Many species of bees, ants and wasps within the Order Hymenoptera live in colonies that require complex social behaviors rivaling that of human societies. Social behaviors within these colonies are largely mediated by chemical communication (Wilson and Holldobler, 2005), and the social Hymenoptera have evolved complex systems for chemical communication that include large numbers of exocrine glands that produce complex chemical mixtures used as pheromones. Despite the fundamental basal importance of sexual reproduction and mating behavior, very few sex pheromones have been identified from the social Hymenoptera due to their sophisticated behavior and the difficulty of behavioral assays (Ayasse et al. 2001). We will use state-of-the-art methods to address this deficit in a model species that represents the social Hymenoptera, the honeybee *Apis mellifera*. Our approach has potential for use in many other contexts in chemical ecology.

For several millennia human society has been intrigued by the honeybee *Apis mellifera*, to exploit their honey production and simply to observe their complex social structure. Its genome has been sequenced (The Honeybee Genome Sequencing Consortium, 2006) and the honeybee is being developed as a molecular genetic model to study the mechanisms and evolution of social behavior (Robinson et al., 2005; Wilson, 2006). Termed eusociality (Crespi, and Yanega, 1995; Wilson and Holldobler, 2005), honeybees live in colonies averaging 10 to 20 thousand individuals that cooperatively care for their offspring by a division of labor between reproductive and nonreproductive biological castes: A single female queen bee solely responsible for reproduction, several hundred male drone bees that exist only to mate, and all remaining females that are workers responsible for maintaining the colony (Winston, 1987). The different castes are specialized towards their function in the colony, for example, drones are strong fliers with enhanced olfactory and visual senses for mating. To regulate the complex social interactions within their colonies honeybees have evolved an intricate system of chemical communication that includes numerous glands that produce complex blends of pheromones (Slessor et al., 2005). For example, mandibular, hypopharyngeal, labial, wax, tergite, poison, nasanov, Dufour’s and Koschevnikiv’s glands produce pheromones that inhibit the development of worker ovaries, inhibit queen rearing, stimulate the queen retinue response, stimulate alarm and defense, and facilitate swarming, orientation and foraging, among many other behaviors (Free, 1987). Currently, as many as 50 honeybee pheromones have been identified (Keeling et al., 2004; Slessor et al., 2005). The queen retinue pheromone (QRP) is composed of at least 8 components (Keeling et al., 2003) while the alarm pheromone is made up of more than 12 chemicals (Breed et al., 2004). However, only a single sex pheromone component has been identified from honeybees to date.

In the early 1900s caged virgin queens were used to attract male drones and demonstrate the role of scent and olfaction in mating behavior (Salmon, 1938). Gary (1962) identified 9-oxodecenoic acid (9-ODA) as the scent that attracts male drones to virgin queens. 9-ODA was discovered two years previously in 1960 as the “queen substance” that stimulates the worker retinue that tends to the queen and that inhibits worker ovary development (reviewed in Winston, 1987). Therefore, a single pheromone mediates both mating behavior and social organization. Forty years later, no other sex pheromone component has been described from the honeybee, and the extent to which sex pheromones and social pheromones are shared remains unknown. The difficulty of conducting behavioral assays has without doubt contributed to this deficit. We have developed an *in vitro* receptor-based assay to circumvent this technical hurdle. Sexual dimorphism of the honeybee olfactory system provided a strategy to identify the honeybee
pheromone receptors as a component of our ongoing research into the molecular mechanisms of insect chemoreception.

Honeybee mating behavior: The olfactory system is sexually dimorphic

Drone rearing is coordinated with the emergence of virgin queens in the spring season. During summertime afternoons generally between 2:00 to 4:00 PM sexually mature drones and virgin queens depart on mating flights where they meet in discrete aerial congregation sites (typically 30-200 m in diameter and 10-40 m above the ground) (reviewed in Free, 1987). The same congregation area can be used for several years, but the specific characteristics that define a congregation area remain a mystery. When a virgin queen enters the congregation area the drones (from a few hundred to thousands) begin pursuit, first orienting to chemical cues but also using vision at closer distances. Drones mount virgin queens during mid-flight and copulation is complete within a few seconds. In the process the drone’s endophallus breaks off and remains in the queen’s vagina and the drone quickly dies. Queens may mate with several drones during the same and different mating flights. Drones exist only to mate with virgin queens, they perform no other task within the colony, and when the mating season has passed worker bees force drones out of the colony to die. As a result, drone morphology, including their olfactory system, has become specialized towards successful mating (Winston, 1987).

Drone antennae are larger, with 11 annuli (segments) compared to 10 in workers (Figure 1). The most common type of olfactory sensilla on honeybee antennae are oval “pore plate” shaped placoid sensilla and hollow hair-like trichoid sensilla. Electrophysiological recordings have demonstrated that olfactory neurons within the placoid sensilla respond to 9-ODA (Kaissling and Renner, 1968; Vareschi, 1971), the main component of the queen retinue.

Figure 1. A) The olfactory system of male drone bees is specialized for the detection of sex pheromone produced by virgin queens during mating flights. The sexual dimorphism is apparent when compared to B) female worker bees. Drone antennae are longer, have a greater density of pheromone sensitive placoid sensilla (p), and fewer trichoid sensilla (ta) compared to female antennae. Pictures were provided by Dr. Axel Brockmann, Entomology Department, University of Illinois at Urbana-Champaign, Urbana.
pheromone (QRP) that also attracts drones during mating. Drone antennae have about seven times as many pore plate sensilla (~18000 compared to ~2700) and seven times fewer trichoid sensilla (~400 compared to ~3000) relative to worker antennae (Figure 1) (Esslen and Kaissling, 1976; Brockmann and Bruckner, 2001). Due to this sexual dimorphism drone antennae have more olfactory neurons tuned to 9-ODA and their antennae as a whole are more sensitive as measured by electroantennography (EAG) (Vetter and Visher, 1997; Brockmann et al., 1998). The increased number of pheromone sensitive olfactory neurons has resulted in the development of four enlarged macroglomeruli in the drone antennal lobe (Figure 2) (Arnold et al., 1985; Brockmann, 1999; Sandoz, 2006). Within its genus the sex pheromone system of Apis mellifera appears to be more complex. The drone antennae from five other species have fewer pore plate sensilla, and the antennal lobe of A. florea drones has only two macroglomeruli (Brockmann and Bruckner, 2005).

