

Multidisciplinary approach to determining the behavioral and genetic basis of chemosensation in
Plodia interpunctella (Lepidoptera: Pyralidae) for improved management

by

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B.S., Austin Peay State University, 2016
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AN ABSTRACT OF A DISSERTATION

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Abstract

The management of stored-product pests (SPPs) poses a critical challenge to global food security, as these pests thrive in human-modified environments associated with harvested or processed food supplies. In post-harvest landscapes, chemosensation acts as the primary physiological interface governing survival-critical behaviors in these pests, including resource acquisition and reproductive success. In the Indian meal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae), semiochemical based monitoring or behavioral management tactics serve to either the presence and abundance of adults in an environment or reduce the population through tactics such as mating disruption and attract and kill; however, the efficacy of these strategies remains limited by an incomplete understanding of the mechanisms used by *P. interpunctella* perceive and respond to stimuli and how different stimuli, such as pheromones and kairomones, influence behaviors. This dissertation synthesizes current knowledge of SPP chemosensory biology and investigates the multiple physiological mechanisms underlying *P. interpunctella* behavior, using a multidisciplinary approach that spans the biological hierarchy to identify how different volatiles influence dispersal and flight behavior, to identify key behaviors in courtship that could be disrupted to prevent mating, and to identify candidate genes that influence behavioral responses to food volatiles and pheromones.

At the organismal level, flight and dispersal are characterized as dynamic decision-making processes shaped by internal physiological states and external semiochemical cues. Using tethered flight mills, we studied flight capacity and determined that flight distance was highest in unmated males, while the major pheromone component, (*Z*, *E*)-9,12-tetradecadienyl acetate (ZETA), acted as a behavioral catalyst by increasing flight bout frequency. While combinatorial cues of ZETA and flour together resulted in arrestment, which could be exploited

to manipulate behavior in field settings such as encourage longer exposure to toxicants at attract and kill stations. Moreover, exploring the close-range reproductive interactions, Markov chain frameworks revealed that courtship operates as a probabilistic communication system. Within this architecture, head-to-head contact emerged as a critical behavioral bottleneck and a mandatory gateway for mating success, suggesting that mate choice is governed by the probabilistic control of behavioral sequences rather than discrete actions. This behavior represents a keystone in determining the outcome of reproductive behavior in *P. interpunctella* that could be disrupted to reduce successful mating and copulation to augment traditional mating disruption programs.

To identify the life history parameters, the influence of physiological responses to volatiles and molecular drivers that influence behavioral outputs, we also characterized antennal olfactory responses of males and females at varying ages, reproductive statuses, and prior behaviors to ZETA. Electroantennography revealed that pheromone perception is highly plastic influenced mainly by sex and mating status. Furthermore, we determined that the antennal response exhibited lateralization, with the right antenna exhibiting a 17% stronger response that varies according to *P. interpunctella* reproductive state and age within males and females. At the transcriptomic level, multi-factorial analysis showed that mating strongly influences gene expression: mated males exhibited a tenfold increase in ZETA-responsive differentially expressed genes, characterized by the downregulation of mate-seeking machinery in favor of broader environmental sensors. Finally, these functional shifts were anchored in the genomic architecture by manually curating 132 chemosensory genes from a chromosome-scale assembly, which revealed species-specific expansions of ionotropic receptors on chromosome 8. Collectively, this research underscores that the ecological success of *P. interpunctella* as a pest is

driven by its behavioral plasticity and genomic architecture, which work simultaneously with underlying physiological processes associated with sex, mating status, and age to regulate the integration of chemosensory information, which provides frameworks for improving semiochemical-mediated behavioral pest management strategies.

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To identify the life history parameters, the influence of physiological responses to volatiles and molecular drivers that influence behavioral outputs, we also characterized antennal olfactory responses of males and females at varying ages, reproductive statuses, and prior behaviors to ZETA. Electroantennography revealed that pheromone perception is highly plastic influenced mainly by sex and mating status. Furthermore, we determined that the antennal response exhibited lateralization, with the right antenna exhibiting a 17% stronger response that varies according to *P. interpunctella* reproductive state and age within males and females. At the transcriptomic level, multi-factorial analysis showed that mating strongly influences gene expression: mated males exhibited a tenfold increase in ZETA-responsive differentially expressed genes, characterized by the downregulation of mate-seeking machinery in favor of broader environmental sensors. Finally, these functional shifts were anchored in the genomic architecture by manually curating 132 chemosensory genes from a chromosome-scale assembly, which revealed species-specific expansions of ionotropic receptors on chromosome 8. Collectively, this research underscores that the ecological success of *P. interpunctella* as a pest is

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Dedication

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Chapter 1 - From sensory chemicals to pest management:

Chemosensory genes shaping behavior in stored product pests

Introduction

Chemosensation is the physiological mechanism by which organisms perceive, receive, and process chemical stimuli that elicit or modify behavioral responses. All unicellular (Kleerebezem and Quadri 2001, Schmidt et al. 2015) and multicellular organisms engage in chemosensation to some extent, as the ability to detect environments with resources (Nguyen et al. 2008) or lead them away from unfavorable conditions (Bell 1990) is paramount for survival. Thus, key evolutionary behaviors linked to chemosensation include but are not limited to the initiation of feeding, food selection, mate-finding, and mate (Schneider 1969) or oviposition site selection (Cox and Collins 2001). Insects are no exception, using semiochemicals or chemicals that transmit information between members of the same or different species through two main modalities 1) olfaction, which is the detection of volatile compounds in the air, or 2) gustation, the perception of chemicals that come into direct contact with taste receptors expressed on sensory organs. Olfaction and gustation are not mutually exclusive sensory processes because behavioral responses to semiochemicals can have a continuum of outcomes, bracketed on either extreme by full attraction (e.g., the movement of an organism towards a stimulus) or repulsion (e.g., the movement of an organism away from a stimulus), with many volatiles eliciting intermediate behavioral responses (Morrison et al. 2020). Although most organisms recognize and respond to diverse volatiles, not all elicit clear behavioral responses especially when compounds are separated from their derived blends. Semiochemicals are compounds that are released by an organism to modify the behavior of another organism. The factors influencing behavioral outcomes are complex and often involve various interacting molecular factors that

can be influenced by the physiological status of the individual, genetics, environmental conditions, previous experiences (i.e., learning), and population dynamics.

Because olfactory responses strongly influence habitat selection, mate location, food preferences, and can occur over varying distances without direct contact with the volatile source, they are often exploited for semiochemical-mediated behaviorally-based management (SMBM) strategies to disrupt an insect's ability to locate food and mates. SMBM is applied to a range of insect species, including stored product pests (SPPs), in an attempt to reduce pest colonization of commodities. At least 96 commercially available semiochemical lures that are efficacious for 25 coleopteran and 14 lepidopteran SPPs (Table 1.1) for use in integrated pest management (IPM) programs are available. However, the efficacy of these semiochemicals is low under certain conditions, and the identification of semiochemicals that elicit clear behavioral responses for many SPP species is lacking.

The complexity of behavioral outcomes suggests that chemosensation is not simply a behavioral reflex, but a dynamic process mediated by chemosensory molecules transduced and influenced by the internal physiology of the insect. While genomic resources for several SPP species have become available, the functional bridge between genomic-level information and behavioral response remains unclear. This dissertation aims to synthesize current knowledge of SPP chemosensory biology in this chapter, while subsequent chapters use *Plodia interpunctella* (Lepidoptera: Pyralidae) as a model to address the following knowledge gaps: 1) how semiochemicals influence *P. interpunctella* flight capacity, 2) the complexity and predictability of *P. interpunctella* courtship, 3) how *P. interpunctella* perceives pheromonal mating cues, 4) how gene transcription in *P. interpunctella* changes in pheromone-rich environments, and 5) the relationship of *P. interpunctella* chemosensory receptors to those of close relatives.

How are olfactory signals perceived from the environment?

Genetic factors significantly influence olfactory perception and downstream behavior through the form and function of the anatomical structures, receptors, and binding proteins involved in recognizing and processing volatile cues and the physiological signals they transduce. Olfactory sensilla are the primary sensory organs responsible for detecting volatiles and are distributed throughout different sensory structures and tissues, including antennae, legs, mandibles, maxilla, labrum, wings, and ovipositors. The sensilla contain pores that allow volatile compounds to diffuse into the sensillum lymph, where chemosensory proteins (CSP) and odorant-binding proteins (OBP) bind to the odorants and deliver them to olfactory receptors (Leal 2013, Alabi et al. 2014). Subsequently, the OBPs or CSPs facilitate the binding of the volatile to odorant receptors (OR) or ionotropic receptors (IR) located on the dendrites of the olfactory sensory neuron (OSN), triggering a transduction cascade that results in insect behavior.

Sensillar morphology and diversity

Despite this seemingly straightforward mechanism, many aspects of the olfactory sensory pathway remain unclear in both model and non-model species. Sensillar morphology and roles associated with chemosensation were first described in the model *Drosophila melanogaster* (Diptera: Drosophilidae) (Couto et al. 2005, Song et al. 2012, Grabe et al. 2015), where it was identified that insect basiconic and trichoidea sensilla are often associated with housing ORs that are linked to food and mate location behaviors, respectively, and coeloconic sensilla typically house IRs used to locate amines, alcohols, and organic acids (Benton et al. 2009, Ai et al. 2010). These findings of similar sensilla morphology and distribution across sensory tissues have been extended to other species, including SPPs like *P. interpunctella* (Ndomo-Moualeu et al. 2014), *Sitophilus granarius* (Coleoptera: Curculionidae) (Ali et al. 2016), and *Tribolium castaneum*

(Coleoptera: Tenebrionidae) (Ali et al. 2016). For example, based on scanning electron microscopy (SEM) of the antenna, Ndomo-Moualeu et al. (2014) determined that *P. interpunctella* possesses eight main types of sensilla, including Böhm bristles, basiconic, coeloconic, auricillica, chaetic, styloconic, squamiformia, and trichodea. Though the downstream effects and functions of these sensilla in *P. interpunctella* chemosensation are not yet fully understood, research in other pyralid moths indicates that trichoidea sensilla may be important for mate location, while basiconic, auricillica, or coeloconic sensilla may play roles in food location. Chaetic sensilla are likely crucial for gustation (Anderson and Hallberg 1990), and styloconic sensilla may contribute to thermo-hydrosensation (Hallberg et al. 1994, Ndomo-Moualeu et al. 2014). Similarly, based on transmission electron microscopy (TEM) and SEM, Ali et al. (2016) determined that the *S. granarius* antennae possess two subtypes each of basiconic and coeloconic sensilla, and five subtypes of chaetic sensilla, though their molecular functions remain unclear. In contrast, *T. castaneum* primarily has two main sensilla on the antennae, trichodea and basiconic sensilla situated throughout the antenna and densely on the last three antennal segments, respectively (Suliman A. I. Ali et al. 2016, Seada and Hamza 2018). The basiconic sensilla of *T. castaneum* play a significant role in perceiving plant volatiles (ethyl-1-hexanol, heptacosane, nonacosane, and (-)-limonene) and the microbial/fungal volatile 1-octen-3-ol. Removing these sensilla renders the insect unresponsive to these cues, emphasizing their importance (Ali et al. 2016). These findings highlight the diverse roles that sensilla play in chemosensation and their potential roles in behavior for at least two SPPs; however, sensillar morphology has yet to be described for the majority of SPPs, which is important for ascertaining function.

Molecular mediators: OBPs and CSPs

Once odorants have entered the sensilla, they interact with several proteins that mediate the interaction between odorants and the receptors on OSNs, including OBPs and CSPs. OBPs are a small family of soluble proteins (10–30 kDa) with six conserved α -helical domains creating a large hydrophobic cavity (Sandler et al. 2000) that contains multiple cysteine residues that are stabilized through disulfide bonds (Wojtasek and Leal 1999) for the binding of odorants. The number of OBPs varies widely among insects, ranging from 0 to 83 (Vieira and Rozas 2011); however, reports of zero OBPs may reflect limitations in genome and transcriptome sequencing as well as gene annotation rather than their true absence. In SPPs, as many as 49 OBPs have been reported in *T. castaneum* (Dippel et al. 2014), whereas as few as nine have been reported in *Amyelois transitella* (Lepidoptera: Pyralidae) (Leal et al. 2009). Similarly, *Rhyzopertha dominica* (Coleoptera: Bostrichidae), *Callosobruchus chinensis* (Coleoptera: Bruchidae), *P. interpunctella* (Lepidoptera: Pyralidae), and *Sitophilus zeamais* (Coleoptera: Curculionidae) exhibit 10, 25, 29, and 41 OBPs, respectively (Mandiana Diakite et al. 2015, Zhang et al. 2017, Jia et al. 2018, Tang et al. 2019). In contrast, CSPs are generally smaller (12–15 kDa) with six or seven α -helical domains and four cysteine residues resulting in two disulfide bonds (Lartigue et al. 2002). Studies of *A. transitella*, *C. chinensis*, *R. dominica*, *P. interpunctella*, and *T. castaneum* have identified 1, 5, 8, 15, and 20 different CSP copies, respectively (Leal et al. 2009, Dippel et al. 2014, Mandiana Diakite et al. 2015, Zhang et al. 2017, Jia et al. 2018) with diverse functions. Although binding proteins are predominantly expressed in chemosensory structures like the antennae, they have also been found in non-chemosensory tissues, though their functions there often remain poorly understood. Additionally, unresolved ligand specificity, pH, and odorant-

degrading enzymes affect semiochemical-induced transduction delivery (Leal 2013, Pelosi et al. 2018).

Despite their extensive copy number variation, some CSPs and OBPs have conserved functions and amino acid sequences across multiple species. For example, OBP59a is conserved in many insect lineages (Sánchez-Gracia et al. 2009) and has been linked to behavioral responses to humidity in *D. melanogaster* (Xiao et al. 2019). However, most OBPs are lineage specific, where they may mediate responses to diverse odors and substrates found in complex landscapes, such as pheromones, host plant volatiles, or even pesticides (Lin et al. 2018). For example, in the stored product mite, *Tryophagus putrescentiae* (Acari: Acaridae), two CSPs were identified. Three major ligands of CSP1 were identified as (-)-alloaromadendrene, 2-methylnaphthalene, and cyclopentadecane; thus, the authors concluded that this protein might mediate host recognition (Qu et al. 2016). In *Spodoptera litura* (Lepidoptera: Noctuidae), the expressions of *SlituCSP18* and *SlituCSP6* were induced when the insecticides chlorpyrifos, emamectin benzoate or fipronil were administered through feeding, which may influence insect behavioral resistance mechanisms (Lin et al. 2018). Additionally, in *T. castaneum*, *TcCSP7I* and *TcCSP7M* showed high expression in the body exclusively with no expression in the antenna (Dippel et al. 2014), suggesting there could be an alternative function other than peripheral semiochemical perception. Despite the progress made in understanding these intricate molecular processes, there remain significant gaps in our knowledge. One such gap is the interaction dynamics between the OBP/CSP and the odorant within the sensillar lymph, which has yet to be elucidated. The large hydrophobic cavity of these proteins likely allows them to interact with a broad range of volatiles (Sandler et al. 2000, Pophof 2002, Ma et al. 2016), but the sensitivity and compatibility of the binding site can vary depending on the chemical structure of the odorant (Xiao et al. 2019).

Additionally, the pH of the sensillum lymph has been shown to affect the binding activity of the proteins and volatiles (Wojtasek and Leal 1999, Xu et al. 2010, Liu et al. 2023). Furthermore, the presence and efficacy of enzymes (odorant-degrading enzymes, ODEs) within the sensillum lymph (Kaissling 2001, Ishida and Leal 2005) may influence the longevity of the odorant in the sensillum. Therefore, the form and function are influenced by non-genetic factors, which make functional characterization challenging (Shanbhag et al. 2001).

Signal reception: ORs and IRs

The ORs have seven conserved transmembrane regions with the N-terminus facing internally towards dendrites of the OSN and the C-terminus exposed to the sensilla lymph; thus, interacting directly with OBPs and volatile compounds (Benton et al. 2006). The IRs have a similar topology with 3–5 transmembrane domains, but they generally detect short organic acids, amines, and aldehydes (Benton et al. 2009) and are hypothesized to interact with CSPs. Although a general belief has been that each OR generally detects a specific class of volatile, OSNs that contain different ORs can be located within a single sensillum (Song et al. 2012). Additionally, the ORs form heteromeric complexes with co-receptors, including one encoded by the *or83b* gene. This gene is ubiquitous in insects (Nei et al. 2008, Sánchez-Gracia et al. 2009) and was renamed *orco* to reflect its conserved nature as a coreceptor for ORs (Grosse-Wilde et al. 2011, Mitchell et al. 2012). In contrast to ORs, which have a single co-receptor, IR coreceptors are diverse, with several co-receptors identified, including IR25a, IR8a, and IR76b (Missbach et al. 2014). IR25a and IR76b have additional functions in sensing cold temperatures (Ni et al. 2016) and in sodium detection (Zhang et al. 2013) in *D. melanogaster*, both of which are crucial for survival. Transcriptome resources with comparative phylogenetics have detailed the ancient nature of IRs, which are primarily expressed in the antenna and found throughout the insect body

(Anderson et al. 2013). Although the general structure of receptors and co-receptors is well known, the binding sensitivity and specificity of these receptors and associated binding proteins remain poorly annotated or functionally characterized in many insects. Function validation approaches such as heterologous expression, RNA interference (RNAi), or CRISPR-Cas system are largely lacking for these molecules. Additionally, the specific genes and/or signaling cascades that regulate attraction and repulsion to specific volatile stimuli are not functionally characterized in many insects, including the model *D. melanogaster*.

Signal transduction: Neuromodulation and behavioral plasticity

In addition to the receptors and binding proteins involved in olfactory perception, other molecular components likely play a significant role in modulating insect behavior and chemosensory responses. For example, in *Locusta migratoria* (Orthoptera: Acrididae) (Guo et al. 2011), several genes are associated with the transition from gregarious to solitary situations, including CSPs that showed increased expression in gregarious situations, and *LmigT01*, a takeout gene (i.e., regulatory gene that links circadian clock to feeding behaviors, energy metabolism and starvation), was implicated in driving solitary situations (Ben-Shahar et al. 2002, Guo et al. 2011). Genes coding for neuromodulators, such as serotonin, are also responsible for driving aggregation behaviors (Anstey et al. 2009), and in the corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), responsiveness to two pheromone blends was linked to genes associated with neural pathway development (Koutroumpa et al. 2016, Unbehend et al. 2021). Therefore, genes beyond receptors and binding proteins can strongly influence behavioral responses to semiochemicals in insects. Neuromodulator compounds (serotonin or octopamine) can influence the perception and processing of the chemosensory input (Gatellier et al. 2004, Sakurai et al. 2014).

SPPs represent novel opportunities to identify genes that are important for regulating behavior and elucidating the functions and binding partners of receptors and binding proteins. For example, investigating genes linked to pheromonal perception for taxa genera that share the use of pheromonal components and respond to the same food odors could lead to the discovery of receptors and binding proteins that share similar functions (Li et al. 2013, Zhang et al. 2019, Liao et al. 2023, Wang et al. 2026). This is evident in the overlap of several coleopteran and lepidopteran SPPs that respond to identical or chemically similar volatiles (Table 1.2). Furthermore, even distantly related taxa respond similarly to the same food odors, suggesting they have converged on shared chemosensory strategies to exploit stable, resource-rich stored commodity environments (Hansson and Stensmyr 2011 and Andersson et al. 2015).

In addition, chemosensory genes are responsible for perceiving volatiles over a variety of different spatial scales in SPP. The influence of semiochemicals on SPP movement is defined by distinct spatial transitions, where volatile plumes and surface-bound cues dictate different behavioral programs. At long ranges, volatile attractants such as sex and aggregation pheromones facilitate navigation through upwind orientation, or anemotaxis (Bardwell et al. 1994, Kleerebezem and Quadri 2001, Nei et al. 2008, Wyatt 2009). These signals trigger directed movement, allowing SPPs to traverse large distances within storage facilities to locate mates or high-quality food patches (Campbell and Arbogast 2004, Cardé and Willis 2008, Morrison et al. 2021). Conversely, at short ranges, semiochemicals like cuticular hydrocarbons or high-concentration kairomones can function as arrestants or deterrents (Athanasios et al. 2017, Domingue et al. 2020, Harman et al. 2023). Within the immediate vicinity of a source, these compounds induce a transition to area-restricted search behaviors, characterized by increased turning rates and reduced velocity (Bell 1990). This shift ensures that the insect remains

localized for feeding or oviposition once a suitable resource is found (Trematerra et al. 2000, Būda et al. 2016).

Evolution of chemosensory genes and behavioral divergence

Many behaviors show heritability, including adult orientation and local searching to various stimuli (i.e., food, mates, combined cues, etc.), indicating that genetic variation likely influences these responses (Bell 1985, 1990, Nagle and Bell 1987). For example, studies in *T. castaneum* demonstrated that dispersal behavior was dependent on a single, sex-linked locus (Beermann et al. 2001) and differences in responsiveness to food odors were observed among geographically distinct populations of *T. castaneum* (Campbell 2012). Though the number of candidate genes can influence behavior, the number of abiotic and biotic factors that influence the sensitivity to volatiles responsiveness (Blaney et al. 1986) has made linking genetics to behavior difficult for SPPs. For example, copy numbers of ORs can vary tremendously in different insect lineages, with as many as 341 OR genes and 79 pseudogenes identified in *T. castaneum* (Engsontia et al. 2008), 58 ORs in *Cydia pomonella* (Lepidoptera: Tortricidae) (Bengtsson et al. 2012), 47 ORs in *P. interpunctella* (Jia et al. 2018), and 123 ORs in *R. dominica* (Mandiana Diakite et al. 2015). The evolution of IR and OR is complex in insects and is often accompanied by significant lineage-specific gains, losses, and tandem duplications (Vieira et al. 2007, Nei et al. 2008, Sánchez-Gracia et al. 2009). Comparisons between cerambycid beetles and *T. castaneum* have also identified several orthologous pairs of ORs that are speculated to bind to important odorants both species encounter (Mitchell et al. 2012). *Megacyllene caryae* (Coleoptera: Cerambycidae) lacks *T. castaneum* groups 4–6 of OR genes, suggesting that the expansion of those groups is specific to *Tribolium* sp. or Tenebrioids (Engsontia et al. 2008, Mitchell et al. 2012). Comparisons of amino acid sequences confirm a

high level of sequence divergence among paralogous copies within a species (Bengtsson et al. 2012, Mitchell et al. 2012) and among ORs between species, much like OBPs and CSPs. Although the copy numbers of ORs are high, the copy numbers of CSPs are much lower in comparison and tend to be more conserved across taxa (Vieira and Rozas 2011).

Polymorphisms near chemosensory, developmental, and transduction genes have also been associated with the natural variation of chemosensory behavior (Gupta et al. 2000, Mackay 2001, Swarup et al. 2013). Additionally, polymorphisms in chemosensory genes or expression level differences between sexes may contribute to dimorphic responses to volatiles. This collectively makes identifying polymorphisms associated with behavioral phenotypes difficult. Phenotypic traits may be controlled by only a few loci and have variable behavioral effects, whereas other observable traits are minutely controlled by only a few loci (Mackay 2001). Chemosensation is a complex behavior where a simple ratio of phenotype to loci is not easily determined by the segregation of phenotypes or crosses of phenotypes observed to the same stimulus.

Stored product pest molecular resources

The pace of advancement in developing molecular tools for the functional characterization of genes has been exponentially growing since the early 1980s and these new functional tools can be used in conjunction with classical genetics to identify genes that influence responses to pheromones, kairomones, and other semiochemicals. High-quality genomic and transcriptomic data are necessary to link genomic variation to behavior in SPP and other agriculturally important insect taxa. Several SPP genomes have been sequenced and are publicly available, including *T. castaneum*, which was first assembled in 2008 (Richards et al. 2008) and reassembled in 2020 with improvements (Herndon et al. 2020), *R. dominica* (Oppert et al. 2022),

P. interpunctella (GCF_027563975.2-RS_2024_04), and *Callobruchus maculatus* (GCA_040182625.1). Other SPP genomes with scaffold-level assemblies include *Sitophilus oryzae* (Coleoptera: Curculionidae) GenBank Assembly Accession GCA_002938485.2), *Oryzaephilus surinamensis* (Coleoptera: Silvanidae; GCA_004796505.1), *P. interpunctella* (GCA_900182495.1), *A. transitella* (GCA_001186105.1), *Galleria mellonella* (Lepidoptera: Pyralidae; GCA_003640425.2), and *Cydia pomonella* (GCA_003425675.2) (Table 1.2). Additionally, antennal or head transcriptomes have been collected and assembled for *R. dominica* (Mandiana Diakite et al. 2015), *C. pomonella* (Bengtsson et al. 2001), *P. interpunctella* (Jia et al. 2018), *S. zeamais* (Q. Tang et al. 2019), and *C. chinensis* (Zhang et al. 2017) and USDA's Ag100Pest initiative is producing chromosome-scale assemblies for members of the families Ptinidae, Bostrichidae, Dermestidae, Tenebrionidae, Silvinidae, and Pyralidae, which will substantially expand the number of SPP genomes available for comparative chemosensory genomics (Childers et al. 2019). Notably, many chemosensory genes occur in tandem arrays throughout the genome and are difficult to assemble accurately with short Illumina reads or error-prone PacBio continuous long reads (CLR). However, PacBio HiFi circular consensus (CCS) reads are highly accurate and may improve the assembly of these regions, allowing for a more precise assembly of genic regions containing arrays of repeated paralogous chemosensory genes (Hon et al. 2020, Childers et al. 2021).

Advances in molecular tactics have shown promise in teasing out binding protein specificity to both odorants and receptors (Xu et al. 2005, Scheuermann and Smith 2019) and how important odorants are to interact with receptors for firing OSN (Xiao et al. 2019). Though this approach has often been explored following a functional binding screening leveraging the heterologous *Xenopus laevis* oocyte expression to explore substrate binding of *P. interpunctella*

odorant receptors, PintOR5, 11, 18, 23, and 26 heterodimerized with Orco were screened for ligand screening of 52 volatile compounds; however, only PintOR23/Orco, PintOR26/Orco, and PintoOrco11 were found to respond to 10 of the compounds that were screened, with the PintOR23/Orco complex responding most significantly to acetophenone (Chen et al. 2024). Although these docking assays were done using *in vitro* approaches, docking simulations with artificial intelligence (AI) tools like AlphaFold showed promise in predicting ligand-OR interactions and could be used to expand functional characterizations of ORs in SPP and other insect species. Therefore, molecular docking simulations using AlphaFold Colab software revealed a 97% accurate prediction of the binding between the complex and odorant (Chen et al. 2024). The advancement of AI-based tools for functional gene predictions has paved the way *ex vivo* studies for predicting functions of ORs and other chemosensory genes without the need to perform length experiments.

Towards management

As technology becomes more powerful and SPP genomes become available, reverse chemical ecology approaches could expedite the chemical identification of SPP pheromones to increase the number of SPPs with chemically identified pheromones. The efficacy of several behavior-based approaches has been experimentally explored that involve pheromones or food kairomones in tactics like mass trapping (Trematerra and Battaini 1987, Trematerra 1990, Trematerra and Gentile 2010), mating disruption (Ryne et al. 2001, 2007, Burks et al. 2011, Mahroof and Phillips 2014, Amoah et al. 2019, Campbell et al. 2025, Lindenmayer et al. 2025), attracticides (El-Sayed et al. 2009, Campos and Phillips 2010, 2014, Kroschel and Zegarra 2013).

While pheromones are the primary focus of reverse chemical ecology, the behavioral landscape of SPPs is further modulated by complex interactions with cuticular hydrocarbons, food kairomones, and microbial volatiles. Cuticular hydrocarbons, which can facilitate both intra- and interspecific communication, allow species such as *R. dominica*, *T. castaneum*, and *Sitophilus* spp. to exploit resources previously colonized by other insects (Athanassiou et al. 2017, Trematerra et al. 2015, Trematerra et al. 2000, Aulicky et al. 2016). Similarly, food-derived kairomones and microbial volatile organic compounds (MVOCs) act as critical environmental cues, frequently synergizing with pheromones to enhance attraction (Wakefield et al. 2005, Būda et al. 2016, Trematerra and Girgenti 1989) or, at higher concentrations, acting as repellents that signal overpopulation or resource spoilage (Būda et al. 2016, Pierce et al. 1991, Gardiner et al. 2015, Edde et al. 2007). Despite the demonstrated potential of these compounds—ranging from the synergistic effects of carob volatiles (Obeng-Ofori 1993, Collins et al. 2007, Olsson et al. 2005, Wakefield et al. 2005) to the repellent applications of propionic acid in packaging (Germinara et al. 2010, Germinara et al. 2012), their implementation is hindered by species-specific behavioral variances (Collins et al. 2007, Pierce et al. 1990, Green 2014) and the chemical complexity of synthesizing multi-component blends. Consequently, while pheromone-based tactics like mass trapping and mating disruption have seen extensive experimental exploration, rarely are other sources of semiochemicals implemented in semiochemical behavioral-mediated management tactics.

Push-pull strategies may also benefit from using pheromones, food kairomones, or additional behavior eliciting volatiles but are not well studied (Morrison et al. 2021). Additionally, the lures used in applications may need to be evaluated for improved efficacy which could be done by understanding the molecular interactions in the chemosensory

transduction pathway to link behavioral effects that may improve or expand the tools available for SPP managers.

For example, research in mating disruption and application has primarily focused on male responses to the female-produced pheromone alone. However, female movement, calling, spatial distribution, or oviposition may also be influenced by the concentration and presence of female-produced pheromone or management lures. The process of autodetection by females to their pheromone has traditionally been thought not to occur. However, recent research has shown that the presence of other calling females influences the behavior of female *E. kuehniella*, suggesting that female behavior can be controlled or manipulated in some species by using female-based pheromones (Trematerra and Battaini 1987, Holdcraft et al. 2016). Information on both male and female behavior under mating disruption conditions is understudied. However, with the genomes of several SPP becoming available, pheromone binding proteins, pheromone receptors, and other molecular players that influence autodetection behaviors may be identified, modeled, and compared across taxa to better understand an evolutionarily advantageous behavior that could be impacting mating disruption efficacy.

Classical chemical ecology found in *Sitotroga cerealella* (Lepidoptera: Gelechiidae) that the essential oil from garlic had an impact on behavioral effects that result in reduced fecundity (Yang et al. 2012). Once, molecular techniques like RNA-seq and binding affinity studies were used, they found that diallyl trisulfide, a major component of garlic essential oil, had a competitive pheromone blocking ability to the OBP that the pheromone binds to; thus, reducing a positive behavioral response to the pheromone (Ma et al. 2016). Compounds that have been described to have behavioral effects but are not well understood, but with molecular techniques

and genomic resources available, determination as if these compounds are competitive, agonistic, or antagonistic could make them novel candidates for semiochemical lure development.

Conclusions and future directions

The integration of genetic, molecular, behavioral, and chemoecological approaches is the necessary starting point for the next generation of stored product pest management. As this review has synthesized, the molecular and physiological mechanisms by which semiochemicals are perceived or transduced are not well described for SPPs. Additionally, genetic variation within species and between populations can influence behavioral responses to volatiles by SPPs, which requires attention to improve the efficacy of current semiochemical-mediated behaviorally based management (SMBM) strategies. While at least 96 commercial lures exist, their inconsistent performance in complex storage environments highlights the gap between pest chemosensory biology and its behavior.

The recent surge in high-quality genomic resources, driven by the i5K (Evans et al. 2013) and Ag100Pest (Childers et al. 2019) initiatives, marks a pivotal shift in the field, with at least 29 stored product species now targeted for chromosome-scale assemblies (Childers et al. 2019, 2021). These resources directly benefit and promote molecular work such as reverse chemical ecology, RNA-seq, and functional genomics studies. We highlighted in this chapter that we needed to move beyond classical observation and develop new tactics to leverage genomic tools that are being released to improve the connection of gene to function and behavior. By mapping how specific gene variants influence long-range anemotaxis versus short-range arrestment, we can engineer antagonistic compounds, like the pheromone-blockers found in *Sitotroga cerealella*, or optimize mating disruption by accounting for understudied behaviors like female autodetection. To do so, we must first understand the model system holistically to lessen the gap.

This dissertation utilizes *Plodia interpunctella* as a model to bridge gaps in unknown internal state dependency effects in semiochemical-mediated behaviors (i.e., flight and courtship), perception, and connect them to gene expression differences and evolutionary relationships. Ultimately, as genomic resources become more accessible, the ability to predict and manipulate SPP behavior at the molecular level will prove paramount for the sustainability of global food protection and the continued evolution of integrated pest management.

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Table 1.1. Summary of commercially available semiochemical-based monitoring lures for major stored product insect species.

