1.0 Introduction

The *Nasonia vitripennis* genome sequence and new post-genomic tools provide an unprecedented opportunity to functionally characterize large numbers of chemosensory genes that mediate parasitoid host seeking, host selection, and mating behavior. The genome of *N. vitripennis* has been sequenced (a project led by Jack Werren, University of Rochester, and the Nasonia Genome Sequencing Consortium) by the Human Genome Sequencing Center at the Baylor College of Medicine in Houston with funding from the NHGRI at NIH (http://www.hgsc.bcm.tmc.edu/projects/nasonia/). The second assembly (known as Nas_1.0) was recently released and is a high quality assembly derived from 7.5X sequence coverage of the genome with contigs formed into long scaffolds using mate pairs from large insert clones. It will be annotated with gene models and forms the basis for the main genome paper. The *N. giraulti* and *N. longicornis* genomes have been sequenced at 1X coverage. We are members of the Nasonia Genome Sequencing Consortium with responsibility for annotating the chemoreceptor genes.

*Nasonia* are small, 2-3 mm long wasps in the hymenopteran Family Pteromalidae that parasitize blowfly (F. Calliphoridae) and flesh fly (F. Sarcophagidae) pupae (Whiting, 1967; Beukeboom and Desplan, 2003). The genetics, development and behavior of *Nasonia vitripennis* have been studied for more than 50 years due in part to its ease of handling and rearing (comprehensive reviews are available online at http://fire.biol.wwu.edu/leaf/Nasonia/nas.html and http://www.rochester.edu/College/BIO/labs/WerrenLab/nasonia/). *N. vitripennis* has a cosmopolitan distribution and is found throughout North America (NA). The discovery of two new *Nasonia* species native to NA further promoted this genus as an ecological and behavioral model for the study of insect parasitoids. *N. giraulti* is found in Western NA while *N. longicornis* is distributed in Eastern NA and their ranges do not overlap (Darling and Werren, 1990). *N. vitripennis* is a generalist, parasitizing a wide range of host species associated with carrion, while *N. giraulti* and *N. longicornis* appear to be specialists preferring blowflies in the genus *Protopalliphora* that are ectoparasites in bird nests (Darling and Werren, 1990).

*N. vitripennis* is a member of the parasitic Hymenoptera (also referred to as parasitic wasps or parasitoids) that comprise an enormous number of entomophagous species that are beneficial to the global production of food and fiber. Although often used interchangeably, the term “parasitoid” is distinguished from “parasite” by differences in life history. Unlike parasites, ‘Parasitoids are insects whose larvae develop by feeding on or within an arthropod host, and this host individual is almost always killed by the developing parasitoid larva’ (Gauld and Bolton, 1988). Adult parasitoids lay their eggs on or within the juvenile stages of their insect host which their larvae consume and kill. Parasitoids have diversified into a great variety of life histories and they attack the egg, larval, nymph and pupal stages of most insects, including some of the most import pests of agriculture and forestry in the orders Lepidoptera, Coleoptera, Diptera and Hemiptera (Gauld and Bolton, 1988, Godfray, 1994). Two main types of parasitoid life history are recognized: idiobionts that tend to be ectoparasites that kill or immobilize their host, ceasing its further development (usually an immobile stage such as an egg or pupa); and koinobionts that tend to be endoparasitoids that allow the host to continue its development, killing it at a latter developmental stage (Gauld and Bolton, 1988, Godfray, 1994). Parasitoids are important natural enemies of insects pests and help keep their populations in check, and therefore represent a common source of biological control agents. Some parasitoid species are reared and released into agricultural and forest systems as a component of integrated pest management programs (IPM) (Smith, 1996). It has been estimated that, in the US alone,
crop losses totaling $20 billion annually are prevented by biological control programs that utilize parasitic wasps (Proposal to Sequence the Nasonia Genome, www.genome.gov/Pages/Research/Sequencing/SeqProposals/NasoniaSeq.pdf).

After emerging and mating, female parasitoids must find suitable hosts for their offspring, a critical component of their life history and a behavior essential to their use as biological control agents. While parasitoids use various physical and environmental cues to locate suitable hosts in a multi-stage process, chemical cues and the senses of olfaction and gustation are by far the most important (Vinson, 1976). While many examples of parasitoid semiochemicals have been identified during the last five decades, the collective knowledge remains sparse (Keeling et al., 2004), particularly when compared to lepidopteran pest species. A survey of “The Pherobase: Database of insect pheromones and semiochemicals” (www.pherobase.com) covering over 7000 insect species illustrates the deficit of hymenopteran semiochemicals identified relative to the Lepidoptera.

The chemoreceptor superfamily represents one of the most important environmentally responsive gene families, detecting and discriminating chemical stimuli that mediate many critical insect behaviors (Rutzler and Zwiebel, 2005, and Hallem et al., 2006). Significant resources have been invested towards targeting mosquito chemoreceptors in an attempt to develop methods to disrupt their ability to sense and locate their hosts, and we are working towards extending this progress to economically important pest species in the order Lepidoptera. The first genome sequence of a parasitoid species (Nasonia vitripennis) provides an opportunity to extend this research to an enormous group of beneficial insects, with the long-term goal of improving their ability to find and select their hosts.

### 1.1 Parasitoid Host Seeking, Host Selection and Mating Behavior

| Semiochemical: broadly refers to chemical substances that carry a message. |
| Allomone: a chemical produced by one species/organism that affects the behavior of another species/organism to the benefit of the producer. |
| Kairomone: a semiochemical that benefits the receiver to the disadvantage of the producer. |
| Pheromone: a chemical that triggers an innate behavioural and or physiological response in another member of the same species, such as sex, aggregation, alarm and oviposition pheromones. |

The life history of parasitoids is diverse and fascinating and is the subject of many books (e.g. Gauld and Bolton, 1988, Godfray, 1994). Only aspects related to host seeking, host selection and mating behavior will be reviewed in this proposal. Adult parasitoids often feed on nectar or other plant exudates for carbohydrate nourishment, while females commonly feed on the wounds that they inflict on their host during oviposition, a source of protein for the developing eggs. After mating, adult females must seek out new hosts, behavior critical both for their survival and their use as biological control agents. New hosts are located predominantly by the use of chemical cues detected by the parasitoid’s olfactory and gustatory senses.