Figure 2. The drone antennal lobe (schematic illustration) has four macroglomeruli (MG1-4) absent in female antennal lobes. Image provided by Dr. Axel Brockmann, published in Brockmann (1999). The axons from olfactory neurons in the antennae project to the antennal lobe of the brain, the first higher order processing center. Enlarged male-specific macroglomeruli accommodate the large number of olfactory neurons in the antennae thought to be dedicated to detecting sex-pheromones that mediate mating behavior. MG2 with the largest volume is activated by 9-ODA (Sandoz, 2006). MG1, 3 & 4 represent unidentified sex pheromone components.

The honeybee sex pheromone is expected to have at least four components

In the Lepidoptera, male-specific macroglomeruli in the antennal lobe respond exclusively to sex pheromones produced by the female moths (reviewed in Hannson and Anton, 2000; also see Lee et al., 2006 for a recent discussion). The number of macroglomeruli in the male antennal lobe matches the number of different major sex pheromone components produced by the female. Therefore, we expect the honeybee sex pheromone will be composed of at least four components based on the four macroglomeruli in the male drone’s antennal lobe (Figure 2). Only 9-ODA, the major component of the queen retinue pheromone (QRP), has clear sex pheromone activity. The queen retinue pheromone (QRP) consists of the main “queen substance” 9-ODA plus at least seven other components: 9-hydroxydecenoic acid (9-HDA), methyl p-hydroxybenzoate (HOB), 4-hydroxy-3-methoxyphenylethanol (HVA), methyl oleate, coniferyl alcohol, palmitoyl alcohol and linolenic acid (reviewed in Slessor et al., 2005). 9-ODA, 9-HDA, HOB and HVA were isolated from the queen’s mandibular gland and the blend was collectively termed the queen madibular pheromone (QMP) and commercially sold to bee keepers (Winston,
The last four minor components were discovered more recently by Keeling et al. (2003), and the new blend was named the queen retinue pheromone (QRP) since some of the pheromones are not synthesized in the queen’s mandibular gland. In the honeybee colony, QRP communicates the presence of the queen, attracts the retinue that tends to her, attracts and stabilizes swarms, inhibits queen rearing and inhibits worker ovary development. 9-ODA is also attractive to male drones during mating flights (Gary, 1962). 9-HDA was found to be “somewhat” attractive to drones on its own, but did not enhance the attractiveness of 9-ODA (Butler and Fairey, 1964). However, subsequent experiments were unable to confirm the attractiveness of 9-HDA (Blum et al., 1971; Boch et al., 1975). The attractiveness of queen pheromone components to drones was revisited recently. A blend of 9-ODA, 9-HDA and 10HDA together did not increase the attractiveness of baited dummies to drones over a distance, but did increased the frequency of drones that contacted the dummy (Brockmann et al., 2006).

When drone antennae were presented with 9-ODA, 9-HDA, 10-HDA, HOB and HVA, only macroglomerulus #2 (the largest) was activated by 9-ODA; macroglomerulus#1 (the second largest) was not activated (Sandoz, 2006; Figure 2). The sex pheromone that activates macroglomerulus #1 should produce EAG-responses similar in magnitude to that of 9-ODA (the size of the macroglomerulus corresponds to the number of olfactory neurons that project to it). 9-HDA and 10-HDA do not yield significant EAG responses when presented to drone antennae (Brockmann and Bruckner, 1998). Therefore, MG#1, 3 and 4 (Figure 2) likely represent unidentified major sex pheromone components while 9-HDA and 10-HDA are likely minor components processed by ordinary glomeruli. Our experimental results in the following sections support this hypothesis.

Behavioral observations also support the existence of missing sex pheromone components. Gary (1962) isolated two chemical fractions from virgin queens that were attractive to drones, one contained 9-ODA but the other remains unidentified. Furthermore, several behavioral studies have suggested that the queen tergite glands located on the abdomen secrete substances that promote courtship and copulation behavior (Free, 1987; Wossler and Crewe, 1999). An alternate intriguing possibility is the existence of a drone aggregation pheromone that may help establish aerial congregation sites (behavioral observations supporting this hypothesis are reviewed in Free [1987]). Male aggregation pheromones have been identified from the mandibular gland of some hymenopteran species (Ayasse, 2001) and one study suggests a similar gland exists in drone honeybees (Lensky et al., 1985). Research into the role of male aggregation pheromones in mating behavior is lacking.

**Functional genomics approach to resolving honeybee sex pheromone communication**

The difficulty of conducting behavioral assays with an insect that mates only in mid-flight has without doubt contributed to the deficit of sex pheromone research. We propose to identify the hypothesized missing honeybee sex pheromone components using an interdisciplinary strategy that utilizes functional genomics in a “reverse chemical ecology” approach. The honeybee sex pheromone receptors will be identified and used in an *in vitro* receptor-based assay to identify the pheromone chemicals from solvent extracts of virgin queen bees. This will require an interdisciplinary effort; combining the Roberston lab’s expertise in the identification and cloning of chemoreceptors with the Luetje lab’s expertise in receptor pharmacology.