Order	Family	Species	Common Name	No. of Manufactures with Lures on Market
Coleoptera	Anobiidae	<i>Stegobium paniceum</i>	Drug Store Beetle	2
	Bostrichidae	<i>Prostephanus truncatus</i>	Larger Grain Borer	1
		<i>Rhyzopertha dominica</i>	Lesser Grain Borer	2
	Cleridae	<i>Necrobia rufipes</i>	Red-legged Ham Beetle	2
	Curculionidae	<i>Sitophilus granarius</i>	Granary Weevil	1
		<i>Sitophilus oryzae</i>	Rice Weevil	3
		<i>Sitophilus zeamais</i>	Maize Weevil	2
		<i>Sitophilus spp.</i>	Weevils	2
		<i>Anthrenus museorum</i>	Museum Beetle	1
		<i>Anthrenus spp.</i>	Carpet Beetles	1
		<i>Anthrenus verbasci</i>	Varied Carpet Beetle	1
		<i>Attagenus spp.</i>	Black Carpet Beetle	1
		<i>Attagenus unicolor</i>	Black Carpet Beetle	1
		<i>Dermestes maculatus</i>	Hide Beetle	2
	Dermestidae	<i>Trogoderma granarium</i>	Khapra Beetle	1
			Trogoderma beetles (<i>Trogoderma variabile</i> , <i>T. granarium</i> , <i>T. glabrum</i> , <i>T. inclusum</i> , etc.)	3
		<i>Trogoderma variabile</i>	Warehouse Beetle	3
	Laemophloeidae	<i>Cryptolestes pusillus</i>	Flat Grain Beetle	1
	Ptinidae	<i>Lasioderma serricorne</i>	Cigarette Beetle	6
		<i>Cathartus quadricollis</i>	Square-necked grain beetle	1
		<i>Oryzaephilus mercator</i>	Merchant Grain Beetle	3
			Oryzaephilus beetles (<i>Oryzaephilus mercator</i> , <i>O. surinamensis</i> , etc.)	1
	Silvanidae	<i>Oryzaephilus spp.</i>		
		<i>Oryzaephilus surinamensis</i>	Sawtoothed Grain Beetle	3
	Tenebrionidae	<i>Tribolium castaneum</i>	Red Flour Beetle	3
		<i>Tribolium confusum</i>	Confused Flour Beetle	3
Lepidoptera	Gelechiidae	<i>Sitotroga cerealella</i>	Angoumois Grain Moth	3
		<i>Cadra cautella</i>	Almond Moth	4
		<i>Cadra figulilella</i>	Rasin Moth	4
	Pyralidae	<i>Ectomyelois ceratoniae</i>	Carob Moth	3
		<i>Ephestia elutella</i>	Tobacco Moth	3
		<i>Ephestia kuehniella</i>	Mediterranean Flour Moth	4
		<i>Plodia interpunctella</i>	Indian Meal Moth	6
		<i>Nemapogon granella</i>	European Grain Moth	1
	Tineidae	<i>Niditinea fusculla</i>	Brown-dotted Clothes Moth	2
		<i>Tinea pellionella</i>	Casemaking Clothes Moth	3
		<i>Tineola bisselliella</i>	Webbing Clothes Moth	5
		<i>Cryptophlebia leucotreta</i>	False Coddling Moth	3
	Tortricidae	<i>Cydia pomonella</i>	Coddling Moth	4
		<i>Thaumatotibia leucotreta</i>	False Coddling Moth	1

Table 1.2. Summary of semiochemicals identified in stored product pests (Orders Coleoptera and Lepidoptera), including the chemical name, behavioral function, emitting sex, supporting literature (found on Pherobase), and available GenBank genomic resources.

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
Coleoptera	Bostrichidae	<i>Prostephanus truncatus</i>	1-methylethyl (<i>E</i>)-2-methyl-2-pentenoate (trunc-all 1) and 1-methylethyl (<i>E</i>)-2, (<i>E</i>)-4-2, 4-dimethyl-2-heptadienoate (trunc-all 2)	Aggregation	Male	Cork et al. 1991a; Dendy et al. 1989	GCA_025024175.1
		<i>Rhyzopertha dominica</i>	1-methylbutyl (<i>E</i>)-2-methyl-2-pentenoate (dominicalure 1) and 1-methylbutyl (<i>E</i>)-2,4-dimethyl-2-pentenoate (dominicalure 2)	Aggregation	Male	Khorramshahi and Burkholder 1981; Williams et al. 1981	
	Bruchidae	<i>Acanthoscelides obtectus</i>	methyl (<i>E,Z,Z</i>)-2,4,7-decatrienoate, methyl (<i>E,Z</i>)-2,4-decadienoate, (<i>Z,E</i>)- and (<i>E,E</i>)- α -farnesene	Sex	Male	Horler 1970; Vuts et al. 2015a	GCA_963669975.1
	Chrysomelidae	<i>Callosobruchus chinensis</i>	(<i>E</i>)-3,7-dimethyl-2-octene-1,8-dioic acid and straight-chain and methyl-branched hydrocarbons (3-methylpentacosane, 11-methylheptacosane, 3-methylheptacosane, 11-methylnonacosane, 13-methylnonacosane, 11,15-dimethylnonacosane, 13-methylhentriacontane, 9,13-dimethylhentriacontane and 11,15-dimethyltritriacontane	Sex	Female	Tanaka et al. 1981, Tanaka et al. 1982; Yajima et al. 2007	
		<i>Callosobruchus maculatus</i>	3-methyleneheptanoic acid, (<i>Z</i>)-3-methyl-3-heptenoic acid, (<i>E</i>)-3-methyl-3-heptenoic acid, (<i>Z</i>)-3-methyl-2-heptenoic acid, and (<i>E</i>)-3-methyl-2-heptenoic acid	Sex	Female	Shu et al. 1996; Phillips et al. 1996; Ramaswamy et al. 1995	
		<i>Sitophilus granarius</i>	1-ethylpropyl (<i>3R,2S</i>)-2-methyl-3-hydroxypentanoate (sitophilate)	Aggregation	Male	Faustini et al. 1982c; Walgenbach et al. 1987	
	Curculionidae						

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
			3-hydroxy-2-butanone	Attractant	Mixed	Cai et al. 2022	
			1-pentadecene	Repellent	Mixed	Cai et al. 2022	
		<i>Sitophilus oryzae</i>	(4 <i>S</i> , 5 <i>R</i>)-4-methyl-5-hydroxy-3-heptanone (sitophilure)	Aggregation	Male	Phillips and Burkholder 1981; Schmuff et al. 1984	GCA_002938485
		<i>Sitophilus zeamais</i>	(4 <i>S</i> , 5 <i>R</i>)-4-methyl-5-hydroxy-3-heptanone (sitophilure)	Aggregation	Male	Walgenbach et al. 1983; Schmuff et al. 1984; Walgenbach et al. 1987	
		<i>Dermestes maculatus</i>	isopropyl (Z)-9-dodecenoate, isopropyl (Z)-9-tetradecenoate, and isopropyl Z-9-hexadecenoate	Aggregation	Males	Francke et al. 1979; Levinson et al. 1981a	
		<i>Trogoderma granarium</i>	(Z):(E)-14-methyl-8-hexadecenal	Sex	Female	Rodin et al. 1969; Levinson and Levinson 1974	
	Dermestidae	<i>Trogoderma inclusum</i>	(Z)-14-methyl-8-hexadecen-1-ol, (Z)-14-methyl-8-hexadecenoate and (Z)-14-methyl-8-hexadecenal (trogoderma)	Sex	Female	Rodin et al. 1969	
		<i>Trogoderma variabile</i>	(Z)-14-methyl-8-hexadecenal	Sex	Female	Cross et al. 1976; Greenblatt et al. 1976; Cross et al. 1977	
	Nitidulidae	<i>Carpophilus hemipterus</i>	(2 <i>E</i> ,4 <i>E</i> ,6 <i>E</i> ,8 <i>E</i>)-3,5,7-trimethyl-2,4,6,8-decatetraene and (2 <i>E</i> ,4 <i>E</i> ,6 <i>E</i> ,8 <i>E</i>)-3,5,7-trimethyl-2,4,6,8-undecatetraene	Aggregation	Male	Bartelt et al. 1990	

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
	Ptinidae	<i>Lasioderma serricorne</i>	4 <i>S</i> ,6 <i>S</i> ,7 <i>R</i> -dimethyl-hydroxynonan-3-one (serricornin), (2 <i>S</i> , 3 <i>R</i>)-cis-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-oxobutyl)-4Hpyran-4-one (serricorone), (2 <i>S</i> ,3 <i>R</i>)-cis-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-hydroxybutyl)-4H-pyran-4-one (serricorole)	Sex	Female	Chuman et al. 1979; Levinson et al. 1981b; Chuman et al. 1983; Chuman et al. 1985	
		<i>Stegobium paniceum</i>	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-2,3-dihydro-2,3,5-trimethyl-6-(1-methyl-2-oxybutyl)-4H-pyran-4-one and (2 <i>S</i> ,3 <i>R</i> ,1' <i>S</i> ,2' <i>RS</i>)-2,3-dihydro-2,3,5-trimethyl-6-(1'-methyl-2'-hydroxybutyl)-4H-pyran-4-one	Sex	Female	White and Birch 1987; Ansell et al. 1979; Hoffman and Ladner 1979; Mori et al. 1981; Kodama et al. 1987	
	Silvanidae	<i>Oryzaephilus mercator</i>	(<i>Z</i>)-3-dodecen-11-olide and (<i>Z,Z</i>)-3,6-dodecadien-11-olide	Aggregation	Male	Pierce et al. 1984a; Pierce et al. 1984b; Pierce et al. 1985; Oehlschlager et al. 1988	
		<i>Oryzaephilus surinamensis</i>	(<i>Z,Z</i>)-3,6-dodecadien-11-olide, (<i>Z,Z</i>)-3,6-dodecadienolide, and (<i>Z,Z</i>)-5,8-tetradecadien-13-olide	Aggregation	Male	Pierce et al. 1984a; Pierce et al. 1984b; Pierce et al. 1985	GCA_004796505.1
	Tenebrionidae	<i>Gnatocerus cornutus</i>	(+)-acoradiene and alpha-cedren-14-al	Aggregation	Male	Tebayashi et al. 1998	

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
		<i>Tenebrio molitor</i>	4-methyl-1-nonanol		Female	Tschinkel 1970	
		<i>Tenebrio molitor</i>	(Z)-3-dodecenyl acetate	Sex	Male	Bryning et al. 2005	
		<i>Tribolium audax</i>	1,6-pentadecadiene	Unknown	Mixed	Arnaud et al. 2002	
		<i>Tribolium brevicornis</i>	1,6-pentadecadiene	Unknown	Mixed	Arnaud et al. 2002	
			(4 <i>R</i> ,8 <i>R</i>)-dimethyldecanal and (4 <i>S</i> ,8 <i>R</i>)-dimethyldecanal	Aggregation	Male	Suzuki, 1980; Suzuki 1981a; Suzuki et al. 1984; Santangelo et al. 2006	GCA_031307605.1
		<i>Tribolium castaneum</i>	1-pentadecene		Mixed	Keville and Kannowski 1975; Suzuki et al. 1975	
			1-heptadecene, 1,8-heptadecadiene, 1-tetradecene, 1-hexadecene, 1,6-pentadecadiene and heptadecatriene	Repellent	Both	Suzuki et al. 1975	
			1-tridecene	Sex	Male	Senthilkumar et al. 2012; Suzuki, 1980; Suzuki 1981a;	
		<i>Tribolium confusum</i>	(4 <i>R</i> ,8 <i>R</i>)-dimethyldecanal and (4 <i>S</i> ,8 <i>R</i>)-dimethyldecanal	Aggregation	Male	Suzuki et al. 1984; Santangelo et al. 2006	GCA_029207805.1
		<i>Tribolium destructor</i>	(4 <i>R</i> ,8 <i>R</i>)-dimethyldecanal and (4 <i>S</i> ,8 <i>R</i>)-dimethyldecanal				

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
Lepidoptera	Gelechiidae	<i>Tribolium freemani</i>	(4 <i>R</i> ,8 <i>R</i>)-dimethyldecanal and (4 <i>S</i> ,8 <i>R</i>)-dimethyldecanal	Aggregation	Male	Suzuki et al. 1987	GCA_939628115.1
		<i>Tribolium madens</i>	(4 <i>R</i> ,8 <i>R</i>)-dimethyldecanal and (4 <i>S</i> ,8 <i>R</i>)-dimethyldecanal	Aggregation	Male	Arnaud et al. 2002	GCA_015345945.1
		<i>Sitotroga cerealella</i>	(<i>Z,E</i>)-7,11-hexadecadienyl acetate	Sex	Female	Vick et al. 1974; Ando et al. 1985	
		<i>Phthorimaea operculella</i>	(<i>Z,E</i>)-7,11-Hexadecadienal	Sex	Female	Ando et al. 1985	
		<i>Amyelois transitella</i>	trans-4, cis-7-tridecadienyl acetate and trans-4, cis-1, cis-10 tridecatrienyl acetate	Sex	Female	Persoons et al. 1976	
		<i>Cadra cautella</i>	(11 <i>Z</i> ,13 <i>Z</i>)-hexadecadienal, (11 <i>Z</i> ,13 <i>Z</i>)-hexadecadien-1-ol, (11 <i>Z</i> ,13 <i>E</i>)-hexadecadien-1-ol, and (3 <i>Z</i> ,6 <i>Z</i> ,9 <i>Z</i> ,12 <i>Z</i> ,15 <i>Z</i>)-tricosapentaene	Sex	Female	Coffelt et al. 1979; Leal et al. 2005; Kuenen et al. 2010	GCA_032362555.1
		<i>Cadra cautella</i>	(<i>Z,E</i>)-9,12-tetradecadienyl acetate (ZETA) and Z-9-tetradecenyl-acetate (ZTA)	Sex	Female	Brady 1973; Shani and Lacey 1985; Barrer et al. 1987	
		<i>Cadra figulilella</i>	(<i>Z,E</i>)-9,12-tetradecadienyl acetate (ZETA)	Sex	Female	Brady 1972	
		<i>Corcyra cephalonica</i>	6,10,14-trimethyl-2-pentadecanol	Sex	Female	Hall et al. 1987	
		<i>Ephesia elutella</i>	(<i>E,E</i>)-/(<i>Z,E</i>)-3,7,11-trimethyl-2,6,10-dodecatrienal (farnesal)	Sex	Male	Zagatti et al. 1987; Mori et al. 1991	GCA_040436485.1
		<i>Ephesia elutella</i>	(<i>Z,E</i>)-9,12-tetradecadienyl acetate (ZETA) and (<i>Z,E</i>)-9, 12-tetradecadienol (ZETOH)	Sex	Female	Krasnoff et al., 1984	GCA_018467065.1
		<i>Ephesia elutella</i>	3,7,11,15-tetramethyl-2-hexadecenyl-1-ol; γ -decalactone and γ -undecalactone	Sex	Male	Phelan et al. 1986	

Order	Family	Species	Semiochemical	Type of Semiochemical	Emitter	Semiochemical References	GenBank Resource
		<i>Ephestia kuehniella</i>	(Z,E)-9,12-tetradecadienyl acetate (ZETA)	Sex	Female	Kuwahara et al. 1971; Brady et al. 1971	GCA_921024065.1
		<i>Galleria mellonella</i>	5,11-dimethylpentacosane	Long-range sex	Male	Svensson et al. 2014	GCA_026898425.1
			nonanal and undecanal	Short-range sex	Male	Lebedeva et al. 2002	
		<i>Plodia interpunctella</i>	(Z,E)-9,12-tetradecadienyl acetate (ZETA), (Z,E)-9,12-tetradecadien-1-ol (TDO), and (Z,E)-9,12-tetradecadienal (ZETAL)	Sex	Female	Kuwahara et al. 1971a; Brady et al. 1971; Dahm et al. 1971; Teal et al. 1995	GCA_027563975.2
			palmitic, oleic, linoleic acids, and ethyl esters	Sex	Male	Phelan et al. 1992	
		<i>Pyralis farinalis</i>	(11Z,13Z)-hexadecadienal, (11Z,13Z)-hexadecadien-1-ol, (11Z,13E)-hexadecadien-1-ol, and (3Z,6Z,9Z,12Z,15Z)-tricosapentaene	Sex	Female	Leal et al. 2005; Kuenen et al. 2010	GCA_947507595.1
	Tineidae	<i>Tineola bisselliella</i>	(E,Z)-2,13-octadecadienal and (E,Z)-2,13-octadecadienol	Sex	Female	Takacs et al. 2001	

Chapter 2 - How is *P. interpunctella* flight capacity influenced by semiochemicals?

In preparation as: Jacqueline M. Maille, Jaden R. Reed, Matthew Hetherington, Nicholas Sixbury, William Rust, Daniel Brabec, Melanie Kessler-Mathieu, Kun Yan Zhu, Erin D. Scully, and W.R. Morrison III. State-dependent flight capacity by Indian meal moth varies by age, mating status, and sex: implications for optimal foraging.

Introduction

Dispersal carries both risks and benefits critical to the ability to survive and reproduce. For example, dispersal can incur fitness and energy costs but can lead to the discovery of food and mates that are critical for survival and reproduction (Emlen 1966, MacArthur and Pianka 1966). Optimal foraging theory (OFT) provides a conceptual framework linking these behavioral decisions to ecological and evolutionary contexts given life-history constraints and predicts that individuals adjust movement strategies based on physiological statuses and external environmental cues (Charnov 1976, Smith 1978).

In insects, flight represents a specialized but widespread form of movement, enabling rapid relocation and exploration of the environment. Insect flight provides specific advantages, such as escaping predators (Humphreys and Ruxton 2019), locating resource-rich patches in a patchy landscape (Fragoso et al. 2021), or finding mates (Schumacher et al. 1997, Abshire et al. 2024), but sustained flight is energetically costly (Suarez 2000). An individual flight bout may be directed as in dispersal, or it may be arrested, as in the case when attractive mates or high-quality resources are nearby. Dispersal occurs when competition is high, mates are scarce, or resources are limited, allowing individuals to relocate to higher-value patches. Arrestment, by contrast, involves shorter bouts of movement with higher turning radii and may be advantageous when

resource patches and/or mates are abundant, and competition is low (Bell 1990). Therefore, individuals face a trade-off between dispersal and arrestment, modulated by the perceived value of the current patch (Zera and Denno 1997). Volatile cues play a critical role in communicating patch quality and modulating decisions about whether to remain or leave (Kennedy 1978). For example, pheromone emissions from conspecifics typically indicate mating opportunities and facilitate mate-finding through directed anemotaxis (Cardé and Willis 2008). Similarly, food odors or kairomones provide information about resource availability and offspring suitability, allowing individuals to assess patch value. Such cues can influence multiple stages of the foraging process, including patch detection, acceptance, and the initial decision to disperse (Andersson et al. 2013).

Optimal foraging theory (OFT) also predicts state-dependent variation in movement. Decisions to move or disperse may differ based on sex, physiological shifts that occur after mating, or age-related energetic and reproductive constraints. For example, unmated individuals may invest more in flight in an attempt to find mates, whereas mated females may reduce dispersal effort to focus on localized searching for oviposition opportunities (Rosenheim et al. 2008, Sambaraju et al. 2016). Older insects may have reduced flight capacity due to energy limitations, altering their dispersal decisions (Sohal 1976).

The Indian meal moth (*Plodia interpunctella* [Hübner]; Lepidoptera: Pyralidae) provides an ideal model for evaluating questions about flight and OFT. As a stored-product pest, *P. interpunctella* navigates discontinuous environments characterized by variable resource availability, such as those found in food facilities (Campbell et al. 2002, Semeao et al. 2013). Adults do not feed, meaning that responses to food cues reflect decisions to possible mating opportunities, reproductive success, and oviposition site selection rather than immediate nutrition

(Sambaraju and Phillips 2008). Although the flight behavior and chemosensory responses of *P. interpunctella* are partially characterized (Mankin et al. 1983), few studies have simultaneously manipulated multiple physiological and environmental conditions to quantify dispersal outcomes. By contrast, such factorial approaches are common in other non-stored product Lepidoptera (Foley 1985, Schumacher et al. 1997, Sarvary et al. 2008, Qin et al. 2018). Finally, only two studies have systematically investigated the flight capacity of stored product insects (Cui et al. 2016, Abshire et al. 2024), but this type of work is important for assessing risk and informing management programs for insects after harvest.

Here, we used a tethered flight mill system to examine *P. interpunctella* behavior under controlled laboratory conditions. Our aims were to assess the effects of mating status (mated vs. unmated), age (1, 3, or 5 days old), sex (male vs. female), and olfactory cues (food, sex pheromone, both, or neither) on flight behavior. We predicted that exposure to pheromone and food cues would shorten flight distance due to increased patch attraction, while unmated individuals would fly longer distances in search of potential mates. This study allowed us to explore whether *P. interpunctella* flight behavior follows state-dependent OFT predictions, quantifying the decision to invest in sustained flight versus arrestment in response to ecologically relevant olfactory cues.

Materials and Methods

Source insects and staging

The Savannah laboratory colony of *P. interpunctella* was used in this study, which has been maintained in continuous culture at the USDA-ARS Center for Grain and Animal Health in Manhattan, KS, USA, for over 60 years. To maintain colonies, approximately 100 newly emerged adults (1–3 days old) were placed into 473 mL glass jars fitted with an 8.26 cm

diameter steel mesh lid to allow ventilation and future egg removal. Adults were allowed to mate and oviposit for 72 h, after which jars were gently shaken to dislodge eggs. The eggs were collected, weighed (~0.05 g), and placed on 200 g of standard diet consisting of wheat shorts, cracked wheat, wheat germ, brewer's yeast, glucose, water, and honey in 473 mL glass jars. All life stages were maintained at 27°C, 65% relative humidity (RH), and a 16:8 light: dark (L:D) photoperiod.

In staging adults for experimentation, we incorporated three factors: sex (male or female), mating status (unmated or mated), and post-eclosion age (1, 3, or 5 d). To generate unmated adults, fourth-instar larvae (Figure 2.1A) were sexed and individually isolated into 59.2 mL (2 oz) plastic cups (Precisely Plastic, Amazon) containing 2 g of diet. They were then held under standard rearing conditions until adult emergence. Emergence was checked daily after 1200 h, and adults were aged from the day of eclosion (day 0). For mated treatments, a single female and male were placed into a single 59.2 mL plastic cup and given a mating opportunity under standard conditions for 12–18 h prior to experimentation. Mating status was confirmed post-experiment by dissection of females to assess the presence of a spermatophore (Figure 2.1B).

Stimuli sources

Four different olfactory stimulus treatments, including pheromone, kairomone (food), their combination, and a control (e.g., no stimuli), were presented in a 100 cm diameter Petri dish (Fisher Scientific, Hampton, NH, USA) to standardize surface area and odor availability. The pheromone stimulus consisted of the commercial *P. interpunctella* pheromone lure (IL-103; Insects Limited, Westfield, IN, USA; Batch No. 1625011023), which contains a proprietary blend of *P. interpunctella* sex pheromone components, including <5% (by weight) (Z, E)-9,12-tetradecadienyl acetate (hereafter: ZETA), for which we quantified the emission rate in Section

2.4. Due to technical constraints, the volatile emissions from the food stimuli could not be quantified; however, the exposure consisted of 20 g of unbleached wheat flour to ensure consistency across trials. For the combined treatment, pheromone and food stimuli were presented together in the same Petri dish, which consisted of a lure (as above) and 20 g of flour. The control contained no stimuli and consisted of an empty Petri dish. A smoke test confirmed diffusion of odors to the flight mill. Stimuli were maintained in a -30°C freezer until testing.

Flight experiments

Flight capacity was measured using a custom tethered flight mill system (Scribner et al., 2026) that recorded eight moths simultaneously through four flight mills arranged each in two arrays housed within a walk-in environmental chamber set to rearing conditions. Each flight mill consisted of a horizontal flight arm constructed from 19-gauge, non-magnetic hypodermic steel tubing (22 cm in length) mounted on a central pivot via a 10 μ L micropipette tube and secured with hot glue. The arm was effectively weightless and stabilized by strong neodymium magnets positioned dorsally and ventrally. One end of the arm held the tethered insect, while the opposite end supported a small flag designed to interrupt an LED/phototransistor sensor (OPB800W; Optek Technology Inc., Texas, USA). This flag design allowed for the recording of each revolution and, due to its design, also recorded the flight direction. Flights that were determined to be in the opposite direction of the forward moving flight path were removed due to the moth not engaging in flight actively but resting and the mill moving in a reverse direction autonomously. Sensor signals were recorded using a DATAQ Model DI-2108-P data acquisition system (DATAQ Instruments, Ohio, USA) and were streamed to a computer via USB using custom-built software. The system converted analog voltage changes into digital signals, allowing continuous logging of flight metrics for each channel. Two shelves of plexiglass (25.5 $\times$

24.5 × 12 cm, L × W × H) housed the flight mill arrays, with each shelf accommodating up to four mills vertically unit (Figure 2.2A). Sensors were powered by +5 V DC adapters through the interface, and wiring from the LED sensors connected via screw terminals to the data acquisition.

Before tethering, moths were cold-immobilized by placing their 59.2 mL (2 oz) cups on ice for 10 min. The pronotum was lightly descaled with a silicone artist's brush (Masterstouch, Hobby Lobby, Oklahoma City, OK, USA), and a No. 2 insect pin (Willbond Technology Ltd., Amazon) was affixed using instant adhesive (T-7000, Guangdong Zhanlida New Material Co., Ltd., Foshan, China), taking care to avoid the eyes, antennae, and wing articulation. The tethered moth was mounted on one end of the flight arm (Figure 2.2B), and olfactory stimuli were placed 136 cm from the center of the flight mill.

Moths of the same sex, mating status, and age were blocked together. Insects were given a 24 h flight opportunity, and trials began between 1300 and 1800 h. Consecutive revolutions of the flight arm were considered a single flight bout except where the span between revolutions exceeded 6 s, then the revolution was split into two separate bouts. Metrics reported included channel (e.g., flight mill number), time of day, flight direction, and revolution time, which were logged continuously over the entire 24 h duration. Post-trial data processing included retaining only forward flights and calculation of key flight capacity metrics, including total distance flown, total revolutions, flight velocity, total flight duration, continuous bout flight distance, number of continuous flight bouts, and bout duration.

Headspace collections and GC-MS analysis

Headspace collections were conducted to quantify volatile emissions from independent IL-103 pheromone lures in the study over 24 h to ensure consistent emission. Three of four 500 mL PTFE-lined glass headspace chambers (10.2 × 12.7 cm D: H; Research Facility Solutions,

Gainesville, FL, USA) were loaded with an IL-103 lure, while the fourth chamber served as a negative control. Volatiles were collected over three durations (3, 6, 18, and 24 h), with four replicates per duration ($n=16$). For volatile capture, chambers were connected in parallel to four flow meters via 5 mm ID PTFE tubing. The incoming air was scrubbed through an activated charcoal filter and controlled at a flow rate of 1 L/min using flow meters (Sigma Scientific, LLC, Micanopy, FL, USA). Headspace volatiles were captured using 7.62 cm Porapak-Q™ volatile collection traps (20 mg; Volatile Collection Systems, Gainesville, FL, USA). After collection, volatiles were eluted with 150 μ L of dichloromethane into 2 mL GC vials equipped with magnetic screw-top caps and 250 μ L vial inserts (Agilent Technologies, Santa Clara, CA, USA) using solvent pushed through by inert extra dry N₂ gas (Matheson Tri-Gas, Irving, TX, USA). An internal standard of tetradecane (2 μ L, 190.5 ng, 99% purity; MilliporeSigma, Burlington, MA, USA) was added to each vial using a microsyringe (Hamilton Co., Reno, NV, USA) for quantification. Traps were cleaned between collections with three successive rinses of 700 μ L dichloromethane. GC vials were sealed with PTFE tape and stored at -30 °C until analysis.

Volatile extracts were analyzed using an Agilent 7890B gas chromatograph (GC) equipped with a Durabond HP-5 column (30 m $\times$ 250 μ m $\times$ 0.25 μ m) and coupled to an Agilent 5997B single-quadrupole mass spectrometer (MS). Helium was used as the carrier gas at a constant flow of 1.2 mL/min (linear velocity 39 cm/s). Samples (1 μ L) were injected in splitless mode with the inlet maintained at 250°C. The oven program started at 30°C for 1 min, then ramped at 10°C/min to 300°C over 27 min, and was held for 0.5 min. A 4 min solvent delay was applied, and mass spectra were scanned from 50–550 atomic mass units. Compounds were preliminarily identified by comparison with the NIST 14 spectral library using deconvolution software, specifically Unknowns Analysis (Agilent Technologies). A technical standard (*Z, E*)-

9,12-tetradecadienyl acetate (ChemCruz Biochemicals, Santa Cruz, CA, USA; CAS 31654-77-0) was injected on the same column to confirm compound identity via retention time.

Statistical analyses

The flight capacity data were analyzed using generalized linear mixed models (GLMMs) to assess the effects of sex (male, female), mating status (unmated, mated), age (1, 3, or 5 d post-eclosion), and olfactory treatment (pheromone, food, pheromone/food combination, control) by *P. interpunctella*, including all relevant interaction terms. The experimental design followed a factorial structure with block nested within experimental run. Sample sizes were unbalanced between mating status treatments for unmated *P. interpunctella*, $n = 24$ per sex $\times$ treatment $\times$ age combination, whereas for mated individuals $n = 16$ per sex $\times$ treatment $\times$ age combination.

All statistical analyses were performed in R (version 4.4.3; R Core Team) using the packages *glmmTMB* (Brooks, Mollie et al. 2017) for model fitting and *emmeans* (Lenth and Piaskowski 2025) for post-hoc analysis. Because total distance flown, total flight duration, and metrics with continuous-positive data exhibited overdispersion, we primarily employed a Tweedie distribution with a log-link function. For total flight velocity and bout distance, a gamma distribution with a log-link was utilized. The number of continuous flight bouts was modeled using a negative binomial distribution (Type II) to account for overdispersion in count data. Distributions that reflected each of the response variables were used in each case to avoid well-known artifacts from transformation (Bolker et al. 2009, Warton and Hui 2011). To address substantial heteroscedasticity during initial diagnostics and to account for the unbalanced design, conditional dispersion submodels were included for total distance flown, bout duration, and flight velocity models allowing residual variance to vary by mating status and sex. This

modeling approach significantly improved model fit, as evidenced by AIC reductions compared to constant variance models.

To account for the hierarchical structure of the data and variation among experimental runs and flight mill units, block was included as a random effect. In the flight bout-level models, which utilized a larger longitudinal dataset derived from individuals that took continuous flight bouts ($n = 101,104$), moth id was included as a random intercept to account for repeated measures on the same individual. Model assumptions were evaluated using the DHARMA package (Hartig 2025) to inspect residual diagnostics and goodness-of-fit measures.

Fixed effects were assessed using Wald z-tests. When significant main effects or interactions were detected, pairwise comparisons among estimated marginal means were conducted. Significance levels were controlled for multiple testing using Sidak or Tukey adjustments as specified in the figure captions per model, with $\alpha = 0.05$.

The headspace data collected from independent lures were analyzed separately to determine the emission rate during flight duration. Since these measurements were obtained from independent sampling at each time point, a one-way analysis of variance (ANOVA) was utilized, with $\alpha = 0.05$. Prior to analysis, data were assessed for normality and homogeneity of variance using Levene's test from the car package (Fox and Weisberg 2019).

Results

Cumulative flight capacity

We first evaluated the total flight capacity of *P. interpunctella* over the course of 24 h. Overall, total flight capacity was more strongly influenced by mating status and sex rather than olfactory treatments or age (Table 2.1).

Total distance flown

Overall, the total distance flown in 24 h by the average male and female *P. interpunctella* was 7.3 ± 0.4 (SE) and 5.3 ± 0.3 km, respectively. The 5% of the longest distance dispersals flew an average total distance of 17.0–22.7 km in 24 h. After modeling heterogeneity in variance, total distance flown differed by mating status and sex, with unmated individuals flying about 20% farther than mated individuals ($\beta = 0.701 \pm 0.102$ (SE), $z = 3.774$, $p < 0.001$) and males flying ~38% farther than females ($\beta = 0.326 \pm 0.086$, $z = 6.896$, $p < 0.001$; Figure 2.3). In the conditional dispersion model, the distance flown by unmated moths was less variable than that of mated moths ($\beta = -0.298 \pm 0.092$, $z = -3.239$, $p < 0.001$). By contrast, the presence of both pheromone and food reduced total flight distance by 35% when compared to the control treatment ($\beta = -0.445 \pm 0.199$, $z = -2.235$, $p < 0.025$; Figure 2.4). The other treatments (e.g., pheromone alone or food alone), day, and all treatment $\times$ day interactions did not significantly influence total distance flown (all $p > 0.07$; Table 2.1). Variation attributable to block was negligible ($\sigma^2 = 0.002$), indicating that observed differences in flight distance primarily reflected biological differences among individuals rather than experimental structure.

Total flight duration

Overall, the mean flight duration by unmated male and female *P. interpunctella* was 10.4 ± 0.5 and 7.2 ± 0.3 h, respectively. While flight duration for mated males and females was 5.9 ± 0.5 and 4.3 ± 0.4 h, respectively. After accounting for heterogeneity in variance, total flight duration differed significantly by mating status and sex, with unmated individuals remaining in flight 72% longer than mated individuals ($\beta = 0.5444 \pm 0.085$, $z = 6.37$, $p < 0.001$) and males flying 47% longer than females ($\beta = 0.387 \pm 0.071$, $z = 5.48$, $p < 0.001$; Figure 2.5). In the conditional dispersion model, the time unmated moths spent flying was more consistent and less variable than mated moths ($\beta = -0.426 \pm 0.093$, $z = -4.573$, $p < 0.001$). The combined stimulus

treatment (e.g. pheromone and food) reduced total flight duration by 30% when compared to the control treatment ($\beta = -0.342 \pm 0.164$, $z = -2.078$, $p < 0.025$; Figure 2.5). No significant effects of day, or treatment $\times$ day interactions were found on total flight duration (all $p > 0.17$; Table 2.1). Block-level variance was negligible ($\sigma^2 < 0.001$), indicating minimal influence of experimental design effects on total flight duration.

Total flight velocity

Total flight velocity (m/s) was analyzed using a gamma distribution with log-link, including block as a random effect. We allowed the residual variance to vary by sex and mating status within the model, improving model fit. Unmated moths flew significantly faster (23%) than mated moths ($\beta = 0.211 \pm 0.036$, $z = 5.899$, $p < 0.001$), while males flew slightly slower (7%) than females ($\beta = -0.071 \pm 0.032$, $z = -2.197$, $p < 0.05$; Figure 2.6). In the conditional dispersion model, the flight velocity of unmated moths was more consistent than that of mated moths ($\beta = -1.209 \pm 0.106$, $z = -11.380$, $p < 0.001$). Flight velocity was not significantly affected by treatment, day, or their interaction (Table 2.1). The block random effect accounted for minimal variance ($\sigma^2 < 0.001$).

Flight bout dynamics

To understand how the olfactory stimuli influenced the structure of flight, we analyzed continuous flight bouts. Unlike the cumulative flight capacity metrics, flight bout-level dynamics were significantly influenced by the presence of food and pheromone cues (Table 2.2).

Number of flight bouts

The number of flight bouts in the top 5% of females and males ranged from 5,108 to 7,577 bouts; whereas the number of flight bouts in the bottom 5% ranged from 2 to 6 bouts, showing both sexes engage in sustained flight. After accounting for variability, males flew in

45% more continuous flight bouts than females ($\beta = 0.375 \pm 0.096$, $z = 3.89$, $p < 0.001$). No significant effects of treatment, day, or mating status were found (all $p > 0.4$, Table 2.2). Interestingly, the pheromone $\times$ day 3 interaction was significant, which resulted in a 120% greater number of flight bouts compared to other ages and treatment interactions ($\beta = 790 \pm 0.345$, $z = 2.29$, $p = 0.022$) (Figure 2.7). Most of the unexplained variance in the number of flight bouts was attributable to individual-level heterogeneity rather than experimental treatments or block effects ($\sigma^2 < 0.001$).

