (Macherey-Nagel) were used for analysis. The modified solvent system requires 3 different solvent systems of decreasing polarity (Figure 3.1). The plates are cut rectangularly (5cm × 7cm) so that they may be developed along a different direction for each solvent system. The plate is spotted with a single extract in the bottom left corner 1cm from all edges. The plate is dried and developed in 62:34:4 CHCl_3/MeOH/H_2O. The plate is dried at 35-40˚C and developed 90˚ perpendicular to the first development in 4:5:0.1 benzene/ether/acetic acid. Again, the plate is dried at 35-40˚C and developed for the last time in the same direction as the first development. The final development is in 8:2:0.2 hexane/ether/acetic acid.
Copper (II) Acetate – Phosphoric Acid reagent was used as a general lipid stain (Jork 1990). This reagent will stain polar lipids, glycolipids, esters of fatty acids, phospholipids and sterols. Copper (II) acetate (3 g) is dissolved in 100 mL 15% phosphoric acid.

Several functional group specific stains were used to get an overall idea of the class of lipids present in the *L. dispar* extracts. An α-naphthol/sulfuric acid reagent was used to identify any glycosylated lipids (Christie 1982). α-naphthol (0.5 g) is dissolved in water/MeOH (1:1). The developed TLC plate is sprayed with the stain until wet and then air-dried. The plate is then lightly sprayed with 95% sulphuric acid and dried with a heat gun. Hexoses will dehydrate to 5-(hydroxymethyl)-furfural under acidic conditions and can then condense with two molar equivalents of α-naphthol to form a colored complex as part of what is known as a Molisch reaction. Complexes with any lipid containing a sugar stains purple and all other complex lipids will stain yellow.

Phosphorus-containing lipids were identified using a molybdenum reagent (Christie 1982). MoO₃ was dissolved in 70% sulphuric acid. The mixture was refluxed...
until all molybdic oxide was dissolved. After cooling, 100mL of the reflux mixture added to 0.4072 g of molybdenum powder and refluxed for 1 hour. The first mixture and the second containing molybdenum powder are combined after everything is cooled to room temperature. The solution is diluted with 200mL of water and filtered. The reagent for staining is prepared by mixing the molybdenum solution/water/acetic acid (1:2:0.75). The reagent is left to stand at room temperature for 3 days before use.

A ninhydrin reagent was used to stain plates for free amino groups such as those found on phosphotidylethanolamine. Ninhydrin (0.2 g) was dissolved in 100 mL of water saturated with butanol. The developed plate was sprayed and the dried using a heat gun over a water bath. Lipids containing a free amino group stain as red-violet.

Figure 3.2

(A) Colored complex formed from a hexose and α-naphthol under acidic conditions. (B) Colored complex formed from a free amino group and ninhydrin
3.3.4. **Lipid-bound fatty acid analysis by GC/MS**

Fatty acid standards were esterfied (Figure 3.3) in order to make them more volatile and produce more highly resolved GC/MS chromatogram peaks. DMAP (0.2 molar equivalent) and EDC (1 molar equivalent) were dissolved in 5mL DCM. MeOH (4 molar equivalents) and the fatty acid were combined. The EDC/DMAP mixture was added to the round-bottom with the fatty acid and stirred overnight at room temperature. The reaction mixture was washed with NH$_4$Cl (2x10mL) and brine (2x10mL) and subsequently dried over Na$_2$SO$_4$. The methyl ester was purified by silica gel with 5% ethyl acetate in hexane as the mobile phase.

Lipid bound fatty acids were trans-esterfied (Figure 3.3) under basic conditions (Plettner 1995). To 1mL of the lipid extract, 100 µL of 5% KOH in MeOH was added and allowed to sit at room temperature for 1 hour. The reaction was quenched with 120 µL of 1N HCl. The methyl esters were extracted with distilled hexane (3x100µL). The extract was washed with 50 µL of 5% NaHCO$_3$ and dried over Na$_2$SO$_4$.
Analysis was carried out with Varian CP-3800/Saturn 2000 Gas Chromatograph/Mass Spectrometer equipped with a Supelco SPB-5 capillary column. Chromatographic conditions were as follows: Injector temperature: 220°C, Oven temperature: Hold 100°C for 5 minutes, ramp to 140°C at 10.0°C/min and hold for 2 minutes, finally ramp to 260°C at 5.0°C/min and hold for 8 minutes. Molecules were ionized in EI mode and mass analyzed in a 3D quadrupole ion trap.