Doutt (1959), Vinson (1976) and Vinson and Iwantsch (1980) described host location and selection by parasitoids as series of successive stages: A) Host habitat location; B) host location; and C) host acceptance. Each stage serves to reduce the subsequent area searched, and all three stages primarily depend on the parasitoid’s chemical senses. While this model does not incorporate recent theories of parasitoid leaning that influence host searching behavior (for example, the process can be more dynamic, and the parasitoid can learn to skip particular
stages), it is a sufficient framework for the identification of semiochemicals and the receptors that mediate these behaviors.

**A) Host habitat location.** When ready to oviposit, female parasitoids search environmental niches that typically harbor their host, whether or not the host is actually present. While physical cues such as light intensity, humidity and altitude may be used to narrow the environmental search area, semiochemicals detected by olfaction are the primary stimuli. Female parasitoids often are attracted by their host’s food source, such as plant volatiles in the case of phytophagous hosts (in some cases, insects feeding on unusual hosts may escape parasitism). In many cases, odors that indicate host habitat lead to arrestment behavior, and rather than following the odor to its source, the female parasitoid alters its searching behavior within the vicinity of the host habitat. For example, after mating, *N. vitripennis* females are attracted to meat that has been infested by fly pupae, and this technique is used to trap *N. vitripennis* in the field (Edwards, 1954).

**B) Host location.** Upon entering the host’s habitat a parasitoid’s flight pattern may change as it searches for olfactory cues that indicate the actual presence of a potential host. Visual, acoustic, and even infra-red stimuli can be used at this stage to locate the host, however, the majority of parasitoid species use semiochemicals and olfaction. Examples include volatiles released as a result of the host feeding on a plant, odors produced directly by the host (or its frass, silk etc.) and odors produced indirectly by host-associated microorganisms. In some cases plants will release herbivore-specific volatiles (allomones) that subsequently attract parasitoids specific to that herbivore (e.g. Turlings et al., 1995). Parasitoids will also exploit the semiochemical communication system of its host. A chemical that is used by the host as a pheromone may become a kairomone for the female parasitoid (reviewed in Keeling et al., 2004). For example, aphid parasitoids are attracted to sex and alarm pheromones produced by their hosts (reviewed by Powel and Picket, 2003).

**C) Host acceptance.** The location of a potential host leads to further contact and examination to identify the host (characteristics of the host that elicit egg release) and to determine its acceptability for oviposition (Vinson, 1976). During this examination the size, shape and surface texture of the potential host can be important, but contact chemical cues are paramount. Host acceptance often involves frequent contact with the antennae (antennation) and probing with the ovipositor, both are forms of contact chemoreception that could involve either the olfactory or gustatory senses. After locating potential host pupae, adult female *N. vitripennis* first examine the pupae with their antennae before drilling into it with their ovipositor (Edwards, 1954). Many female parasitoids probe a prospective host with their ovipositor, and in some cases an egg is laid only if the appropriate chemical cues are present (host identification). Host discrimination (after identification) is often considered a mechanism to avoid superparasitism (multiple parasitism of a single host by the same parasitoid species). Many parasitoids will mark their host with a pheromone that may be applied externally or injected internally to indicate it has been parasitized (reviewed in Keeling et al., 2004). In other cases, contact sensilla on the parasitoids ovipositor (likely gustatory) appear to detect internal physiological conditions of the host during probing, that leads to discrimination. However, it should be noted that while many parasitoids examine the host before egg laying, some simply use the same host-associated semiochemical for host finding, identification and acceptance (reviewed in Gauld and Bolton, 1988, Godfray, 1994).

Female parasitoids of some species will produce sex pheromones for long distant attraction (female calling), and courtship pheromones can be produced by both species (Ayasse et al., 2001). Short range pheromones are thought to be spread by antennal contact between
males and females during courtship behavior. The antennae of male parasitoids often have sex glands that produce secretions thought to promote female receptivity (Ayasse et al., 2001; Keeling et al., 2004). *N. vitripennis* courtship behavior has been well characterized and includes a series of stereotypic movements (Assem and Werren, 1994), including antennal contact. *N. vitripennis* males are believed to transfer an aphrodisiac to the female by antennal contact, that causes the female to signal sexual receptivity (van den Assem et al., 1980) and recent unpublished results have identified the male-produced aphrodisiac as hydroxyl-4-decanolide (Jurgen Gadau, poster presented at Cold Spring Harbor Laboratory, Biology of Genomes). In addition, a recent study suggests that female-specific cuticular hydrocarbons act as contact sex pheromones, inducing male courtship behavior (Steiner et al., 2006).

1.2 Chemoreceptors Mediate Behavior by Detecting and Discriminating Chemical Stimuli
The last decade has witnessed tremendous progress in our knowledge of the molecular and neurological mechanisms of olfaction (reviewed in Dahanukar et al., 2005, Rutzler and Zwiebel, 2005, and Hallem et al., 2006) including the large families of chemoreceptors (Crs). The term chemoreceptor refers collectively to both the odorant receptor, Or, and gustatory receptor, Gr, families. Insect Ors and Grs are transmembrane proteins averaging 380-420 amino acids in length. Expressed in the periphery (on the dendrites of sensory neurons), they detect chemical stimuli in the environment resulting in the activation of secondary messaging systems and nerve impulses. Sensory neuron dendrites are surrounded by a sensillum lymph filled with proteins that transport (OBPs and CSPs) and modify (P450s, CEs and GSTs) the chemical stimuli (reviewed in Rutzler and Zwiebel, 2005). While all of these components can influence chemoreception at the periphery, the critical nature of the Cr superfamily of Ors and Grs has been eloquently demonstrated in the fruit fly using molecular genetic techniques. John Carlson’s Lab developed transgenic flies with an olfactory neuron that lacked its Or (“empty neuron system”, reviewed in Hallem et al., 2006). Different fruit fly Ors can be expressed in this empty neuron and their odor-binding characteristics analyzed *in vivo* by single-sensillum electrophysiology. A significant outcome of this research was the demonstration that the electrophysiological characteristics of a “wild type” sensillum can be replicated simply by expressing its chemoreceptor in the empty neuron (Hallem et al., 2004). The preeminent role of Crs in determining sensory neuron activity (and the link between sensory neuron activity and behavior) has been demonstrated in fruit flies using molecular genetic tools. Gustatory neurons that express the *Drosophila* trehalose sugar receptor Gr5a were either selectively inactivated by tetanus toxin, or selectively activated by capsaicin-sensitive TRP channels. Sensory neurons expressing DmGr5a mediate behavioral attraction to sweet stimuli but not behavioral aversion to bitter stimuli (Thorne et al., 2004; 2005; Marella et al., 2006). Similarly, gustatory neurons expressing the caffeine bitter receptor DmGr66a (Moon et al. 2006) mediate aversion to bitter stimuli, and not attraction to sugars (Thorne et al., 2004; 2005; Marella et al., 2006). Subsequently, significant public and private funds have been invested towards developing methods to disrupt the ability of mosquitoes to locate their hosts by targeting their chemoreceptors.