The last decade has witnessed tremendous progress in the molecular and neurological mechanisms of chemoreception, including the identification of the transmembrane
chemoreceptor (Cr) genes (members of the GPCR chemoreceptor superfamily). The term chemoreceptor refers collectively to the odorant and gustatory receptor subfamilies (Ors and Grs). The molecular model of insect olfaction at the periphery has been reviewed recently by Dahanukar et al. (2005), Rutzler and Zwiebel (2005) and Hallem et al. (2006). Briefly, odors diffuse into olfactory sensilla that are filled with aqueous lymph that surrounds the dendrites of specialized olfactory neurons. Odorant binding proteins transport the hydrophobic stimuli to the sensory neuron membrane where the Ors are located. Odor binding to odorant receptors triggers cellular signaling pathways thought to be mediated by G-proteins, resulting in sensory nerve impulses. The preeminent role of the Or family in the molecular perception of odors is clearly illustrated using transgenic Drosophila that lack Or gene expression in a specific olfactory neuron. Drosophila Ors can be expressed in the “empty neuron” and their odor binding characterized by single sensillum electrophysiology (reviewed in Hallem et al., 2006). The electrophysiologial characteristics of a “wild type” sensillum can be replicated in the empty neuron simply by juxtaposing its chemoreceptor into the empty neuron (Hallem et al., 2004).

Recently, the Ors that bind and perceive the silkworm (Bombyx mori) sex pheromone (Nakagawa et al., 2005) and the Drosophila melanogaster aggregation pheromone (Ha and Smith, 2006) have been identified. Therefore, we (Robertson lab) annotated the complete repertoire of honeybee odorant receptors and characterized their gene expression levels in male drone and female worker antennae. Candidate sex pheromone receptors were identified based on high expression levels in drone compared to worker antennae, exploiting the sexually dimorphic olfactory system. Chemical extracts from virgin queens will be screened for activity in vitro using Xenopus oocytes expressing the candidate pheromone receptors (Luetje lab) to ultimately identify the active chemicals.

The honeybee genome encodes 170 odorant receptor genes

The honeybee genome sequence was recently published (Honeybee Genome Sequencing Consortium, 2006) and HMR was an integral member of this effort that represents his broad research in the field of insect genomics. During the past decade HMR has led the effort to annotate chemoreceptor genes from the nematode (Robertson, 1998; 2000; and 2001; Robertson and Thomas 2005), fruit fly (Robertson et al., 2003), mosquito (Hill et al., 2002) and honeybee (Robertson and Wanner, 2006) genomes as part of his general interest in the molecular mechanisms of insect olfaction (Robertson et al., 1999 and Nardi et al., 2003 for example). The superior olfactory abilities of honeybees has stimulated the curiosity of many scientists over the years (including the 1973 Nobel Laureate von Frisch, for example), and publication of their odorant receptors (Robertson and Wanner, 2006) was an anticipated event noted by the Faculty of 1000 for its significance. An important insight into the evolutionary plasticity of chemoreceptor families in response to their “chemical niche” resulted. Consistent with the critical role of honeybee olfaction in floral foraging and social pheromone communication, its odorant receptor genes have expanded to 170 (compared to 62/79 in fly/mosquito) (see phylogenetic tree in Robertson and Wanner, 2006). In contrast, the honeybee genome encodes very few gustatory receptors, only 10 compared to 68 and 76 in flies and mosquitoes, a reflection of its symbiotic relationship with plants as nectar and pollen feeding pollinators (as opposed to herbivores that must decode botanical feeding deterrents). Identifying the receptor sequences provides the foundation for subsequent scientific discoveries. Annotation of the Drosophila melanogaster odorant receptor genes in 1999/2000 propelled this field towards the tremendous progress reviewed in Dahanukar et al. (2005), Rutzler and Zwiebel (2005) and Hallem et al.
Honeybee pheromone research during the last 40 years illuminates their complexity; many honey bee pheromones exert both primer and releaser effects to regulate both social organization and other behaviors (Slessor et al., 2005). However, during this time only a single sex pheromone has been identified. We have an unprecedented opportunity to unravel the complexities of honeybee sex pheromone communication from the perspective of the receptors that perceive these chemicals.

Of 170 honeybee Or genes, four are expressed at higher levels in male drone antennae

We hypothesized that the Ors that perceive the sex pheromone will be expressed at much higher levels in the male antennae compared to females due to their sexual dimorphism. This strategy of exploiting the sexual dimorphism of the olfactory system was used successfully to identify the first candidate moth sex pheromone receptors (Krieger et al., 2004; 2005; Nakagawa et al., 2005). To screen the gene expression levels of 170 Ors we constructed an olfactory-specific custom microarray. Oligonucleotide probes (70 bp in length) were designed for approximately 400 olfactory-related genes including the 170 odorant and 10 gustatory receptors described in Robertson and Wanner (2006). Other honeybee genes that might be involved in odor transport (odorant binding and chemosensory proteins) and odor degradation (P450s, glutathione s-transferases and esterases) were included, as well as control house-keeping genes. Microarrays have not been particularly successful for studying insect chemoreceptors (John Carlson’s lab with fruit flies, personal communication; our experience and Biessmann et al. (2005) with mosquitoes), however we believed they would work for honey bees because we anticipated that the Ors expressed in male antennae involved in sexual communication would be expressed at unusually high levels.

Foraging worker bees returning to the hive with pollen loads, and mature drones within hives, were collected during the summer months of 2005 and 2006 from colonies maintained by the University of Illinois Bee Research Facility (Urbana, IL). Antennae were dissected from pooled batches of 50 bees that were collected independently, immediately frozen on dry ice, and stored at -80°C (six batches of foragers, and six batches of mature drones were collected in total). Total RNA was isolated from each batch of antennae using the Qiagen RNeasy Mini Kit and stored at -80°C. 1st cDNA was synthesized and labeled with single-use CyDye label (Cy5/Cy3, GE Healthcare). After probe hybridization and washing the microarray slides were scanned on an Axon 4000B Scanner using GenePix v6.0 software.