3.3.5. Free fatty acid analysis by GC-FID

Fatty acid standards were prepared in 1:1 distilled (ethyl acetate/hexane). A 100 mM stock containing palmitic acid (27.24 mg/mL), linoleic acid (26.38 mg/mL), oleic acid (32.21 mg/mL) and stearic acid (25.85 mg/mL) was prepared. The required volume of stock standard was evaporated to dryness in glass ampoules and silylated with 5 µL BSTFA, sealed, and allowed to react for 1 hour at room temperature. The derivitized standards were diluted to the appropriate volume with distilled hexane to prepare working standards of 100 µM, 50 µM, 25 µM, 10 µM and 1 µM. The tissues analyzed for free fatty acids included the antennal sensilla, the head, the wings and the legs. The extract for each tissue (100 µL) was transferred to a glass ampoule prepared from disposable pipets. The solvent was evaporated to dryness under nitrogen, 5 µL of BSTFA was added, the ampoules were sealed and allowed to react at room temperature for 1 hour. The silylated extracts were diluted with 95 µL of distilled hexane and further diluted 1/100 in distilled hexane before injection.

Analysis was carried out by gas chromatography under the following conditions: Inj. Temp. 250°C; Det. Temp. 300°C; Splitless mode 20 mL/min total flow; Initial column temp. 100°C held for 5 min, 20°C/min to 205°C and held 10 min, 5°C/min to 250°C. The GC was fitted with a Supelco SPB-5 capillary column.
3.3.6. Isolation of major lipid classes and analysis of polar lipids

3.3.6.1. Chromatography

The lipid extract can be separated into its polar lipid components by silica gel chromatography. The column was built on the microscale using a disposable pipet. The mobile phase is CHCl₃/MeOH/H₂O (62:34:4). The column is packed with approximately 20 mm of silica. Fractions are monitored by TLC using the same mobile phase and stained using a copper acetate/phosphoric acid reagent. The fractions should not exceed 300-400 μL as some of the polar lipids are not well resolved.

3.3.6.2. NMR

Dr. Andrew Lewis performed instrument shimming and optimization for all NMR experiments.

The isolated glycosphingolipid was run in CDCl₃ (99.96%) on a Bruker AVANCE II 600 MHz digital NMR spectrometer equipped with a Bruker 5 mm TCI cryoprobe with Z-gradient. The sample temperature was maintained at 298 K (Bruker BVT 3000 temperature controller with Bruker BCU-05 cooler unit). Manual shimming was performed by adjusting the currents in the various room-temperature shim coils to optimize the ¹H spectrum in real-time. ¹H decoupling was achieved using WALTZ-16
composite pulse decoupling with the $^1$H transmitter was offset to 4 ppm. $^1$H and $^{13}$C spectra were referenced using the known chemical shifts of organic solvents.

The $^1$H experiment was optimized for a 3 mm NMR tube. The 90° pulse was 7.6 $\mu$s at 3.00 dB (12.6 W), D1 = 2s, 400 scans, LB = 0.05. CHCl$_3$ set to 7.26 ppm. 2D experiments included $^1$H-$^1$H COSY, $^1$H-$^{13}$C HSQC, $^1$H-$^{13}$C HMBC and $^1$H-$^1$H TOCSY with two mixing times of 120 ms. Additionally, a gradient double quantum filtered-COSY was performed (Appendix Table A3.7).

### 3.3.6.3. Mass Spectrometry

MS acquisition was completed by Hongwen Chen on the instrument belonging to the SFU Chemistry department Mass Spectrometry facility.