The effort to identify and annotate the large families of insect chemoreceptor genes in insects beyond *Drosophila* has been led by HMR, and represents a significant contribution. The discovery of 62 Or (Clyne et al. 1999; Vosshall et al. 1999; Gao and Chess 1999; Vosshall et al. 2000; Robertson et al 2003) and 68 Gr (Clyne et al., 2000; Scott et al., 2001; Dunipace et al. 2001; Robertson et al., 2003) sequences in the fruit fly genome was a landmark event. Along with Larry Zwiebel, HMR annotated the complete repertoire of 79 Or and 76 Gr genes of *A.
*gambiae* (Hill et al., 2002). HMR was a member of the honeybee genome consortium and he annotated 170 Ors and 10 Grs from its genome (Robertson & Wanner, 2006), and KWW has annotated more than 40 Ors from the silkworm genome (Wanner et al. 2007). The Robertson lab is currently responsible for Cr annotation from several insect genomes, including the flour beetle *Tribolium castaneum*, the pea aphid *Acyrthosiphon pisum*, the body louse *Pediculus humanus* and the jewel wasp, *Nasonia vitripennis*.

### 1.3 Identifying Candidate Receptors that Mediate Significant Insect Behaviors

Sex, tissue and development specific Cr gene expression can identify candidates that mediate specific behaviors. The antennae of male insects are often specialized for the detection of female-produced sex pheromones, including the Ors that detect the pheromone chemicals with very high specificity (Krieger et al., 2004; 2005; Nakagawa et al., 2005). Screening for male-biased Or expression led to the identification of the first moth pheromone receptors, and we used this strategy to identify the honeybee Queen pheromone receptor. Male drone bees exist in a hive only to mate with virgin queens, and their antennae are specialized to detect 9-oxodecenoic acid (9-ODA), the main component of the queen pheromone blend. We used an olfactory-specific custom microarray to screen the expression of 180 honeybee Ors genes (Figure 1) to identify four candidate pheromone receptors expressed at higher levels in male antennae, AmOrs 10, 11, 18 & 170, confirmed by quantitative real-time PCR (qPCR) (Figure 2). qPCR can also be used as an accurate method to screen the expression levels of large numbers of Cr genes. Using qPCR we have identified several female-biased Ors expressed in adult silkworm antennae (Wanner et al., 2007), and one is activated by the plant volatile linalool (unpublished data). We have also identified a lineage of silkworm Ors that is expressed specifically in the larval antennae, and

**Figure 1.** Four candidate sex pheromone receptors are expressed at higher levels in drone antennae. Sex-biased expression of olfactory-related genes in drone and worker antennae determined by microarray analysis (2-way dye swap design, n = 4 replicates). The volcano plot indicates that the majority of the genes were not differentially expressed (fold change in expression was >0.5 and < 2.0 and the p-values were > 0.05). Ten genes were expressed at higher levels in worker antennae (upper left quadrant, p<0.05 and fold change in expression <0.5) and 6 were expressed at higher levels in drone antennae (upper right quadrant, p<0.05 and fold change in expression > 2.0), including four Ors.
Figure 2. qPCR analysis of AmOr2, 10, 11, 18 and 170 expression in the antennae of drone, worker and queen bees, relative to the control gene AmRPS8 (plotted on a logarithmic scale). Bars represent the 95% confidence interval of the mean expression value, n = 3 replicates. Strikingly, the expression levels of AmOr11 and 18 in drones are comparable to that of AmOr2, the DmOr83b ortholog. In moths, the pheromone receptors are among the few genes that are expressed at levels comparable to this broadly expressed receptor component.

expect that they will detect odors specific to host seeking behavior by the larvae (unpublished results). Functionally characterizing the ligand binding specificity of the candidate Ors identified by gene expression profiling is the next challenge of this project.

1.4 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 (the *Drosophila* “empty neuron” developed in the Carlson lab, reviewed in Hallem et al. 2006). However, Or genes from other insect species 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. For our 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 the Luetje laboratory 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., 2006; 2007). Recently, the Luetje laboratory has successfully used this assay to characterize several mouse odorant receptors (Abaffy et al., 2006; 2007). 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 3). 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 also important to demonstrate that insect odorant receptors expressed in this system display accurate ligand recognition properties. In Figure 3, we show the ligand specificity of DmOr35a expressed in the *Xenopus* oocyte system and assayed under two-electrode voltage clamp. Functional responses of
Figure 3. 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*, dose-response relationship for hexanol activation of DmOr35a + DmOr83b (EC$_{50}$ = 670 ± 70 nM, mean ± SEM, n = 3). D) *Left*, an oocyte expressing DmOr35a and DmOr83b is challenged with 30 µM of 1-alkohols of varying carbon length. *Right*, results from 6 oocytes are presented. The DmOr35a receptor also responded to 30µM hexanal (26±1.3% of hexanol, n=6), heptanal (23±0.9% of hexanol, n=6) and octanal (12±0.4% of hexanol, n=6). All applications were 25 sec and are indicated by bars (panel A) or arrowheads (panels B-D).

DmOr35a to the cognate ligand (hexanol) only occur when DmOr83b is co-expressed (Fig. 3A). 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; Benton et al., 2006). 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. 3B), or to 30 µM propionic, butanoic, pentanoic, hexanoic, heptanoic or nonanoic acids, or to linalool (data not shown), 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. 3C) 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. 3D). 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. Next, the assay was used to identify the honeybee Or that detects 9-ODA, the main component of Queen pheromone.