Sixteen genes were expressed at differential levels in male and female honeybee antennae with statistical significance (p-value < 0.05) (Table 1). Six were expressed at higher levels in male antennae while 10 were higher in female antennae. The sex-biased expression of six odorant binding protein genes and one carboxyl esterase gene (AmOBPs 2, 4, 11, 13, 14, and 19 and AmCEst01, Table 1) agrees with published results (Kamikouchi et al., 2004; Foret and Maleszka, 2006) and provides a validation of this approach. The sex-biased expression of five odorant receptor genes was detected at p-values < 0.05, four were higher in male drone antennae and therefore were putative sex pheromone receptors (AmOr10, 11, 18 and 170).

To validate the microarray results, differential expression of the AmOr genes in Table 1 was assayed by quantitative real-time PCR using methodology reported in Robertson and Wanner (2006) and Wanner et al. (In Press). Because moth pheromone receptors form a conserved phylogenetic subfamily (Krieger et al., 2004; 2005; Nakagawa et al., 2005; Wanner et al., In Press), we included AmOrs with sequence similarity to the male-biased Ors detected in the microarray analysis. First, total RNA (genomic DNA removed by DNAse I digestion) was
isolated from pooled male and female antennae and 1st strand cDNA was synthesized and used as template to screen the expression of 43 honeybee Ors (Figure 3). Supporting our microarray results, of 43 AmOrs tested, only AmOr10, 11, 18 and 170 were expressed at higher levels in drone antennae (15-25 times higher). Next, we repeated the qPCR assay using single pairs of antennae dissected from individual insects for biological replication to confirm the male-biased expression of AmOr10, 11, 18 and 170. In this experiment we compared male drones to female workers and to female queen bees (Figure 4). The gene expression levels of AmOrs10, 11, 18 and 170 are significantly higher in male drone compared to female worker and queen antennae. AmOr11 and 18 are expressed most highly (Figure 4), at levels comparable to AmOr2, the DmOr83b ortholog. DmOr83b and its orthologs act as a chaperone and dimer partner for all regular Ors (Larsson et al., 2004; Nakagawa et al., 2005; Neuhaus et al., 2005; Benton et al., 2006) and therefore, AmOr2 is expressed in all olfactory neurons and its overall level in antennae is high. In moths, only the pheromone receptor genes in male antennae are expressed at levels comparable to this ubiquitously-expressed receptor (Wanner et al., In Press). Next, an assay to functionally characterize the sex pheromone receptors was developed in collaboration with the Luetje lab (manuscript in preparation).

Table 1. Genes expressed differentially between male drone and female worker bee antennae, p-value = 0.05, microarray analysis, n = 4 replicates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Male : Female</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmCyp006BE1</td>
<td>0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AmOBP19</td>
<td>0.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AmAAR2</td>
<td>0.11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AmCestB1</td>
<td>32.6</td>
<td>0.001</td>
</tr>
<tr>
<td>AmOBP11</td>
<td>0.08</td>
<td>0.001</td>
</tr>
<tr>
<td>AmOBP02</td>
<td>0.16</td>
<td>0.002</td>
</tr>
<tr>
<td>AmOr011</td>
<td>12.8</td>
<td>0.006</td>
</tr>
<tr>
<td>AmOBP04</td>
<td>0.28</td>
<td>0.007</td>
</tr>
<tr>
<td>AmCg5</td>
<td>0.31</td>
<td>0.02</td>
</tr>
<tr>
<td>AmOr010</td>
<td>5.1</td>
<td>0.03</td>
</tr>
<tr>
<td>AmOr018</td>
<td>4.5</td>
<td>0.04</td>
</tr>
<tr>
<td>AmOBP14</td>
<td>0.32</td>
<td>0.04</td>
</tr>
<tr>
<td>AmOr170</td>
<td>4.3</td>
<td>0.05</td>
</tr>
<tr>
<td>AmOr151</td>
<td>0.26</td>
<td>0.05</td>
</tr>
<tr>
<td>AmOBP13</td>
<td>0.26</td>
<td>0.05</td>
</tr>
<tr>
<td>Amlip1</td>
<td>3.4</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Figure 3. Male to female expression ratio of 43 AmOr genes was determined by qPCR, AmOrs 2, 4, 5, 6/7, 8 - 25, 29, 30, 35, 42, 49, 50, 60, 63, 68, 78, 81, 89, 95, 109, 126, 132, 149 -152 and 170 sequentially on the x-axis). CT values for each gene expressed in male drone and female worker antennae were normalized to AmRPS8 before calculating the male to female ratio (y-axis).
The ligand-binding properties of insect Ors can be assayed in Xenopus oocytes