The lipids isolated from the silica gel chromatography were analyzed with a Bruker Daltonics micrOTOF ESI-TOF mass spectrometer. The MS was run in positive ion mode from 50-3000 m/z. Capillary bias was set to 5000 V and endplate offset was set to -500 V. Dry heater temperature was set to 200°C, nebulizer pressure was 4.0 bar and dry gas flow was 8.0 L/min. Samples were introduced by direct infusion to the nebulizer with an acetonitrile/water solvent mixture containing 0.1% formic acid. The instrument was calibrated with Agilent ESI-L low concentration tuning mix in positive ion mode.
3.4. Results

3.4.1. Qualitative 2D-TLC analysis

TLC analysis provided a general overview of the lipids present in *L. dispar*. Lipid patterns were consistent between tissues for less polar lipids (Figure 3.4).

![2D-TLC developed with 3 solvent systems of decreasing polarity for various tissues from L. dispar. Brightness and contrast enhanced for sharper image.](image)

The blue spot at the center of each plate was determined to be cholesterol by reference standard. The more polar membrane lipids are separated along the initial vertical solvent vector, the relatively medium polarity lipids are separated along the horizontal vector and the low polarity lipids along the second vertical solvent vector. TLC is a type of normal-phase chromatography which works on the principle of adsorption. The composition of the mobile phase dictates how well the solutes will separate. Normal-
phase chromatography works much better to resolve polar compounds as these can adsorb to silica much better. As the polarity of the mobile phase increases, solvent molecules will begin to out-compete solute molecules for adsorption sites and this will speed up elution of the analyte. This principle is clear from looking at the resolution of the polar membrane lipids separated by the first solvent system (Figure 3.4) compared to that of the non-polar lipids separated by the third solvent system.

The polar lipids of the antennal sensilla were of most interest because they are the primary site for olfaction. These lipids were stained with various reagents geared to identify specific functional groups which would later be helpful for more detailed analysis.

*Figure 3.5*  
Antennal sensilla lipid extract stained with various reagents. The TLC plates were scanned and imported to PowerPoint. The scan quality limited the contrast between spots and background so brightness and contrast were optimized with PowerPoint to improve visualization of the spots.
3.4.2. **Analysis of Lipid-bound fatty acids**

The lipids extracted from *L. dispar* contained phospholipids, glycerides, cholesterol, free fatty acids and other polar lipids possibly with esterfied fatty acyl chains (Figure 3.6). Methanalysis will only produce fatty acid methyl esters from lipid bound fatty acids.

**Figure 3.6**

_Esterfied fatty acids and other lipids from *L. dispar* antennal sensilla. These fatty acids are esterfied to parent lipid head groups found in the olfactory biomembrane. For analysis, these fatty acids were trans-esterfied to form the methyl ester analog. Fatty acids were assigned based on retention time of standards and diagnostic fragment ions._
Unlike fatty acids esterfied to a large parent lipid head group, such as a phosphocholine group, free fatty acids lack a good leaving group and will revert back to their carboxylate form upon attack by methoxide. Free fatty acids will not elute from a GC column because of their significantly lower volatility and ability to H-bond with the stationary phase. The reaction with the chloroform/methanol extract and therefore other compounds extracted appeared in the chromatograms of this analysis. For example, the chromatogram of the lipid-bound fatty acid methyl esters from the antennal sensilla contain various hydrocarbons as well as cholesterol (Figure 3.6) (Appendix Figure A3.3). Bis(2-ethylhexyl)phthalate (Appendix Figure A3.4) and retinal (Appendix Figure A3.5) were assigned based on comparison of their fragmentation pattern against a mass spectral database.

Hydrocarbons were identified based on fragmentation pattern (Appendix Figure A3.2) and assigned based on retention time (Appendix Figure A3.1). Esterfied fatty acids were also identified by fragmentation pattern (Figure 3.7) and assigned based on retention time comparison.
Figure 3.7

*Methyl myristate (Me-14:0) mass spectrum displaying fragment ions typical of a fatty acid methyl ester.*

with standards. The most prevalent fatty acids were palmitic acid, linoleic acid and oleic acid (Figure 3.8).