Taking a functional genomics approach based on sexual dimorphism, we identified four honeybee Ors (AmOr10, 11, 18 &170) as candidate sex pheromone receptors (Section 1.3). We screened oocytes injected with RNA encoding AmOr10, 11, 18 or 170 (Fig. 4 & 5), in
Figure 4. 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 + AmOr2 responds to 100 µM 9-ODA. All applications were 25 sec and are indicated by arrowheads (panel A) or bars (panel B). AmOr11+AmOr2 also appeared to respond to 100 µM 9-HAD (panel A). However, 9-HDA is synthesized from 9-ODA and a slight contamination of 9-HDA with 9-ODA is expected (~0.3%, Pherotech, personal communication).

Figure 5. Characterization of the 9-ODA receptor, AmOr11 + AmOr2. A) An oocyte expressing AmOr11 + AmOr2 is challenged with a range of 9-ODA concentrations. B) Dose-response relationship for 9-ODA activation of AmOr11 + AmOr2 (EC50 = 280 ± 31 nM, mean ± SEM, n = 10). C) An oocyte expressing AmOr11 + AmOr2 responds to 100 µM 9-ODA, but not to 100 µM methyl oleate (MO), linolenic acid (LA), coniferyl alcohol (CA) or 1-hexadecanol (HD). D) An oocyte expressing AmOr11 + AmOr2 responses to 100 µM 9-ODA, but not to 100 µM hexanol (HEX), linalool (LIN), geraniol (GER) or citral (CIT). All applications were 25 sec and are indicated by arrowheads (panels A, C, D).
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 Queen Mandibular Pheromone (QMP, a commercially available subset of QRP components), as well as the QMP preparation itself (Figure 4). The AmOr11+AmOr2 receptor responded to 100 µM 9-ODA and to the QMP preparation (Figure 4A). Similar to what has been observed with DmOr83b (see above), we found that responses to 9-ODA can only be observed when the AmOr2 putative dimerization partner is coexpressed with AmOr11 (Fig. 4B). Dose-response analysis (Fig. 5A&B) showed that the AmOr11+AmOr2 receptor is highly sensitive to 9-ODA, with an EC\textsubscript{50} of 280 ± 31 nM. The AmOr11+AmOr2 receptor did not respond to any of four additional minor components of QRP that have been recently described: methyl oleate, linolenic acid, coniferyl alcohol and 1-hexadecanol (Keeling et al., 2003) (Fig. 5C, n = 5). We further examined the specificity of the AmOr11+AmOr2 receptor by screening floral odorants (linalool and hexanol) and social pheromones (geraniol and citral). AmOr11+AmOr2 did not respond to any of these compounds (Figure 5D, n = 8). Thus, the AmOr11+AmOr2 receptor is highly selective for 9-ODA. With these results, we have identified AmOr11+AmOr2 as a receptor for 9-ODA, the main component of QMP and QRP. This work demonstrates the utility of the \textit{Xenopus} oocyte assay in the functional characterization of insect Ors.

1.5 \textit{Xenopus} oocytes expressing an insect Or can be used as a biosensor to screen chemical extracts

The semiochemicals used by many insects remain unknown, particularly in the order Hymenoptera. Traditional chemical ecology techniques such as GC-EAD analysis have proven to be one powerful approach. In Figure 6, we demonstrate that we can use an insect Or (in this case, honeybee AmOr11+AmOr2) expressed in \textit{Xenopus} oocytes, to screen a chemical extract. The front half of four queen heads (including the mandibular glands) were dissected and ground in 100 µl of analytical grade methanol in a dounce homogenizer on wet ice. The sample was spun in a microcentrifuge at 13,000 RPM for 3 minutes. The solvent was removed from the tissue pellet, brought back up to 100 µl with

![Figure 6. Screening a chemical extract. A) An oocyte expressing AmOr11+AmOr2 responds to 10µM 9-ODA and to a methanol extract of queen bee mandibles (0.5%). B) An oocyte expressing AmOr11+AmOr2 responds to 10µM 9-ODA, but not to 0.5% methanol. Coapplication of methanol has no effect on the response to 9-ODA. C) An oocyte expressing AmOr11+AmOr2 responds to a series of dilutions of a methanol extract of queen bee mandibles (0.5% to 0.0125%). All applications were 25 sec and are indicated by arrowheads.](image-url)
analytical grade methanol and stored at -20°C in a glass vial. The AmOr11+AmOr2 receptor responded to the extract (Fig. 6A), with 0.5% extract (2.5µl extract in a 0.5 ml application volume) yielding a response that was similar in amplitude to the responses obtained with 10µM 9-ODA (this is a maximal response, see Fig. 5B). Based on the preliminary data shown in Figure 6C, the EC$_{50}$ for activation of AmOr11+AmOr2 by the mandibular extract is ~0.0125%. Thus, an application yielding a half-maximal response requires only one quarter of one percent (0.25%) of the extract obtained from a single queen. These results demonstrate that our proposed use of a “reverse chemical ecology” approach to identify novel *Nasonia* Or ligands is feasible.

2.0 Progress Report  N/A  
3.0 Rationale and Significance

This proposal is aligned with USDA CSREES FY2007 priority 3: Characterization on a large scale the function of genes of arthropods having a completely sequenced genome. Our combination of EST sequencing using the 454 platform to construct custom microarray chips represents a new approach to characterizing gene families that are expressed at low levels. It will allow new hypotheses concerning the chemosensory basis of generalist vs specialist host selection in the genus *Nasonia* to be tested (FY2007 priority 1).

This proposal directly meets the USDA’s long-term goal of reducing pesticide use through the development of sustainable, integrated pest management techniques. Parasitic Hymenoptera represent the largest group of beneficial insects. They are commonly used in IPM programs; a 1997 survey listed more than 100 companies as suppliers of beneficial organisms in North America, many of which included parasitoids (www.cdpr.ca.gov/docs/ipminov/bensup.pdf). Parasitoids are also used in classical biological control programs to control invasive insect pests; in the US it is estimated that $20 billion annually is saved as a result of controlling invasive pest species with parasitic wasps (Proposal to Sequence the Nasonia Genome, www.genome.gov/Pages/Research/Sequencing/SeqProposals/NasoniaSeq.pdf). Enhancing parasitoids’ ability to locate and select their insect pest hosts was one rationale behind more than five decades of research towards elucidating the semiochemicals that mediate these behaviors (Gauld and Bolton, 1988, Godfray, 1994). Parasitoid semiochemicals can be used in “push-pull” strategies of pest management (Cook et al., 2007). Parasitoid pheromones are useful for monitoring their populations in the field (Keeling et al., 2004). Plant biotechnology holds particular promise (Powell and Pickett, 2003); *Arabidopsis* plants have been engineered to emit aphid alarm pheromone as a volatile, and it acts as an attractive kairomone to aphid parasitoids (Beale et al., 2006). It is clear that post genomic tools will be an essential component of the research to further this exciting field (Powell and Pickett, 2003).