Flight bout distance

The distances flown in a bout by the average male and female *P. interpunctella* were 52.0 ± 3.1 and 58.0 ± 2.7 m, respectively. Bout distance flown was reduced by 53% in the presence of the combined stimuli treatment ($\beta = -0.765 \pm 0.366$, $z = -2.092$, $p < 0.001$) when compared to the control (Figure 2.8) and reduced by 57% by moths that were 5-d old ($\beta = -0.3851 \pm 0.367$, $z = -2.321$, $p = 0.020$) compared to 1-d old moths. Unmated adults flew 79% farther per flight bout than mated moths ($\beta = 0.584 \pm 0.155$, $z = 3.778$, $p < 0.001$), while sex and other treatments, and all treatment $\times$ day interactions did not significantly affect flight bout distance (Table 2.2). There was no blocking effect on bout distance (both, $\sigma^2 < 0.001$).

Flight bout duration

Flight bout duration differed across olfactory treatments and days, with 41% and 49% shorter flight bouts observed in the presence of food ($\beta = -0.530 \pm 0.244$, $z = -2.173$, $p = 0.030$) and pheromone + food (combined) ($\beta = -0.685 \pm 0.244$, $z = -2.807$, $p = 0.005$) relative to controls, respectively (Figure 2.9). Flight bout duration also declined with age, with flight bouts lasting 52% less on day 5 ($\beta = -0.735 \pm 0.245$, $z = -3.001$, $p = 0.002$) compared to day 1. Unmated individuals exhibited 66% longer bout durations than mated individuals ($\beta = 0.507 \pm$

0.104, $z = 4.893$, $p < 0.001$), whereas sex had no detectable effect ($p = 0.471$). Interestingly, interactions of food $\times$ day 5 ($\beta = 0.843 \pm 0.350$, $z = 2.411$, $p = 0.016$) and combined $\times$ day 5 ($\beta = 0.773 \pm 0.346$, $z = 2.235$, $p = 0.025$) exhibit shifts in behavioral response as the moths aged, since these same stimulus treatments suppressed flight bout durations on day 1.

Headspace

Concentrations of (Z, E)-9,12-tetradecadienyl acetate emissions were consistent over time, determined by a one-way ANOVA ($F(3,13) = 0.64$, $p = 0.603$) (Table 2.3). During 24 h collections, emissions were an average of 66.7 ng/ L h (Figure 2.10).

Discussion

Animal movement reflects dynamic cost–benefit trade-offs shaped by physiological condition and environmental context. Although optimal foraging theory predicts that dispersal decisions should integrate internal state with external cues, empirically disentangling these interacting drivers remains challenging, particularly in small insects. Controlled experimental systems provide a powerful framework for resolving these mechanisms. In this study, we demonstrate that flight behavior for *P. interpunctella* is strongly influenced by internal physiological states (status and sex) and modulated by olfactory cues, showing consistency with predictions from state-dependent OFT.

In particular, we found *P. interpunctella* flew up to 22.7 km in a 24 h period. This finding highlights the risk posed by *P. interpunctella* to food facilities, given the long distances moths are capable of flying in 24 h, which is similar to another stored product moth, the Mediterranean flour moth, *Ephestia kuehniella* (Hübner) (Lepidoptera: Pyralidae) (Abshire et al. 2024). This suggests that populations of *P. interpunctella* may not be isolated in the landscape and can move long distances to locate mates or resources. Work with other insects, such as the stored product

insect *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), indicates that populations exhibit frequent gene flow aided by active flight across food facilities in Australia (McCulloch et al. 2022). Other stored product insects also disperse over large distances, including lesser grain borer, *Rhyzopertha dominica* (L.) (Coleoptera: Bostrichidae), which is regularly trapped miles away from food facilities (Scheff et al. 2011), while *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) is often found in forested areas and is preferentially attracted to stimuli from acorns and other resources found in these landscapes (Ming et al. 2025). Although it is unknown to what extent *P. interpunctella* traverses the landscape in search of resources, its flight capacity and behavior suggest that this species may exhibit similar tendencies. Further studies should focus on the landscape-level dispersal of *P. interpunctella* to understand the risk that it poses to adjacent storage facilities. Overall, this highlights the landscape-scale risk posed by *P. interpunctella* as well as other stored product insects.

Mating status was the strongest predictor of flight capacity by *P. interpunctella*. Unmated moths generally exhibited flight distances and velocities that were farther, longer, and faster, and they took more flights than mated individuals, highlighting the role of reproductive motivation in shaping dispersal. These observations support what Sambaraju *et al.* (2016) observed in determining time budgets for *P. interpunctella* in the laboratory, where most of the time was spent away from food sources. In the current study, the physiological and flight behavior of the moths shifted after mating. For females, the reduction in flight distance likely reflects the transition from mate-finding to localized ovipositional patch assessment with the reduction of energy following copulation, whereas the reduction in distance flown by mated males could be a consequence of energy reduction after copulation. This, in turn, may prompt shorter flight bouts by mated males. Interestingly, our conditional submodels showed that unmated moths were more

consistent and less variable in their flight patterns, suggesting unmated moths are capable of sustained dispersal to new areas, while mated moths exhibit more behavioral plasticity in searching. In pro-ovigenic species like *P. interpunctella* and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), the transition from consistent to variable flight is not limited by oocyte maturation (Schumacher et al. 1997, Beckemeyer and Shirk 2004) as described in the oogenesis flight syndrome theory (Johnson 1960, 1963). Therefore, mating may act as the trigger of shifting behavioral tactics from dispersal (continuous flight bouts) to searching (variable flight bouts) through the reception of the sex peptides contained within the spermatophore transfers (Rafaeli 2005). This transfer initiates a cascade that likely suppresses sustained flight while activating host-seeking or oviposition in *P. interpunctella* (Rafaeli 2005). Furthermore, since *P. interpunctella* females fertilize their eggs as they pass through the vestibulum during oviposition (Lum et al. 1981), the exact onset of mated flight may be more variable. This behavioral transition aligns with observations in another pro-ovigenic long-distance disperser, *Helicoverpa armigera* (Armes and Cooter 1991, Liu et al. 2021), where a 28-fold decrease in the longest continuous flight bout after mating was observed in females.

For males, the observed reduction in flight consistency and velocity aligns with the tradeoff in mate-finding compared to ejaculate investment identified in *P. interpunctella* (Lewis et al. 2013). Lewis et al. (2013) suggested that males must strategically allocate limited resources between energy-intensive pre-copulatory behaviors, like searching or seducing females, and the metabolic cost of producing high-quality spermatophores. This trade-off is acute in *P. interpunctella* because males eclose with higher lipid reserves than females (Subramanyam and Cutkomp 2017).

Olfactory stimuli modulated flight behavior by *P. interpunctella*. Although there was a limited effect on the total flight capacity over 24 h, there was a significant reduction in flight bout distance and duration with both pheromone and food cues present. This provides evidence for arrestment as the combined cues signal high-value, resource-rich patches, with a corresponding reduction in distance and duration indicative of increasing search patterns within a resource patch (Charnov 1976, Bell 1990). While the upwind flight *P. truncatus* was mediated by the presence of conspecific aggregation pheromone but not food odors (Fadamiro and Wyatt 1996), the flight bout distance of *E. kuehniella* decreased from 207 m to 40 m in the presence of food odors compared to pheromone alone (Abshire et al. 2024). In this study, we found that the combined effect of pheromone and food resulted in a significant reduction in flight by *P. interpunctella*. This has important ramifications for semiochemical-based, behaviorally-based management programs (Morrison et al. 2021). While mating disruption is typically effective for *P. interpunctella* and other stored product moths (Burks et al. 2011, Athanassiou et al. 2016), the application of effective kairomones in mating disruption programs for *P. interpunctella* may decrease the risk of moths immigrating to or emigrating away from treated areas, which is one of the key reasons for mating disruption failure (Miller and Gut 2015). Thus, our work has important implications for the management of *P. interpunctella*, in addition to laying a foundation for understanding conspecific flight behavior in the presence of important olfactory stimuli.

We predicted that older moths would exhibit reduced flight capacity due to energetic limitations, as adults are non-feeding and rely on larval lipid reserves (Imura and Sinha 1986, Subramanyam and Cutkomp 2017). Our results partially supported this, because even though age did not significantly impact flight metrics overall, it significantly reduced individual flight bout

metrics. By day 5 (e.g., at the end of the lifespan of *P. interpunctella*), shorter and less frequent flight bouts were taken by individuals. The observation that older moths were still attempting to fly suggests that they may have less energy or stamina for sustained bouts, leading to a more variable flight pattern. This makes sense considering the high metabolic cost of sustained flight, as explained by Suarez (2000), who demonstrated that insect flight muscles operate a near-maximum catalytic capacity (Suarez 2000). In addition, Fadamiro (1997) found that older *P. truncatus* flew significantly less than interreproductive and pre-reproductive individuals, which reinforces our findings (Fadamiro 1997).

Males exhibited greater flight capacity than their female counterparts. However, while distances flown by males were greater, they flew at slower velocities, which is typical for many lepidopterans, including *Plutheia operculella* (Gelechiidae) (Foley 1985), and *Eucosma giganteana* (Tortricidae) (Scribner et al. 2026), since males often actively seek pheromone emissions by females. However, females flying at higher velocities may be a functional necessity, since they carry a significant egg load and may need higher wingbeat frequencies to offset this weight to maintain flight, even though their endurance is not as great. This contrasts with *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae), where sex did not significantly affect flight capacity (Cui et al. 2016). In addition, there was no significant difference between sexes in flight distance by the moth *C. pomonella* (Schumacher et al. 1997). Thus, sex-specific differences in flight capacity may also be specific to species. In *P. interpunctella*, sex-specific differences in flight capacity appear to reflect functional and sexual-selection-based differences.

However, the food volatile emissions from flour were below the limit of detection threshold of our GC-MS. Nonetheless, given the obvious effects of the combined effect of food

and pheromone in this study and given how highly sensitive insect chemosensory organs are compared to our detection means, it suggests that the moths detect the food volatiles. Our standardized presentation ensured consistent exposure, and we also observed consistent behavioral effects of combined stimuli. Despite these constraints, our fully factorial design allowed clear detection of state-dependent and stimulus-dependent effects, providing insight into dispersal and arrestment behavior.

These findings enhance our foundational understanding of *P. interpunctella* dispersal ecology, demonstrating that the intrinsic state of individuals and the olfactory environment jointly shape flight behavior. Such knowledge can inform integrated pest management (IPM) by identifying conditions under which moths are most likely to disperse and encounter control measures. For instance, the high velocity flight of unmated moths shows us the critical physiological target for landscape-level pheromone disruption to intercept colonizers into facilities. Furthermore, the reduction in flight distance when moths encountered combined pheromone and food cues also shortened their bout distance, suggesting that arrestment occurred, which is ideal for attract-kill or push-pull tactics. Although push-pull tactics have not been studied for *P. interpunctella*, previous attract-kill (Nansen and Phillips 2004, Campos and Phillips 2010, 2013, 2014) studies have not used multi-cues. Incorporating multiple cues into attract-and-kill systems could induce arrestment in moths to increase the exposure time interacting with the toxicant. Therefore, the combined stimuli would be best deployed in environments where active dispersal dominates. Targeting passive dispersal is also important in a robust integrated pest management program. Our study was limited to quantifying active flight, but future studies accounting for both passive and active flight, management could move toward a more dynamic and multi-cue model for pest suppression. Additional, future work should

explore natural variation in pheromone and food emissions, abiotic modulation of flight responses, and interactions with landscape-level factors to better predict population risk assessment, spread, and mitigation opportunities. Ultimately, this study highlights the flight behavior of *P. interpunctella* is an internal physiological state dependent response to sensory landscapes. The capacity for this pest to utilize active flight dispersal provides necessary behavioral data to optimize lure-based monitoring and supports exploring alternative arrestment-based suppression strategies beyond mating disruption.

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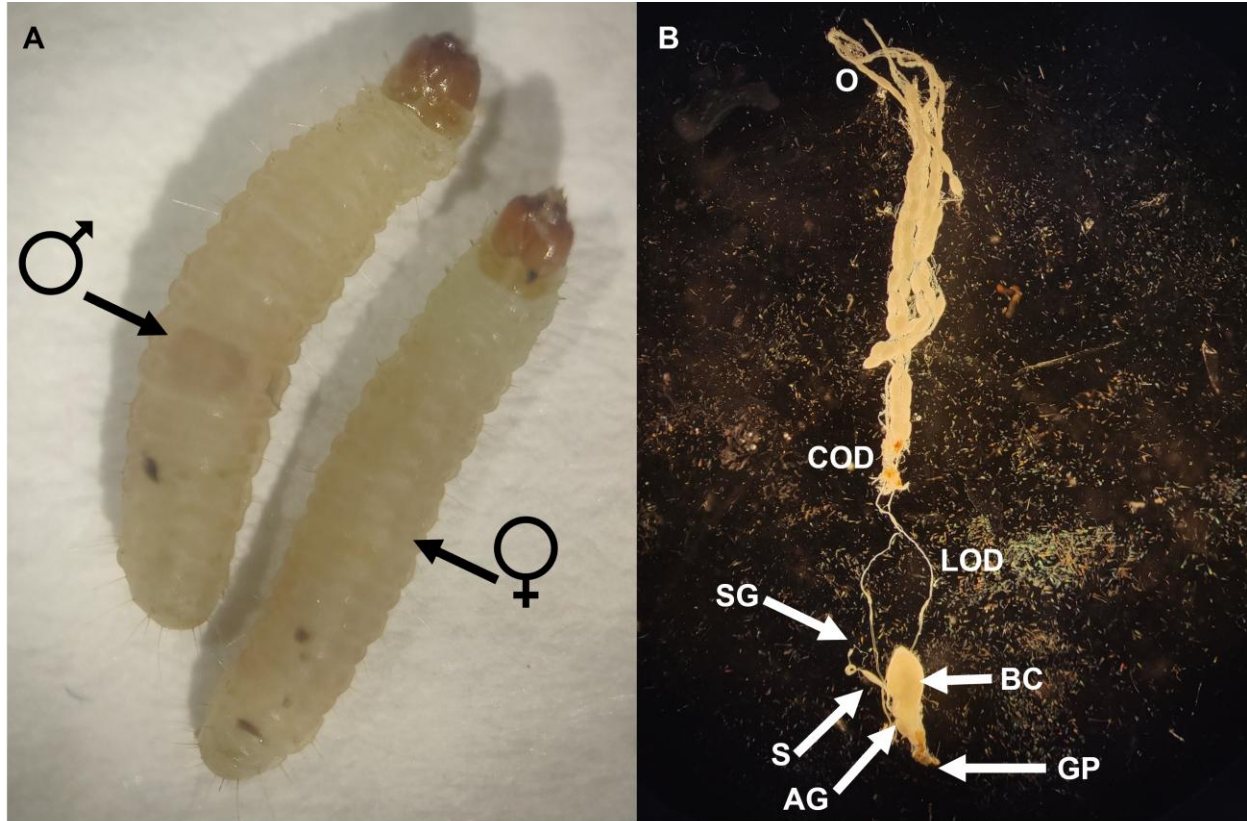


Figure 2.1. Morphological sexing and internal reproductive anatomy of female *P. interpunctella*. A) Sexual dimorphism in fourth instar larvae (L4); the male (♂) is identified by the visibility of the internal testes through the integument, whereas these are absent in the female (♀). B) Dissected female reproductive system: ovaries (O), common oviduct (COD), lateral oviducts (LOD), spermathecal gland (SG), spermatheca (S), which is enlarged as can be seen by widening since it has a spermatheca package inside, accessory gland (AG), bursa copulatrix (BC), and gonopore (GP). Microscopy for imagery was conducted at 20x and 400x magnification (Nikon SMZ18 stereoscope, Tokyo, Japan).

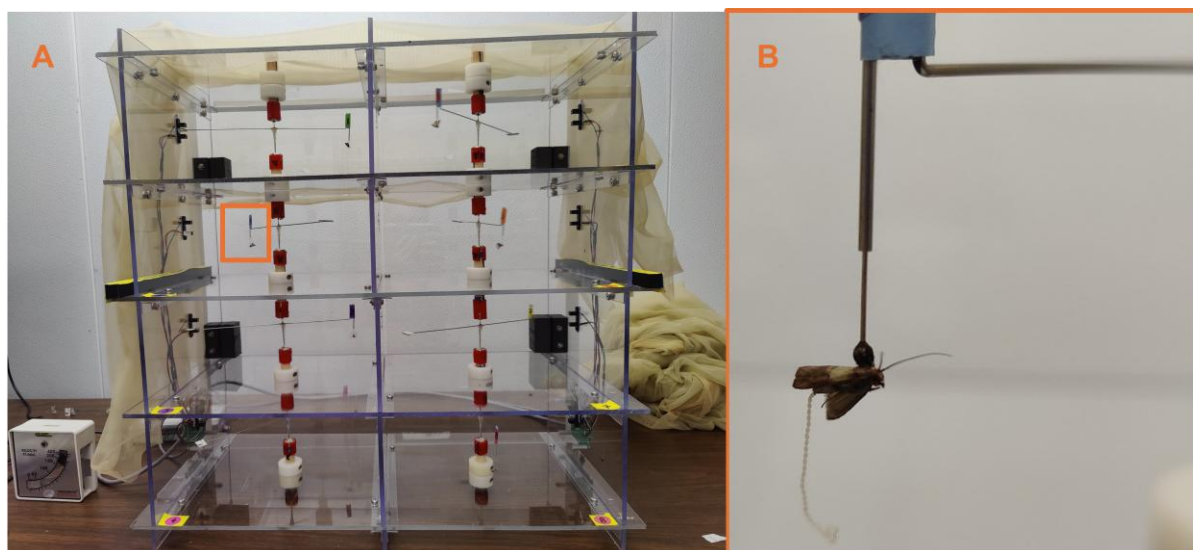


Figure 2.2. The tethered flight mill system used to measure *P. interpunctella* flight capacity. A) The eight-channel array housed in a plexiglass rack, showing the vertical and horizontal distribution of the individual flight mills. B) Close-up of a mated female laying eggs while tethered to the hypodermic steel flight arm by the No. 2 insect pin and adhesive.

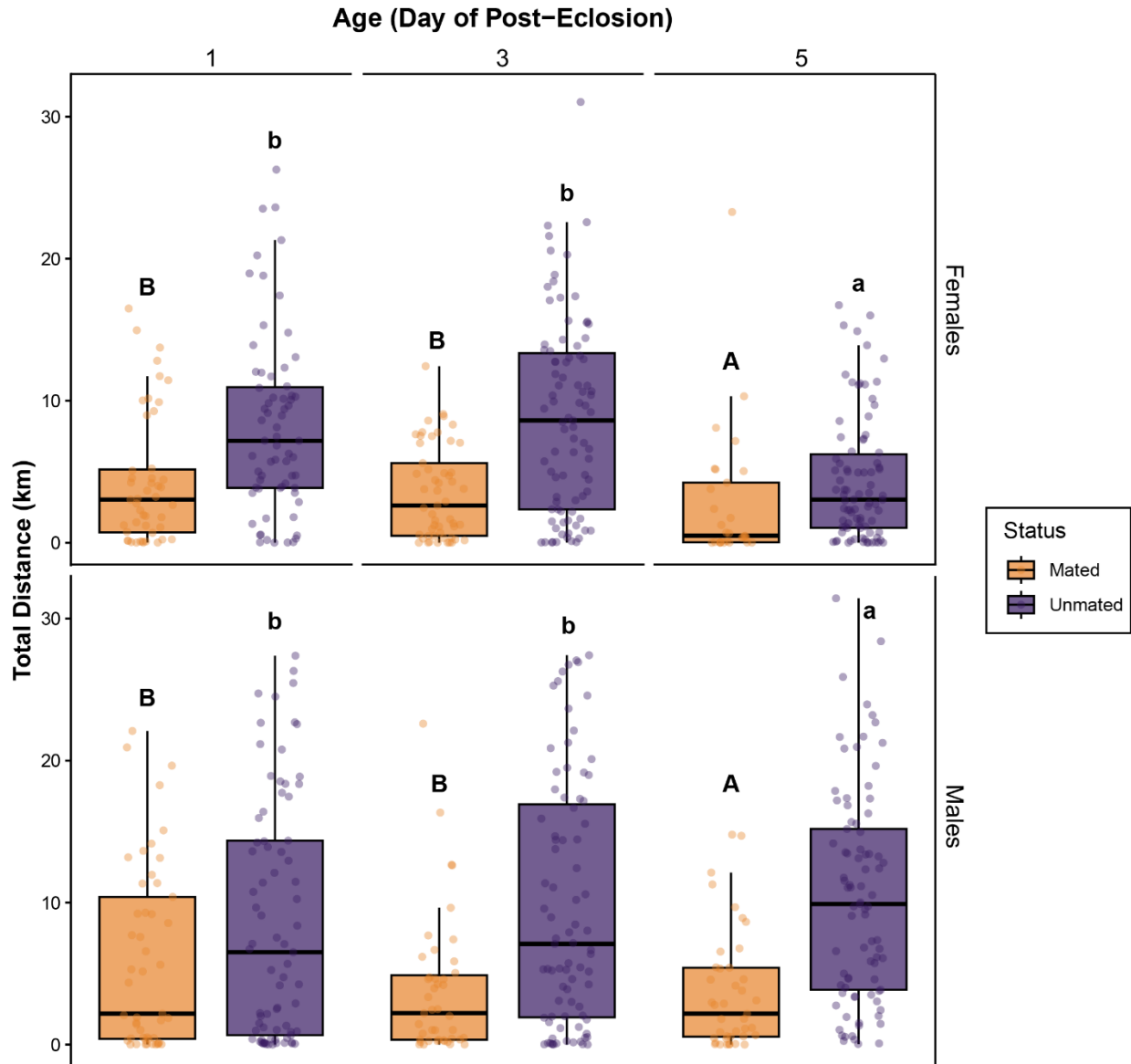


Figure 2.3. Interaction of sex and mating status on total distance flown (km) over 24 h for three adult age cohorts (1, 3, or 5 post-eclosion). Boxplots represent the median and interquartile range (IQR), with whiskers extending to 1.5 x IQR. Individual data points are jittered to show the distribution. Letters represent significant differences between age cohorts within each sex and mating status combination (capital letters for mated; lowercase letters for unmated) (emmeans, Sidak-adjusted, $p < 0.05$); shared letters within unique sex and mating status combinations indicate no significant difference. While the main effect of age was not significant in the global model ($p = 0.07$), the interaction results highlight a consistent decline in dispersal capacity by day 5 across all age cohorts.

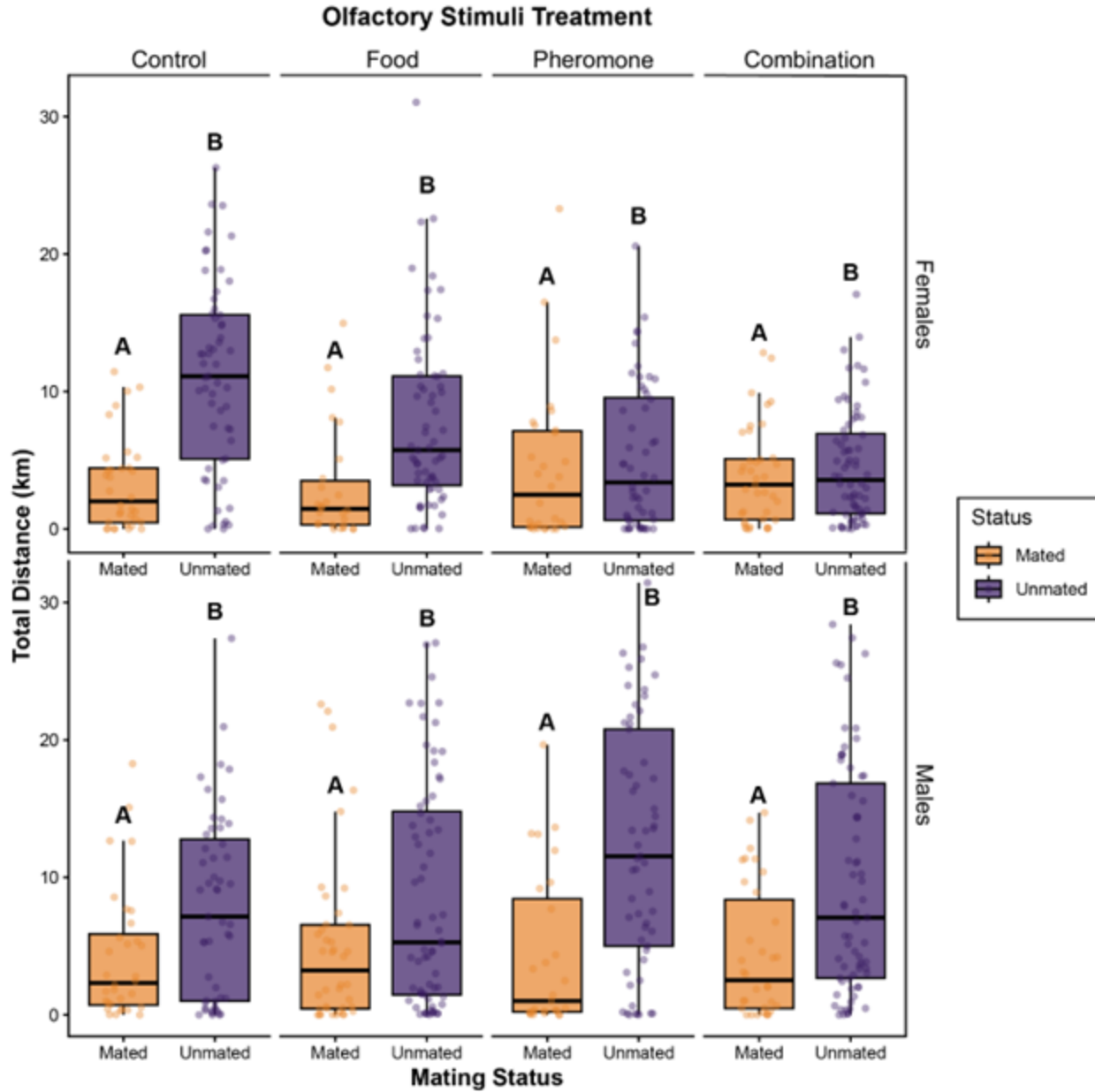


Figure 2.4. Interaction of sex and mating status on total distance flown (km) when exposed to olfactory stimuli (food, pheromone, both, or control). Boxplots represent the median and interquartile range (IQR), with whiskers extending to 1.5 x IQR. Individual data points are jittered to show the distribution. Unmated moths (purple) exhibited significantly higher dispersal capacity than mated individuals (orange) across both sexes ($p < 0.001$), regardless of treatment. Boxplots within each olfactory stimulus with shared letters are not significantly different from each other (emmeans, Sidak, $\alpha = 0.05$).

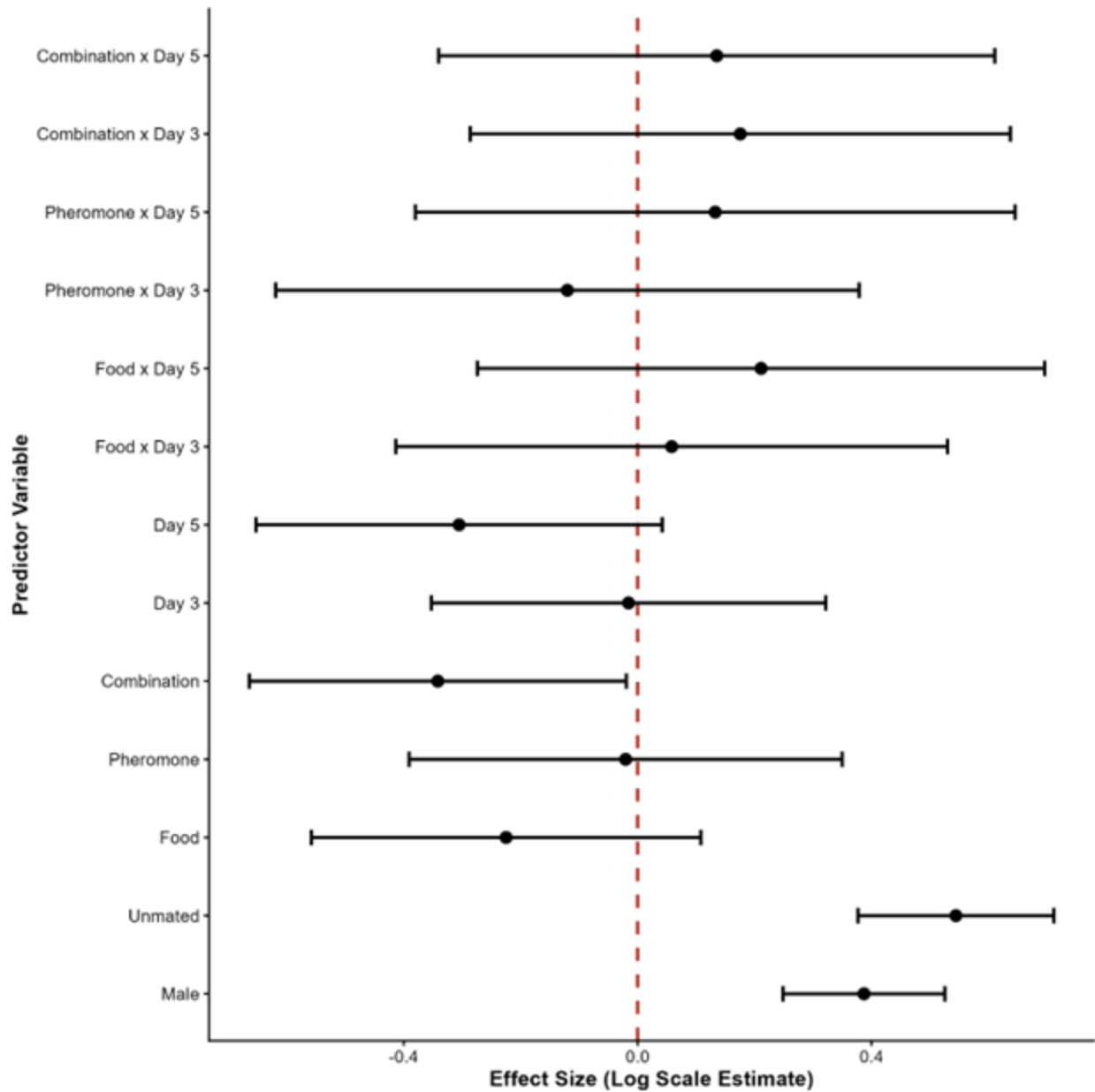


Figure 2.5. Interaction of sex and mating status on total distance flown (km) when exposed to olfactory stimuli (food, pheromone, both, or control). Boxplots represent the median and interquartile range (IQR), with whiskers extending to 1.5 x IQR.

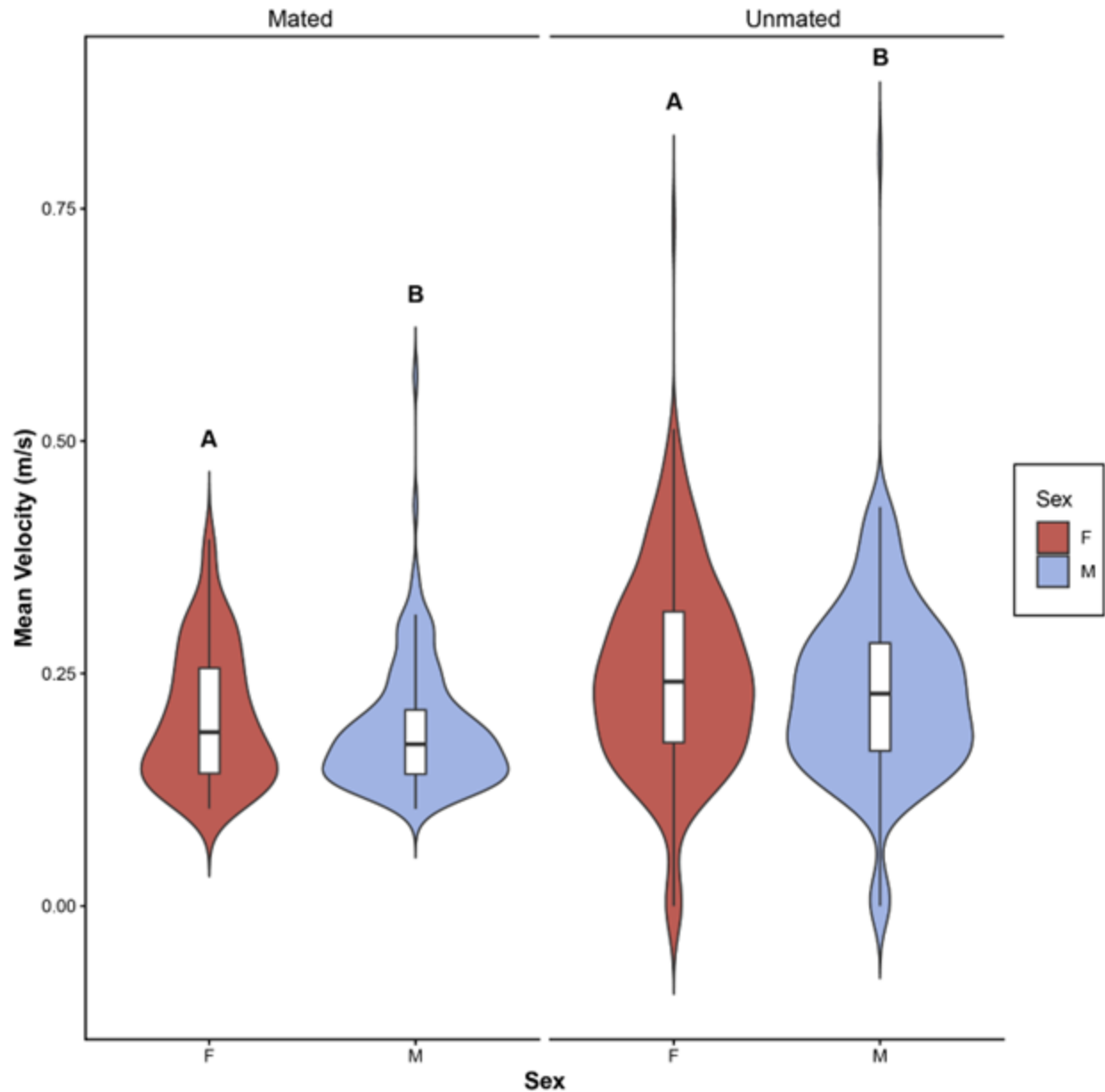


Figure 2.6. Distribution of flight velocity (m/s) between male (blue) and female (dark red) moths, depending on whether they are mated (left column) or unmated (right column). Violin plots illustrate the probability density of the data, with internal boxplots indicating medians and quartiles. Females exhibited more variability in velocities despite lower total dispersal distances, suggesting a sex-specific trade-off between flight speed and search duration. Plots with shared letters are not significantly different from each other (emmeans, Tukey, $\alpha = 0.05$).