To carry out the proposed project, it is important for us to have established a robust functional expression system for in vitro characterization of insect odorant receptors, which until recently has proved elusive. Drosophila molecular genetic tools have enabled the characterization of many of its Ors using transgenic flies and single sensillum electrophysiology in the Drosophila “empty neuron” developed in the Carlson lab (Hallem et al. 2006). However, moth and bee Or genes that the Robertson lab has sent to the Carlson lab for testing have not worked in this system (unpublished data). Incompatibility between the Ors from different insect orders and the Drosophila downstream signaling components may prevent efficient signal transduction. Recently, a significant study demonstrated the function of a moth pheromone receptor in the Drosophila “empty neuron” system (Syed et al., 2006). However, the activity was weak and the pheromone had to be delivered at very high concentrations. For the proposed study, we require an in vitro assay that is not restricted by the phylogenetic source of the receptors, and one that is amenable to high throughput screening methods. Recently there have been a few reports of functional expression of insect odorant receptors using the Xenopus oocyte expression system (Wetzel et al., 2001; Nakagawa et al., 2005). Xenopus oocytes are commonly used in pharmacology research and CWL has extensive experience with this expression system, having used Xenopus oocytes to characterize a variety of receptors and channels (for example, Hsiao et al., 2006; Abaffy et al., 2006a; 2006b). Recently, the Luetje laboratory has successfully used this assay to characterize several mouse odorant receptors (Abaffy et al., 2006a; 2006b). To assess the utility of the Xenopus oocyte expression system for characterization of insect odorant receptors, we chose to examine the Drosophila odorant receptor DmOr35a (Figure 5). The ligand specificity of this receptor has been extensively characterized in an in vivo context (Hallem et al., 2004; Hallem and Carlson, 2006), offering an ideal test of the oocyte expression approach. In addition to determining whether oocytes offer a reliable functional expression system, it is important to demonstrate that insect odorant receptors expressed in this system display accurate ligand recognition properties. In Figure 5, we examine the ligand specificity of DmOr35a expressed in the Xenopus oocyte system and assayed under two-electrode voltage clamp. We find that functional responses of DmOr35a to the cognate ligand (hexanol) only occur when DmOr83b is co-expressed (Fig. 5A). This is consistent with reports that the widely expressed DmOr83b serves as a dimerization partner for many different DmOrs (Larsson et al., 2004; Nakagawa et al., 2005; Neuhaus et al., 2005; Benton et al., 2006).
Figure 5. The ligand-binding characteristics of insect odorant receptors can be assayed in *Xenopus* oocytes. A) *Left* trace, an oocyte injected with RNA encoding DmOr35a fails to respond to 30 µM hexanol (HEX). *Right* trace, a different oocyte expressing DmOr35a and DmOr83b responds to 30 µM hexanol. B) An oocyte expressing DmOr35a and DmOr83b responds to 1 µM hexanol, but not 30 µM geraniol (GER) or 30 µM octanoic acid (OCT). C) *Left*, an oocyte expressing DmOr35a and DmOr83b is challenged with a range of hexanol concentrations. *Right*, results from 3 oocytes are presented. D) *Left*, an oocyte expressing DmOr35a and DmOr83b is challenged with 30 µM of 1-alcohols of varying carbon length. *Right*, results from 6 oocytes are presented.

While the DmOr35a+DmOr83b receptor responds to a low concentration of hexanol (1 µM), no responses are seen upon application of 30 µM geraniol or 30 µM octanoic acid (Fig. 5B), consistent with the ligand specificity of this receptor *in vivo* (Hallem and Carlson, 2006). Using the *Xenopus* oocyte assay, we can characterize various aspects of the ligand sensitivity and specificity of the DmOr35a+DmOr83b receptors. For example, dose-response analysis yields an EC$_{50}$ for hexanol activation of 670 ±70 nM (Fig. 5C) and a brief screen with a series of aliphatic alcohols shows that while this receptor is most responsive to hexanol, it also responds to both shorter and longer ligands (Fig. 5D). Whether the lower responsiveness to these other ligands is due to lower potency or lower efficacy (or both) is not currently known, but this information can be derived from further dose-response analyses.

*AmOr11 binds 9-ODA, the main component of QRP. AmOr10, 18 and 170 are orphan receptors*  
The complete open reading frames (ORF) of AmOr2, 10, 11, 18 &170 were cloned (from cDNA synthesized from total RNA isolated from male drone bee antennae) into the pGEMHE plasmid vector and confirmed by sequencing. We screened oocytes injected with RNA encoding AmOr10, 11, 18 or 170, in combination with RNA encoding AmOr2 (thought to serve the same role, as a dimerization partner, as DmOr83b). Oocytes were challenged with each of the 4 components of a commercial QMP preparation, as well as the QMP preparation itself (Figure 6). The AmOr11+AmOr2 receptor responded to 100 µM 9-ODA and to the QMP preparation.
Figure 6. 9-ODA activates AmOr11 + AmOr2. A) Oocytes injected with RNA encoding AmOr10 + AmOr2, AmOr11 + AmOr2, AmOr18 + AmOr2 or AmOr170 + AmOr2 are challenged with 100µM methyl-p-hydroxybenzoate (HOB), E-9-oxo-2-decenoic acid (9-ODA), 4-hydroxy-3- ethoxyphenylethanol (HVA) and E-9-hydroxy-2-decenoic acid (9-HDA). Each oocyte is also challenged with queen mandibular pheromone (QMP) prepared such that the concentration of 9-ODA is approximately 100 µM. B) Left trace, an oocyte injected with RNA encoding AmOr11 fails to respond to 100 µM 9-ODA. Right trace, a different oocyte expressing AmOr11 and AmOr2 responds to 100 µM 9-ODA. C) Dose-response relationship for 9-ODA activation of oocytes expressing AmOr11 and AmOr2. Data are the mean ± SEM of 4-10 oocytes. D) An oocyte expressing DmOr35a and DmOr83b responds to 30 µM hexanol, but not to 100 µM 9-ODA.

(Figure6A). Similar to what was observed with DmOr83b (see above), we find that responses to 9-ODA can only be observed when the AmOr2 putative dimerization partner is coexpressed with AmOr11 (Fig. 6B). Dose-response analysis (Fig. 6C) shows that the AmOr11+AmOr2 receptor is highly sensitive to 9-ODA, with an EC50 of 430 ± 60 nM. With these results, we have identified AmOr11+AmOr2 as a receptor for 9-ODA, the main component of QMP and QRP. The other 3 candidate sex pheromone receptors appear not to respond to any of the applied compounds. This result is consistent with the report that while 9-ODA activates MG#2, no QMP components activate MG#1 (Sandoz, 2006; Figure 2). However, it remains a possibility that these receptors simply failed to express in the oocyte system. We will confirm surface expression of these receptors using an epitope tag approach (see below), before ruling out activation by any compound.