The insect Cr superfamily has emerged as the preeminent gene family responsible for detecting stimuli in the environment. Our research to functionally characterize the Cr genes that mediate parasitoid host seeking, host selection and mating behavior will synergize the efforts of a large research community (a query of the AGRICOLA data base with the term “parasitoid” yields almost 7,000 hits). Not only will our research further the understanding of these behaviors in the emerging model *N. vitripennis*, it will also extend this knowledge broadly to economically important parasitoid families. Our use of *Xenopus* oocytes expressing insect Crs as a biosensor to screen chemical extracts and its application to “reverse chemical ecology” will provide the template for many labs globally to continue to identify parasitoid semiochemicals, an important task for continued progress. Finally, we have demonstrated that our proposal is viable, and that
we possess the expertise to successfully complete it, including an interdisciplinary approach that includes entomology, molecular biology, functional genomics and pharmacology.

4.0 Approach
The genome sequence of *N. vitripennis* and new technological developments will for the first time allow the functional characterization of large numbers of Cr genes (and other sensory-related genes) that mediate parasitoid host seeking, host selection and mating behavior. New high throughput sequencing technology combined with *in vitro* functional assays will identify phylogenetically conserved gene lineages responsible for detecting important classes of semiochemicals (sex pheromones, host marking pheromones, kairomones and alemones), extending this research broadly within the parasitic Hymenoptera, including economically important species.

Objectives:
1) Annotate the Or, Gr and other sensory-related genes from the *Nasonia vitripennis* genome sequence (approximately 300 genes expected).
2) Sequence antennal cDNA from 12 parasitoid species representing economically significant taxonomic group, to identify conserved Or, Gr and other sensory-related gene lineages (approximately 600 genes expected).
3) Profile species, sex and tissue specific gene expression patterns (of approximately 900 genes) to identify candidates that mediate host seeking, host selection and mating behavior.
4) Screen 75-100 Cr genes for *in vitro* activation by known parasitoid semiochemicals.
5) Screen candidate *N. vitripennis* Crs with solvent extracts from male and female insects and host pupae to identify semiochemicals that mediate the host seeking, host selection and mating behavior of this model parasitoid.

4.1 Annotate the Or, Gr and other sensory-related genes from the *Nasonia vitripennis* genome sequence
Many sensory-related genes have been annotated from the *N. vitripennis* genome by colleagues, such as the OBP and CSP genes (Ryszard Maleszka) and the P450 genes (May Berenbaum) as part of the genome annotation effort. HMR and KWW are members of the *Nasonia* genome annotation group, HMR will annotate the Grs while KWW is responsible for annotating the Ors. This task will be completed by August 2007 and the sequences will be submitted as an update to this proposal. Insect Or and Gr sequences are highly divergent, particularly in species from different orders, typically sharing only 10-30% amino acid identity (Robertson and Wanner 2006). For this reason, automated software used to annotate genome sequences rarely predicts the correct Cr sequence, and manual annotation is required. HMR has lead this effort and his expertise is commonly requested by genome annotation groups (Mosquito, honeybee, beetle, louse and aphid genome annotation groups for example). Although laborious, Cr gene family annotation by the HMR lab has become routine, benefiting from a detailed knowledge of the subtle characteristics of Or and Gr genes that comes with experience. Methodology for annotation is described in Robertson et al. (2003) and Wanner et al. (2007). Also belonging to the Order Hymenoptera, the 180 honeybee (*Apis mellifera*) Cr genes annotated by HMR (Robertson and Wanner, 2006) will be used to search the *Nasonia* genome sequence since Cr sequence identity tends to increase with taxonomic relatedness (see Wanner et al. 2007 for example). Briefly, candidate genes will be identified from the Nas_1.0 assembly of the *N.*
vitripennis genome sequence using BLAST search tools. A tBLASTn search compares the protein query with all translations of the genome sequence of interest. Contiguous genome sequences that encode peptides that share amino acid similarity with the query sequence are identified. The candidate genomic sequences are downloaded from GenBank and the genes constructed manually in a text editor. Or and Gr genes typically have six or more introns, ranging in size from 100 nucleotides to many thousands. Software programs, such as intron splice site predictors, are helpful. As new sequences are identified, they will in turn be used as BLAST queries in an iterative process.

4.2 Sequence antennal cDNA from 8 parasitoid species that represent economically significant taxonomies, to identify conserved Or, Gr and other sensory-related gene lineages. Two recent developments are essential to this proposed objective: 1) Evidence for significant phylogenetic conservation of Or lineages within the Lepidoptera that mediate the detection of specific classes of semiochemicals (Wanner et al., 2007), and 2) New technology that allows high throughput sequencing directly from cDNA, dramatically increasing the ability of EST projects to identify rare transcripts.

4.2.1 Parasitoid species. Twelve candidate species have been identified for antennal cDNA sequencing (of which 8 will be selected), representing the two parasitoid superfamilies that contain the greatest number of species beneficial to food and fiber production, the Chalcidoidea (http://www.tolweb.org/Chalcidoidea/11183) and Ichneumonoidea (http://www.tolweb.org/Ichneumonoidea/11174). The superfamily Chalcidoidea includes 19 families while the Ichneumonoidea has two extant families, the Braconidae and the Ichneumonidae. Parasitoid species were selected on the basis of availability and phylogenetic representation. In terms of classical biological control, Greathead (1986) summarized the number of successful parasitic introductions by taxonomic family: Aphelinidae (90), Braconidae (53), Encyrtidae (53), Eulophidae (23), Ichneumonidae (22), Pteromalidae (17) and Mymaridae (9). The Braconidae is a large family that includes many economically beneficial species and its phylogeny has been resolved to a greater extent (http://www.tolweb.org/ Braconidae/23447) by Jim Whitfield in the UIUC Entomology Department (J. Whitfield will provide technical assistance related to working with parasitoid, see attached letter). To give us depth within this important parasitoid family, we identified five species each representing a different braconid subfamily: *Aphidius colemani*, *Lysiphlebus testaceipes*, *Bracon hebetor*, *Cotesia vestalis* (synonymous with *C. plutellae*; Shaw, 2003) and *Wroughtonia ferruginea*. *Diadegma insulare* represents the Family Ichneumonidae. Six species represent four chalcid families: *Encarsia formosa* and *Aphytis melinus* (F. Aphelinidae), *Metaphycus helvolus* (F. Encyrtidae), *Pediobius foveolatus* (F. Eulophidae), *Muscidifurax raptor* (F. Pteromalidae) and *Trichogramma pretiosum* (F. Trichogrammatidae). Sensory-related genes will be identified from antennal EST sequences formatted as a searchable data-base using a stand alone version of BLAST software available on the NCBI website (http://www.ncbi.nlm.nih.gov/Ftp/). We have used this software to BLAST search trace file databases. Conserved gene lineages will be identified by constructing phylogenetic trees using the PAUP software package (for examples, refer to Robertson et al., 2003, Robertson and Wanner 2006).