The C16 and C18 ratios of esterfied fatty acids did not vary significantly between insect parts. With all the different insect parts, oleic acid was the present in greatest amounts followed by linoleic acid and palmitic acid. Among the minor fatty acids present, palmitoleic acid was present in the greatest amounts except in the case of the antennal sensilla where behenic acid was present in approximately equal amounts.
Figure 3.8

**Distribution of assigned esterfied fatty acids extracted from different tissues of L. dispar.** Top left panel compares ratios of minor fatty acids from each tissue. Top right panel compares ratios of major fatty acids from each tissue. Bottom panel shows GC chromatograms of tissue known to contain olfactory sensilla and the thorax, not known to contain sensilla hairs. Fatty acid ratios should not be compared between tissues from this data as the experiment was done qualitatively.

### 3.4.3. Free fatty acid quantitative analysis

The free fatty acids are those which are not esterfied to a parent head group. Free fatty acids were silylated in order to be able to complete analysis using gas chromatography. Lipid-bound fatty acids will not become silylated because the ester oxygen on the fatty acid is too hindered and not nucleophilic enough to attack the
BSTFA silicon. Palmitic acid, linoleic acid, oleic acid and stearic acid were the fatty acids which were analyzed because they were the ones found to bind PBP1 and PBP2.

<table>
<thead>
<tr>
<th></th>
<th>$C_{\text{palmitic}}/C_{\text{stearic}}$</th>
<th>+/-</th>
<th>$C_{\text{oleic}}/C_{\text{linoleic}}$</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>sensilla</td>
<td>1.3</td>
<td>0.1</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>head</td>
<td>2.1</td>
<td>0.2</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>legs</td>
<td>2.9</td>
<td>0.3</td>
<td>1.9</td>
<td>0.4</td>
</tr>
<tr>
<td>wings</td>
<td>1.7</td>
<td>0.2</td>
<td>1.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Throax</td>
<td>1.6</td>
<td>0.04</td>
<td>1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Abdomen</td>
<td>1.7</td>
<td>0.1</td>
<td>1.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Figure 3.9*  
*Free fatty acids per mg of extracted lipid. The inset table describes how free fatty acid ratios fluctuate between different tissues analyzed*

The head contained the highest overall concentration of fatty acids. Oleic acid was found in highest concentrations in all the tissues analyzed and stearic acid was found to be in lowest concentration. Ratios of the saturated fatty acids and unsaturated fatty acids were compared between tissues (Figure 3.9). Palmitic acid was found in higher concentrations than stearic acid in all tissues analyzed except the sensilla where the ratio between the two was much closer. The average ratio of palmitic/stearic was 2.2 for all tissues except the antennal sensilla where the ratio was $1.3 +/- 0.1$. This could
mean that the antennal sensilla contains greater relative concentrations of free stearic acid than the other tissues analyzed.

### 3.4.4. NMR structure determination

The isolated glycosylated lipid found in the wing extract with \( R_f \) similar to that found in the antennal sensilla and staining positive for sugars was analyzed by various NMR techniques. It was confirmed by mass spectrometry that this lipid fraction is identical to that in the antennal sensilla (Figure A3.2). The wing fraction was used because of a larger available extract mass. The \(^1\)H NMR revealed overlapping signals of shielded non-exchangeable protons from the CH\(_2\) envelope at about 1.25 ppm. A singlet at 8.10 ppm was exchangeable and appeared in a galactocerebroside standard, which is a glycosphingolipid containing a single galactose bound to a ceramide group. The amide proton seen at 8.1 ppm comes from the N-linked fatty acyl chain on the ceramide. The carbohydrate ring protons appear between 3 and 5 ppm in a \(^1\)H NMR. The most deshielded of the ring protons are the anomeric protons because they are the only protons on the ring adjacent to 2 oxygens. A 1H-1H COSY (Figure 3.10) was able to identify the proton signals for H2 based on coupling to the anomeric protons.
The three overlapping anomeric proton signals on the f2 axis (Figure 3.10) couple with protons at 3.4 ppm. A $^1$H-$^1$H TOCSY showed further coupling of H1 with protons H2, H3 and H4 (Figure 3.11). The dqf-COSY (Figure 3.12) was used to assign geminal carbohydrate protons at H6. The two doublet-of-doublets show geminal coupling of 11.97 Hz and 11.92 Hz. The dqf-COSY shows that these signals are coupled together. Multiplets at 3.70 ppm and 3.77 ppm are also gem-coupled to each other. These protons are not coupled to ring other ring protons in the TOCSY. The HSQC (Figure 3.13) shows that both signals are coupled to the same carbon at 64 ppm. These protons have therefore been assigned as the H7 protons. The HSQC also showed coupling of both H6 protons to the same carbon at 62 ppm. Finally, the HSQC showed that the signals assigned H2, H3, H4 and H5 are all coupled to different carbons.
Figure 3.11
TOCSY spectrum of a glycosphingolipid isolated from the lipid extract of L. dispar wings