The AmOr11+AmOr2 receptor also appears to respond to a high concentration (100 µM) of 9-HDA (Fig. 6A). This slight activation by 9-HDA could be the result of its similarity to 9-
ODA or possibly due to a slight contamination of the 9-HDA sample with a small amount of 9-ODA. We will examine this further by GC-MS analysis of the commercially purchased 9-HDA and 9-ODA chemicals. Preliminary results indicate that none of four drone-biased receptors binds any of the four minor QRP components (methyl oleate, coniferyl alcohol, palmityl alcohol and linolenic acid). Further results will be provided as an update.

In the preceding sections we have outlined a clear deficit in the knowledge of honeybee mating behavior, and summarized our progress towards developing a multidisciplinary approach to address this problem using state-of-the-art scientific methodology. Deciphering the chemicals that mediate the great range of social behaviors of bees, wasps and ants is difficult due to their complex ethology and their use of complex chemical blends. For example, Keeling et al. (2003) were forced to breed honeybee colonies with particular behavioral traits to enhance the sensitivity of their assay to discover four minor components of the QRP pheromone, and more remain to be discovered. The fact that as many as 35 different olfactory neurons are housed within a single poreplate sensilla (Kelber et al., 2006) has inhibited the application of single cell electrophysiology. Imaging the activity of macroglomeruli in the antennal lobe is an alternate approach, but at present, the technology is limited to imaging only two of the four macroglomeruli (Sandoz, 2006). Our research will employ “reverse chemical ecology”, using the receptor to identify the pheromone. There is a wealth of knowledge concerning the chemical composition of many honeybee glands, however, the difficulty of behavioral assays has precluded their testing. An in vitro assay amenable to high throughput screening is clearly advantageous.

**Goal:** To resolve sex pheromone mediated mating behavior in honeybees using a “reverse chemical ecology” approach based on functional genomics tools and a pheromone receptor-based assay.

**Objectives:**
1) Screen all known honeybee pheromones (alarm, swarm and brood pheromones) for binding to the three orphan sex pheromone receptors (AmOr10, 18 and 170) to eliminate them as possible ligands (using the *Xenopus* oocyte assay);
2) Validate the use of the *Xenopus* oocyte assay to screen solvent extracts from virgin queen honey bees using AmOr11 as a control;
3) Localize the source of the sex pheromone components. Screen solvent extracts from whole virgin queens and specific queen bee glands implicated in sex pheromone communication for activity on the three orphan sex pheromone receptors expressed in *Xenopus* oocytes;
4) Identify the active sex pheromone chemicals; and
5) Confirm their sex pheromone function using behavioral assays.
6) As an additional objective we will identify the QRP pheromone receptors that we hypothesize will be expressed at higher levels in worker compared to drone antennae (drones do not exhibit the retinue response of workers). This will require continued gene expression profiling using our olfactory-specific microarray and qPCR and represents our continuing work to characterize the honeybee odorant receptors.

**Methodology**

We will express the three orphan honeybee sex pheromone receptors in *Xenopus* oocytes and use this in vitro assay to guide the identification of the missing sex pheromones. Our strategy is simple: first, exclude all known pheromones as ligands; second, locate the anatomical source/gland that produces the missing sex pheromone components; third, purchase and screen all known chemicals produced in the identified gland; and finally, fractionate chemical extracts
to identify the active component. This approach utilizes the wealth of knowledge describing the chemicals produced in honeybee glands with our unique capacity to screen many chemicals in vitro. Candidate sex pheromones will be validated using behavioral assays. The Robertson lab will take the lead in objectives related to the identification and cloning of honeybee Ors genes, chemical extractions from bees, behavioral assays and overall project management. The Luetje lab will take the lead in characterizing receptor ligand binding and screening chemical extracts for activity.

**Functional analysis of insect odorant receptors in Xenopus oocytes:** The Luetje laboratory has extensive experience in studying various receptors and channels expressed in *Xenopus* oocytes using two-electrode voltage clamp (TEVC). Detailed methods can be found in previous publications (Abaffy et al., 2006a; 2006b; Hsiao et al., 2006). All functional experiments will be conducted using an OpusXpress 6000A Parallel Oocyte Voltage Clamp system (Molecular Devices) in the Luetje laboratory. This robotic system performs TEVC experiments on 8 oocytes simultaneously. Perfusion and robotic pipetting of compounds are controlled through a software interface. This system has been in operation in the Luetje laboratory for over 3 years and all functional data presented in Figures 5 and 6, as well as in our recent publications (Abaffy et al., 2006a; 2006b), were obtained using this system. Data will be captured with the OPUSXPRESS 1.1 software that controls the experiment. Initial analysis will be done with CLAMPFIT 9.1 (Molecular Devices). Detailed analysis will be done using PRISM 4 (Graphpad). Statistical significance will be assessed using a two-tailed unpaired t-test, or one-way ANOVA followed by Dunnett’s post-test, as appropriate.

Using this system, our proposal to screen a large number of compounds is feasible. Approximately 50 known pheromone components will be screened, as well as a similar number from honeybee glands, against four receptors. In our assay, it is routine for oocytes to be maintained for 1.5 to 2 hours (see Figure5C), although oocytes can sometimes last for much longer. We generally apply a compound once every 10 minutes, allowing 8 to 12 compounds to be tested with each set of oocytes (this system screens 8 oocytes simultaneously, allowing suitable replication with each run). Several runs can be accomplished in a day, allowing approximately 20 – 30 compounds to be screened against one receptor in a day. In recently published work from the Luetje lab, several mouse odorant receptors were screened with 40 compounds (Abaffy et al., 2006a).