4.2.2 EST sequencing: Historically Cr genes have not been amenable to discovery by EST sequencing of cDNA libraries (while OBP, CSP and P450 transcripts are abundant, Robertson et
al., 1999 for example). The low expression of Cr genes in specific tissues makes their occurrence in cDNA libraries rare, and the depth of EST sequencing required was prohibitively expensive. Typically anywhere from 3-10 Ors genes have been discovered using traditional cDNA libraries and EST sequencing (HMR unpublished data and R. Newcomb and E. Jacquin-Joly, personal communications). The development of new high throughput sequencing technology provides a solution to this technical problem. Pyrophosphate sequencing technology is the basis of new high throughput sequencers (454 Life Sciences™ Genome Sequencer 20 System) that can produce 200,000 EST reads averaging 100 bp in length in one afternoon. Roche Diagnostics in collaboration with 454 Life Sciences has developed the next generation of this technology, the Genome Sequencer FLX System, capable of 300,000 EST reads averaging 200-250 bp each. In addition, up to four different samples can be processed in parallel at the same time, producing 50,000-70,000 reads for each of the four samples. 454 Life Sciences™ offers sequencing services, charging $24,000 for a single run on the new Genome Sequencer FLX System (ie $24,000 per 300,000 x 200 bp reads = 60 Mbp of sequence). The University of Illinois at Urbana-Champaign W.M. Keck Center DNA Sequencing Facility has also purchased the new Genome Sequencer FLX System from Roche and it will be installed during August of 2007.

4.2.3 cDNA synthesis: The 454 Life Sciences™ platform represents two significant technical breakthroughs for Cr discovery from ESTs. First, traditional EST projects sequence clones from cDNA libraries, and the process of cloning cDNA into vectors often creates a bias towards more abundant transcripts. The 454 system can sequence ds cDNA synthesized from antennal RNA directly, eliminating cloning steps that can introduce bias. In addition, cost and effort is dramatically reduced, synthesizing cDNA is the simplest step compared to actually constructing and maintaining a library. Second, ds cDNA can be synthesized from amplified RNA, an approach suited to small tissue and limited RNA. For example, laser assisted microdissection (LAM) has been coupled with RNA amplification from limiting tissue to synthesize ds cDNA for sequencing by the 454 platform (Emrich et al., 2007).

Many of the ichneumonid and braconid parasitoids will be of sufficient size to collect enough antennae to isolate 200-300 ug of total RNA (~ 1000 individuals), enough to synthesize 1-5 ug of ds cDNA required for 454 sequencing, based on our experience with the European corn borer (ECB). Total RNA will be isolated using Trizol reagent (Invitrogen), mRNA purified using the Qiagen Oligotex mRNA Kit, and ds cDNA synthesized using the Clontech SMART™ cDNA Synthesis Kit. The smaller chalcid species will require an amplification step. The antennae from several hundred individuals will be required to isolate 1-3 ug of Total RNA, based on our experience isolating total RNA from *Nasonia vitripennis* antennae. This amount of total RNA allows two options for amplification: 1) PCR amplification using the Clontech SMART™ PCR cDNA Synthesis Kit and 2) RNA amplification using the Genisphere SenseAmp Plus kit. Both require no more than 1 ug of total RNA to make 5 ug of ds cDNA. Dr. Alvaro Hernandez at the UIUC WM Keck center has experience with both systems and synthesized 5 ug of ds cDNA from 1-3 ug of ECB total RNA. Many kits are now marketed based on the original “eberwine” concept that incorporates a T7 RNA polymerase promoter into ds cDNA synthesized from as little as 100 ng of total RNA (typically uses 1 ug total RNA). T7 RNA polymerase produces ss RNA from the DNA template that can subsequently be converted to 1st and 2nd strand cDNA. Unlike PCR, many studies have demonstrated that RNA amplification by T7 RNA polymerase is linear and therefore not biased to abundant transcripts. Amplified RNA commonly averages 500-1000 bp, sufficient for the 454 Life Sciences™ platform that produces reads of 250 bp. Most
RNA amplification kits incorporate the T7 promoter into the poly dT primer used for 1st cDNA synthesis, and the resulting amplified RNA does not have a 3’ polyA tail to prime 1st cDNA synthesis. The Genisphere SenseAmp Plus kit incorporates the T7 promoter into the 5’ end of the 1st cDNA, producing amplified RNA with the normal polyA tail at the 3’ end, making it easier to synthesize 1st and 2nd strand cDNA using traditional methods.

It should be noted that *T. pretiosum* and *E. formosa* are extremely small (~1 mm) and *M. helvolus* is small and its antennae are stubby-shaped. In these cases we will dissect heads with antennae from ~1000 wasps to gain sufficient RNA for amplification. Ten of 12 species can be purchased commercially (Suppliers of Beneficial Organisms in North America, [www.cdpr.ca.gov/docs/ipminov/bensup.pdf](http://www.cdpr.ca.gov/docs/ipminov/bensup.pdf); we have verified the websites of current suppliers). *Cotesia vestalis* can be reared from ECB with assistance from Marianne Alleyne in the UIUC entomology department (see attached letter), who currently rears two other *Cotesia* species. *Wroughtonia ferruginea* will be collected locally from its beetle hosts with assistance from the Hanks lab in the UIUC entomology department, who have conducted chemical ecology studies using this parasitoid (unpublished data).