Figure 3.12
dqf-COSY spectrum of a glycosphingolipid isolated from the lipid extract of L. dispar wings
3.4.5. Mass Spectrometry of isolated polar lipids from the antennal sensilla

The polar lipids were isolated from the antennal sensilla for mass spectrometry analysis using a silica gel column and fractions were monitored using TLC. I was most interested in the glycosphingolipids. The first glycosphingolipid eluted at Rf = 0.71 and will be noted as fraction ‘S5’. The second glycosphingolipid which could be resolved eluted at Rf = 0.63 at will be identified as fraction ‘S6’. The mobile phase for TLC was
CHCl₃/MeOH/H₂O (62:43:4). A phospholipid was isolated on the same type of column and TLC system with a Rf=0.31 and will be referred to as ‘F5’. The glycosphingolipids with similar retention factors were isolated from the wing extract and compared those from the antennal sensilla (Appendix Figure A3.6).

There are more than one (M+H)⁺ precursor ion species for S5 and S6. This is because each fraction contains a glycosphingolipid with various species containing different fatty acyl chains. The most common sphingosine base is d18:1 and therefore structures with this base will be given preference. S5 contains a cluster of (M+H)⁺ ions around 1404.14 Da (Figure 3.14).
Figure 3.14

ESI-TOF MS in positive ion mode for glycosphingolipid fraction S5 isolated from antennal sensilla extract.

The Lipid Maps structure database (LMSD) was used to help with structure assignment. LMSD belongs to the online community Lipid MAPS (Lipid Metabolites and Pathways Strategy) and is supported by the National Institute of General Medical Sciences (NIH). The composition and sequence of the attached sugars is not known and will be identified as a generic hexose (Hex). N-acetylhexosamines have a different molecular weight and will be designated as (HexNAc).
There are 5 clusters of peaks showing in the close-up of the S5 precursor ions (Figure 3.14). All the ions identified have a sphingosine base d18:1 and have varying fatty acyl chains ranging from 18:0 to 26:0. I identified saturated fatty acids C18, C20 and C22 from the lipid-bound fatty acid analysis. Each hexose adds 162 mass units to the glycosphingolipid so I believe that S5 has 5 hexose sugars attached. The sugar fragment ions are not easily recognized from the mass spectrum (Figure 3.15). The ions corresponding to the hexose fragment ions are present although not very intense. Slightly less harsh ionization conditions may be able to improve the relative intensity of these ions.
Figure 3.15
Mass spectra of fragment ions from isolated L. dispar sensilla GSL. Loss of a single hexose sugar amounts to loss of 162 mass units. Fragment ion for 1080.84 ion was not observed.

The doubly charged species (M+2H)^+^2 and (M+H+Na)^+^2 were identified for many of the precursor ions (Figure 3.16). The glycosphingolipid likely contains 5 hexose sugars and the sphingosine base d18:1.

The second extracted glycosphingolipid, S6, has a cluster of precursor ions around 1063.68 Da (Figure 3.17). The possibilities of glycosphingolipids involving this precursor all include one amide hexose (HexNAc) and two hexoses. The sphingosine base d18:1 was again included because it is the most common. Fatty acyl chains for the precursor ions included 16:1 and 16:0. The sodium adduct for 1063.68 Da can be seen at 1085.68 Da.
Figure 3.16

*Doubly-charged species and sodium adducts of several precursor ions for S5*

The loss-of-sugar fragments for S6 are seen in Figure 3.18. Loss of HexNAc carries a loss of 203 mass units which is seen at 860.68 Da. Loss of the remaining hexoses is also seen among the fragment ions in Figure 3.1
Figure 3.17
Precursor ions for lipid extract fraction S6.
3.5. Discussion