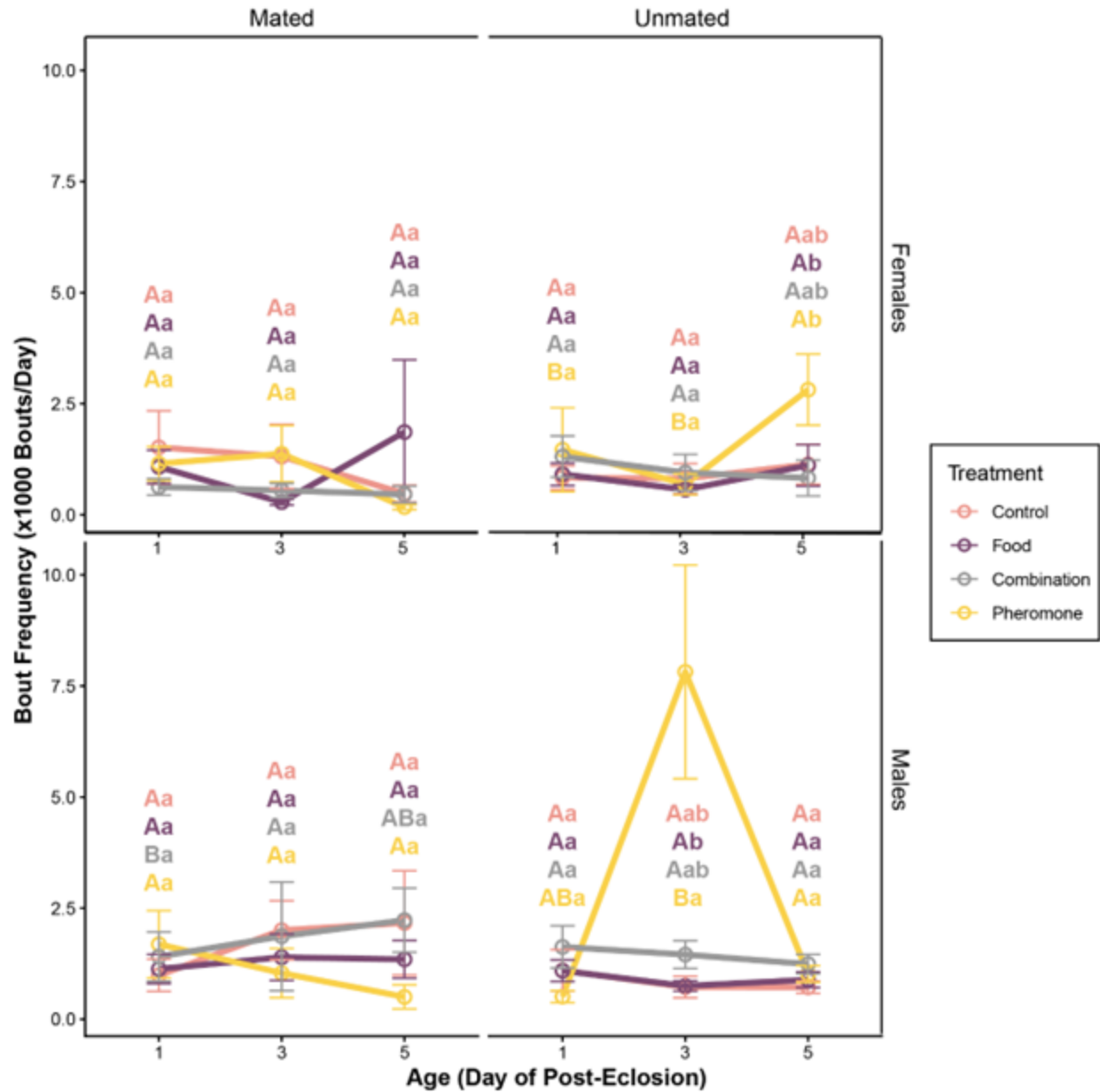


Figure 2.7. The effect of olfactory treatment on the number (frequency) of continuous flight bouts in a 24 h period across both sexes and mating status for adults on day 1, 3, or 5 post-eclosion. Error bars represent 95% confidence intervals. Lower case letters represent treatment effects within day and are in the color of the treatment in the legend, while capitalized letters represent comparisons among treatments across days. Plots with shared letters are not significantly different from each other (emmeans, Sidak, $\alpha = 0.05$).

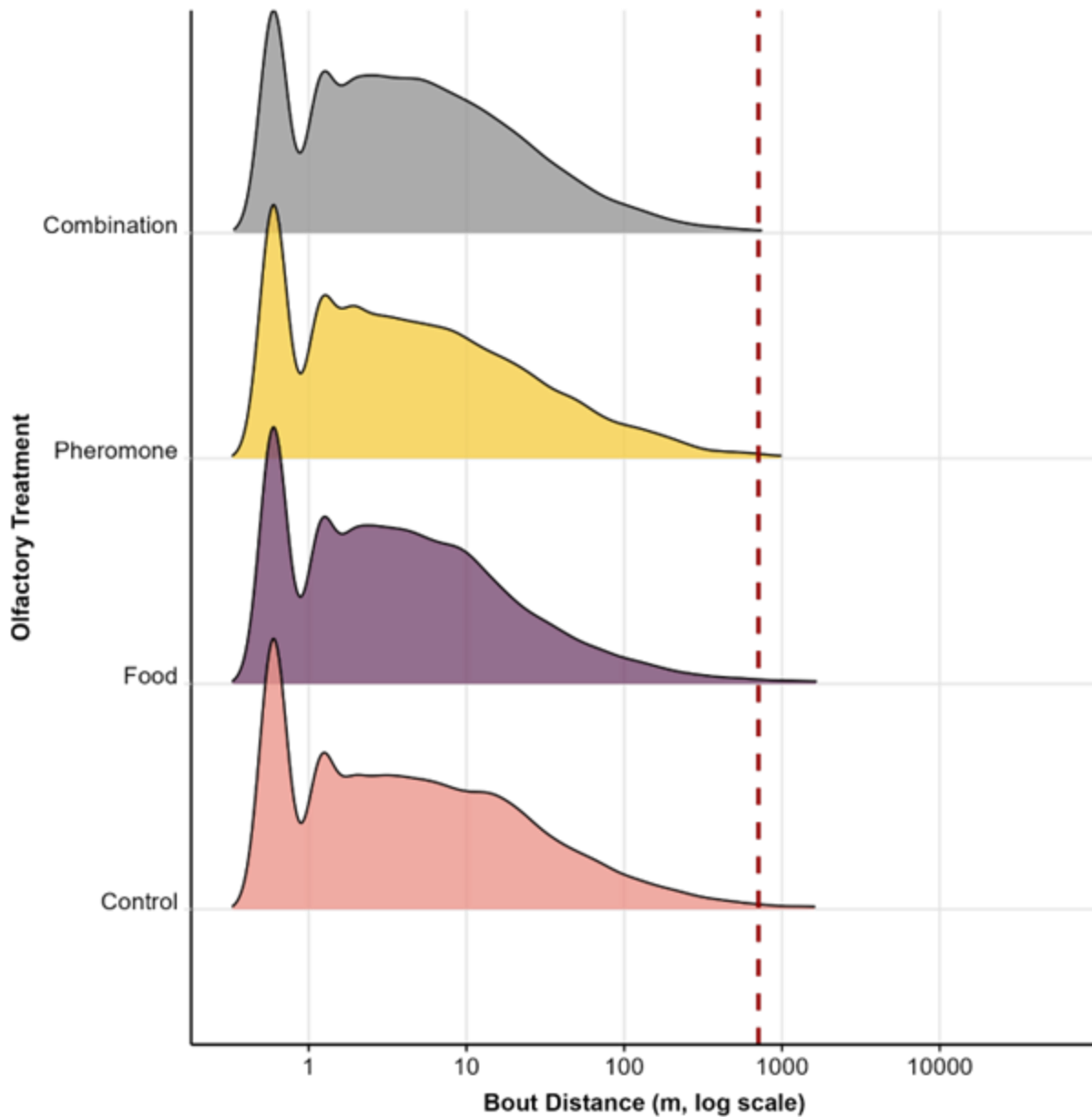


Figure 2.8. Dispersal kernel (individual bout distances in m) for *P. interpunctella*, depending on presence of olfactory stimuli. The x-axis is presented on a log scale to account for the continuous-positive skew of flight data. Exposure to combined cues (grey) resulted in a shift in the distribution toward shorter, more localized flight bouts. This restructuring of flight dynamics represents a transition from directed dispersal to intensive, patch-focused searching behavior. Dashed red line provided at the termination of the dispersal kernel for *P. interpunctella* when both pheromones so termination of other dispersal kernels can easily be compared.

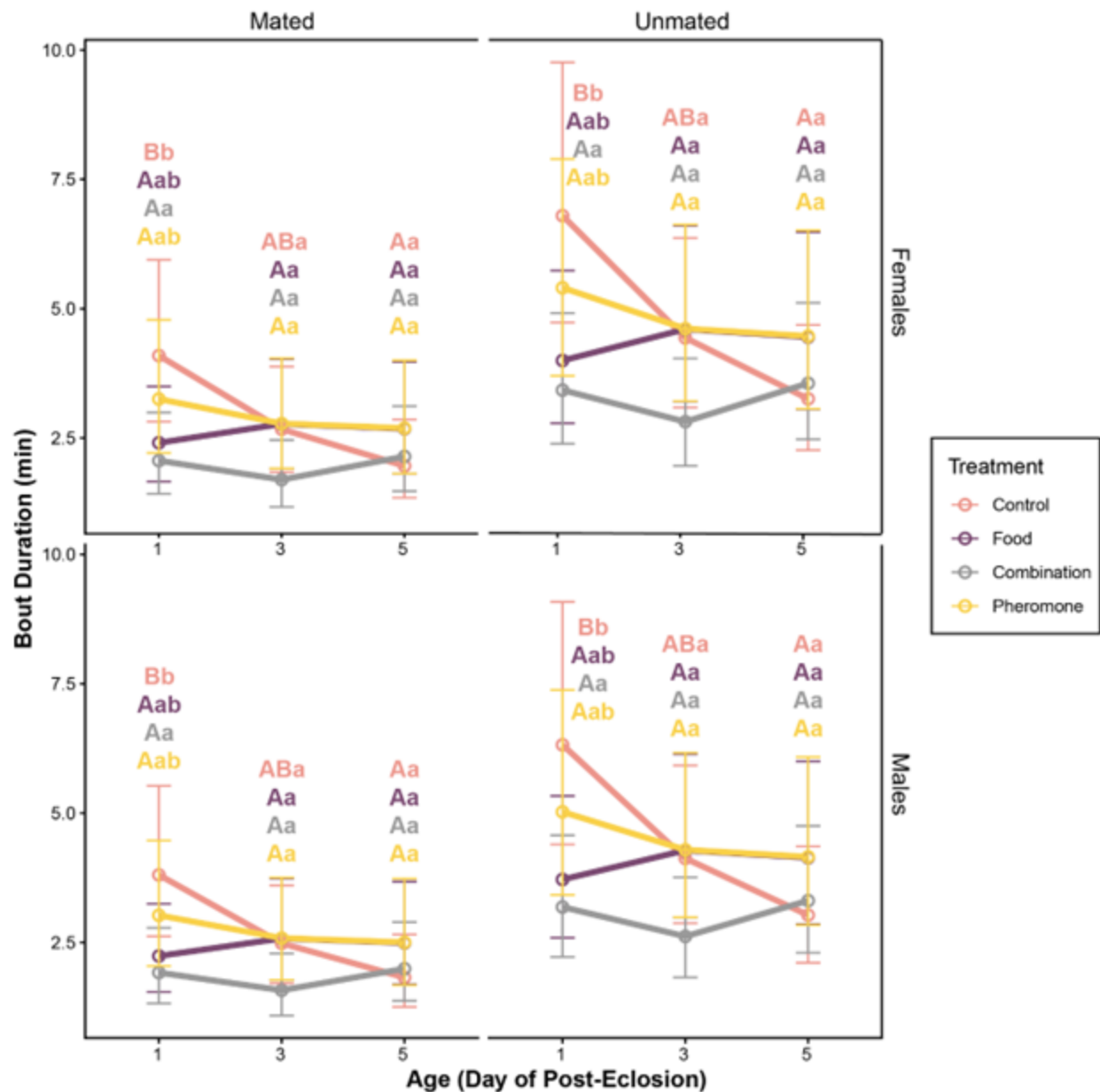


Figure 2.9. Interaction between age (days post-eclosion) and olfactory treatment on the mean duration of individual flight bouts (min). Lines represent the back-transformed marginal means from a Tweedie GLMM; error bars represent 95% confidence intervals. Lower case letters represent treatment effects within day and are in color of the treatment in the legend, while capitalized letters represent comparisons among treatments across days. Plots with shared letters are not significantly different from each other (emmeans, Sidak, $\alpha = 0.05$).

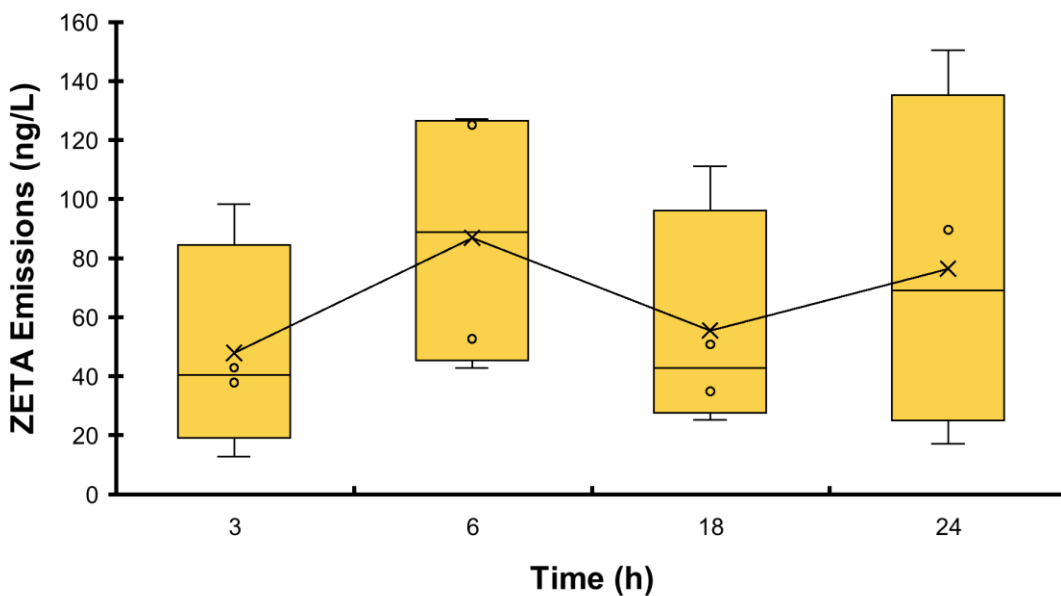


Figure 2.10. Emission rates of ZETA (ng/L) over a 24 h period. Boxplots represent the median and interquartile range of observed values. The line connects the average emission rates (indicated by 'x') at each time interval to show the release pattern observed.

Table 2.1. Summary of generalized linear mixed models (GLMM) evaluating the effects of treatment, day, mating status, and sex on total flight capacity metrics (distance, duration, and velocity) in *P. interpunctella*. The distance and time models used a Tweedie distribution with a log-link function, while the velocity model used a gamma distribution with log-link function. Estimates (β) and standard errors (SE) are reported on the log scale. Bolded rows represent significant factors ($\alpha = 0.05$).

Response Variable	Fixed Factor	df	β	SE	z	p-value
Distance (km)	Status (Unmated)	1	0.584	0.091	6.417	< .001
	Sex (Male)	1	0.339	0.082	4.134	< .001
	Day (5)	1	-0.409	0.213	-1.916	0.054
	Treatment (Food)	3	-0.185	0.211	-0.876	0.381
	Treatment (Pheromone)	3	-0.043	0.231	-0.186	0.852
	Treatment (Combined)	3	-0.407	0.181	-0.248	0.025
	Status (Unmated)	1	0.554	0.085	6.37	< .001
Time (h)	Sex (Male)	1	0.387	0.071	5.479	< .001
	Day (5)	1	-0.305	0.177	-1.723	0.085
	Treatment (Food)	3	-0.225	0.170	-1.324	0.186
	Treatment (Pheromone)	3	-0.020	0.189	-0.0108	0.914
	Treatment (Combined)	3	-0.342	0.165	-2.078	0.038
Velocity (m/s)	Sex (Male)	1	-0.071	0.032	-2.197	0.028
	Status (Unmated)	1	0.211	0.036	5.899	< .001
	Day	1	-0.083	0.078	-1.062	0.288

Table 2.2. Summary of GLMM results evaluating the effects of treatment, day, mating status, and sex on flight bout characteristics (frequency, duration, and distance) for *P. interpunctella*. Bout frequency was modeled using a negative binomial distribution; bout duration and distance were modeled using a Tweedie distribution with a log-link function. Estimates (β) and standard errors (SE) are reported on the log scale. Bolded rows represent significant factors ($\alpha = 0.05$).

Response Variable	Fixed Factor	df	β	SE	z	p-value
Bout Frequency	Sex (Male)	1	0.375	0.096	3.89	< .001
	Status (Unmated)	1	-0.019	0.103	-0.19	0.85
	Day 3	1	0.080	0.23	0.35	0.728
	Day 5	1	0.006	0.234	0.02	0.981
	Treatment (Pheromone) x Day 3	1	0.790	0.345	2.29	0.022
Bout Duration (min)	Status (Unmated)	1	0.503	0.103	4.867	< .001
	Treatment (Combined)	3	-0.686	0.243	-2.824	0.005
	Day 5	1	-0.734	0.244	-3.008	0.003
	Treatment (Food)	3	-0.531	0.243	-2.185	0.029
	Sex (Male)	1	-0.073	0.101	-0.729	0.466
	Treatment (Food) $\times$ Day 5	1	0.851	0.348	2.443	0.015
	Treatment (Combined) $\times$ Day 5	1	0.771	0.344	2.238	0.025
Bout Distance (m)	Status (Unmated)	1	0.584	0.155	3.78	<.001
	Day 3	1	-0.507	0.366	-1.39	0.166
	Day 5	1	-0.851	0.367	-2.32	0.020
	Treatment (Combined)	3	-0.765	0.366	-2.09	0.036

Table 2.3. One-way analysis of variance (ANOVA) for the effect exposure time on ZETA emissions (ng/L) ($n=16$).

Source of Variation	Df	Sum of Squares (SS)	Mean Square (MS)	<i>F</i> -value	<i>p</i> -value
Exposure Time (h)	3	3,924	1,308	0.641	0.603
Residuals (Error)	12	24,489	2,041		
Total	15	28,413			

Chapter 3 - How complex and predictable is *P. interpunctella* courtship?

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Reinforced learning approach to the Markov chain-based probabilistic model of close-range mating behavior of *Plodia interpunctella* (Lepidoptera: Pyralidae) for understanding and predicting mating success.

Introduction

Courtship is a pre-copulatory behavior evident across the animal kingdom that evolves under strong sexual selection because mating requires effective communication between the senders and the receivers of signals within a taxon (Andersson and Iwasa 1996). In insects, these signal interactions unfold across both long- and short-range distances, and both spatial scales impose distinct constraints on signal reliability. Long-range communication for attraction typically depends on a sender producing volatile pheromones or acoustic cues that must be distinguished by a receiver through turbulent and noisy environments, whereas short-range communication is often rapid, multimodal, and used to assess mate quality (Cardé and Willis 2008). Modalities for close-range communications can be visual, tactile, or chemical in nature, which is critical for species recognition, mate assessment, and coordination of mating decisions (Mayr 1974, Candolin 2003). Since courtship is a sequence of behaviors composed of several behavioral states where each state provides only partial information about mate quality and suitability, the order, transitions, and timing of courtship behavioral states ultimately shape information flow and determine reproductive fitness (Griffith and Ejima 2009).

Overall, courtship is historically seen as a linear, sequentially progressive communication behavioral system, meaning that any single behavior is contingent on its placement within the broader behavioral sequence. A given behavioral action cannot be fully interpreted outside of the context provided by the prior behavioral state or receiver response. Therefore, delineating behaviors associated with courtship has often been described using traditional ethological approaches, which describe the interaction and identify factors that influence the behavior or interaction. While informative, these methods struggle to capture stochasticity, branching pathways, and dynamic environmental conditions where selection favors evolutionary flexibility (Rodrigues et al. 2018). As a result, nonlinear and flexible courtship architecture cannot be fully captured by static ethograms; therefore, sequential state-based models provide powerful means to quantify the complexity of branching courtship architecture (Hebets et al. 2016). Markov chain models are particularly well-suited for this task because they encode behavior as a series of discrete states connected by probabilistic transitions, allowing courtship to be analyzed as a dynamic process rather than as a static summary of observed frequencies (Billingsley 1961). Under this framework, the likelihood of subsequent behavior depends primarily on the current state rather than the full behavioral history, reflecting how animals continuously modify decisions based on immediate cues.

Markov-chain approaches have been successfully applied in other biological contexts. For example, state-transition models have been used to characterize premating, mating, and post-mating behavioral pathways in scarab beetles (*Cyclocephala* spp.), demonstrating the utility of probabilistic frameworks for revealing sequential structure in insect courtship (Rodrigues et al., 2018). Beyond entomology, Hidden Markov Models (HMMs) have been widely used to model animal movement (Leos-Barajas et al. 2017, Jannetti et al. 2025) and habitat selection (Invernizzi

et al. 2024), identifying important latent behavioral states and transitions that are not apparent from direct observation alone (McClintock and Michelot 2018). These approaches demonstrate how probabilistic models can reveal behavioral organization that is not entirely obvious in descriptive analyses.

Recent advances at the intersection of probabilistic modeling and machine learning further highlight the broader relevance of these approaches for behavioral ecology. HMMs have been applied to high-resolution video data to decode sub-second behavioral dynamics in rodents, linking observable movements to latent neural and behavioral states (Wiltshko et al. 2015). Similarly, reinforcement learning frameworks combined with stochastic control have been shown to predict complex sequences of actions in dynamic environments (Wang et al. 2020). While these approaches differ in scale and application, they collectively demonstrate the power of probabilistic models to describe, predict, and interpret complex behavioral sequences.

Understanding the architecture of courtship also has real-world relevance. For example, if a behavioral sequence can be disrupted at a key behavioral event, mating success can be reduced, and pest population growth can be lowered. Mating disruption is a management strategy that interferes with chemosensory perception by creating additional, false olfactory signals through the abundant application of synthetic pheromones, exploiting the behavioral vulnerability of the receiver that is in tune with the much lower natural concentrations of pheromone emitted by a sender (Carde 1995). However, the effectiveness of this behavioral management technique heavily relies on impairing or masking the redundant and compensatory behaviors within the courtship sequence (e.g., pheromone calling), and without proper and consistent coverage of the synthetic pheromones, a chance for behavioral recovery or breakthrough in the courtship sequence can happen (Miller et al. 2006, Miller and Gut 2015). Therefore, to improve mating

disruption tactics, it is necessary to identify other vulnerable behaviors and sequence transitions that, when interrupted, minimize the probability of disrupting the life cycle and preventing successful copulation.

The Indian meal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae), is a polyandrous cosmopolitan pest that significantly contributes to losses in the billions of dollars annually after harvest (FAO 2019). *Plodia interpunctella* is easily reared in the laboratory and has a well-characterized pheromone biology by females releasing a long-range attractant [major component (Z, E)-9,12-tetradecadienyl acetate] and males releasing an unknown arrestment compound during courtship (Brady et al. 1971; Kuwahara et al. 1971; McLaughlin 1982; Zhu et al. 1999; Gerken et al. 2022). Moreover, has transcriptomic resources underpinning its chemosensory biology (Jia et al. 2018, Li et al. 2023, Chen et al. 2024), and has a defined suite of courtship behaviors (Grant and Brady 1975, Phelan and Baker 1990). Therefore, *P. interpunctella* has been a target for mating disruption commercially (Ryne et al. 2007, Burks et al. 2011, Trematerra et al. 2011, Lindenmayer et al. 2025) and is an excellent system for studying sequential courtship architecture with potentially significant implications for applied pest management. In this study, we used Markov-chain models to delineate the architecture of courtship sequences that lead to successful mating for *P. interpunctella*. Our research objectives were to: 1) identify and validate the behavioral states and transitions most strongly associated with mating success, 2) determine the sequences or transitions that exhibit flexibility, and 3) evaluate what behaviors or transitions may be the most susceptible to disruption in enhanced mating disruption.

Materials and Methods

Insect colonies and rearing

The *P. interpunctella* Savannah colony used in this experiment has been in culture in the laboratory at the USDA-ARS Center for Grain and Animal Health Research (CGAHR), Manhattan, KS, USA, for over 60 years and has previously been used for behavioral mating studies (Lum and Flaherty 1970). The Agronomy colony of *P. interpunctella* was established as a laboratory colony at CGAHR in 2019 from a warehouse collection in Manhattan, KS. These two colonies were examined to ensure courtship behaviors that could have been lost to extended colony maintenance were captured. To subculture colonies monthly, approximately 100 (1–3 day old) emerged adults were placed into a 473 mL glass jar with a 1 cm diameter opening, and a steel screen mesh to secure the jar for 72 h. The mating jar was shaken to gather eggs. When 0.05 g of eggs were collected, they were placed on 200 g of diet consisting of wheat shorts, cracked wheat, wheat germ, brewer's yeast, glucose, water, and honey in a 473-mL glass jar. Each population was maintained at 27 °C and 65% relative humidity (RH) on a 16:8-hour light to dark (L: D) cycle.

Close-range mating behaviors

Males and females from colonies were sexed as larvae during their 4th instar of development (Figure 2.1A) and isolated in 2-oz (~59.2-mL) plastic cups (Precisely Plastic, Amazon.com) on 2 g of rearing moth diet and allowed to pupate in isolation. Individual pairs of age-matched shared colony unmated males and females from each *P. interpunctella* colony were placed in a 3D printed mating chamber (470 × 370 × 300 mm, L: W:H) that was fabricated with a Lulzbot IC3D (Fargo Additive Manufacturing Equipment 3D, LLC, Fargo, ND, USA) using 2.85 mm acrylonitrile butadiene styrene filament. The mating chamber design contained a

removable glass barrier ($480 \times 3 \times 370$ mm L: W:H) to separate the pairs and disrupt pheromonal communication. A Dino-Lite digital microscope (Dunwell Tech, Inc., Torrance, CA, U.S.A.) was used to record behavior. Pairs were placed in the mating chamber with the barrier in place to allow for acclimation for five minutes. The barrier was removed, and individuals were given five minutes to interact with each other and the environment. Each pair of moths was given one opportunity to interact for mating behavior observation. Courtship behaviors (described below and in Table 3.1) were documented throughout the total recording period until successful mating was observed or at the end of 5 minutes from removing the barrier, whichever came first. Successful mating was defined as viewed copulation success (Table 3.1), which priorly when viewed had shown success in spermatophore transfer (Figure 2.1B). Recordings were conducted in an environmental chamber with the same conditions as the rearing chamber (27 °C and 65% RH) between 0900 and 1200 CST (Figure 3.1). Mating chambers were chemically cleaned between every recording with methanol and hexane and given 10 min to dry before a new pair was introduced. One-hundred and twenty pairs in total were observed, with sixty being from the Savannah colony and sixty from the Agronomy colony.

Three independent observers analyzed the videos to determine the time and order of timing of fourteen different behaviors linked to courtship in pyralid and related moths (Phelan and Baker 1990, Curkovic et al. 2006, Girling and Cardé 2006), which are described in detail in Table 1. These behaviors included: Female Call in Acclimation (FCA), Female Call in Free period (FCF), Female Pursuit (FP), Male Pursuit (MP), No Contact (NC), Male Wing Hitting (MWH), Female Wing Hitting (FWH), Antenna–Wing Interaction (AW), Antenna–Antenna Interaction (AA), Head–Head Contact (HH), Copulation Attempt (CA), Head–Head plus Copulation Attempt (HH_CA), Female Directed Clasp (FDC), and Joining (J). The start time

of each behavior was recorded. Behaviors were not mutually exclusive, meaning two behaviors could be documented simultaneously during the interactions.

Data pre-processing and model building

To prepare the courtship sequences for computational modeling, behaviors were encoded into discrete states. All timestamps were standardized into seconds to ensure a uniform time resolution across trials. For pairings where behaviors did not occur, they were recorded as zeros; downstream analyses then skipped these empty states and proceeded with analysis. In instances where recordings were incomplete (e.g., due to moth escape), all valid behaviors before interruption were retained for analysis. This approach preserved the variability of the behavioral sequences while ensuring the dataset remained in the appropriate structure for Markov chain probabilistic modeling.

Since the Markov chain framework relies on sequential transitions between behavioral states, each dataset was chronologically ordered to reflect the flow of courtship actions, which allowed us to describe probabilistically if/how one action gives rise to the next. From the ordered behavioral sequences, a transition count matrix was created by documenting every observed movement from one state to another. Once aggregated, these counts were standardized by the total number of observations to produce a transition probability matrix (P), representing the likelihood of a moth moving from one behavioral state to another. The matrix design reflects the structured and sequential progression of courtship, where only certain transitions are feasible, as determined by the manual scoring efforts, although most behavioral states were given the latitude to transition freely to any other state. Each behavioral state was defined as a discrete node in the Markov chain, while transitions between states were encoded as probabilities derived from observed sequences. To quantify the dynamics of moth courtship, it was necessary to formalize

how behavioral transitions were counted as a chronological chain of events. By using state-to-state pairs across trials, we captured the frequency with which one behavior directly led to another. This step provided the foundation for constructing the transition matrix, which in turn formed the probabilistic Markov framework, implemented using MATLAB (R2024a).

To quantify the dynamics of moth courtship interactions, we calculated transition counts of behaviors and the probabilities based on the observed sequences of behaviors moving from one state to another. The transition count defines the frequency with which the initial behavior S_i is followed by the behavior S_j that follows across all replicates, calculated as:

Equation 3.1

$$C_{ij} = \sum_{r=1}^R \sum_{t=1}^{T_\gamma-1} 1 \{ X_t^{(\gamma)} = S_i, X_{t+1}^{(\gamma)} = S_j \}$$

Where C_{ij} is the number of observed transitions from state S_i to S_j ; R is the number of trials (replicates); T is the number of behavioral events in trial (γ); $X_t^{(\gamma)}$ is the behavioral state at time t in the sequence γ ; and $1 \{ \dots \}$ is the indicator function, returning 1 if the condition is true, otherwise returning 0.

Transition probabilities (P_{ij}) were derived through row-normalization, representing the likelihood of moving from behavior S_i to behavior S_j , relative to all outgoing transitions from S_i .

Equation 3.2

$$P_{ij} = \frac{C_{ij}}{\sum_{k=1}^n C_{ik}}$$

Here, n represents the total number of behavioral states (e.g., $n=14$), and k is the index used to sum across all possible subsequent states. Where the denominator, $(\sum_k C_{ik})$ function is the total outgoing transitions from state S_i . Finally, we defined the absorbing states, specifically “Success” or “Joining”, to denote the termination of the sequence. For absorbing state, a , the

probability of the remaining in that state is $P_{aa} = 1$, while the probability of transitioning to any other state j is $P_{aj}=0$ for all $j \neq a$.

Equation 3.3

$$P_{aa} = 1, P_{aj} = 0 \text{ for } j \neq a$$

Results

Transition probability matrix and sequential pathways

Thirty-one percent of pairs resulted in successful mating (37/120) within the five minute free period. The raw counts of behavioral states observed across all trials in the dataset are in Table 3.2 and visualized in Figure 3.2. These frequencies provide an essential reference point for understanding the distribution of courtship behaviors prior to analyzing their sequential transitions. Among these, HH, CA, and HH_CA were the most observed behaviors, indicating that most pairs interacted frequently. Behaviors AW and MWH also occurred often, as signals of courtship receptivity or attempts to attract the female.

The total number of transitions observed for each behavioral state (Table 3.2) provides insight into the most dominant pathways within the courtship sequence. The highest-frequency pathways include $CA \rightarrow HH_CA$ and $HH \rightarrow CA$ and highlight the key behavioral junctions driving progression toward successful mating outcomes. In addition, counts of transitions confirmed that certain behaviors acted as keystone events within the sequence (Figure 3.2). Behaviors that occur early in the courtship sequence, such as $FCA \rightarrow FCF$ occurred 13 times, $FCF \rightarrow FP$ two times, and $FP \rightarrow MP$ two times, while $MP \rightarrow NC$ was not directly observed. Although lower in frequency, these early transitions reflect the structured initiation and pursuit stages that were observed across trials. Later transitions, such as $FWH \rightarrow AW$, occurring four times, or $HH \rightarrow FDC$, which was rare but predictive and was more indicative of progression toward Joinings.