### 4.3) Profile species, sex and tissue specific expression patterns of parasitoid Cr and sensory-related genes

We have extensive experience using custom microarrays and qPCR to profile Cr gene expression in various insect species, such as the honeybee and silkmoth, research summarized in section 1.3 (also see Robertson and Wanner 2006, Wanner et al., 2007). We will use the parasitoid Cr and sensory-associated genes identified in sections 4.1 and 4.2 to construct a custom microarray chip that will be used to survey gene expression.

#### 4.3.1 Microarray analysis.

A custom microarray containing approximately 900 sensory-related parasitoid genes (~300 from the *N. vitripennis* genome and 600 from parasitoid antennal ESTs) will be constructed to screen gene expression. Oligonucleotides (70-mer sense-strand) will be designed for the Or, Gr, OBP, CSP, P450, CE and GST genes annotated from the *N. vitripennis* genome and the parasitoid antennal ESTs using ArrayOligoSelector3.5 software (http://sourceforge.net) using default settings and the *N. vitripennis* genome Assembly 1. The Functional Genomics Unit at the W.M. Keck Center will print the oligonucleotides (IDT, Coralville, IA) onto glass slides using a GeneMachines OmniGrid 100 microarray printer. Four house-keeping genes (positive controls) and ArrayControl sense 70-mer oligonucleotides (Ambion, negative controls) will be distributed throughout the array. Spot quality will be assessed using a Cy3-labeled 9-mer (Operon) hybridized to a test microarray.

Total RNA from male and female parasitoid tissue (antennae, ovipositor, head minus antennae, and body) will be isolated and probes prepared from amplified RNA (Ambion MessageAmp™ RNA Amplification Kit). This kit is commonly used by our colleague Gene Robinson (UIUC Entomology Department) with the honeybee genome microarray. In addition to tissue comparisons, we will compare sensory-related gene expression in the antennae of all three *Nasonia* species, to screen for differences that may be associated with host range (specialist vs generalist).

Microarray slides are prehybridized (20% formamide, 5X Denhardt’s, 6X SSC, 0.1% SDS, 25μg/mL tRNA) for 1 hour in a 42°C waterbath with occasional shaking. After rinsing, slides are placed in Corning hybridization chambers with a LifterSlip (Erie Scientific) positioned over the area of the spotted oligonucleotides. The probes are suspended and heated to
95°C for 2-3 min, chilled on ice for 5 min and kept at room temperature until they are mixed with pre-warmed (50°C) 2X hybridization buffer (50% formamide, 10X SSC, 0.2% SDS). The probe and hybridization buffer is pipetted under the Lifterslip edge of the hybridization chamber and incubated in a light-proof hybridization oven at 42°C overnight. Microarray slides are washed with agitation: Wash 1 (1X SSC, 0.2% SDS) at 42°C for 8 minutes; Wash 2 (0.1X SSC, 0.2% SDS) for 8 min at room temperature; Wash 3 (0.1X SSC) for 8 min at room temperature, repeated twice. The microarray slides are scanned on an Axon 4000B Scanner using GenePix v6.0 software. Microarray data will be analyzed using SAS software v.9.1 (SAS Institute Inc., Cary, North Carolina). After assessing spot quality, normalization for common mean and variance between microarrays will be achieved by scaling and centering the loess-transformed values. Spots that are more than 3 SD from the data set mean will be removed as outliers.

**4.3.2 Quantitative real-time PCR (qPCR) analysis:** The relative method of qPCR (Relative Quantitation of Gene Expression, ABI PRISM 7700 Sequence Detection System, User Bulletin #2, Applied Biosystems) will be used to validate leads identified in the microarray analysis. In general primers will be designed using coding sequence close to the 3’ end of the gene, using ABI Primer Express 2.0 software (Applied Biosystems) with default settings. Real-time quantitative PCR will be performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and SYBR Green dye (SYBR Green PCR Master Mix, Applied Biosystems) following the manufacturer’s protocol. Each primer set will first be validated by constructing a standard curve of the $C_T$ values (the cycle number at which the fluorescence intensity crosses the threshold line determined by the ABI Primer Express 2.0 software) at 10x dilutions of template. A no-template control will be included and all reactions performed in triplicate. Dissociation curves will be used to assess the purity of the PCR reactions. $Or$ gene expression levels will be calculated relative to the control gene ribosomal protein S8 using the formula $2^{-\Delta C_T}$.

**4.4 Screen 75-100 Cr genes for in vitro activation by known parasitoid semiochemicals.** Expression profiling will identify sensory-associated genes with species, sex and tissue biased expression patterns providing clues towards their function. For example, genes expressed at higher levels in male antennae may detect female-produced sex pheromones; those expressed at higher levels in female antennae may detect host kairomones and/or male-produced sex pheromones; and those expressed specifically in the female ovipositor may detect oviposition cues and/or marking pheromones. Crs are the preeminent gene family responsible for detecting and discriminating chemical cues in the environment; we adapted the *Xenopus* oocyte assay to characterize $Or$ receptor activation in vitro. In addition, our collaborator Jack Werren (see attached letter) may identify Cr candidates by mapping loci involved in host seeking and selection behavior.

**4.4.1 Xenopus oocyte assay.** 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., 2006; Abaffy et al., 2007; 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 4 years and all functional data presented in Figures 3-6, as well as in our recent publications (Abaffy et al., 2006; 2007), 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 against a large number of candidate Ors is feasible. In our assay, it is routine for oocytes to be maintained for 1.5 to 2 hours (see Figures 3 & 5), 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 40 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., 2006).

4.4.2 Parasitoid semiochemicals. Selecting appropriate semiochemicals to screen against the candidate Cr genes will be important. The objective is to determine Cr responses to classes of semiochemicals, not to test an exhaustive list of chemicals. We expect to find conserved Or and Gr lineages that respond to specific semiochemical classes, and we simply require examples of semiochemicals from different species, not from all species. For example, a conserved lineage of Ors expressed highly in female parasitoid antennae may detect its host pheromone, and this can be tested using a few (not all) species. Since an exhaustive review is not within the scope of this proposal, we have utilized “The Pherobase: Database of insect pheromones and semiochemicals” (www.pherobase.com/) covering 7000 insect species to illustrate semiochemicals available for this objective. In addition, we will continue to network with chemical ecologists to obtain appropriate chemicals.