The qualitative TLC helped to identify substituents on the separated lipids. Further more detailed analysis would not have been possible without having this visual map of the lipidome. The lipid patterns were similar between the different tissue extracts. Cholesterol was clearly visible as a different colour and was confirmed by TLC of a standard and again thorough GC/MS analysis.
The goal of the lipid-bound fatty acid analysis was to identify as many lipid-bound fatty acids as possible. Analysis revealed that there were not significant differences between the different extracted tissues in terms of their fatty acid composition, with the exception of the antennal sensilla having larger amounts of 20:0 and 22:0 relative to its other fatty acids. This was also seen in the leg extracts and wings but not in the abdomen and thorax. It was interesting to see in the ESI-TOF mass spectra of the glycosphingolipids that possible fatty acyl chains included these long chain fatty acids. It is interesting to note that the wing and leg extracts were also found to have free fatty acid concentrations similar to that of the antennal sensilla. Future work would likely include attempting to isolate PBPs from these tissues. The combination of similar lipid components and possibly PBPs could mean pheromone olfaction occurs elsewhere than the antennal sensilla.

It is not surprising that C16 and C18 saturated and unsaturated fatty acids were found as these are present in many orders of insects (Stanley-Samuelson, Jurenka et al. 1988). It was interesting to learn that *L. dispar* also contains lipid-bound fatty acids with odd-number carbon backbones which are less common. The large array of hydrocarbons which were assigned are likely coming from the waxy cuticle of the sensilla. Cholesterol is acquired through the moths’ diet as they are not able to biosynthesize it themselves. Retinal was identified by its fragmentation pattern. Its presence is not clear. The plasticizer Bis (2-ethylhexyl) phthalate was also identified by a specific fragmentation pattern which was verified against a standard database. This may have been acquired into the moths’ body from being reared in plastic containers.

Free fatty acid analysis quantified palmitic acid, linoleic acid, oleic acid and stearic acid. These were the fatty acids found to bind PBP endogenously (Honson 2006). I was looking for any patterns of fatty acids composition found in the antennal sensilla not found elsewhere. A particular ratio of fatty acids or a specific one of those fatty acids may be critical to PBP acquiring an optimal conformation for pheromone binding. The head contained the largest concentration of free fatty acids of the tissues analyzed which is not surprising because after the fat body, the brain is the next largest source of lipids in insects. The fatty acid ratios in the sensilla contained a larger
concentration of stearic acid relative to palmitic acid and a larger concentration of linoleic acid relative to oleic acid. The wings had similar ratios for oleic/linoleic within error. A binding assay could be set up to prove or disprove this hypothesis similar to the one carried out in chapter 2.

The NMR data was needed to clearly show that the lipids were in fact glycosphingolipids. Beyond that, the spectra were too complex and involved too much signal overlap to be able to propose detailed structure. Mass spectrometry helped propose structures for the 2 isolated glycosphingolipids. In the case of S6, several structures involving 2 hexoses and a HexNAc with several different length fatty acyl chains were proposed. Glycosphingolipids belonging to the arthropod series are known to exist in insects (Abeytunga, Oland et al. 2008). These are glycosphingolipids with the a carbohydrate core of glucose and mannose followed by a GlcNAc or GalNAc. The other larger glycosphingolipid was proposed to have a structure containing 5 hexose sugars. The precursor ions also suggest that this lipid carries longer fatty acyl chains.

One potential function of these glycosphingolipids is to stabilize the membranes of dendrites in the presence of such high free fatty acid concentration. Fatty acids and their salts can act as bilayer membrane disruptors (Luckey 2008). The sensillar membrane lipids found here included sterols (found by Honson 2006) as well as glycosphingolipids. Sterols and glycosphingolipids are known to coalesce in microdomains which are known to strengthen the membrane (Parkin, Turner et al. 2001). Establishing the identity and sequence of the sugars in each of these lipids would require further analysis. Sugar sequences are almost exclusively determined by exoglycosidase incubation followed by mass analysis. An analysis which is simplified once the sugar composition is known.
3.6. Conclusion

The lipidome of *L. dispar*, and likely all insects, is functional. Its function can be better determined by studying the structural features of those lipids. A further experiment to investigate the relevance of the isolated glycosphingolipids towards bilayer stability could be to build a monolayer of phospholipid components and monitor isotherms in the presence of fatty acid micelles. If including the glycosphingolipids in the monolayer creates a detergent resistant membrane, then the monolayer would not dissolve from adding micelles.