**In vitro RNA synthesis and injection of RNA into Xenopus oocytes:** Capped cRNA will be synthesized in vitro from linearized template DNA encoding Ors using mMessage mMachines kits (Ambion). Isolation, injection and culturing of oocytes are described in detail in previous publications (Abaffy et al., 2006a; 2006b; Hsiao et al., 2006). Oocytes are maintained for 3-7 days in Barth’s saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 15 HEPES, pH 7.6 and 12µg/ml tetracycline).

**Immunofluorescent localization of AmOrs on the oocyte surface:** While we know that AmOr11+AmOr2 is functionally expressed in *Xenopus* oocytes (see Fig. 6), it is important to confirm that the orphan receptors AmOr10, 18 and 170 are successfully expressed on the oocyte surface before drawing final conclusions about whether these receptors are activated by particular compounds. We will confirm surface localization using the methodology described in the recent publication from the Luetje laboratory (Abaffy et al., 2006a). Briefly, vitelline membranes are removed from oocytes 2-3 days after cRNA injection and incubated in blocking solution (Barth’s saline + 1mg/ml BSA) for 15 min at room temperature. After rinsing, the oocytes are incubated with either 1) a dilution of hybridoma supernatant containing the anti-
rhodopsin antibody 4D2 (Hicks and Molday, 1986); or 2) the commercially available anti-myc-tag antibody 4A6 (Upstate/Millipore) for 15 min. Oocytes are then washed into cryo-embedding medium and frozen overnight. 15 µm sections are cut on a Leica microtome, and the sections incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a 1:400 dilution. Sections are then rinsed, fixed with 40% glycerol, and then visualized at 400x magnification on a Leica DMIRB Epifluorescent inverted microscope equipped with a Q-Imaging color CCD camera and imaging software. This microscope is part of the University of Miami Analytical Imaging Core, which is maintained by trained, full-time staff and is available to the members of the Luetje laboratory.

Confirmation of cell surface localization requires an epitope tag on an extracellular portion of the receptor protein. Until recently, insect Ors have been assumed to be G-protein coupled receptors similar to the Ors of mammals. Thus, a strategy of tagging the N-terminus could suffice. However, recent work has challenged this assumption (Benton et al., 2006) by demonstrating that insect Ors have a membrane topology that is divergent from that of mammalian Ors. While insect Ors most likely possess 7 transmembrane domains (Nakagawa et al., 2005; Benton et al., 2006), the orientation of the N- and C- termini is currently a matter of debate. Therefore, to ensure success, we will test each of two different epitope tags at the N- and C-termini of both AmOr11 and AmOr2 (for a total of 8 constructs). We will use the human rhodopsin epitope tag (N-terminus = MNGTEGPNFYVPFSNATGVVR; C-terminus = NGTEGPNFYVPFSNATGVVRstop), with which we have experience (Abaffy et al., 2006a) and for which we possess the 4D2 antibody (Hicks and Molday, 1986). We will also use the myc-tag (N-terminus = MEQKLISEEDL; C-terminus = EQKLISEEDLstop), for which an antibody is commercially available (4A6, Upstate/Millipore). The tagging approach that yields the best result with AmOr11 and AmOr2 will then be used to test AmOr10, 18 and 170.

Validating the *Xenopus* oocyte assay to detect ligands in chemical extracts: The AmOR11+AmOr2 receptor, expressed in *Xenopus* oocytes, will serve as a control to validate the use of this bioassay to screen chemical extracts from insects. Reproductively mature virgin queens (6-8 days old) will be collected from colonies maintained by the UIUC bee research facility (or purchased from commercial sources). The heads from 10-20 mature virgin queens will be ground three times in diethyl ether on ice (Keeling et al., 2003; C. Keeling, personal communication) in order to extract pheromone components (including 9-ODA) from their mandibular gland. The extract will be concentrated by evaporation on wet ice and quantified as volume per number of insects extracted (queen equivalents). The chemical composition of the extract will be analyzed by GC-MS along with 9-ODA as a standard. The activity of the extract will be assayed using *Xenopus* oocytes expressing AmOr11+AmOr2.

*Xenopus* oocyte collection: The Luetje laboratory (University of Miami) will conduct experiments involving the use of *Xenopus laevis* frogs. Female *Xenopus laevis* clawed frogs (2 to 4 year old) will be used as a source of oocytes for the odorant receptor expression studies. Thirty frogs will be purchased and used each year – no good alternatives are available. Surgery will not be performed on any one frog more than 4 times per year or four times total. At the time of the 4th surgery, the frog will be euthanized. *Xenopus laevis* frogs are housed within an AAALAC accredited animal care facility. The frogs are maintained at a density of no more than 1 frog/gallon. For oocyte isolation, a frog is anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester. Depth of anesthesia is judged by loss of nasal flare and swallow reflexes. Oocytes are surgically removed. The incision is treated with gentamicin sulfate and sutured. The frog is allowed to recover from surgery in a humid chamber before being placed back in the
holding tank. Oocytes are freed from the follicle cells by treatment with collagenase B for 2 hours at room temperature. When euthanasia is required it will be conducted following the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The care and use of Xenopus frogs described here has been reviewed and approved by the University of Miami Animal Research Committee.

**Localizing the source of sex pheromone production:** The Luetje and Robertson labs do not have previous experience collecting solvent extracts from insects. However, this routine procedure is commonly conducted in chemical ecology labs, including Dr. Larry Hanks lab (Ginzel et al., 2006, for example) that is located directly across the hall from Dr. Robertson’s lab at the UIUC entomology department. Molecular biology equipment in the Robertson lab and chemical ecology equipment in the Hanks lab (including a GC-MS) is commonly shared. In addition, we have sought the advice of chemists who work specifically on honeybee pheromones, including their extraction and discovery (Plettner et al., 1996; Keeling et al., 2003) (Dr. C. Keeling and Dr. E. Plettner). Additionally, we have the advantage of having identified AmOr11 as the 9-ODA receptor which will provide a positive control for our assays.