The empirical transition probability matrix (Table 3.3) summarizes the probabilities of transitions among any courtship behavioral state observed during courtship interactions. Each matrix entry represents the likelihood that one behavioral state (row) is directly followed by another (column), providing a quantitative description of sequential structure. High transition probabilities were observed from NC to female calling (FCA/ FCF) behaviors, with NC transitioning to FCA ($p = 0.60$) and FCF ($p = 0.40$). These transitions indicate frequent re-entry into the signaling phase following disengagement. Early stages of courtship also showed consistent progression from FCA to FCF ($p = 0.22$) and from FCF to MWH ($p = 0.47$). Transitions involving pursuit behaviors, such as FP to MP ($p = 0.13$), were also observed, reflecting escalation from signaling to active movement-based interaction.

Later stages of the sequence exhibited transitions characteristic of convergence toward mating. HH showed high probabilities of transitioning to FDC ($p = 0.45$) and Joinings (J; $p = 0.45$). Joinings functioned as an absorbing state within the model, receiving high incoming probabilities from FDC ($p = 0.86$) and MWH ($p = 0.36$). These patterns indicate that while early-stage behaviors are frequently revisited, successful mating is disproportionately associated with a small number of late-stage transitions.

A heatmap visualization of the transition matrix (Figure 3.3) shows the dominant behavioral pathways. Strong probability intensities were observed in transitions such as FCA → FCF, FCF → FP, FP → MP, and MP → NC. These sequences align with the natural progression toward mating, wherein female signaling initiates the interaction, followed by male pursuit behaviors. The heatmap also revealed bottlenecks, where progression either stalled (e.g., prolonged NC) or advanced toward interaction states such as MWH and FWH. These findings suggest that reciprocity between sexes is critical for maintaining the momentum of courtship.

The total outgoing and incoming behavioral transitions (Figure 3.4) revealed asymmetries in the directional flow of the courtship sequence. States such as MP and FCF exhibited the highest number of outgoing transitions, indicating their role in driving movement through the behavioral sequence. In contrast, HH and Joinings showed the highest number of incoming transitions, reflecting convergence of multiple pathways onto these states. This distinction between states with high outgoing versus high incoming transitions highlights functional differentiation within the courtship sequence, with early-stage behaviors facilitating exploration of multiple pathways and late-stage behaviors acting as convergence points.

Ethogram

The behavioral pathway graph shown in Figure 3.5 provides a visual representation of the transitional architecture of moth courtship based on the Markov chain analysis. Each node corresponds to a discrete behavioral state, while edges represent the probability-weighted transitions between states. The network structure highlights recurrent loops in early behaviors such as FCA or FCF and MP, while downstream convergence occurs at HH, FDC, and ultimately Joinings.

Discussion

Courtship in insects has commonly been described as a stereotyped and sequential progression of behaviors culminating in copulation. However, our results challenge this deterministic view by demonstrating that courtship in *P. interpunctella* operates as a probabilistic communication system shaped by repeated transitions, reversals, and behavioral bottlenecks. Specifically, our study shows that moth courtship behavior can be effectively modeled as a probabilistic state-transition system using a Markov chain framework. By encoding observed behavioral events into discrete states and analyzing their sequential flow, we reveal the dominant

pathways, critical transitions, and absorbing states that characterize successful mating interactions by and identify likely target states to enhance mating disruption of *P. interpunctella*. The sequential architecture revealed by the model aligns with predictions within sexual selection theory, including signal redundancy through the multiple messaging theory and convergence (Pomiankowski and Moller 1995, Candolin 2003), so that early signal detection and species recognition have multiple cues and behavioral pathways to funnel toward a decisive terminal action such as copulation. While previous work on *P. interpunctella* courtship primarily focused on identifying or using these sequences for taxonomic comparisons of courtship behavior (Grant and Brady 1975, Phelan and Baker 1990), our study provides a functional analysis to identify specific behaviors and transitions vulnerable to pest management.

In particular, the behavioral states and transitions that were most strongly associated with mating success were early-stage behaviors such as female calling (FCA, FCF) and male pursuit (MP), which serve as robust initiators into the courtship sequences and signal receptiveness for engaging with potential courtship partners. These behaviors exhibited high outgoing transition probabilities and frequent repetition, indicating that initiation and engagement are comparatively unconstrained stages of the interaction. In contrast, late-stage behaviors such as HH and FDC act as strong convergence points with high incoming probabilities and close association, with successful Joinings leading to copulation. Notably, HH emerged as a bottleneck within the sequence, meaning that once HH occurred in the sequence, the probability of progressing to successful copulation through the states of FDC or Joining sharply increased. Failure to reach HH resulted in a repeated cycle of prior behavioral states or termination of the interaction into NC with a reset in FCF. This defining behavior demonstrates that mating success is appropriately dependent on passing through a late-stage gateway rather than accumulating several early-stage

behaviors. This late-stage constraint therefore represents a biologically meaningful decision point within the courtship sequence, following the sequential assessment model (Enquist and Leimar, 1983).

Moreover, the sequential assessment model indicates that animals may use a series of increasingly stringent filters to reduce suboptimal mating, which has already been demonstrated in *P. interpunctella* (Grant and Brady 1975, Phelan and Baker 1990) and is common in other complex insect courtship sequences. For example, repeated early-stage behaviors of the acoustic, visual, or chemical variety are followed by late-stage bottlenecks, which are often tactile. *Drosophila melanogaster* (Diptera: Drosophilidae) uses repeated acoustic wing songs, extensions and circling followed by a late-stage genital licking leading to a copulation attempt (Spieth 1974, Markow and Hanson 1981, Ning et al. 2022), and parasitic jewel wasps (*Nasonia* sp. [Hymenoptera: Pteromalidae]) assess repeated head bobbing followed by antennal touching that leads to female receptivity (van den Assem, 1986). Additionally, the navel orangeworm (*Amyelois transitella* [Walker]; Lepidoptera: Pyralidae) has repeated early behaviors of walking and wing hitting until the male reaches the female and has contact by wing hitting or head-to-wing contact (Girling and Cardé 2006). In all these cases, the late-stage bottleneck serves as a safeguard to ensure that copulation can only occur after mutual verification of a mating partner.

Furthermore, our results indicate that mate choice and rejection are expressed through probabilistic control over sequence progression rather than explicit antagonistic behavioral states. The first indicator of this control is observed through the frequent repeat transitions from no contact (NC) to female calling (FCA or FCF), which served as a feedback loop within the courtship structure. This feedback loop demonstrates sustained effort from females to reinitiate communication when interactions fail or if signals are lost and the courtship does not escalate.

Given that females are controlling the signal (calling), this pattern of behavior could be a cycle of female-mediated termination (female-choice) rather than a random breakdown of an interaction. In *Cyclocephala barrerai* (Coleoptera: Melolonthidae), there is a premating phase in which the female will walk and kick at the male to reject the advancement from the male, but return to walking in a searching state. In turn, the male then restarts the sequences of walking for pursuit of the female, re-engaging in antennation on her body, and then attempting a body hold to rub the antenna. The body hold in this species is a test of grasping efficiency which helps the female determine male partner fitness (Sanchez-Cruz et al. 2025). Likewise, *P. interpunctella* female calling was repeated without a guaranteed advancement of pursuit or interaction, which could establish that calling not only functions as an attractant but also as a filtering mechanism for further assessing males. Male wing-hits often seen in male-pursuit may result in arrestant cues (McLaughlin 1982) in addition to ultrasonic vibrational cues up to 80 kHz (Trematerra and Pavan 1995), which females may be using to assess male attractiveness for heritable traits, and stopping the pursuit for repeated assessments could be a way to determine the quality of the male. Ultimately, HH requires close-range tolerance and mutual positioning, conditions that are biologically consistent with female approval. In our data, failure to reach HH despite persistent male pursuit seems to reflect rejection through a non-progressive gate rather than explicit refusal from a female. Importantly, some interactions also terminate after tactile contact between individuals, indicating that rejection can occur even late in the sequence after assessment has taken place. Mate choice seems to be directed by females through a multi-stage process rather than a single acceptance decision.

This sequential architecture has important consequences for the implementation of mating disruption. The current mating disruption tactics target early signaling behavior (calling,

pursuit), which is shown to be highly redundant in our study, and thus these behaviors are resilient to interference unless partners reach signal fatigue or fail to overcompensate to find the true signal. Though it has yet to be explored in *P. interpunctella*, autodetection of synthetic pheromones by females can alter behavior through shifting the timing of their calling behavior to not compete with synthetics or disperse to new areas, as seen in *Grapholita molesta* (Busck) and *Chroistoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Stelinski et al. 2014). Additionally, other species that autodetect their own pheromone can overcompensate in the field by increasing their calling (Kuhns et al. 2012). In these cases, targeting early courtship behaviors for mating disruption would not be effective if individuals are in close proximity and can overcome or compensate for the excess pheromone. However, the identification of HH and FDC as late-stage bottlenecks suggests that mating disruption strategies targeting close-range interactions could be effective in enhancing current mating disruption tactics. In the laboratory, the use of ultrasonic noise (99–119 dB at 0.43 m) reduced mating frequency by 58% of *P. interpunctella* (Huang and Subramanyam 2004), but effects combined with mating disruption lures have yet to be seen. Unfortunately, the application and development of tools to disrupt these behaviors are reliant on a limited understanding of visual and tactile courtship cues within this species. For example, Nordlund and Brady (1974) demonstrated that adjustments in photoperiod increased female calling and therefore increased the frequency of female-directed mate finding (Nordlund and Brady 1974). A combined acoustic or visual interference with long-range pheromone mating disruption could be a multi-faceted tactic to target both early and late-stage courtship behaviors and reduce mating significantly.

Future work could extend the courtship framework to more complex ecological contexts, incorporating various physiological or sensory measures, examining individual or population-

level variation, and exploring repeated interactions or choice scenarios. Such extensions would further refine our understanding of how courtship architecture evolves and operates under natural selection. In addition, continual examination of courtship behaviors from newly caught populations can help generalize these findings in the field. The use of semi-supervised learning with artificial intelligence (AI) and machine learning could also expand our understanding of the static behavioral states based on human observations and previous literature. The implementation of AI and machine learning with semi-supervised learning has proven successful in elucidating behaviors in bumblebee flight (Williams et al. 2021), Angoumois grain moth (*Sitotroga cerealella* [Lepidoptera: Gelechiidae]) attraction (Guru et al. 2024), and unsupervised learning methods have been applied in fruit fly courtship (Ning et al. 2022). Observations of these behaviors extended to different times of day, stages of life, temperature, or season could also expand our understanding of how to best target these courtship behaviors for pest management. However, this study highlights the major behavioral changes that occurred in many of the interactions that lead to successful copulation, providing a comprehensive look at how insect pests use courtship behaviors to secure mates.

Our findings reveal that *P. interpunctella* courtship is governed by probabilistic rules that balance persistent signaling with decisive transitions. Female mate choice and rejection are expressed sequentially through control over progression rather than discrete refusal behaviors. By integrating behavioral observation with Markov chain modeling, this study moves beyond description to reveal the underlying logic of mating interactions, offering a powerful framework for understanding the evolution, function, and vulnerability of complex communication systems for pest management exploitation.

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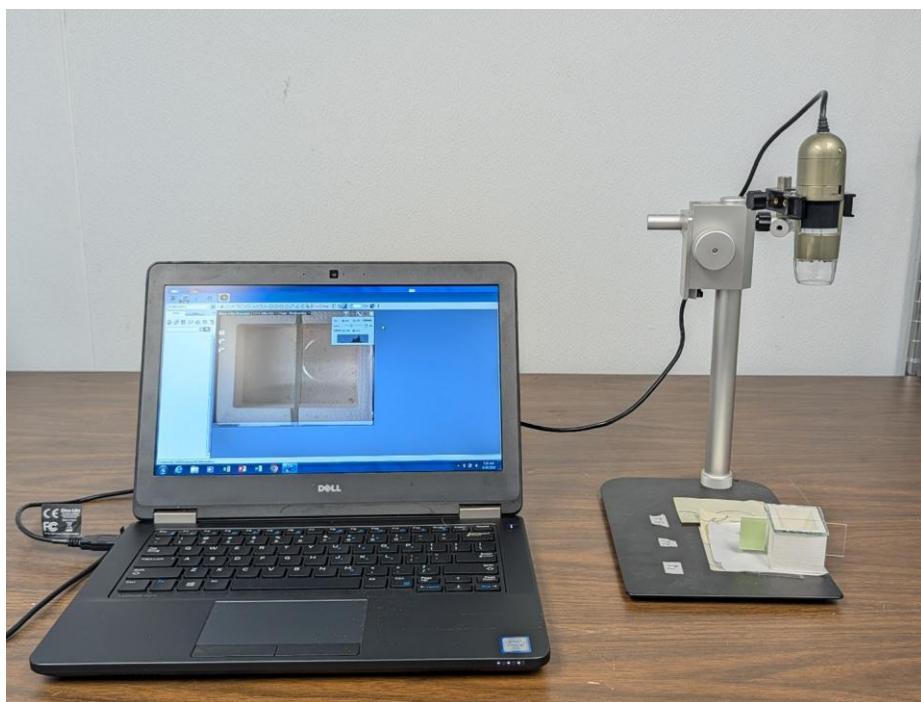


Figure 3.1. Experimental courtship box set-up of the bioassay arena from a top-down view with female on the left and male on the right (top image) and the recording set-up with the Dino-lite software open on the screen utilized for real-time behavioral capture and post-recording frame-by-frame review (bottom image).

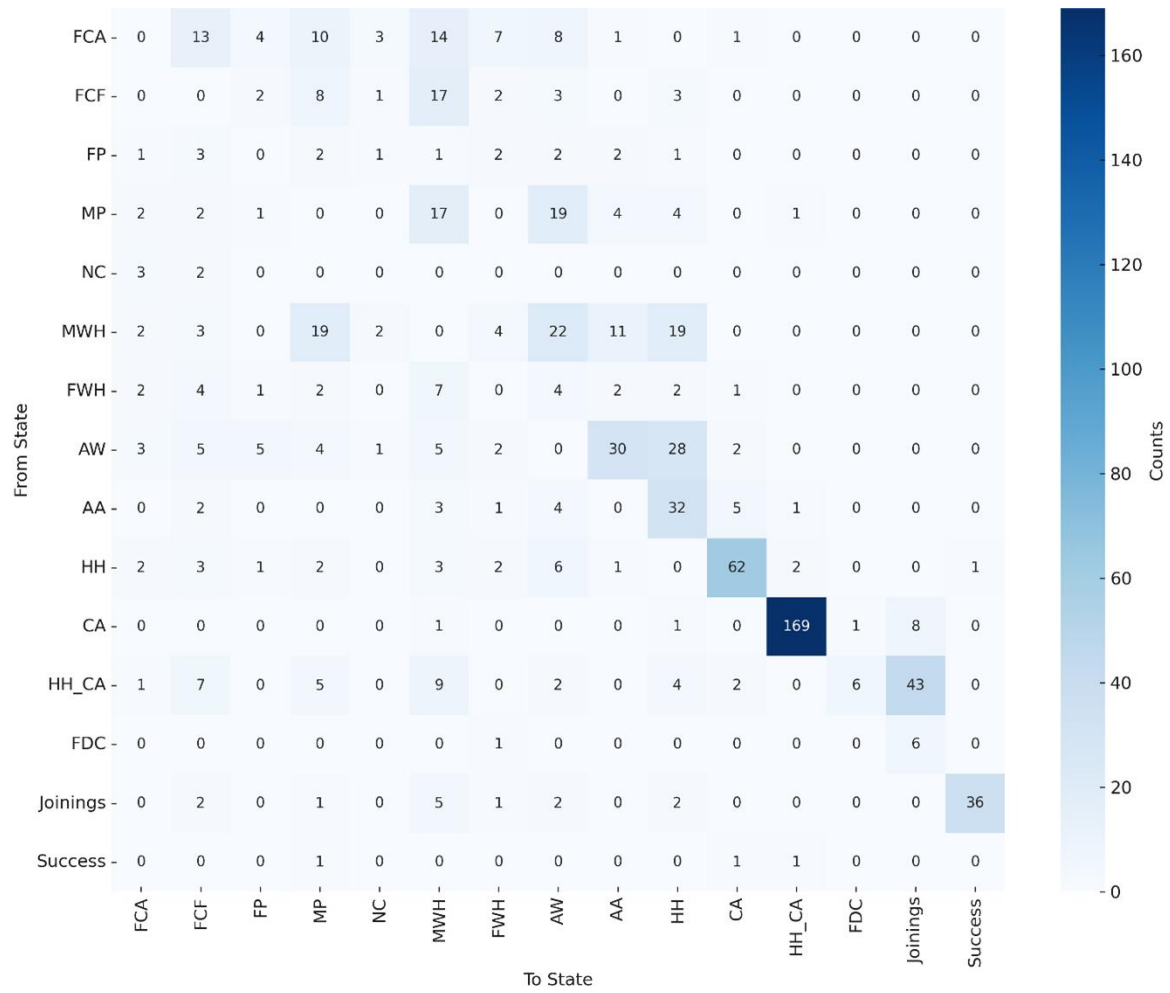


Figure 3.2. Heat map of total transition counts (C_{ij}) per behavioral state. This transition matrix visualizes the total observed frequency of transitions between 14 behavioral states across all trials ($n = 120$). The intensity of the color indicates the raw transition count, highlighting dominant behavioral pathways. Darker regions represent high-frequency, while light regions represent low frequency.

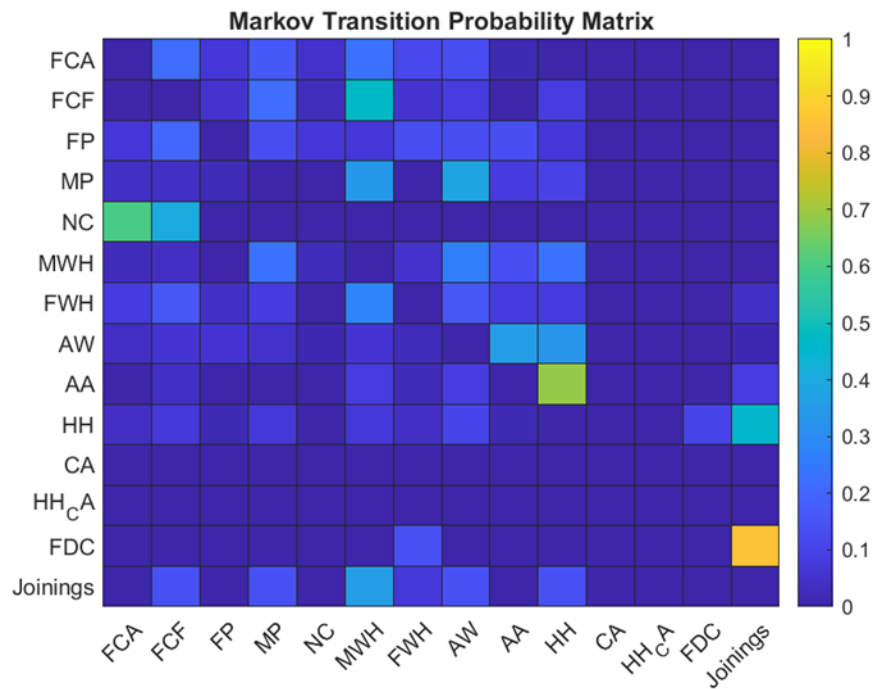


Figure 3.3. Heatmap of behavioral transitions ($n = 120$). Darker intensities represent higher transition probabilities, emphasizing recurrent signal–pursuit loops and convergence into joining behaviors.

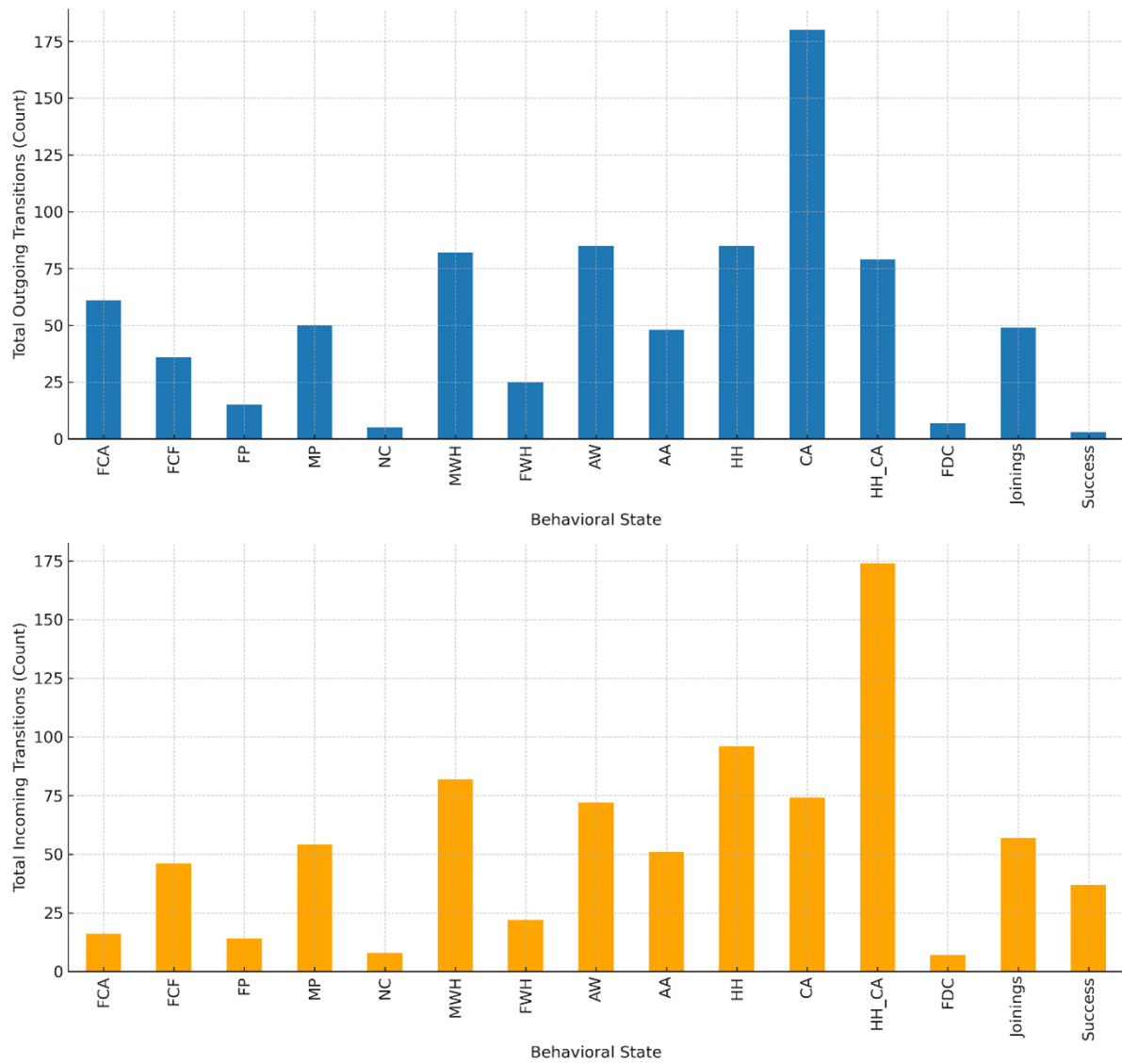


Figure 3.4. Outgoing transition counts per behavioral state (top graph), and incoming transitions (bottom graph).

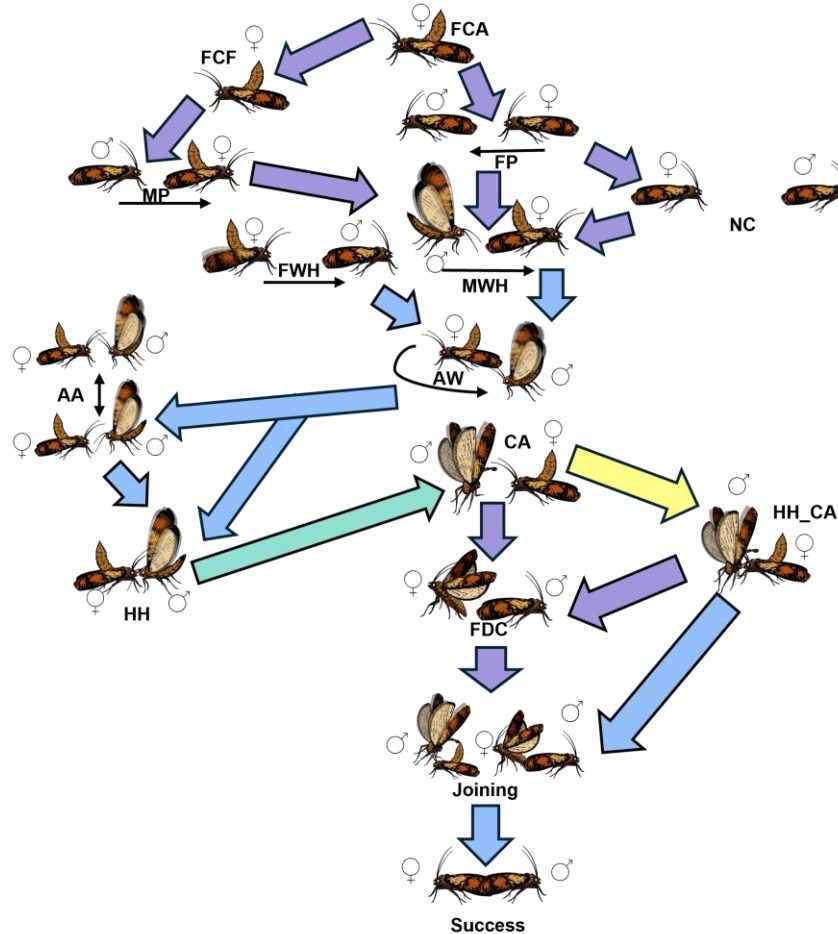
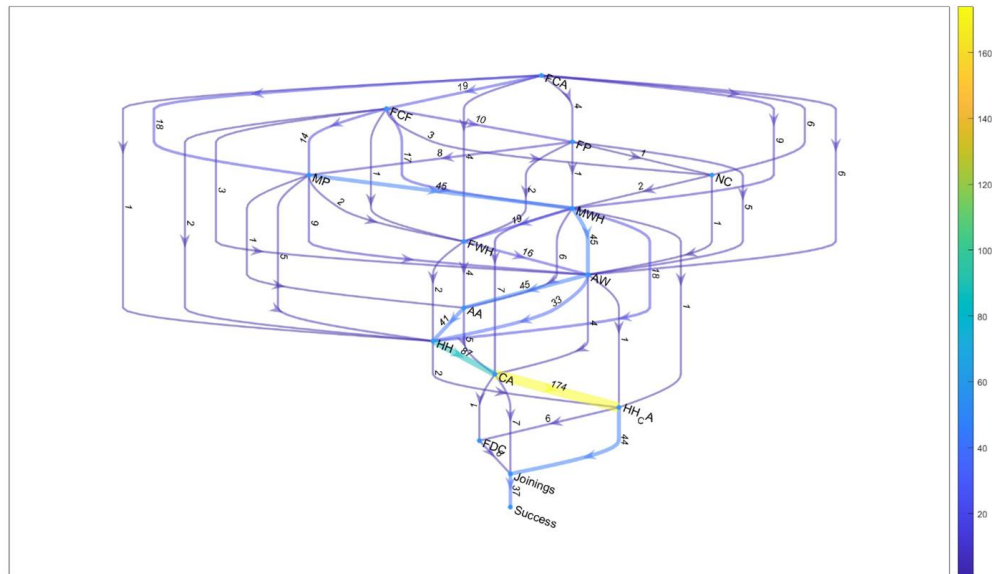


Figure 3.5. Behavioral pathway graph of moth courtship sequences represented in a KEGG-style network (top image). Nodes denote discrete behavioral states, and directed edges represent observed transitions with their frequencies. Using the same scale, the coloration graphics of behavioral states are represented (bottom image) with males (♂) and females (♀).

Table 3.1. Descriptions and definitions of the scored *P. interpunctella* courtship behaviors. Behaviors are categorized by courtship state, illustrating the transition from long-range signaling to close-range interactions.

Term	Abbreviation	Description
Female Call in Acclimation	FCA	Female remains stationary, raises her abdomen, and emits ZETA pheromone during the acclimation period of the video.
Female Call in Free Period	FCF	Female remains stationary, raises her abdomen, and emits ZETA pheromone during the free period of the video.
Male Wing Hitting	MWH	Male repeatedly beats his wings without flying, often in pursuit or close contact with the female.
Female Wing Hitting	FWH	Rare female behavior resembling male wing-hitting was observed only in the free period.
Male Pursuit	MP	Male tracks or follows the female, often while wing-hitting or after physical contact.
Female Pursuit	FP	Female tracks or responds to the male, may call or wing hit while doing so.
Antenna-Wing Interaction	A-W	Antenna touches the other's wing, often eliciting a response (e.g., wing hitting or copulation attempt).
Antennae-Antennae Interaction	A-A	Males and females face each other and "bat" antennae, indicating intentional interaction.
Head-to-Head Contact	HH	Intentional head contact before copulation; the female may "bite" the male.
Copulation Attempt	CA	Attempt to clasp abdomens, either facing toward (HH + CA) or away (FDC) from each other.
Head-to-Head Copulation Attempt	HH + CA	During head contact, males and females lift their abdomens to attempt clasping.
Female-Directed Clasping	FDC	Clasping attempt while facing away; often more successful.
Joinings	JS	Pair successfully clasps and remains joined for an extended duration.
No Contact	NC	No physical contact between individuals during the free period, even if calling occurs.

Table 3.2. Total raw counts of behavioral states observed across all recorded courtship trials ($n = 120$). These counts represent the total number of times each discrete state was initiated during the study period, providing the basis for the transition probability matrix.

Behavioral States	Raw Counts
FCA	74
FCF	54
FP	22
MP	63
NC	30
MWH	114
FWH	30
AW	142
AA	59
HH	115
CA	191
HH_CA	213
FDC	7
Joinings	57
Success	37

Table 3.3. Transition probability matrix for *P. interpunctella* courtship sequences. Estimated transition probabilities between 14 discrete behavioral states. Rows (S_i) represent the initial behavior and columns (S_j) represent the subsequent behavior. Each cell (P_{ij}) indicates the probability of transitioning from the row to the column, normalized by the total number of outgoing transitions from S_i . Values of 0 indicate transitions that were not observed in the dataset.

	FCA	FCF	FP	MP	NC	MWH	FWH	AW	HH	CA	HH_CA	FDC	Joinings
FCA	0	0.22	0.07	0.17	0.05	0.23	0.13	0.13	0	0	0	0	0
FCF	0	0	0.06	0.22	0.03	0.47	0.06	0.06	0.08	0	0	0	0
FP	0.07	0.2	0	0.13	0.07	0.07	0.13	0.13	0.07	0	0	0	0
MP	0.04	0.04	0.02	0	0	0.34	0	0.38	0.1	0	0	0	0
NC	0.6	0.4	0	0	0	0	0	0	0	0	0	0	0
MWH	0.02	0.04	0	0.2	0.02	0	0.05	0.18	0.23	0	0	0	0
FWH	0.08	0.18	0.04	0.08	0	0.28	0	0	0.08	0	0	0	0.04
AW	0.04	0.06	0.06	0.05	0.01	0.06	0.02	0.08	0.33	0	0	0	0.01
AA	0	0.04	0	0	0	0.08	0.02	0.11	0.68	0	0	0	0.08
HH	0.04	0.07	0.02	0.07	0	0.07	0.04	0	0	0	0	0.11	0.45
CA	0	0	0	0	0	0	0	0	0	0	0	0	0
HH_CA	0	0	0	0	0	0	0	0	0	0	0	0	0
FDC	0	0	0	0	0	0	0.14	0	0	0	0	0	0.86
Join	0	0.14	0	0.14	0	0.36	0.07	0.14	0.14	0	0	0	0

Chapter 4 - How does *P. interpunctella* perceive pheromonal mating cues?

In preparation as: Jacqueline M. Maille, Danielle Dryer, James Campbell, Kun Yan Zhu, Erin D. Scully, and W.R. Morrison III. Physiological and reproductive modulation of electroantennographic responses to sex pheromone in *Plodia interpunctella* (Lepidoptera: Pyralidae).

Introduction

The Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) is a globally distributed stored product pest that contributes to extensive economic loss in over 177 grain and grain-based commodities within the post-harvest supply chain (Hagstrum and Subramanyam 2009). These insects infest a variety of products that are stored in chemically complex landscapes where food resources are often patchy and need to locate mates and suitable oviposition sites (Campbell et al. 2002; Semeao et al. 2013). Interestingly, *P. interpunctella* adults do not feed and are short-lived; therefore, the ability to rapidly find mates and suitable oviposition substrate is key to sustaining population levels (Silhacek et al. 2002; Sambaraju and Phillips 2008).

Importantly, management of *P. interpunctella* exploits the known long-range, female-produced pheromone, (Z, E)-9,12-tetradecadienyl acetate (e.g., ZETA) (Brady et al. 1971; Kuwahara et al. 1971; Zhu et al. 1999). Existing research has explored the effects of ZETA on long- and close-range behaviors, including dispersal (Campbell et al. 2002; Maille et al. 2026 [Chapter 2]), courtship sequences (Gerken et al. 2022), usage in attract-and-kill (Nansen and Phillips 2004; Campos and Phillips 2010, 2013, 2014), and commercial mating disruption (Ryne et al. 2007; Burks et al. 2011; Trematerra et al. 2011; Lindenmayer et al. 2025; Campbell et al.

2025). Although lures containing ZETA have been successfully deployed to monitor and manage *P. interpunctella* populations in various retail stores, food processing facilities, and storage bins, less is known about how life history and exposure to stimuli and mates influence the efficacy of these pest management tactics and the responsiveness of the peripheral olfactory system of *P. interpunctella* to ZETA (Kuwahara and Casida 1973; Zhu et al. 1999; Ding et al. 2022). Hence, there is a critical need to explore how internal states influence responsiveness to pheromone signals. This approach is advantageous because they mimic the complexity of life history traits that would be encountered in a management system, which this pest may encounter under field conditions.