The free fatty acid analysis suggests that a particular fatty acid may be more effective at producing a conformationally favorable state for pheromone binding in PBP. It could be an explanation for the variation in free fatty acid concentrations found in the antennal sensilla compared to the other tissue extracts.
4. Summary and Perspectives

4.1. Concluding Remarks

Historically, lipids have been ignored when discussing insect olfaction. Research has focused on pheromone binding proteins and receptor proteins that are involved (Plettner, Lazar et al. 2000; Xu, Atkinson et al. 2005). While it is known that PBPs can bind molecules other than their native pheromone (Du, Ng et al. 1994; Du and Prestwich 1995) as well as endogenous ligands when available (Honson 2006), a deeper understanding for some of these interactions has not been studied.

With this project I have shown that endogenous ligands which bind \textit{L. dispar} PBPs cause increased binding affinity for pheromone. This increased affinity does not appear to be specific to (+)-disparlure as the other stereoisomer and racemic mixture also showed increased affinity. Similar results with the C-terminally truncated PBPs indicated that the increased affinity is not related to interactions between the fatty acids and the C-terminus. Circular dichroism experiments revealed that the C-terminus does alter its conformation in a hydrophobic environment, which led me to believe that it may serve to dock PBP at the lipid bilayer. Other circular dichroism experiments showed that PBPs and tPBPs increase their global $\alpha$-helix content in the presence of fatty acids in the same ratios found endogenously. This suggested to me that the increased binding affinity observed is related to the conformational change of the protein and not to conformational change in the C-terminus. To try and justify this hypothesis, docking simulations were run using homology modeled \textit{L. dispar} PBPs. The docking simulations
showed that among the available exterior binding sites, sites closest to the pheromone binding cavity entrance (which also happen to be adjacent to the C-terminus) showed the lowest estimated free energy of binding (approximately 5 kcal/mol). Conformational change near the entrance may be a reasonable explanation for the increase in pheromone binding seen with the data from the binding assays. Altered conformation in the rest of the protein may change the shape of the interior cavity and be just as relevant to the increase in binding affinity.

The analysis of lipid-bound fatty acids showed a range of fatty acids including some longer chain ones up to 22:0. These fatty acids were later assigned as the fatty acyls belonging to the glycosphingolipids isolated from the antennal sensilla. Polyunsaturated fatty acids were difficult to identify because of the high degree of fragmentation. Many hydrocarbons likely belonging to the waxy cuticle of the sensilla were also identified as were some other lipids. These lipids included cholesterol, retinal and Bis(2-ethylhexyl) phthalate and were identified based on their fragmentation patterns. Among the major lipid classes separated by TLC, the polar lipids were focused on for structure determination because of their importance to biomembranes. Structures were proposed for both of the isolated glycosphingolipids based on ESI-TOF MS. NMR supports that these lipids are in fact glycosphingolipids. The determination of structure for these membrane lipids was of interest because of ability of *L. dispar*’s olfactory biomembrane to remain intact in the presence of fatty acid micelles and vesicles. The large carbohydrate moieties belonging to the glycosphingolipids may H-bond with each other adding stability. Further, the long chain fatty acyls which appear to be bound to these glycosphingolipids may provide additional stability in the form of additional van der Waals interactions within the bilayer. Finally, the structure determination of these glycosphingolipids is important because these lipids like to cluster with lipid to form microdomains in bilayers. Membrane proteins like to reside in microdomains (Ostrom and Insel 2004; Insel, Head et al. 2005), which means that these glycosphingolipids are in close contact with the olfactory receptors and any PBP that would approach the receptor.
4.2. Future Directions