A variety of the typical solvents will be used to collect extracts from whole honeybees (male drones, female workers and virgin queens) as well as from their dissected head, thorax and abdomen segments to locate the source of sex pheromone that activates AmOrs 10, 18 and 170. We will begin with a broad approach so as not to bias our expectations as to the source of the sex pheromone components (several investigators suspect the tergite glands on virgin queen bee abdomens as the source of the missing sex pheromone components). In addition, we will investigate the possibility that AmOr10, 18 or 170 may be a receptor for a male aggregation pheromone produced by other males. Chemical extracts will be concentrated, analyzed by GC-MS and screened against AmOrs 10,11, 18 and 170 expressed in Xenopus oocytes.

**Identifying the sex pheromone chemicals:** First, we will screen all known honeybee pheromones to exclude them as ligands. Approximately 40-50 honeybee pheromones with demonstrated behavioral activity (retinue attraction, swarm attraction, alarm pheromone etc.) have been identified (Keeling et al., 2003; Slessor et al., 2005). Almost all can be purchased from Sigma. These pheromones will be screened against the four candidate sex pheromone receptors (AmOr 10, 11, 18 and 170) expressed in Xenopus oocytes as outlined above.

Next, we will target chemicals produced by glands that we identified as possible sources of the sex pheromone. The chemical components of different honeybee glands have been thoroughly characterized. For example, more then 20 volatile chemicals have been identified from queen tergite glands (Wossler and Crewe, 1999); more then 30 from queen Dufour’s gland (Katzav-Gozansky et al., 1997; 2001); and more then 100 compounds from the Queen’s madibular gland (Engels et al., 1997). The robotic electrophysiology system in the Luetje lab provides the capability to screen hundreds of compounds against the candidate sex pheromone receptors. However, we may also use a combinatorial screening methodology being developed in Dr. Luetje’s lab. If required, assay directed fractionation will be used to further identify pheromones from active fractions.

**Confirming the behavioral activity of candidate sex pheromones:** Candidate sex pheromones must be verified using behavioral assays. First, we will locate a drone congregation area in the vicinity of the UIUC honeybee research facility. A pheromone lure impregnated with 9-ODA will be attached to a helium filled balloon and elevated 10-20 m in height. Upon entering a congregation area, the drones will swarm around and follow the pheromone lure (Free 1987). Candidate pheromone chemicals (alone or in combinations) can be tested using dummies on a
dual choice rotating carousel as described by Brockmann et al. (2006). Dr. Brockmann is currently a member of the UIUC Entomology Department and he has agreed to advise our field experiments. We also benefit from the support of Dr. Robertson’s colleague, Dr. Gene Robinson, who maintains the UIUC honeybee research facility (see attached letters).

**AmOr gene expression and cloning:** The Robertson lab will continue to use microarray and qPCR techniques described in this proposal to identify additional female-biased Or genes. Female-biased Ors will be cloned and screened by the Luetje lab to identify the queen retinue pheromone receptors as a natural extension of this research.

**Future research:** The proposed work has the potential for major impact towards understanding bee mating behavior. If the above research agenda is as rapidly fruitful as we believe it will be, we will continue work on the orphan receptors that are relatively over-expressed in workers compared to drones (Figure 3) in the context of the possibility that they might be involved in detection of other components of the queen retinue pheromone, as well as other ligands of particular relevance to worker behavior, e.g. prominent plant odorants. Identification of the receptors for the former will go a long way to fleshing out the complex pheromonal communication system underlying eusocial bee colonies, while identification of ligands for plant odors will lead us into interesting questions concerning the receptors that detect different enantiomers and chiral forms of plant odors. Finally, development of this frog oocyte assay for insect chemoreceptors, in combination with ongoing insect genome projects that provide the substrate for functional genomics approaches, will open the door to many other studies illuminating the molecular biological underpinnings of insect chemical ecology.

**Broad impacts:** The broader impacts from the proposed work range from development of an important new assay in insect chemical ecology, to teaching and training, to public education. The frog oocyte assay adapted in the Luetje laboratory for studies of insect chemoreceptors has the potential to be widely adopted in this field. Previous cell-based assays have proven inconsistent and the in vivo Drosophila “empty neuron” assay developed in the Carlson lab is tedious and intensive, might not work for receptors from highly divergent insects, and is not suitable for high-throughput work. There is a need for in vitro assays that are reasonably high-throughput, not only for ligand identification, but for subsequent structure-function studies and for identification of antagonists and super-agonists that might be employed in insect control strategies in innumerable contexts from agriculture to medical and veterinary entomology. Our research activities are well integrated into teaching and training, including training of graduate students and involvement of undergraduates in research projects under the supervision of both graduate students and postdocs. They also contribute considerably to our formal teaching roles, e.g. Robertson includes lectures on smell and taste and on genomics for his introductory biology class that incorporate this research. The odorant and taste receptors are major gene families in most animal genomes, nicely linking these two fields. Our laboratories contribute to the diversity of biological science students, e.g. the Robertson laboratory currently has three graduate students and three undergraduates, all female, one African-American, and one Muslim. We will make special efforts to disseminate our results and communicate them beyond the usual scientific publications. The remarkable breeding biology of honey bee queens and drones lends itself to popular articles in newspapers and magazines. The Robertson lab will also participate in the annual local Brain Awareness Week organized by our Neuroscience Graduate Program (Robertson is an affiliate) at the local Orpheum Children’s Science Museum with a presentation on smell and taste. Robertson has helped with other scientific presentations at this museum in the past (Biographical Sketch).