C. plutellae and D. insulare are important larval parasitoids of the diamond back moth (DBM), Plutella xylostella; B. hebetor parasitizes the larvae of Indian meal moth, Plodia interpunctella; and T. pretiosum parasitizes the eggs of many lepidopteran species. The Pherobase lists 19 scientific papers describing semiochemicals used by these parasitoids: www.pherobase.com/database/genus/genus-Cotesia.php, -Bracon.php and -Trichogramma.php. In some cases specific semiochemicals have been identified, for example 9,10-dihydroxy-z12-octadecanoic acid induces ovipositon behavior by B. hebetor. The Pherobase also lists 26 papers describing the semiochemicals used by the parasitoid’s hosts, that may serve as kairomones: www.pherobase.com/database/species/species-Plutella-xylostella.php and genus/genus-Plodia.php. Host sex pheromones are commonly used by female parasitoids as kairomones to locate their host. Sex pheromone chemistry in the Lepidoptera is very well known (including the DBM and the Indian meal moth), and many pheromones are commercially available from companies such as Bedoukian (www.bedoukian.com/products/searchpheromones.asp), ISCA technologies (www.iscatech.com/exec/finechemicals.htm) and Pherobank (www.pherobank.com/).

A. colemani and L. testaceipes both parasitize aphids, including the green peach aphid, Myzus persicae and the cotton aphid, Aphis gossypii. A. colemani is attracted by the aphid alarm

*A. melinus* parasitizes the California red scale (*Aonidiella aurantii*) and *M. helvolus* parasitizes soft scales such as *Coccus hesperidum*. *A. melinus* uses o-caffeoyltyrosine as an oviposition stimulant and its host’s sex pheromones have been identified as 3-Methyl-6-isopropenyl-9-decenyl acetate and (Z)-3-Methyl-6-isopropenyl-3,9-decadienyl acetate ([www.pherobase.com/database/genus/genus-Aphytis.php](http://www.pherobase.com/database/genus/genus-Aphytis.php) and -Aonidiella.php).

*P. foveolatus* and *Wroughtonia ferruginea* represent parasitoids whose hosts are beetles, the Mexican bean beetle and the red headed ash borer, respectively. Three papers describe the semiochemicals used by the genus *Epilachna*, ([www.pherobase.com/database/genus/genus-Epilachna.php](http://www.pherobase.com/database/genus/genus-Epilachna.php)) and the Hanks lab has identified hexanediol as a male aggregation pheromone that attracts the red headed ash borer (Lacey et al., 2004) as well as its parasitoid *W. ferruginea* (Larry Hanks, personal communication). Little is know of the chemical ecology of *E. formosa*, but it is the most commonly used biocontrol agent for whitefly control in greenhouses (Hoddle et al., 1998). Similarly, *M. raptor* represents an important group of parasitoids used for control of stable flies, flesh flies and blow flies found in and around farms, and it is in the same family as *Nasonia*.

### 4.4.3 in vitro RNA synthesis and injection of RNA into Xenopus oocytes

Each Cr will be cloned into the pGEMHE plasmid vector and confirmed by sequencing. We have developed a reliable method for amplifying and cloning the Ors that can easily be scaled up to clone large numbers. Without ramping up our efforts we have cloned dozens of receptors to date. Capped cRNA will be synthesized *in vitro* from linearized template DNA encoding Ors using mMessage mMachine kits (Ambion). Each *Nasonia* Or will be coexpressed with NvitOr2, the *Nasonia* equivalent of DmOr83b. The isolation, injection and culturing of oocytes are described in detail in previous publications (Abaffy et al., 2006; 2007; 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 CaNO₃, 0.41 CaCl₂, 0.82 MgSO₄, 15 HEPES, pH 7.6 and 12µg/ml tetracycline).

### 4.4.4 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. Ten 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.

4.5 Screen candidate *N. vitripennis* Crs with solvent extracts from male and female insects and host pupae to identify semiochemicals that mediate the host seeking, host selection and mating behavior of this model parasitoid.

We will utilize the *N. vitripennis* male-produced courtship pheromone hydroxyl-4-decanolide that was recently identified. We will also use solvent extracts of male and female insects and host pupae to screen Crs whose expression is sexually biased or specific to the female ovipositor. Our ability to screen honeybee Ors with solvent extracts has been summarized in section 1.5. Solvents such as methanol, dichloromethane and hexane are commonly used for this purpose. We have access (and have used) a GC-MS across the hall from us, in Larry Hanks lab. The Hanks lab also has volatile collecting equipment that we can use to analyze components of host infested food that are attractive to the females (flies are reared in the UIUC Entomology Department insectary). Solvent extracts will be sufficient to determine the response of specific Crs to functional categories of semiochemical. For example, a Gr receptor expressed specifically in the female ovipositor might respond to solvent extracts from its pupal hosts, and not to extracts from the host food.

4.6 Timeline

**Year 1:** We expect to have candidate receptors from the *N. vitripennis* genome based on preliminary qPCR expression profiling. The Robertson lab will clone 25-35 *N. vitripennis* receptors and send them to the Luetje lab for preparation (midipreps and RNA expression) and functional screening in the *Xenopus* oocyte assay (against sex pheromones and solvent extracts of adults and pupae collected by Robertson and sent to the Luetje lab). Robertson will synthesize and sequence the cDNA from the antennae of 4 parasitoid species. Robertson personnel will visit the Luetje lab to assist in project coordination.

**Year 2:** Robertson will synthesize and sequence cDNA from the antennae of 4 more parasitoid species (for a total of 8). Sensory-related genes from the EST sequences (all 8 species) and the *N. vitripennis* genome will be used to design and construct a sensory-specific microarray that will be used to screen for sex and tissue biased gene expression of *N. vitripennis* and the first 4 parasitoid species. The Robertson lab will clone 25-35 candidate parasitoid receptors and send them to the Luetje lab for preparation (midipreps and RNA expression) and functional screening in the *Xenopus* oocyte assay against a panel of known parasitoid semiochemicals. Robertson personnel will provide a list of parasitoid semiochemicals for the Luetje lab to purchase, and will visit assist in project coordination. Data will be analyzed and publications prepared.

**Year 3:** Robertson will use the sensory-specific microarray to screen for sex and tissue biased gene expression of *N. vitripennis* and the last 4 parasitoid species and will clone 25-35 candidate parasitoid receptors and send them to the Luetje lab for preparation (midipreps and RNA expression) and functional screening in the *Xenopus* oocyte assay against a panel of known parasitoid semiochemicals. Data will be analyzed, final reports written and publications prepared. Results will be presented at scientific meetings by both groups.