The work for the two projects undertaken in this thesis can be expanded. Significant progress was made in mapping *L. dispar*’s lipidome, however the analysis was not completed. The total lipid mapping of any biological species is a huge undertaking and requires significant skills with many analytical techniques, with mass spectrometry being the most vital as I have learned. While NMR is a valuable technique for structure determination of organic molecules and there has been some lipid analysis carried out using advanced NMR techniques, the complexity, large size, microscale sample weights and diversity of closely related lipid species is simply too much for current NMR techniques to deal with. I have identified several lipid-bound fatty acids but I believe there are many which could not be identified by electron ionization or chemical ionization, especially the polyunsaturated fatty acids which break apart easily under harsh ionization conditions. The completion of this analysis is possible, but will need to be done with various nanospray-LC/MS/MS techniques. Resolution on a silica gel column is not high enough to be able to separate the closely related compounds, which makes interpreting the mass spectra of already complicated molecules more difficult.

With the knowledge gained from previous work on binding kinetics of PBPs (Gong 2009) and structure determination (Honson 2006), and now a better understanding for the role of these endogenous ligands, the next project with this work should be to study how PBPs interact with lipid bilayers. We have shown that the C-terminus does not affect pheromone binding and may therefore serve some other purpose such as docking with a lipid bilayer. With an already better understanding of the composition of *L. dispar*’s olfactory bilayer composition, liposomes can be prepared and used to study this interaction.
Appendices
Figure A2.1  Delipidation of PBP2 (2T3) with HIC-Methyl Macro Prep beads
Figure A2.2  Mean molar ellipticity per residue for CPBP2 (50µM) as a function of wavelength under phosphate buffered conditions (30mM, pH 8) and under 95% isopropyl alcohol 5% phosphate buffer conditions.
Figure A2.3  Sample chromatograms from Pheromone binding assay. 1. Extract from PBP2 bound with disparlure. 2. Extract from PBP2 bound with fatty acids. 3. Extract from PBP2 bound with fatty acids and disparlure.
Figure A2.4  Sample chromatograms from Pheromone binding assay. 1. Extract from Phosphate buffer, fatty acids and *disparylure*. 2. Extract from PBP2 bound with fatty acids and *disparylure*. Both samples were passed through a P2 polyacrylamide gel prior to extraction. Panels show the efficacy of the gel at retaining non-bound *disparylure*.
Figure A2.5 CPBP2 from homology model 2WCJ is unstructured in an aqueous solution and becomes an α-helix in isopropyl alcohol. The length of CPBP2 as an α-helix is 17.67 Å as calculated by MOE compared to the thickness of a dipalmitoylphosphotidylcholine bilayer which is 44.5 Å. It’s interesting to note that the PBP2 sequence right before the C-terminus is –Lys-Ala-His-Val-His-Lys-Leu-Asn—C-terminus. This positively charged α-helix sequence could electrostatically interact with the phospho-headgroups. The amphipatic C-terminus might intercalate into the hydrophobic part of the bilayer. The length of oleic acid is 17.75Å as calculated by MOE.
Figure A3.1

Bottom: Hydrocarbon standards ranging from C17H36 to C30H62. Top: Lipid-bound fatty acid methyl esters and hydrocarbons from L. dispar sensillar extract.
**Figure A3.2**

$C_{29}H_{60}$ from antennal sensilla extract (23.115 min) and from a standard (23.122 min)

$C_{29}H_{60}$ from extract

RT= 23.115 min

$C_{29}H_{60}$ from standard

RT=23.122 min
Figure A3.3  MS$^1$ of peak at 28.169 min from antennal sensilla extract assigned as cholesterol based on comparison of fragmentation pattern against a spectral database.
Figure A3.4  MS\textsuperscript{1} of peak at 19.115 min from antennal sensilla extract assigned as Bis(2-ethylhexyl)phthalate based on comparison of fragmentation pattern against a spectral database.
Figure A3.5  MS$^1$ of peak at 17.346 min from antennal sensilla extract assigned as retinal based on comparison of fragmentation pattern against a spectral database.
Figure A3.6

ESI-TOF MS of lipid extract fraction S5 and S6 from the antennal sensilla compared with fractions W4 and W6W7 from the wings extract.
Table A3.7 Parameters for NMR experiments on WinFrac3 run on Bruker AVANCE II 600 MHz spectrometer with 5mm TCI cryoprobe (operating at 600.42 MHz for 1H and 150.98 MHz for 13C)
References


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