State-dependent variations (sex, age, circadian rhythms, mating status, experience, satiety, etc.) are known to affect responsiveness to olfactory cues in insects. For example, in silk moths (*Bombyx mori* [L.]; Lepidoptera: Bombycidae), mating status directly modulates the transductive behavioral threshold for pheromone-triggered searching behavior (Sakurai et al. 2014), which in turn is affected by the circadian rhythm-associated serotonin levels in the brain (Gatellier et al. 2004). Likewise, pheromone perception in the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is variable, and both age and mating status significantly influenced the male electrophysiological responsiveness of antennae to the female-produced sex pheromone (*E*, *Z*, *Z*)-3,8,11-tetradecatrienyl acetate and (*E*, *Z*)-3,8-tetradecadienyl acetate (Domínguez et al. 2019). By contrast, female *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) that were pre-exposed to their pheromone, (*Z*)-11-tetradecenyl acetate, showed reduced sensitivity in electrophysiology responses following both a 2-hour or no delay since exposure, demonstrating autodetection of pheromone may alter subsequent antennal sensitivity (Kuhns et al. 2012).

Beyond state dependency, insects may exhibit lateralization in the left and right antennae during chemosensation. Lateralization may be beneficial to insects because it allows for efficient resource location (e.g., mates and food), while minimizing metabolic costs by streamlining searching (Parthasarathy and Willis 2021). For example, antennal lateralization was demonstrated in *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with above 70% accuracy in discriminating the specific side (left or right) of odor arrival from delivery, which was necessary for following odor plumes. Thus, exploring whether *P. interpunctella* exhibits asymmetric antennal sensitivity can support an enhanced understanding of how behavioral modulation occurs and how internal state-dependent factors shift the detection threshold of ZETA, which may be important for predicting how a mixed population of moths detects mates in patchy and ZETA-rich environments (e.g., environments that contain monitoring lures or mating disruption dispensers).

Therefore, in this study, we characterized the antennal responsiveness of adult *P. interpunctella* through a multifactorial approach across its reproductive lifespan. We sought to determine how sex, mating status, age, and antennal lateralization interact to shape the perception of four progressively higher concentrations of synthetic pheromone (ZETA) to *P. interpunctella*. To achieve this, we tested *P. interpunctella* for 1) sensitivity to ZETA by testing responsiveness to serial dilutions of pure compound, 2) how reproductive state affected the antennal response to ZETA, and 3) lateralization in antennal sensitivity in response to ZETA using electroantennography (EAG). We hypothesized that the antennal responses would increase proportionally with increasing ZETA concentrations but would vary significantly among biological states (e.g., age, sex, mating status) and that response symmetry would be bilateral. Further, we predicted that antennal sensitivity would be altered by mating status and

reproductive state with peak responsiveness reflecting critical life-history transitions for *P. interpunctella*. Lastly, we predicted bilateral symmetry in antennal response and thus expected no differences would be found between left and right antennae regardless of the internal state or experience of the moth.

Materials and Methods

Source insects

The source of the insects, rearing, and sexing was conducted the same as Chapter 2. Then, adults were standardized and blocked for experimentation to account for three factors: sex (male or female), mating status (unmated or mated), and post-eclosion age (1, 3, or 5 d). Emergence of adults was checked daily after 1200 h, with the day of emergence reflecting day 0 of post-eclosion and day 1 representing 24 hours after eclosion. Mated adults were produced by placing a single female and male into a single 59.2 mL plastic cup and allowing them to mate under rearing conditions for 12–18 h prior to antennal recordings. Successful mating was confirmed after the antennae were removed by dissecting females and determining whether spermatophores were transferred (Figure 2.1B).

Stimuli

Six stimuli were prepared and delivered to the antenna including: air (filter paper only, e.g., negative control), methanol (CAS 67-56-1, >99.9% purity; Sigma-Aldrich, Burlington, MA, USA), and four concentrations of (*Z,E*)- 9,12-tetradecadienyl acetate (CAS 31654-77-0, >98% purity; ChemCruz, Lot G022, Santa Cruz Biology Inc., Dallas, TX, USA) diluted in methanol. To prepare the ZETA concentrations, neat ZETA was mixed with methanol to make a stock solution of 100 mg/mL and then decadic dilutions with additional methanol were made to reach final concentrations of 0.1, 1, 10, 100 ng/μL of ZETA: methanol. At the center of a 2.5-cm

diameter filter paper, a 10- μ L aliquot from one of the stimuli was delivered by a 50- μ L Hamilton microliter syringe (Reno, NV, USA) and allowed to dry for 30 s before being utilized in the EAG experiment. Filter papers and glass pipettes were replaced for every recording.

Electroantennography (EAG) experiment

All recordings were conducted between 0900 and 1200 CST. Adults were immobilized in a -20 °C freezer for up to 5 min. The antenna of each moth was excised with surgical microscissors (World Precision Instruments, Sarasota, FL, USA) between the scape and pedicel, alternating between the left and right antenna. Right and left antennae were identified by positioning the insect on its dorsal side and with the anterior/head region of the insect pointed away from the observer. In addition, we recorded whether the following behaviors were observed when we collected the antennae: 1) calling, defined as when females were actively calling 2) laying, defined as after a female oviposition event, which was evidenced by the presence of eggs), and 3) neutral, which conspecifics were not observed engaging in any reproductive behaviors immediately prior to testing). We then mounted each antenna on an antenna holder (Syntech GmbH, Buchenbach, Germany) using electroactive gel (Spectra 360 Electrode gel, Parker Laboratories INC., Fairfield, NJ, USA) to ensure connection when attached to the EAG combi probe (Syntech GmbH, Buchenbach, Germany).

Neuroelectrical signals from the antenna were amplified and directly recorded by an IDAC4-USB box (Syntech GmbH, Buchenbach, Germany). The antennal preparation was held in a continuous air stream (2 L/min) that was charcoal-filtered and humidified then delivered in a glass mixing tube. Stimuli were introduced by a CS-55 stimulus controller (Syntech GmbH, Buchenbach, Germany) containing an inline 1 L flowmeter (Dwyer Omega, Michigan City, IN, USA), restricting puff rates to 0.4 L/min to reduce mechanical agitation of the antennae. Stimuli

were gently puffed into humidified air through a 10- μ L Pasteur pipette for 1 s by triggering the stimulus controller pedal after the antenna had 1 min to establish baseline EAG signal. Each stimulus was presented twice in succession with a 30 s delay before the first puff or from initially reaching baseline followed by a 1 min delay between the first and second puff. Stimuli were changed following the same presentation order within 1 min following the second puff during which antennae were allowed to return to their baseline, and then the sequence repeated (Figure 4.1). Presentation order followed air, methanol, 1, 10, 100, 1000 ng/ μ L of ZETA: methanol. All recordings were analyzed with GcEAD 2014 v.1.2.5 software (Syntech GmbH, Buchenbach, Germany) and changes in antennal response was recorded in the 5 s following a stimulus exposure. Testing was not performed on days in which nearby prairies were being burned or in which air quality was particularly bad in Manhattan, KS. In addition, all trials were conducted at room temperature.

Statistical analyses

Before data were analyzed, each recording was standardized to isolate the depolarization of ZETA and to account for variation in baseline responsiveness between individual antennal preparations and recording dates. A series of progressive standardizations was used. First, the raw amplitude (A_{raw}) for each antennal response (i) to ZETA was subtracted from the average amplitude for the air treatment ($\bar{A}_{air}$) for a given insect (i) in order to standardized amplitudes without mechanical stimulation (A_{mech}):

Equation 4.1

$$A_{mech,i} = A_{raw,i} - \bar{A}_{air,i}$$

Next, in every antennal recording, the ZETA amplitudes (z) without mechanical stimulation (A_{mech}) were subsequently subtracted from the average standardized ($\bar{A}_{mech}$) methanol

response (m) to ensure that the response to the solvent was removed and only the ZETA response (A_{zeta}) remained:

Equation 4.2

$$A_{zeta} = A_{mech,z} - \bar{A}_{mech,m}$$

This resulted in $n = 2,304$ ZETA-induced antennal responses (A_{zeta}). These values were analyzed using linear mixed models (LMMs) to assess the overall effects of sex (male, female), mating status (unmated, mated), age (1, 3, or 5 d post-eclosion), ZETA concentration (log-transformed [1, 10, 100, or 1000 ng]), and antennal lateralization (left or right) on responsiveness (Figure 4.2), where date was included as a random intercept in a five-way interaction model. Because technical rep (puff number) was not a significant factor in the initial model and did not significantly contribute to the observed variance, it was removed from the final model. In addition to these overall effects model, separate models were created for each sex to analyze the influence of specific reproductive states (neutral, calling, and laying) at the time of the EAG recordings on the change in antennal response to ZETA. In these models, mating status was embedded in reproductive state to resolve the confounding effect of mating history (e.g., states included: mated-neutral, mated-calling, mated-laying, unmated-neutral, unmated-calling). Unmated laying was never observed during the experiment, so this level was not included. This allowed us to test whether the antennal response to increasing pheromone concentrations differed between reproductive states or across age groups. The experimental design followed a factorial structure blocked by date. Finally, to understand how reproductive state modulated the antennal lateralization, we analyzed the four-way interaction between antenna, sex, reproductive state, and pheromone concentration. Sample sizes of moths were $n = 16$ replicates per sex $\times$ treatment $\times$ age combination with $n = 8$ per antenna $\times$ sex $\times$ treatment $\times$ age combination.

Statistical analyses were performed in R (version 4.4.3; R Core Team) for all analyses with $\alpha = 0.05$. Standardized antennal response was analyzed using the packages *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017) for model fitting and *emmeans* (Lenth and Piaskowski 2025) for post-hoc analysis comparisons across explanatory factors. The *emtrends* function was used to capture changes in antennal responses with increasing pheromone concentrations. The degrees of freedom for each model were estimated using Satterthwaite's approximation, while *p*-values for fixed effect interactions were obtained using a Type III Analysis of Variance (ANOVA). Post-hoc comparisons were adjusted for multiple testing using Tukey's HSD method with degrees of freedom estimated by the Kenward-Roger method to provide conservative estimates for the complex interaction terms within our models.

Results

Effect of physiological status on antennal response

Exposure to ZETA resulted in 6.64-fold and 1.11-fold higher responses than to air or methanol, respectively. The random effect of date was included as a random factor and explained a significant portion of the variance ($\sigma^2 = 0.222$) of the data, likely because of fluctuations in environmental conditions and at the time of recording. (Table 4.1). Male *P. interpunctella* had a 28% greater antennal response than females ($\beta = 0.246$; SE = 0.115; df = 1,445; $p = 0.032$). Overall, mating status had no significant effect on antennal responsiveness (Table 4.1), but the right antenna of *P. interpunctella* was 17% more responsive in standardized amplitude to ZETA compared to the left antenna ($\beta = 0.159$, SE = 0.003, df = 1,505, $p < 0.0001$). The age of moths affected antennal responses and its interaction with sex was also significant, with greater responses for males on day 5 (Male $\times$ Day 5 $\times$ LogConc) ($\beta = 0.179$; SE = 0.009; df = 1,445; $p = 0.039$) compared to females on day 1. The strength of the antennal response increased as males

aged from day 1 to day 5 ($t_{1447} = -0.132, p = 0.006$) (Figure 4.3; Table 4.2). Although female *P. interpunctella* were less variable across treatment combinations relative to males, responses tended to be higher in unmated females, which exhibited a window of enhanced antennal response from day 3 to day 5 ($t_{205} = 0.532, p = 0.047$) (Table 4.3).

Effects of reproductive states on antennal response

We found antennal responsiveness to ZETA was significantly affected by sex ($\beta = 0.233$; $SE = 0.008$; $df = 1,445$; $p = 0.002$), lateralization of antenna ($\beta = 0.142$; $SE = 0.003$; $df = 1,500$; $p < 0.001$), and the interaction of mating status and ZETA concentration (Table 4.4). Post-hoc contrasts revealed that reproductive state significantly influenced male responsiveness at the highest concentration (1000 ng). Specifically, for day 5 males at the 1000 ng concentration, the mated males paired with laying females (laying state) showed the lowest sensitivity, with responses being significantly lower than mated neutral (a decrease of 0.71 mV, $p < 0.001$) and mated males paired with calling females (calling) (a decrease of 1.30 mV, $p = 0.003$). Conversely, unmated males paired with calling females showed the highest overall sensitivity, outperforming mated males paired with laying females by 1.60 mV ($p = 0.001$) (Table 4.5). Therefore, the overall model was split by sex to tease apart the effect that reproductive state uniquely had on males and females. The female-specific model (Table 4.5; Figure 4.6) showed that reproductive status significantly influenced female responsiveness, especially on day 5 ($\beta = -0.315$; $SE = 0.113$; $df = 704$; $p = 0.005$) compared to day 1. The highest overall sensitivity was observed in day 1 unmated neutral females, where the steepest response slope to ZETA was observed ($\beta = 0.207$; $SE = 0.069$; $df = 704$; $p = 0.003$) relative to all other female groups (Table 4.7). There was high complexity in the interactions among factors in the male-specific model (Table 4.8), with the day 5 mated males paired with laying females (laying) ($\beta = 0.365$, $SE =$

0.094, $df = 698$, $p \geq 0.001$) showing significantly higher antennal response to ZETA compared to day 1 (Table 4.9; Figure 4.5). With the increased sensitivity at day 5, significantly higher than the responses observed on day 3, males of the same reproductive status were affected by age.

Modulation of responsiveness during reproductive states by antennal lateralization

The antennal responses by *P. interpunctella* to ZETA did not always remain asymmetric when the influence of reproductive state on ZETA responsiveness was considered. (Table 4.10). For mated males that were exposed to females that laid eggs (male mated laying), the right antenna was significantly more responsive than the left ($t_{1435} = -2.33$, $p = 0.019$). Similarly, significant right-sided lateralization was observed in unmated females that did not engage in reproductive behaviors at the time preceding recording (female unmated neutral) ($t_{1435} = -2.02$, $p = 0.043$). However, females that were unmated and calling prior to the time of recording demonstrated a symmetrical response (Left $\beta = -0.015$; Right $\beta = 0.006$; $p = 0.674$) (Figure 4.6.; Table 4.10).

Discussion

Pheromone-mediated communication plays a central role in the reproductive ecology and management of many moth pest species, including *P. interpunctella*. Understanding how individuals perceive and respond to pheromone signals is therefore critical for both interpreting behavioral ecology and improving pheromone-based monitoring of pest populations and management of pest insects. However, pheromone perception is not static and can be influenced by multiple intrinsic biological factors that shape sensory responsiveness. Nevertheless, few studies have investigated the perception of *P. interpunctella* to its sex pheromone (e.g., ZETA) in a holistic manner, and none have comprehensively considered combined effects of physiological status, age, reproductive state, and antennal lateralization. Yet this information is critical for

improving our understanding of the chemosensory biology of *P. interpunctella* and for improving the efficacy of existing systems or informing the design of novel tactics for a more effective management program.

In this study, we found that antennal responsiveness was significantly modulated by the interaction of sex, age, mating status, and reproductive state. Most notably, we documented functional antennal lateralization for the first time in this species with a 17% bias towards increased responsiveness to ZETA in the right antenna. However, we also demonstrated that antennal lateralization is not fixed, but that age and reproductive state interact to symmetrize antennal responses to this compound. Other key trends in our study included an increase in antennal response to ZETA as they aged, an effect that was amplified in mated males and in males that were paired with females that had oviposited. In addition, we also observed peak antennal responsiveness to ZETA in unmated females at day 1 that were not engaged in calling or egg laying behaviors.

Antennal responses to ZETA were observed across all tested *P. interpunctella*. While generally higher than the lowest concentration, the amplitude did not follow a concentration-dependent relationship across the 1–1000 ng ZETA range (Table 4.4), unlike prior characterizations of the effect of ZETA on antennal responsiveness (Kuwahara and Casida 1973; Zhu et al. 1999; Ding et al. 2022). However, these prior studies limited responses to unmated males. Our multifactorial analysis revealed that both sexes responded to ZETA; males generally had an elevated response to pheromone above and beyond females, which conforms to the functionality of ZETA as a female-produced sex pheromone. Importantly, our findings physiologically confirm the autodetection of ZETA by female *P. interpunctella*, with the highest sensitivity on day 1 post-eclosion. The ability of females to autodetect ZETA had been

previously hypothesized based on altered behavior and flight in the presence of ZETA (Gerken et al. 2022; Maille et al. 2026 [Chapter 2]). Specifically, Gerken et al. (2022) observed that exposure to synthetic pheromone lures induced strain-specific changes in calling behavior where one strain increased calling duration to outcompete perceived rivals, while another delayed calling to avoid competition. Furthermore, both strains exhibited behavioral arrestment, characterized by a significant decrease in mobility and an increase in antennal grooming (cleaning) when pheromone was present. These findings, combined with the observation of reduced flight bout distances in the presence of ZETA (Maille et al. 2026 [Chapter 2]), provide strong evidence that females actively detect and behaviorally respond to their own sex pheromone, and it alters their behavior. Furthermore, we found high responsiveness of unmated males to ZETA was observed between day 3 and day 5 post-eclosion, compared to day 1 unmated males or mated males of the same ages. In contrast, a significant reduction in ZETA responsiveness was observed in aging females; day 5 females exhibited lower antennal amplitudes than day 1 females. This decline may reflect a shift in life-history strategy. Early in the lifespan (day 1), high sensitivity to ZETA likely facilitates autodetection for spacing and dispersal to avoid conspecific competition and inbreeding, a hypothesis supported by the high flight capacity of young, unmated moths (Maille et al. 2026 [Chapter 2]). By day 5, however, the reduction in sensory sensitivity aligns with the observed decrease in flight bout frequency and duration (Maille et al. 2026 [Chapter 2]). At this late stage, females may lack the lipid reserves (Subramanyam and Cutkomp 2017) necessary for sustained dispersal flight, leading to a sensory reduction as they prioritize localized oviposition over long-range movement.

Our reproductive state model demonstrated that the reproductive behavior of the moth was more important in predicting the sensitivity of the antennal response to ZETA by *P*.

interpunctella than mating status alone. We observed distinct sexual dimorphism in how reproductive state modulates sensory physiology. While females remained largely consistent in responsiveness across all reproductive states, male responsiveness varied significantly. Specifically, the mated laying state males (those paired with a female that oviposited) exhibited the highest overall responsiveness compared to males that were exposed to both females that were calling and or in neutral behaviors states (Table 4.9). This heightened antennal responsiveness by males after mating and exposure to cues from offspring suggests a reproductive priming effect, where a reproductively successful event or exposure to offspring-related volatiles triggers hyper-attunement (Vickers 2000) to female pheromones. The increased sensitivity in *P. interpunctella* males likely reflects a form of experience-dependent plasticity. As seen in *S. littoralis* (Lepidoptera: Sphingidae), exposure to reproductive cues can trigger a rebound heightening sensitivity (Anderson 2003) and a physical enlargement of the antennal lobe (Guerrieri et al. 2012). This strategy could allow the males to remain competitive in high-density populations by locating additional mates while females are actively ovipositing (Edward and Chapman 2011). Though it is important to note that our mated laying males were exposed to these cues for an indeterminate amount of time, and thus we expect this is the response following a rebound period. Since, in many lepidopterans, mating induces a desensitization to cues to save energy, as seen in *B. mori* (Lepidoptera: Bombycidae) (Sakurai et al. 2014). Therefore, our observations of male laying could have been observed outside the desensitization window, and future research should explore the breadth of this window further to see if there are effects on experienced males being less affected by pheromone-based monitoring or management strategies, having implications on their efficacy (Morrison et al. 2021). As this effect, in addition to the lateralization in male *P. interpunctella* may allow the males to remain competitive in high-

density populations by locating additional mates while females are actively ovipositing (Edward and Chapman 2011).

Parallel to these male shifts, we observed distinct lateralization reset in females by reproductive state and age. Unmated females that were not calling and had not laid eggs at the time of recording had a dominant right-handed response in antennal sensitivity; whereas the mated females that had laid eggs showed no lateralization in response to ZETA. The right-handed bias in unmated females could be important for searching for new host patches to avoid sibling mating and assisting in autodetecting to maximize space from competing females for mating opportunities. The loss in right-handed lateralization after mating by females *P. interpunctella* could be attributed to a switch to prioritizing oviposition, but this hypothesis requires further testing. Behavioral impacts on dispersal and searching have been well documented and attributed to the antennal lateralization sensitivity and transduction in *Apis mellifera* (Hymenoptera: Apidae) (Letzkus et al. 2006; Frasnelli et al. 2010b, a); however, research in non-social species is limited.

The reduced responsiveness to ZETA in males that have been exposed to certain stimuli could allow them to evade detection in monitoring traps deployed in food facilities, posing high risks. This challenge is compounded by the ability of females to autodetect their own pheromone, which can cause them to disperse away from pheromone-rich areas to reduce competition and optimize genetic mixing (Holdcraft et al. 2016). Similar behavioral patterns have been documented in *Ephesia kuehniella* (Zeller) (Lepidoptera: Pyralidae), where pheromone-baited traps repelled females (Trematerra and Battaini 1987). To address these sex-based challenges in trap catches, research is focusing on the development of integrated high-precision surveillance programs. By leveraging image-based traps, researchers are developing algorithms to

automatically classify individuals to species level and determine sex in real-time (Serfa Juan et al. 2026). However, improving detection through AI is only a part of the solution, as we must also improve attraction for individuals that are less responsive to ZETA. Drawing on successful management strategies for the codling moth (*Cydia pomonella*), which utilize a combination of kairomonal pear-derived volatiles and sex pheromones (Preti et al. 2021), the development of a multi-component lure for *P. interpunctella* offers a promising pathway to capture both sexes and achieve a more accurate census of facility infestations.

The difference in responsiveness also raises an interesting question, which is whether plume reach for *P. interpunctella* varies by sex, mating status, or reproductive state. Plume reach is based on the underlying biology and neuroethology of the perceiving species (Miller et al. 2015). While we did not specifically test this, Agrafioti et al. (2025) showed that the plume varied by life stage of the quarantined stored product insect, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), and the types of stimuli presented in traps (Agrafioti et al. 2025). Other work has found that the presence of host commodities significantly reduces plume reach in adult *Haylomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Kirkpatrick et al. 2019). In *P. interpunctella*, female pheromone release is altered by photoperiod and declines with age (Nordlund and Brady 1974). Thus, variation in the perception of ZETA by different sexes and reproductive states of *P. interpunctella* raises the possibility that plume reach may also vary more dynamically than previously thought.

While antennal electroantennography provides a valuable measure of total sensory potential, it does not assess physiological changes within the antennal lobe or mushroom body or behavioral changes that occur as a result of increased/decreased antennal responsiveness. However, the observed restoration of symmetry during female calling behavior strongly points

toward a central neuronal mechanism that regulates ZETA responsiveness, likely mediated by neuromodulators such as octopamine or serotonin, as documented in *B. mori* (Gatellier et al. 2004, Sakurai et al. 2014). To resolve whether these asymmetric responses stem from physical sensillar density or localized neuronal activity, future research should integrate scanning electron microscopy with single sensillar recordings (SSR) (Olsson and Hansson 2013). By deconstructing these state-dependent interactions, we move toward a more holistic understanding of *P. interpunctella* chemical ecology, which allows us to bridge the gap between fundamental neurobiology and the refinement of behaviorally based management strategies.

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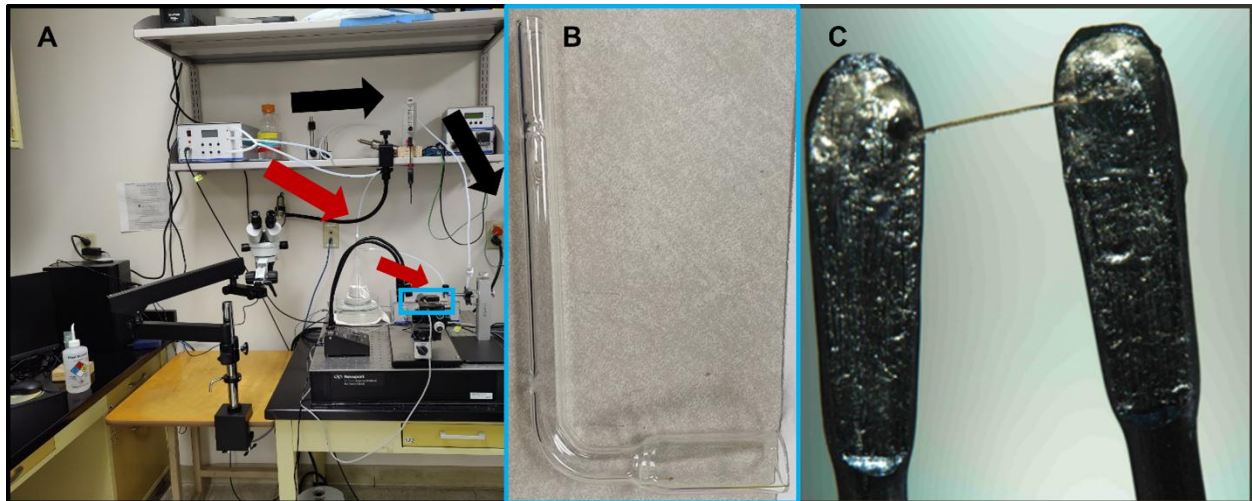


Figure 4.1. General overview of the electroantennogram (EAG). A) Black arrows indicate the stimulus path originating from the stimulus controller to the flowmeter and to the mixing tube. Red arrows denote the delivery of the continuous humidified air stream. The blue box highlights the location of the mixing tube. B) The mixing tube, where the stimulus and carrier converge and the antenna probe sits, is in the enlarged portion of the glass. C) Magnified view (60x, Nikon SMZ18 stereoscope, Tokyo, Japan) of the antenna prepped for recording.

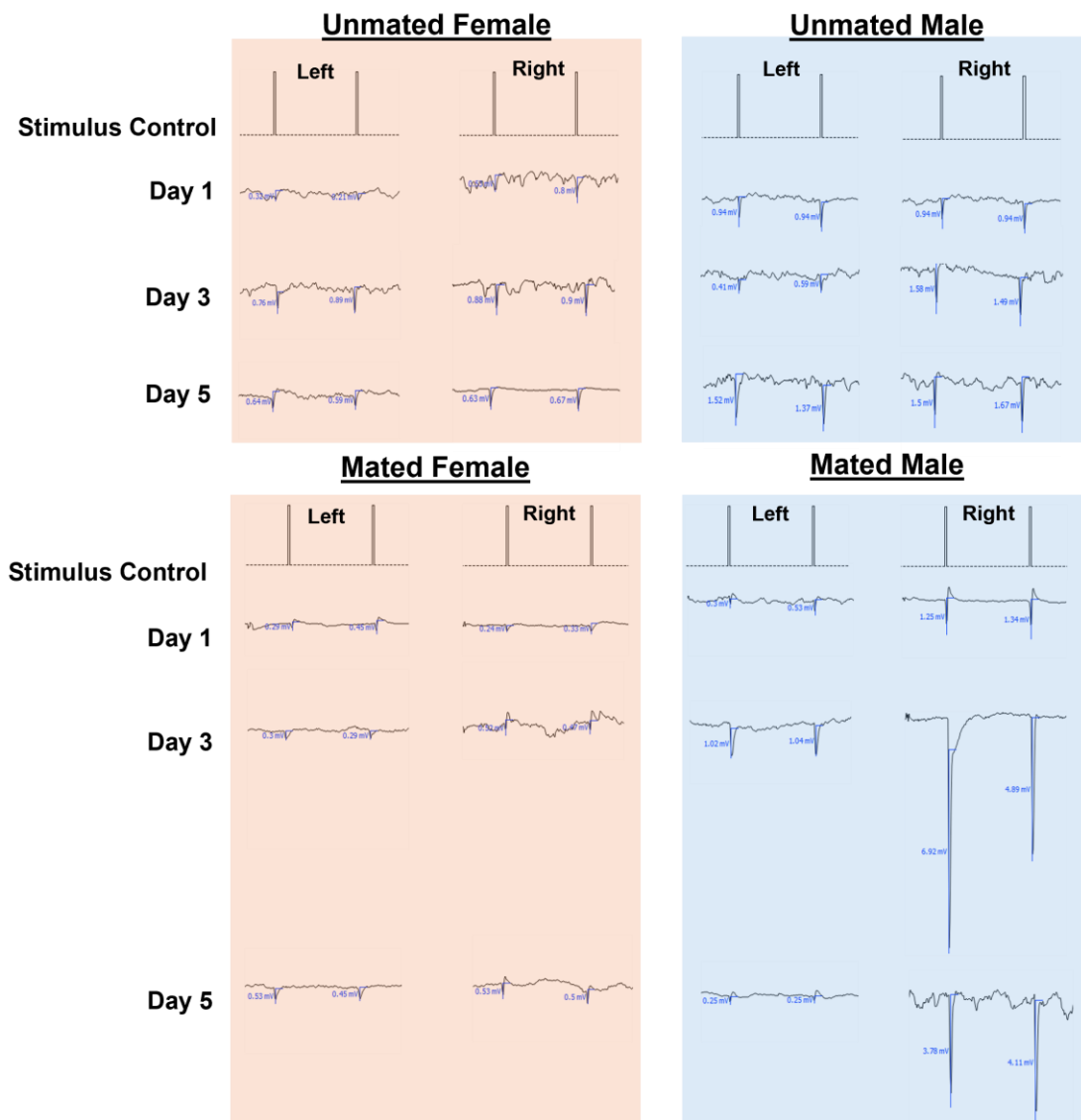


Figure 4.2. Representative electroantennogram (EAG) waveforms. Traces illustrate the change in antennal potential (mV) over a 40 s recording window. Waveforms represent the olfactory response of the sex, mating status, age (day), and antennal side to a 1000 ng/ μ L concentration of ZETA.

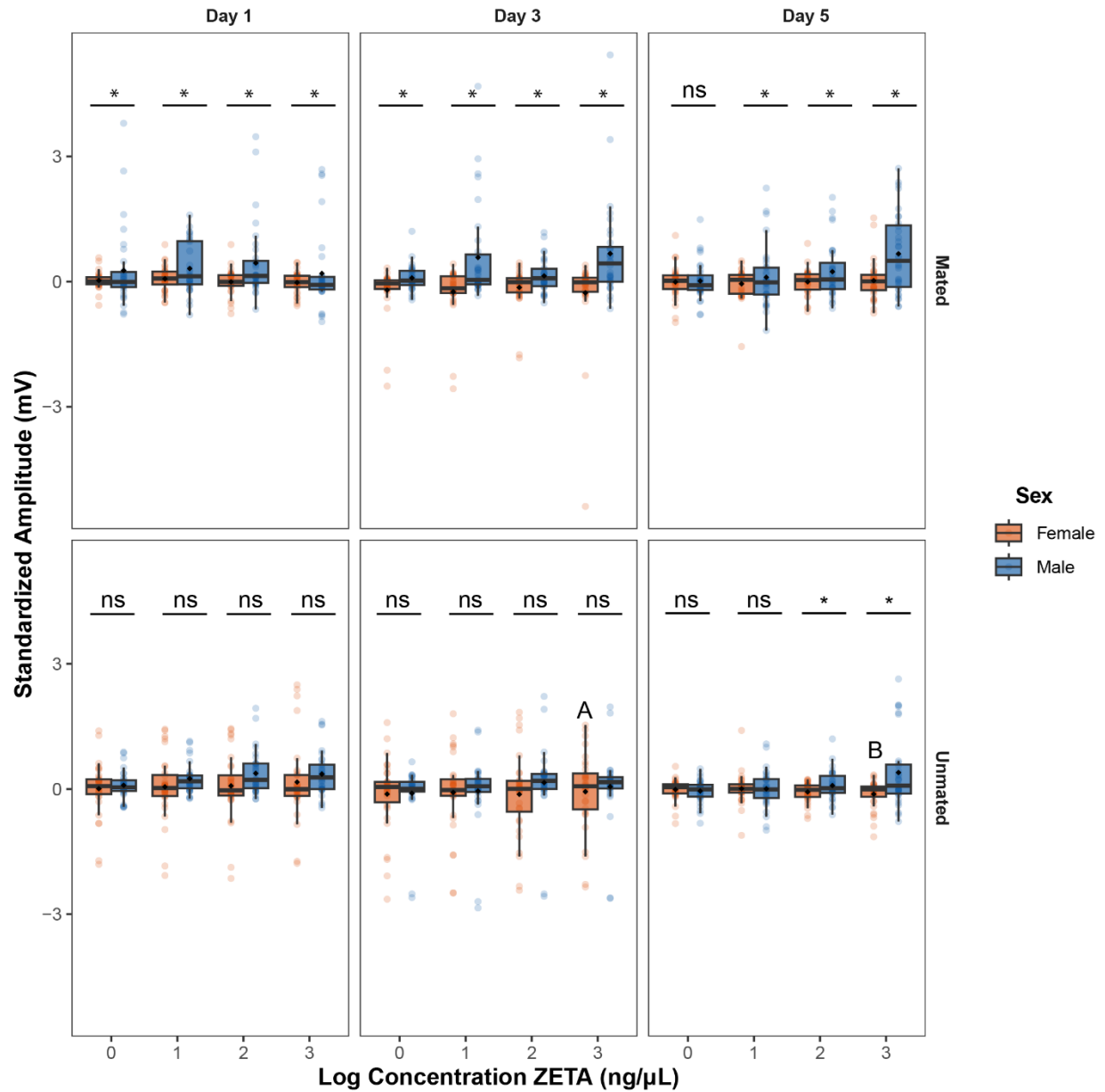


Figure 4.3. Standardized antennal responses (mV) to ZETA across three age cohorts (1, 3, 5 days post-eclosion) and reproductive status (mated or unmated) for female (orange) and male (blue) *P. interpunctella* across four log concentrations (1, 10, 100, 1000 ng/μL). Boxplots represent the median and interquartile range (IQR), with whiskers extending to $1.5 \times \text{IQR}$; black diamonds indicate group means. Individual data points are jittered to show the distribution. Letters represent significant differences between concentrations of ZETA and ages of individuals within each combination of sex and mating status; letters are left off boxplots where there is no significant difference to simplify display. Boxplots with asterisks represent significant differences between sexes within concentrations of ZETA, ages, and mating status; bars with no significant differences are marked with ns (e.g., not significant). All post-hoc comparisons were determined using Tukey-adjusted for pairwise comparisons at ($p < 0.05$).

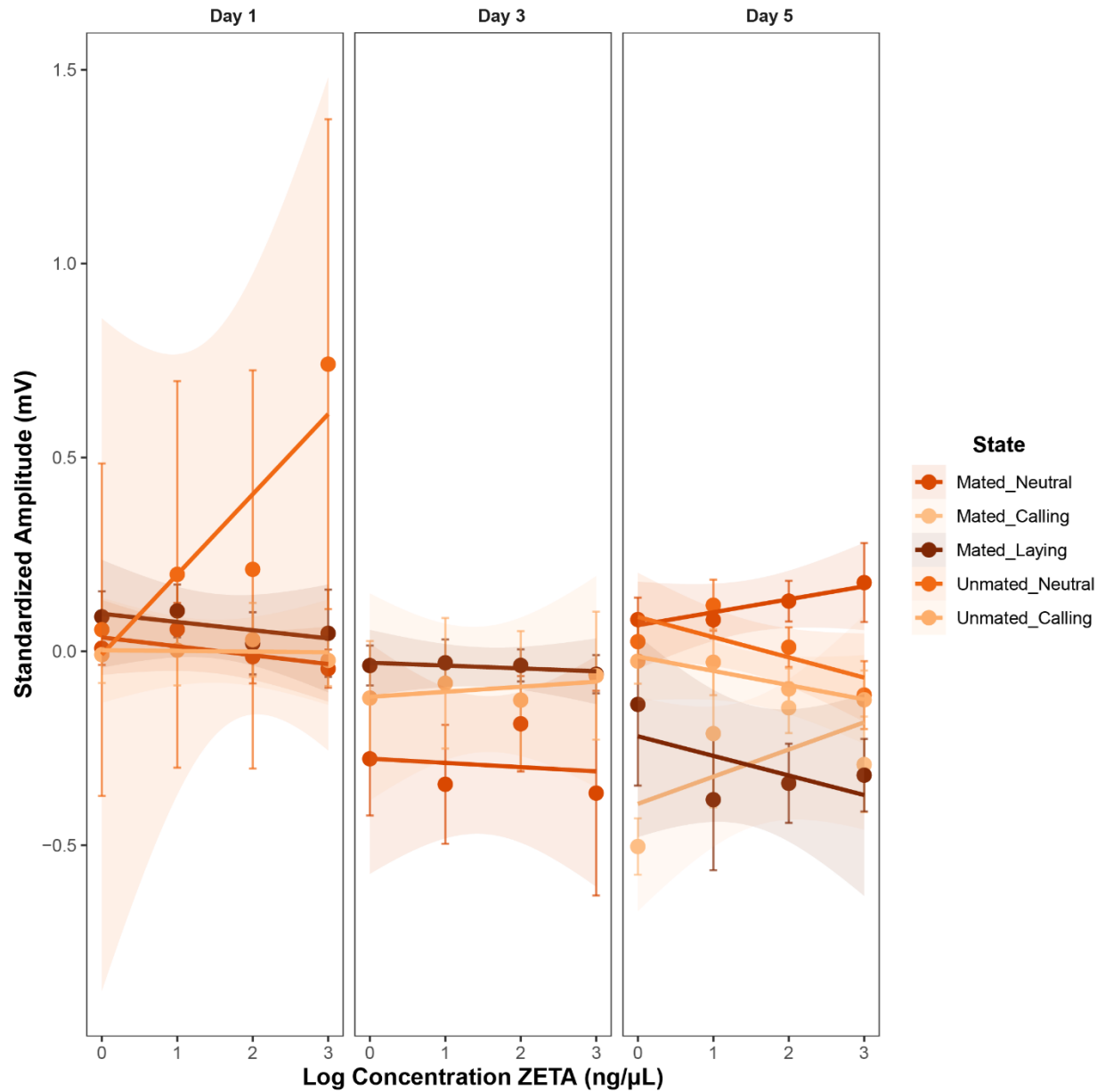


Figure 4.4. Standardized antennal responses (mV) by female *P. interpunctella* to a log-concentration gradient of ZETA (1–1000 ng/μL) for individuals aged 1, 3, and 5 days post-eclosion, depending on mating status (mated or unmated) and reproductive state (neutral, calling, egg-laying). Lines represented fitted linear regressions, and shaded bands represent 95% confidence intervals for each combination of mating status and reproductive state.

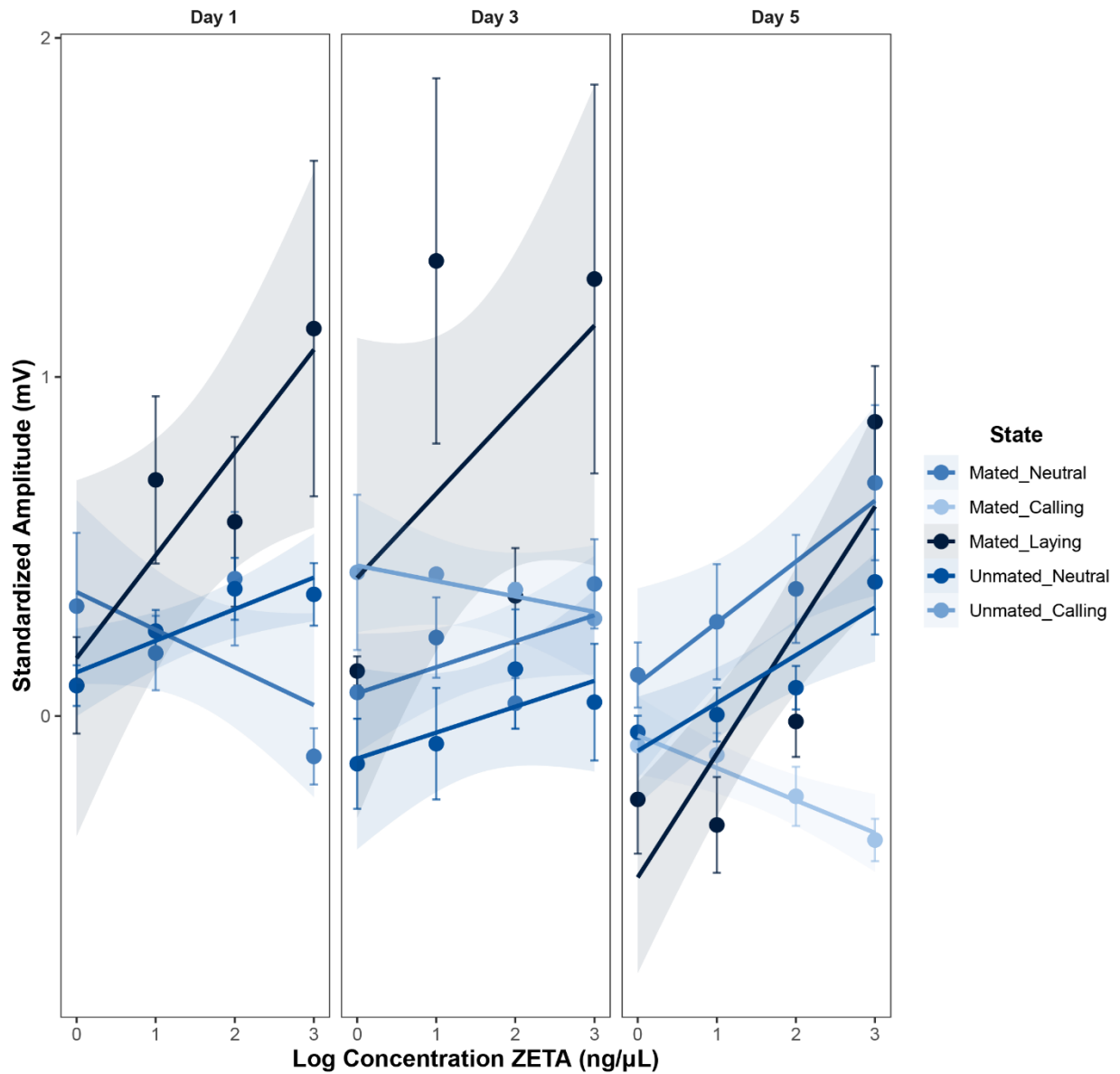


Figure 4.5. Standardized antennal responses (mV) by male *P. interpunctella* to a log-concentration gradient of ZETA (1–1000 ng/μL) for individuals aged 1, 3, and 5 days post-eclosion, depending on mating status (mated or unmated) and reproductive state (neutral, calling, egg-laying). Lines represented fitted linear regressions, and shaded bands represent 95% confidence intervals for each combination of mating status and reproductive state.

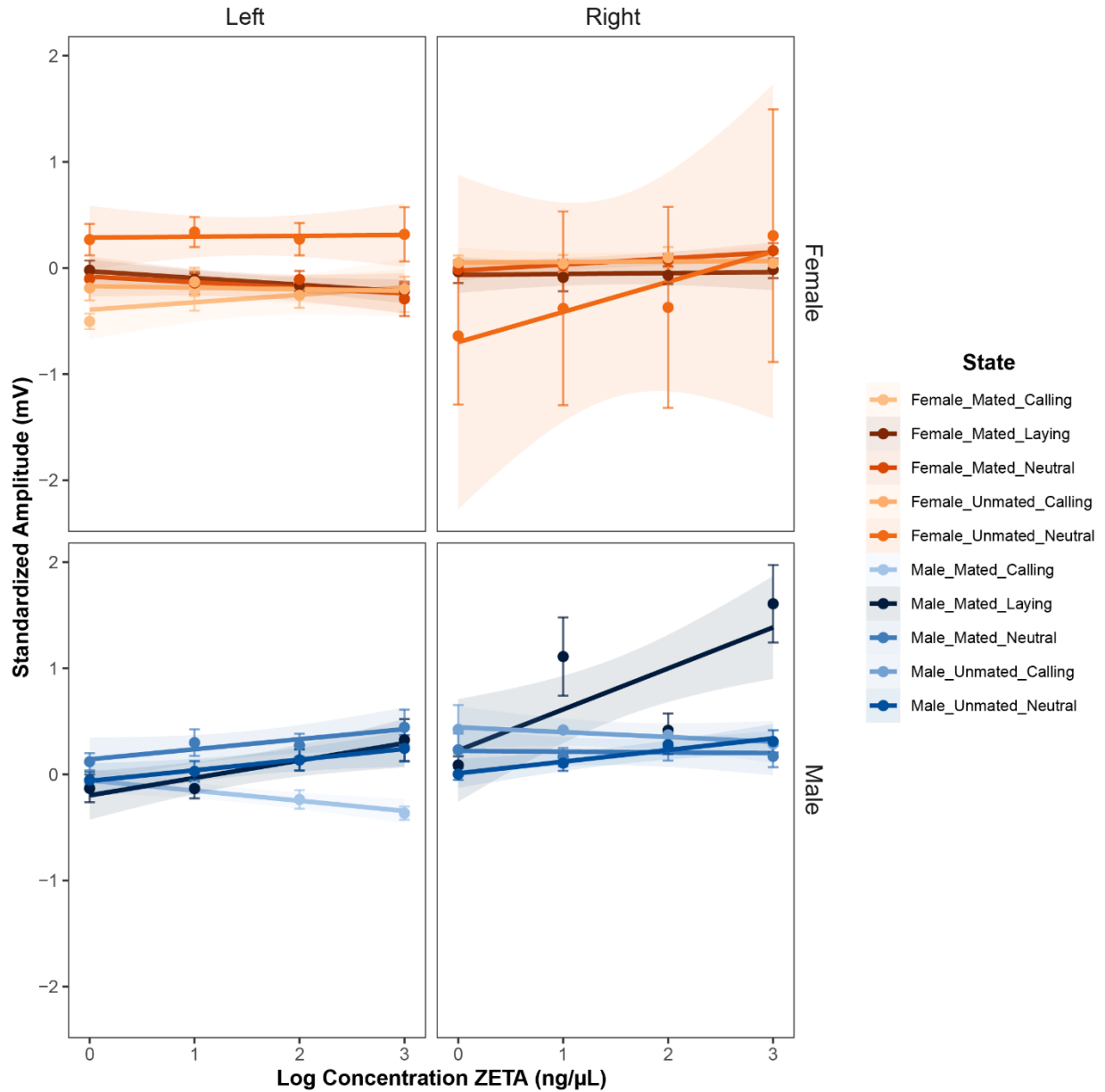


Figure 4.6. Right (right-hand column) and left (left-hand column) antennae standardized responses (mV) by female (orange) and male (bottom row, blue) *P. interpunctella* to a log-concentration gradient of ZETA (1–1000 ng/μL) for individuals aged 1, 3, and 5 days post-eclosion, depending on mating status (mated or unmated) and reproductive state (neutral, calling, egg-laying). Lines represented fitted linear regressions, and shaded bands represent 95% confidence intervals for each combination of mating status and reproductive state.

Table 4.1. Statistical summary of the overall linear mixed-effects model evaluating standardized antennal response by *P. interpunctella* depending on sex (male or female), age (0, 3, or 5 d), mating status (mated or unmated), concentration of ZETA (LogConc of 1, 10, 100, or 1000 ng), antennal lateralization, and the second through fifth order interaction terms. Model parameters were estimated using Restricted Maximum Likelihood (REML) with degrees of freedom and *p*-values calculated by Satterthwaite's method ($n= 1,530$ antennae). Random effects included the collection date. Significance was determined at $\alpha = 0.05$ (grey rows).

Predictor	Coefficient (β)	SE	df	95% CI	p-value
(Intercept: Female $\times$ Day 1 $\times$ Mated $\times$ Left)	-0.027	0.179	77	-0.384 – 0.330	0.883
Sex					
Female	—	—		—	
Male	0.246	0.115	1,445	0.021 – 0.472	0.032
Day					
1	—	—		—	
3	-0.203	0.236	94	-0.672 – 0.266	0.391
5	-0.072	0.235	93	-0.538 – 0.394	0.761
Status					
Mated	—	—		—	
Unmated	0.008	0.235	108	-0.457 – 0.472	0.974
LogConc	-0.022	0.043	1,445	-0.108 – 0.063	0.605
Antenna					
Left	—	—		—	
Right	0.157	0.032	1,505	0.094 – 0.220	<0.001
Sex $\times$ Day					
Male $\times$ 3	0.127	0.162	1,445	-0.192 – 0.445	0.435
Male $\times$ 5	-0.270	0.162	1,445	-0.588 – 0.049	0.097
Sex $\times$ Status					
Male $\times$ Unmated	-0.120	0.213	923	-0.539 – 0.299	0.574
Day $\times$ Status					
3 $\times$ Unmated	0.341	0.306	155	-0.264 – 0.946	0.267
5 $\times$ Unmated	-0.144	0.318	139	-0.773 – 0.485	0.651
Sex $\times$ LogConc					
Male $\times$ LogConc	0.015	0.061	1,445	-0.105 – 0.135	0.807
Day $\times$ LogConc					
3 $\times$ LogConc	0.013	0.061	1,445	-0.108 – 0.133	0.837
5 $\times$ LogConc	0.037	0.061	1,445	-0.083 – 0.158	0.544

<i>Predictor</i>	<i>Coefficient (β)</i>	<i>SE</i>	<i>df</i>	<i>95% CI</i>	<i>p-value</i>
<i>Status x LogConc</i>					
<i>Unmated x LogConc</i>	0.073	0.061	1,445	-0.048 – 0.193	0.235
<i>Sex x Day x Status</i>					
<i>Male x 3 x Unmated</i>	-0.562	0.296	1,112	-1.143 – 0.020	0.058
<i>Male x 5 x Unmated</i>	0.263	0.325	627	-0.376 – 0.902	0.420
<i>Sex x Day x LogConc</i>					
<i>Male x 3 x LogConc</i>	0.125	0.087	1,445	-0.045 – 0.295	0.150
<i>Male x 5 x LogConc</i>	0.179	0.087	1,445	0.009 – 0.349	0.039
<i>Sex x Status x LogConc</i>					
<i>Male x Unmated x LogConc</i>	0.028	0.087	1,445	-0.142 – 0.198	0.748
<i>Day x Status x LogConc</i>					
<i>3 x Unmated x LogConc</i>	-0.044	0.086	1,445	-0.214 – 0.126	0.610
<i>5 x Unmated x LogConc</i>	-0.128	0.087	1,445	-0.298 – 0.042	0.140
<i>Sex x Day x Status x LogConc</i>					
<i>Male x 3 x Unmated x LogConc</i>	-0.118	0.123	1,445	-0.360 – 0.123	0.336
<i>Male x 5 x Unmated x LogConc</i>	-0.040	0.123	1,445	-0.281 – 0.200	0.743
<i>Date (Random Effect SD)</i>	0.449			–	
<i>Residual SD</i>	0.549			–	
<i>No. Obs.</i>	1,530			–	
<i>Sigma</i>	0.549			–	

Abbreviations: CI = Confidence Interval, SE = Standard Error

Table 4.2. Summary of statistical results and marginal means for standardized antennal response by *P. interpunctella* depending on age (day) and sex. Slopes (β) represent the rate of change in antennal responses across a ZETA log-concentration gradient. Trends are partitioned by sex and developmental age, without regard to mating status and handedness of antennae. *P*-values and *t*-ratios test the hypothesis that the slope is significantly different from zero, which are shown in grey (Tukey HSD, $\alpha = 0.05$).

<i>Day</i>	<i>Sex</i>	Slope (β)	SE	df	95% CI (mV)	t-ratio	<i>p</i>-value
1	Female	0.014	0.031	1,446	-0.046 – 0.074	0.455	0.649
3	Female	0.005	0.030	1,446	-0.055 – 0.064	0.149	0.882
5	Female	-0.013	0.031	1,446	-0.073 – 0.047	-0.422	0.673
1	Male	0.043	0.031	1,446	-0.017 – 0.103	1.398	0.162
3	Male	0.099	0.031	1,446	0.038 – 0.160	3.184	0.001
5	Male	0.175	0.031	1,446	0.115 – 0.235	5.701	>.001

Table 4.3. Pairwise comparisons of age-related antennal responsiveness. Estimated marginal mean contrasts of standardized antennal responses (mV) for *P. interpunctella* at maximum concentration (1000 ng/ μ L) across physiological states. Positive estimates indicate a decrease in response as the moth ages, while negative estimates indicate an increase. *P*-values and t-ratios indicate significance of the difference between age groups within each category, with significant findings highlighted in grey and adjusted using Tukey HSD method ($\alpha = 0.05$).

Age Comparison	Status	Sex	Estimate (mV)	SE	df	t-ratio	p-value
Day1 - Day3	Mated	Female	0.166	0.236	96.558	0.700	0.764
Day1 - Day5	Mated	Female	-0.040	0.235	95.143	-0.170	0.984
Day3 - Day5	Mated	Female	-0.205	0.173	799.536	-1.187	0.461
Day1 - Day3	Unmated	Female	-0.043	0.196	433.493	-0.219	0.974
Day1 - Day5	Unmated	Female	0.489	0.216	255.746	2.260	0.063
Day3 - Day5	Unmated	Female	0.532	0.223	205.954	2.383	0.047
Day1 - Day3	Mated	Male	-0.336	0.236	96.462	-1.423	0.333
Day1 - Day5	Mated	Male	-0.307	0.235	95.092	-1.309	0.394
Day3 - Day5	Mated	Male	0.029	0.173	799.536	0.169	0.984
Day1 - Day3	Unmated	Male	0.372	0.188	585.613	1.984	0.117
Day1 - Day5	Unmated	Male	0.079	0.220	203.025	0.361	0.931
Day3 - Day5	Unmated	Male	-0.293	0.235	171.781	-1.246	0.428

Table 4.4. Summary of the statistical results for the linear mixed-effects model examining the effect of reproductive state on the standardized antennal response of both male and female *P. interpunctella* depending on sex, state, ZETA concentration (LogConc), and handedness of antennae, and each second through third order interaction terms. Model parameters were estimated using Restricted Maximum Likelihood (REML) with degrees of freedom and *p*-values calculated by Satterthwaite's method ($n = 1,530$). Random effects included collection date. Significance was determined at $\alpha = 0.05$ (shaded grey).

Predictor	Coefficient (β)	SE	df	95% CI (mV)	p-value
(Intercept)	-0.114	0.109	96	-0.331 – 0.102	0.297
Sex					
Female	—	—		—	
Male	0.233	0.078	1,445	0.079 – 0.387	0.003
State					
Mated_Neutral	—	—		—	
Mated_Calling	-0.275	0.353	1,478	-0.968 – 0.418	0.436
Mated_Laying	0.074	0.116	1,486	-0.155 – 0.302	0.527
Unmated_Neutral	-0.060	0.192	257	-0.438 – 0.319	0.757
Unmated_Calling	0.038	0.147	105	-0.255 – 0.330	0.799
LogConc	-0.001	0.030	1,445	-0.059 – 0.057	0.979
Antenna					
Left	—	—		—	
Right	0.142	0.032	1,500	0.079 – 0.206	<0.001
Sex x State					
Male x Mated_Calling	0.103	0.463	1,445	-0.806 – 1.012	0.824
Male x Mated_Laying	-0.132	0.149	1,445	-0.424 – 0.160	0.376
Male x Unmated_Neutral	-0.028	0.192	960	-0.404 – 0.348	0.885
Male x Unmated_Calling	-0.653	0.379	1,508	-1.397 – 0.091	0.085
Sex x LogConc					
Male x LogConc	0.045	0.042	1,445	-0.038 – 0.127	0.287
State x LogConc					
Mated_Calling x LogConc	0.071	0.175	1,445	-0.273 – 0.414	0.686
Mated_Laying x LogConc	-0.024	0.056	1,445	-0.135 – 0.086	0.668
Unmated_Neutral x LogConc	0.078	0.068	1,445	-0.055 – 0.211	0.250
Unmated_Calling x LogConc	-0.003	0.040	1,445	-0.082 – 0.076	0.937
Sex x State x LogConc					
Male x Mated_Calling x LogConc	-0.210	0.248	1,445	-0.696 – 0.275	0.396
Male x Mated_Laying x LogConc	0.281	0.080	1,445	0.125 – 0.437	<0.001
Male x Unmated_Neutral x LogConc	-0.017	0.078	1,445	-0.171 – 0.136	0.827
Male x Unmated_Calling x LogConc	-0.086	0.180	1,445	-0.439 – 0.266	0.632
Date (Random Effect SD)	0.450				
Residual SD	0.546				
No. Obs.	1,530				
Sigma	0.546				

Abbreviations: CI = Confidence Interval, SE = Standard Error

Table 4.5. Estimated marginal mean contrasts of standardized antennal responses (mV) for *P. interpunctella* at the highest ZETA concentration (e.g. 1000 ng) depending on reproductive states and split by sex. *P*-values and t-ratios test the hypothesis that the slope is significantly different from zero, which are shown in grey (Tukey HSD, $\alpha = 0.05$).

<i>Reproductive State Comparison</i>	<i>Sex</i>	<i>Difference (mV)</i>	<i>SE</i>	<i>df</i>	<i>t-ratio</i>	<i>p-value</i>
Female						
<i>Mated_Neutral vs. Mated_Calling</i>	Female	0.063	0.353	1,480	0.177	1.000
<i>Mated_Neutral vs. Mated_Laying</i>	Female	-0.001	0.116	1,486	-0.011	1.000
<i>Mated_Neutral vs. Unmated_Neutral</i>	Female	-0.175	0.192	270	-0.907	0.894
<i>Mated_Neutral vs. Unmated_Calling</i>	Female	-0.028	0.148	111	-0.190	1.000
<i>Mated_Calling vs. Mated_Laying</i>	Female	-0.064	0.365	1,484	-0.175	1.000
<i>Mated_Calling vs. Unmated_Neutral</i>	Female	-0.237	0.394	1,292	-0.602	0.975
<i>Mated_Calling vs. Unmated_Calling</i>	Female	-0.091	0.375	1,267	-0.242	0.999
<i>Mated_Laying vs. Unmated_Neutral</i>	Female	-0.173	0.209	356	-0.829	0.921
<i>Mated_Laying vs. Unmated_Calling</i>	Female	-0.027	0.169	183	-0.159	1.000
<i>Unmated_Neutral vs. Unmated_Calling</i>	Female	0.147	0.145	1,508	1.011	0.850
Male						
<i>Mated_Neutral vs. Mated_Calling</i>	Male	0.590	0.354	1,480	1.670	0.453
<i>Mated_Neutral vs. Mated_Laying</i>	Male	-0.713	0.116	1,486	-6.122	>.001
<i>Mated_Neutral vs. Unmated_Neutral</i>	Male	-0.096	0.148	109	-0.646	0.967
<i>Mated_Neutral vs. Unmated_Calling</i>	Male	0.883	0.392	1,205	2.252	0.161
<i>Mated_Calling vs. Mated_Laying</i>	Male	-1.303	0.365	1,484	-3.566	0.003
<i>Mated_Calling vs. Unmated_Neutral</i>	Male	-0.686	0.374	1,266	-1.834	0.354
<i>Mated_Calling vs. Unmated_Calling</i>	Male	0.293	0.522	1,437	0.561	0.981
<i>Mated_Laying vs. Unmated_Neutral</i>	Male	0.617	0.169	179	3.648	0.003
<i>Mated_Laying vs. Unmated_Calling</i>	Male	1.596	0.400	1,228	3.986	0.001
<i>Unmated_Neutral vs. Unmated_Calling</i>	Male	0.979	0.384	1,461	2.546	0.081

Table 4.6. Summary of the statistical results for the linear mixed-effects model examining the effect of reproductive state on the standardized antennal response of female *P. interpunctella* depending on reproductive state, age, ZETA concentration (LogConc), handedness of antennae, and select second through third order interaction terms. Model parameters were estimated using Restricted Maximum Likelihood (REML) with degrees of freedom and *p*-values calculated by Satterthwaite's method (*n*= 770). Random effects including the collection date. Significance was determined at $\alpha = 0.05$ (grey).

Predictor	Coefficient (β)	SE	df	95% CI (mV)	p-value
(Intercept)	-0.032	0.181	53	-0.395 – 0.330	0.859
State					
<i>Mated_Neutral</i>	—	—		—	
<i>Mated_Calling</i>	-0.416	0.306	731	-1.015 – 0.184	0.174
<i>Mated_Laying</i>	-0.081	0.167	727	-0.410 – 0.247	0.628
<i>Unmated_Neutral</i>	-0.452	0.284	105	-1.015 – 0.111	0.115
<i>Unmated_Calling</i>	0.124	0.242	63	-0.360 – 0.607	0.611
LogConc	-0.023	0.040	704	-0.101 – 0.055	0.566
Day					
<i>1</i>	—	—		—	
<i>3</i>	-0.264	0.251	55	-0.767 – 0.238	0.296
<i>5</i>	0.024	0.263	51	-0.503 – 0.551	0.928
Antenna					
<i>Left</i>	—	—		—	
<i>Right</i>	0.156	0.037	738	0.083 – 0.229	<0.001
State x LogConc					
<i>Mated_Calling x LogConc</i>	0.037	0.144	704	-0.246 – 0.319	0.799
<i>Mated_Laying x LogConc</i>	0.002	0.080	704	-0.155 – 0.158	0.983
<i>Unmated_Neutral x LogConc</i>	0.230	0.080	704	0.073 – 0.386	0.004
<i>Unmated_Calling x LogConc</i>	0.021	0.056	704	-0.089 – 0.132	0.708
State x Day					
<i>Mated_Laying x 3</i>	0.107	0.227	726	-0.338 – 0.552	0.637
<i>Unmated_Calling x 3</i>	0.258	0.311	86	-0.361 – 0.877	0.410
<i>Mated_Laying x 5</i>	-0.266	0.351	151	-0.959 – 0.427	0.449
<i>Unmated_Neutral x 5</i>	0.449	0.409	85	-0.365 – 1.263	0.276
<i>Unmated_Calling x 5</i>	-0.291	0.362	55	-1.017 – 0.435	0.425
LogConc x Day					
<i>LogConc x 3</i>	0.012	0.058	704	-0.101 – 0.125	0.836
<i>LogConc x 5</i>	0.056	0.058	704	-0.057 – 0.169	0.328
State x LogConc x Day					

Predictor	Coefficient (β)	SE	df	95% CI (mV)	p-value
<i>Mated_Laying x LogConc x 3</i>	0.002	0.109	704	-0.212 – 0.216	0.986
<i>Unmated_Calling x LogConc x 3</i>	0.009	0.078	704	-0.144 – 0.161	0.912
<i>Mated_Laying x LogConc x 5</i>	-0.086	0.113	704	-0.308 – 0.137	0.449
<i>Unmated_Neutral x LogConc x 5</i>	-0.315	0.113	704	-0.537 – -0.093	0.005
<i>Unmated_Calling x LogConc x 5</i>	-0.091	0.080	704	-0.249 – 0.067	0.257
Date (Random Effect SD)	0.461				
Residual SD	0.436				
<i>No. Obs.</i>	770				
<i>Sigma</i>	0.436				

Abbreviations: CI = Confidence Interval, SE = Standard Error

Table 4.7. Estimated marginal trends for standardized antennal response by female *P. interpunctella* depending on reproductive state and age (day). Slopes (β) represent the rate of change in antennal response across a ZETA log-concentration gradient. Trends are partitioned by state and age, without regard to mating status and handedness of antenna. *P*-values and t-ratios test the hypothesis that the slope is significantly different from zero, which are shown in grey (Tukey HSD, $\alpha = 0.05$).

<i>State</i>	<i>Day</i>	Slope (β)	SE	df	95% CI (mV)	t-ratio	<i>p</i>-value
<i>Mated_Neutral</i>	<i>1</i>	-0.023	0.040	704	-0.101 – 0.055	-0.575	0.566
<i>Mated_Laying</i>	<i>1</i>	-0.021	0.069	704	-0.156 – 0.114	-0.307	0.759
<i>Unmated_Neutral</i>	<i>1</i>	0.207	0.069	704	0.071 – 0.342	3.000	0.003
<i>Unmated_Calling</i>	<i>1</i>	-0.002	0.040	704	-0.080 – 0.076	-0.044	0.965
<i>Mated_Neutral</i>	<i>3</i>	-0.011	0.042	704	-0.093 – 0.071	-0.263	0.793
<i>Mated_Laying</i>	<i>3</i>	-0.007	0.062	704	-0.128 – 0.114	-0.120	0.905
<i>Unmated_Calling</i>	<i>3</i>	0.019	0.034	704	-0.048 – 0.086	0.552	0.581
<i>Mated_Neutral</i>	<i>5</i>	0.033	0.042	704	-0.048 – 0.115	0.805	0.421
<i>Mated_Calling</i>	<i>5</i>	0.070	0.138	704	-0.201 – 0.341	0.509	0.611
<i>Mated_Laying</i>	<i>5</i>	-0.051	0.069	704	-0.186 – 0.085	-0.733	0.464
<i>Unmated_Neutral</i>	<i>5</i>	-0.052	0.069	704	-0.187 – 0.083	-0.757	0.449
<i>Unmated_Calling</i>	<i>5</i>	-0.037	0.040	704	-0.115 – 0.041	-0.924	0.356

Table 4.8. Summary of the statistical results for the linear mixed-effects model examining the effect of reproductive state on the standardized antennal response of male *P. interpunctella* depending on reproductive state, age, ZETA concentration (LogConc), handedness of antennae, and select second through third order interaction terms. Model parameters were estimated using Restricted Maximum Likelihood (REML) with degrees of freedom and *p*-values calculated by Satterthwaite's method (*n*= 760). Date was included as a random effect. Significance was determined at $\alpha = 0.05$ (rows are grey).

Predictor	Coefficient (β)	SE	df	95% CI (mV)	p-value
(Intercept)	0.305	0.195	59	-0.085 – 0.696	0.123
State					
<i>Mated_Neutral</i>	—	—		—	
<i>Mated_Calling</i>	0.199	0.414	734	-0.614 – 1.012	0.631
<i>Mated_Laying</i>	-0.198	0.227	731	-0.644 – 0.248	0.383
<i>Unmated_Neutral</i>	-0.291	0.258	63	-0.808 – 0.225	0.264
<i>Unmated_Calling</i>	0.269	0.610	72	-0.948 – 1.485	0.661
LogConc	-0.111	0.054	697	-0.218 – -0.004	0.042
Day					
<i>1</i>	—	—		—	
<i>3</i>	-0.294	0.271	62	-0.837 – 0.248	0.283
<i>5</i>	-0.254	0.282	56	-0.818 – 0.310	0.371
Antenna					
<i>Left</i>	—	—		—	
Right	0.164	0.052	734	0.061 – 0.266	0.002
State x LogConc					
<i>Mated_Calling x LogConc</i>	-0.275	0.197	697	-0.662 – 0.111	0.162
<i>Mated_Laying x LogConc</i>	0.414	0.109	697	0.201 – 0.628	<0.001
<i>Unmated_Neutral x LogConc</i>	0.204	0.072	697	0.063 – 0.345	0.005
<i>Unmated_Calling x LogConc</i>	-0.122	0.197	697	-0.508 – 0.264	0.535
State x Day					
<i>Mated_Laying x 3</i>	0.389	0.307	730	-0.215 – 0.992	0.206
<i>Unmated_Neutral x 3</i>	0.131	0.348	96	-0.559 – 0.822	0.707
<i>Mated_Laying x 5</i>	-0.117	0.421	204	-0.948 – 0.713	0.781
<i>Unmated_Neutral x 5</i>	0.090	0.385	56	-0.680 – 0.860	0.816
LogConc x Day					
<i>LogConc x 3</i>	0.188	0.079	697	0.033 – 0.342	0.017
<i>LogConc x 5</i>	0.291	0.079	697	0.136 – 0.445	<0.001
State x LogConc x Day					
<i>Mated_Laying x LogConc x 3</i>	-0.243	0.149	697	-0.535 – 0.049	0.103

Predictor	Coefficient (β)	SE	df	95% CI (mV)	p-value
<i>Unmated_Neutral x LogConc x 3</i>	-0.204	0.105	697	-0.410 – 0.001	0.051
<i>Mated_Laying x LogConc x 5</i>	-0.229	0.155	697	-0.533 – 0.074	0.138
<i>Unmated_Neutral x LogConc x 5</i>	-0.243	0.103	697	-0.445 – -0.041	0.019
Date (Random Effect SD)	0.460				
Residual SD	0.596				
<i>No. Obs.</i>	760				
<i>Sigma</i>	0.596				

Abbreviations: CI = Confidence Interval, SE = Standard Error

Table 4.9. Estimated marginal trends for standardized antennal response by male *P. interpunctella* depending on reproductive state and age (day). Slopes (β) represent the rate of change in antennal response across a ZETA log-concentration gradient. Trends are partitioned by state and age, without regard to mating status and the handedness of the antenna. P-values and t-ratios test the hypothesis that the slope is significantly different from zero, which are shown in grey (Tukey HSD, $\alpha = 0.05$).

<i>State</i>	<i>Day</i>	<i>Slope (β)</i>	<i>SE</i>	<i>df</i>	<i>95% CI (mV)</i>	<i>t-ratio</i>	<i>p-value</i>
<i>Mated_Neutral</i>	1	-0.111	0.054	698	-0.218 – -0.004	-2.042	0.042
<i>Mated_Calling</i>	1	NA	NA	NA	NA – NA	NA	NA
<i>Mated_Laying</i>	1	0.303	0.094	698	0.118 – 0.488	3.221	0.001
<i>Unmated_Neutral</i>	1	0.093	0.047	698	0.001 – 0.186	1.980	0.048
<i>Unmated_Calling</i>	1	NA	NA	NA	NA – NA	NA	NA
<i>Mated_Neutral</i>	3	0.077	0.057	698	-0.035 – 0.188	1.350	0.178
<i>Mated_Calling</i>	3	NA	NA	NA	NA – NA	NA	NA
<i>Mated_Laying</i>	3	0.248	0.084	698	0.083 – 0.413	2.945	0.003
<i>Unmated_Neutral</i>	3	0.076	0.050	698	-0.022 – 0.175	1.519	0.129
<i>Unmated_Calling</i>	3	-0.046	0.188	698	-0.415 – 0.324	-0.242	0.809
<i>Mated_Neutral</i>	5	0.180	0.057	698	0.068 – 0.291	3.166	0.002
<i>Mated_Calling</i>	5	-0.096	0.188	698	-0.465 – 0.274	-0.508	0.611
<i>Mated_Laying</i>	5	0.365	0.094	698	0.180 – 0.549	3.872	>.001
<i>Unmated_Neutral</i>	5	0.141	0.047	698	0.049 – 0.233	2.994	0.003
<i>Unmated_Calling</i>	5	NA	NA	NA	NA – NA	NA	NA

Table 4.10. Summary of pairwise contrasts (left-right) for antennal response by *P. interpunctella* to increasing ZETA concentrations (LogConc). Rows highlighted in grey indicate states where the antennae functioned with significant asymmetry ($p < 0.05$).

<i>contrast</i>	Sex	State	Difference (L-R)	SE	df	t-ratio	<i>p</i>-value
<i>Left - Right</i>	Female	Mated_Laying	-0.072	0.093	1435	-0.775	0.438
<i>Left - Right</i>	Male	Mated_Laying	-0.222	0.095	1435	-2.334	0.020
<i>Left - Right</i>	Female	Unmated_Neutral	-0.276	0.137	1435	-2.023	0.043
<i>Left - Right</i>	Male	Unmated_Neutral	-0.009	0.049	1435	-0.185	0.853

Chapter 5 - How does *P. interpunctella* gene transcription change in pheromone-rich environments?

Introduction

Insects can be highly responsive to complex semiochemical environments because their fitness, survival, and ability to reproduce depend on their ability to utilize volatiles in complex environments to find food and mate to avoid predators. The sensitivity and responsiveness of their olfactory systems are governed by internal physiological states such as sex, age, and mating status (Anton et al. 2007, Gadenne et al. 2016). The influence of these internal physiological states on behavior allows insects to adjust their olfactory sensitivity to prioritize essential activities such as mate location, food seeking, and oviposition, thereby enhancing survival and reproductive success in species such as the *Plodia interpunctella* (Hübner), a primary contributor to extensive post-harvest losses in grain and grain-based products (Subramanyam and Hagstrum 2011, FAO 2019). As adults, *P. interpunctella* do not feed and have a limited amount of energy for survival and reproduction, including mate-finding, mating, identifying suitable ovipositional locations, and ovipositing to ensure the success of the next generation (Imura and Sinha 1986, Sambaraju et al. 2016, Subramanyam and Cutkomp 2017). Males locate female mates by responding to a long-range female-produced pheromone whose major component is (Z,E)-9,12-tetradecadienyl acetate (e.g., ZETA) (Brady et al. 1971, Kuwahara et al. 1971, Zhu et al. 1999), followed by a complex short-range courtship (Brady et al. 1971, Kuwahara et al. 1971, McLaughlin 1982, Zhu et al. 1999, Gerken et al. 2022, Maille, Serfa Juan, et al. 2026 [Chapter 3]). ZETA perceptiveness at the periphery (i.e., in the antenna) is primarily restricted to unmated males (Kuwahara and Casida 1973, Zhu et al. 1999, Ding et al. 2022) except for the work presented in Chapter 4. Furthermore, the physiological mechanisms that influence response by *P.*

interpunctella to semiochemicals are unknown, especially how those mechanisms interact with internal states such as sex, mating status, and age of the insect.

These knowledge gaps are relevant for semiochemical-mediated behaviorally-based management tactics (Morrison et al, 2021). A primary method used against *P. interpunctella* is mating disruption, whereby high doses of synthetic pheromone are released into the environment to disrupt a pest's life cycle (Athanasassiou et al. 2025). In the case of *P. interpunctella*, mating disruption chronically augments the environment with excess ZETA to confuse males and prevent them from finding mates. These mating disruption programs against *P. interpunctella* and related stored product moths are successful at altering moth behavior and reducing reproduction (Ryne et al. 2001, 2007, Fadamiro and Baker 2002, Burks et al. 2011, Trematerra et al. 2011, Lindenmayer et al. 2025); however, we lack a fundamental understanding of how ZETA exposure interacts with the physiological state of the moth to modulate sensory responsiveness at the molecular level. Prior work has found that perception of ZETA by *P. interpunctella* may be dynamic, depending on physiological state, and not fixed (Chapter 2; Chapter 4). Though not explicitly designed to address these state-dependent shifts under semiochemical pressure, previous studies have explored characterizing the chemosensory transcriptomic repertoire in *P. interpunctella* under specific physiological and environmental constraints to identify chemosensory gene families, the pheromone receptor clade, and their orthologous gene phylogenies (Jia et al. 2018, Li et al. 2023, Chen et al. 2024). Jia et al. (2018) characterized the transcriptomic profile using only 3-day-old unmated moths, omitting sex-, and age- specific transcripts. Li et al. (2023) expanded our understanding by considering mating status, but again the lack of sex-differentiation and age still limited the resolution of their assembly. While Chen et al. (2024) incorporated the distinction of sex and mating status in

females, the mating status of males, age, or prior exposure to semiochemical cues remained undefined. To understand how state dependencies modulate moth transcriptional change in ZETA augmented environments, we conducted a differential expression analysis that simultaneously accounted for sex, age, and mating status while controlling for ZETA exposure.

Materials and Methods

Insect colony and rearing

The insect source, colonies, rearing, and sexing were conducted in the same way as in Chapter 2.

Experimental groups and pre-treatment handling

Adults were assigned to specific age cohorts (day 3 or 5 post-eclosion) based on their eclosion date (day 0). To reduce confounding olfactory noise from the diet, all adults were removed from diet-cups and transferred to clean, empty 52 mL cups 24 h prior to treatment. We also controlled mating status to determine whether mating influenced the expression levels of olfactory genes. To generate pairs of mated individuals for the “mated” treatment, testing pairs consisting of one male and one female were introduced into empty cups 24 h prior and given the opportunity to mate, while unmated individuals were transferred into empty cups to acclimate their olfactory system to the absence of diet. Successful mating was defined by copulation in a pair. For example, an individual that was to be in the treatment factor for mated day 5, was mated on day 4 and had eclosed 5 days prior. Following the 24-h olfactory acclimation period, moths were immobilized by cold anesthesia at -20°C for 5 mins to facilitate handling and exposure to volatiles.

Experimental design, olfactory exposure, and tissue sampling

We studied the effects of sex, mating status, age and exposure to volatiles (e.g., pheromone or no stimulus), and their interactions on antennal gene expression using a factorial experimental design. The same sex, mating status, and age cohort were assigned to olfactory treatments consisting of pheromone or control (e.g., no stimulus). For exposure to stimuli, groups of 50 adult *P. interpunctella* of the same age, mating status, and sex were moved to 150 mL glass media jars and exposed to either pheromone lures containing ZETA (IL-103, Insects Limited, Westfield, IN, USA; Batch No. 1625011023) or no stimulus for 1 h. Prior to exposure, the pheromone lures, which were stored at -20 °C until use, were allowed to equilibrate at room temperature for 30 min before use. Immediately following exposure to ZETA, the media bottles were submerged in liquid N₂ for 1 min to snap freeze the moths. Moths were stored at -80 °C until they were dissected. Thirty pairs of intact antennae were pooled from 15 individuals from each combination of stimulus × sex × age × mating status × treatment were ablated between the scape and pedicel on ice and collected in a 1.5 lock tube (Eppendorf Safe-lock tubes, Fisher Scientific, Bend, OR) containing 300 µL of DNA/RNA shield (cat no. R1100-250; Zymo Research, Irvine, CA). Samples were again stored at -80 °C until they could be extracted and five biological replicates were collected for each treatment combination.

RNA extraction and library preparation

Samples were homogenized with beads that had been treated with RNase Away (Thermo Scientific, Waltham, MA) in a bullet blender (Next Advance, Troy, NY) at setting 12 for 5 min and the supernatant was transferred to new nuclease free tubes. RNA extractions were performed using the Zymo Quick-RNA Miniprep Kit (cat no. R1054; Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA was eluted in 50 µL of DNase/RNase-free

water, then stored at -80°C . RNA purity was evaluated using a NanoDrop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, MA) and RNA concentrations were quantified using an RNA Broad Range Qubit Flex fluorometer (Thermo Fisher Scientific, Waltham, MA).

mRNA-enriched libraries were constructed using the Watchmaker mRNA library Prep Kit (Watchmaker Genomics, Boulder, CO) following the manufacturer's instructions. Poly(A) mRNA was isolated from magnetic capture beads, enzymatically fragmented with Frag&Prime Buffer, and synthesized into cDNA. To facilitate sequencing on the Element Aviti platform, truncated adaptors (Elemental Biosciences, San Diego, CA) were ligated to the cDNA and purified using AMPure XP beads (Beckman Coulter, Brea, CA). Library amplification and indexing were performed by 10 PCR cycles with an initial denaturation at 98°C for 45 s, followed by 10 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. This was followed by two additional cycles of denaturation at 98°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s to increase the yield of libraries with a target insert size of 290–310 bp. The final libraries were evaluated for size using a 4150 TapeStation (D5000 ScreenTape) (Agilent, Santa Clare, CA) and quantified using a Qubit Flex Fluorometer with the dsDNA High Sensitivity Assay; Thermo Fisher Scientific, Waltham, MA). Sequencing was performed to a depth of approximately 10 million reads per sample on an Elemental AVITI System using the AVITI 2 x 150 Cloudbreak chemistry (Elemental Bioscience, San Diego, CA).

Bioinformatic quality control

Sequence data was demultiplexed using Nextflow v24.04.2 (Di Tommaso et al. 2017) pipeline bases2fastq v2.3.0.2116803307 (<https://github.com/ElemBio/bases2fastq-nf>), executed with Apptainer v1.3.1 (Kurtzer et al. 2017), with adapter trimming enabled. Due to the presence

of remaining adapters after running this pipeline, a secondary pipeline, ElementCleanup (10.5281/zenodo.18762052) was used with BBDuk v38.96 (<https://sourceforge.net/projects/bbmap/>) to detect and trim trace adapter artifacts not detected by bases2fastq. FastP v0.24.0 (Chen 2023) was used to trim low-quality bases (average quality score of 20), with a minimum read length of 60 bp. Raw, trimmed, and filtered data was quality assessed using FastQC v0.12.1 (Andrews 2010), and all quality metrics were visualized and explored using MultiQC v1.22.2 (Ewels et al. 2016).

Differential expression

Read mapping and read quantification were performed using the nf-core/rnaseq pipeline v3.20.0 (Patel et al. 2025). In brief, our data was filtered with Trim Galore! V0.6.10 (Krueger et al. 2023) and filtered reads were aligned to the *P. interpunctella* genome assembly (ilPloInte3.2; GCA 027563975.2) using STAR v2.7.11b (Dobin et al. 2013). Transcript expression was then quantified with Salmon v1.10.3 (Patro et al. 2017) using the annotation (GCF 027563975.2-RS 2024 04). Additionally, comprehensive RNAseq quality control metrics were generated, including sequence bias and junction saturation using RSeQC v5.0.2 (Wang et al. 2012), feature coverage and alignment statistics using Qualimap v2.3 (García-Alcalde et al. 2012), and PCR duplication statistics using dupRadar v1.32.0 (Sayols et al. 2016)

Differential expression analysis was conducted in R v4.2.0 (R Development Core Team 2021) using the package *DESeq2* v1.46.0 (Love et al. 2014). Gene quantification from salmon was imported into R using the *tximport* v1.34.0 (Soneson et al. 2016) package and summarized at the gene level. Only genes with pseudocounts that were greater than or equal to 10 in at least three samples were included in the differential expression analysis. Initial quality control was conducted using principal component analysis (PCA) and pairwise Pearson correlations both

based on variance stabilizing transformed (VST) counts. After this initial quality control, 3 samples were removed as outliers because they did not cluster with other biological replicates from the same treatment combination in PCA, and they did not share an $r \leq 0.85$ similarity by Pearson correlation with other biological replicates within their treatment group (Table 5.1). No batch effects were identified between RNA extractions or library preps. Two design formulas were used in DESeq2, a multi-factor, $\sim \text{Sex} \times \text{Mating} \times \text{Stimulus} \times \text{Day}$, and a single-factor, $\sim \text{Stimulus}$. In both designs, the reference level was set to female, unmated, no-pheromone, day three. The multi-factor model was used to identify genes whose expression levels were influenced by factors and combinations of factors, and the single-factor model was used to perform specific treatment contrasts. Significance was determined by Wald hypothesis testing with a null hypothesis of $|\log_2\text{FC}| = 0.58$, ($\text{FC} \geq \pm 1.5$), and resulting p -values were adjusted for false discovery using the Benjamini-Hochberg method.

Term enrichment

AGAT v 1.4.2 (Dainat 2024) was used to standardize the reference annotation, filter the GFF annotation file to contain only the longest isoform of each gene, and extract protein sequences. This set of proteins was then uploaded to STRING v12.5 (Szklarczyk et al. 2025) using the online interface for GO term annotation. The background list of genes included only the protein-coding genes that passed the read mapping threshold for inclusion into the DESeq2 analysis ($n=10,150$). We tested for enrichment among sets of significant up- and down-regulated genes sets were created for each contrast of interest. Term and pathway enrichment (e.g., GO, KEGG, and Reactome) was conducted against the background for each set of factors or comparisons of interest, filtering results to significant results (adjusted p -value ≤ 0.05).

Results

Sequencing and quality control

Sequencing resulted in 1.2 to 26.4 million reads per sample, with an average of 7.0 million reads (standard deviation = 3.42 million). Sample FUF5 R1 (1.2 million reads) was removed due to low coverage compared to other samples. After the trimming of adapters and filtering of low-quality data from the remaining samples, read counts ranged from 2.9 to 25.0 million reads per sample with an average of 6.7 million (standard deviation = 3.21 million). After alignment, all samples had a greater than 99% mapping rate to the genome, with 84.2–87.5% of filtered reads mapping to exons. Salmon quantified read counts for a total of 14,864 putative genes, which were then filtered to a final list of 10,387 putative genes that met the threshold of 10 pseudo-counts in at least three samples. To investigate variability among biological replicates and relatedness of samples, pairwise Pearson correlation coefficients were visualized in a heatmap (Figure 5.1). The clustered heatmap showed two large groups separated by sex, with little clustering evident in relation to mating status, stimulus, or age effects. Moreover, Principal Component Analysis (PCA) showed clear separation by sex in PC1 (19.4%) and PC2 (13.4%) (Figure 5.2A). Although some clustering was observed by age (Figure 2B) in PC 4/5 and mating status (Figure 5.2C) in PC 6/7, overlap was also observed. There was no clear separation related to stimulus on any of the PCA plots.

Differential expression

Differential expression analyses were conducted using two different model formulas, the multifactorial (interaction) model and the single-factor model. In the multi-factor model, all main effects and their interactions had significant genes (adjusted p -value ≤ 0.05), other than the three-way interaction of Mating $\times$ Stimulus $\times$ Day (Table 5.2), which had no significant genes. The

reference level was age three unmated females exposed to the control. Sex, age, and mating status were the primary drivers of differential expression. Sex-biased differential expression (Male vs Female) was most noteworthy, with a total of 2,428 significantly differentially expressed genes (DEGs) (1,145 upregulated, 1283 downregulated). Age (3 vs 5) and mating status (Unmated vs. Mated) followed, with a total of 1,386 (778 upregulated and 608 downregulated) and 1,397 (788 upregulated, 609 downregulated) DEGs, respectively. Stimulus (Control vs Pheromone) resulted in only a total of 49 (16 upregulated and 33 downregulated) DEGs. In terms of interactions, the factors that resulted in the highest numbers of DEGs were the interactions of mating status and day (1,227 DEGs, 589 upregulated and 638 downregulated), sex and day (383 DEGs, 247 upregulated and 136 downregulated), and sex and mating status (191 DEGs, 108 upregulated and 83 downregulated), while the two-way interaction between sex and stimulus resulted in only two DEGs being downregulated. However, interestingly, when mating was added, the three-way interaction of sex, mating status, and pheromone had 15 DEGs.

We employed a single-factor model to identify the DEGs in response to ZETA (adjusted p -value < 0.05) for each tested pairwise comparison (Table 5.3). The transcriptional responses to the pheromone were highly dependent on the physiological state of the individual. The day 3 age cohort of mated males had a total of 137 DEGs compared to its age-matched control (8 upregulated and 129 downregulated), which includes approximately 10-fold more genes than the total number of DEGs detected in the unmated treatment 3 DEGs (2 upregulated and 1 downregulated) (Figure 5.3). Conversely, mated and unmated females exposed to ZETA displayed a relatively similar number of DEGs on day 3, with 32 DEGs observed in unmated vs control and 30 observed in mated (Figure 5.3). Transcriptional changes between all sex $\times$ mating

status reduced in total significant DEGs as moths aged, except in unmated males, where the total number of DEGs increased 5x (Table 5.3).

Within the chemosensory gene families, sex was the most significant driver of differential expression (Table 5.4). Expression levels of a total of 44, six, and five chemosensory genes (CGs) were influenced by sex, age, and mating status, respectively. Among the chemosensory genes whose expression was influenced by sex, there were 25 odorant receptors (ORs), 12 odorant binding proteins (OBPs), three gustatory receptors (GRs), three ionotropic receptors (IRs), and one chemosensory protein (CSP) (Table 5.4). In the multifactorial model, the presence of pheromone did not impact gene expression in any CGs. However, the single-factor contrasts suggested a limited effect on chemosensory gene expression in the presence of pheromone in mated females at day 5, which showed four differentially expressed chemosensory genes, including one OBP, one OR/GR, and one OR. These genes are biologically relevant as they encode a pheromone binding protein (*pbp*), gustatory/odorant receptor (*gr63a*), and an uncharacterized odorant receptor (LOC128683189). Mated males on day 3 showed two differentially expressed chemosensory genes, one IR (*ir8a*) and one OBP (*obp56a*). Interestingly, there were no differentially expressed CGs in any unmated individuals. Also, no sensory neuron membrane proteins were differentially expressed.

Functional enrichment

We performed enrichment analysis on contrasts related to pheromone exposure that showed differential expression of chemosensory genes. To understand the biological relevance of these changes, we identified enrichment of functional terms of the different contrasts relating to ZETA exposure and limited our analysis to contrasts that showed differentiation in chemosensory genes. In day 3, enrichment terms in DEGs associated with mated males exposed

to ZETA showed a robust overrepresentation of pathways important to the nervous system and cellular architecture. Cell-cell adhesion ($\text{FDR} = 5.78 \times 10^{-6}$) and neuroactive ligand-receptor interaction ($\text{FDR} = 6.60 \times 10^{-6}$) were the most significant, while G-protein-coupled receptor signaling showed strong overrepresentation ($\text{FDR} = 0.0016$) (Figure 5.4). The network for this contrast contained 123 nodes and 146 edges, with the average node degree of 2.37 (PPI enrichment $p\text{-value} < 0.001$), and DBSCAN clustering returned one cluster in this contrast. The cluster contained four neuropeptide-coding genes, including *dh31* (diuretic hormone), *tk* (tachykinins), *dh44* (diuretic hormone), and *sNPF* (short neuropeptide F) (Figure 5.5), which are involved in hormonal activity and mesenchymal cell differentiation. However, for pheromone- and control-exposed mated females on day 5, no enrichment terms were significant, and no gene networks were identified.

Discussion

While the long-range attraction of *P. interpunctella* and other lepidopterans to the sex pheromone ZETA is well-documented, the molecular mechanisms that modulate antennal sensitivity across different physiological states remain elusive. Understanding this plasticity requires a multifaceted approach to identify how internal signals are integrated at the transcriptional level. Using a factorial transcriptomic analysis, we demonstrated that the antennal gene expression in *P. interpunctella* is primarily shaped by sex and age, and mating status, to a lesser extent, which collectively modulate the molecular response to ZETA exposure.

Sex was the most significant driver of differential expression, with over 2,400 DEGs observed between males and females. Forty-four of these genes belonged to chemosensory gene families, likely representing a molecular basis for the divergent chemosensory responses to pheromones and other volatile stimuli between the sexes, and reinforcing findings by Chen et al.

(2024). Sexually dimorphic differences in gene expression are also likely reflected in the structures of *P. interpunctella* antennae as the sensilla vary in size, distribution, and density in males and females (Ndomo-Moualeu et al. 2014). Although the functional implications of these morphological and gene expression differences are currently not well-described for *P. interpunctella*, differences are better understood in a closely related pest of cotton. In the cotton bollworm moth (*Helioverpa armigera* [Hübner], Lepidoptera: Noctuidae), the chemosensory system exhibits sexual dimorphism in antennal morphology (Diongue et al. 2013), gene expression (Zhang et al. 2015), semiochemical transduction pathways, and glomerular response profiles in the macroglomerular complex (Wu et al. 2015, Xu et al. 2016). As demonstrated in *H. armigera*, Xu et al. (2016), pheromone discrimination is achieved through spatial separation of ORs across discrete sensilla types. For instance, HarmOR13 (detects the major pheromone component, (Z)-11-hexadecenal) in sensilla type A, while HarmOR14b (detects the minor component, (Z)-9-tetradecenal) are sensilla types B1 and B2.

Mating status significantly modulated the male transcriptional response to ZETA. At day 3, mated and unmated males showed a ten-fold difference in the number of DEGs to ZETA. But the transcriptional response was not uniformly downregulated between the mating statuses; rather, it reflected a strategic shift in the expression of chemosensory genes. For example, the gene coding for *OBP56a*, which is an odorant binding protein involved in an unknown semiochemical transport, was downregulated, but there was significant upregulation of IR8a (Ionotropic receptor co-receptor 8a), which is known to aid in detecting environmental acids and amines (Benton et al. 2009, Abuin et al. 2011). Mating status has been shown to affect the expression levels of IRs and chemosensory genes in other moths with repercussions for pheromone binding protein 1 in *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Zhang et al.

2009), and chemosensory proteins 5, 14, and 26 in *Dendrolimus punctatus* (Walker) (Lepidoptera: Lasiocampidae) (Zhang et al. 2018).

The inverse relationship of downregulating genes coding for pheromone-related transport protein while upregulating genes associated with environmental sensing suggests a post-mating shift in sensory strategy. This transition is further supported by our interaction network, which showed the enrichment of downregulated genes *sNPF* (short neuropeptide F), *dh31*, *44* (diuretic hormone), and *tk* (tachykinin). Functional studies suggest these neuropeptides act as critical behavior switches. *sNPF* has been shown to regulate peripheral olfactory sensitivity and *Orco* expression (Li et al. 2022), while *dh31* mediates the rapid transition between feeding and courtship priorities (Lin et al. 2022). Furthermore, *dh44* is a known regulator of post-copulatory physiology, specifically controlling sperm retention and ejection behaviors (Lee et al. 2015), while *tk* acts in the antennae to modulate the sensitivity of olfactory receptor neurons (Jung et al. 2013).

While these results only reflect the status difference in the male response, these transcripts are often co-expressed or functionally linked with biogenic amines like octopamine and serotonin, which are conserved regulators of insect behavior across sexes and mating status (Gatellier et al. 2004, Sakurai et al. 2014, Nässel and Zandawala 2019). Notably, octopamine agonists have been shown to suppress calling behavior in day 1 unmated females of *P. interpunctella* (Hirashima et al. 2003), suggesting that these genes associated with amine pathways may respond to mating to reconfigure behavioral responses to pheromones. There was a general reduction in the number of significant DEGs as moths aged from day 3 to day 5. By day 5, the limited energy reserves may require conservation by downregulating non-essential pathways to prioritize basic survival over any remaining reproductive efforts (Boggs 2009).

Our multifactorial results showed that the effect of pheromone on gene expression was relatively low (49 DEGs), suggesting that short-term exposure to ZETA does not result in large transcriptional changes. However, in the individual factor modeling, the day 3 mated male response was largely downregulated (129 of 137 DEGs), which supports pheromonal desensitization, especially when the effects in other internal states are considered. Interestingly, more muted transcriptional changes were observed in day 5 males. These transcriptional changes could influence the success of mating disruption tactics. Although resistance to mating disruption has not been observed in the lab or the field (Svensson et al. 2002), its continued use to modulate *P. interpunctella* populations in the field leads experts to be concerned about the evolution of resistance in field populations (Cardé 1995, Harari and Sharon 2022). Our findings support the molecular explanation for the lack of observed mating disruption resistance so far is likely due to the response of ZETA being deeply integrated into the G protein-coupled receptor (GPCR) signaling and cell-cell adhesion pathways, which are fundamental to nervous system function (Hynes and Zhao 2000, Caers et al. 2012).

To further elucidate how ZETA-rich environments influence chemosensation, future studies should compare *P. interpunctella* reared or exposed to ZETA-managed versus unmanaged environments to assess the effects of long-term exposure on antennal and chemosensory gene expression, while accounting for the potential decoupling of mRNA levels from physiological sensory adaptations. Additionally, post-translational modifications such as the phosphoproteomics of modulating odorant receptors could have an impact and should be further explored (Clark and Chen 2018). Moreover, neurophysiological investigations are needed to map the specific transduction pathways that modulate behavioral responses to pheromones. Integrating these molecular and physiological insights with behavioral analyses will deepen our

understanding of how mating disruption strategies exert their effects and clarify how internal physiological states dynamically shape pheromone perception in *P. interpunctella*.

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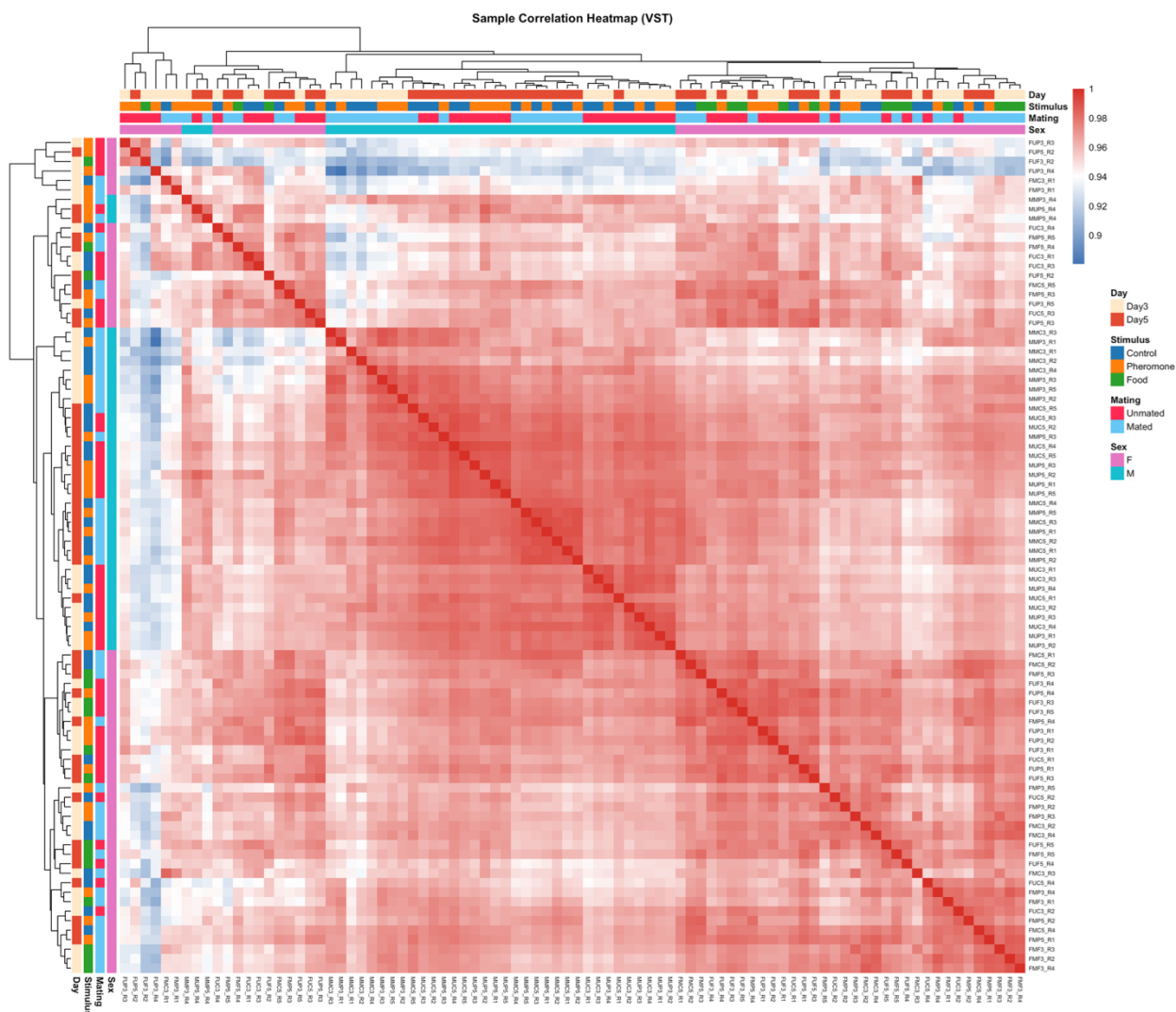


Figure 5.1. Heatmap of pairwise correlations of variance-stabilized counts of RNA-Seq libraries. Pairwise Pearson's correlation coefficients were calculated using SummarizedExperiment (Morgan et al. 2025). Read counts were normalized with the variance stabilizing transformer from DESeq with bling=TRUE, and then visualized using Phetmap (Kolde 2019) in R. Samples were clustered using complete linkage (clustering method = "complete") with Euclidean distance calculated from the pairwise Pearson correlations.

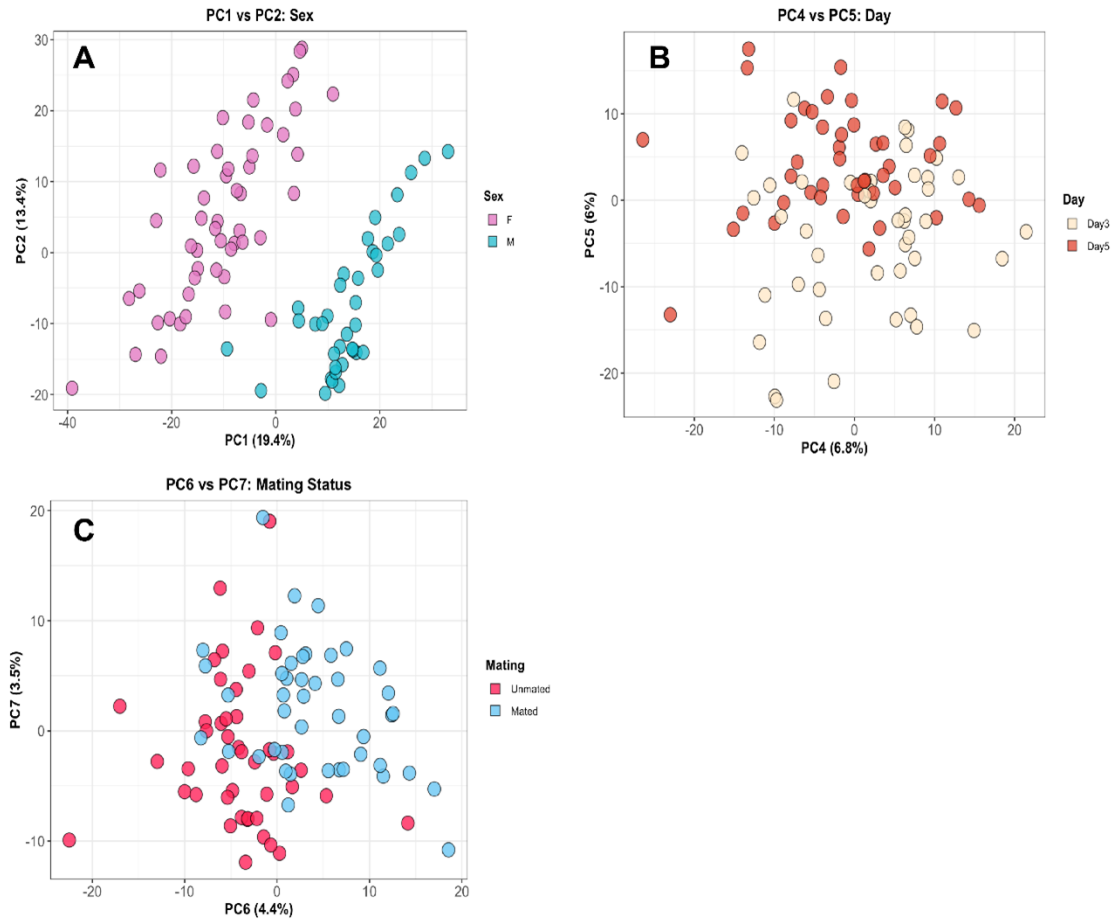


Figure 5.2. Principal component analysis (PCA) across treatments. Ordination of samples with post-hoc labeling of sample type. PC1 (19.4%) and PC2 (13.4%) (A), PC 4 (6.8%) and PC5 (6.0%) (B), and PC6 (4.4%) and PC7 (3.5%). Data points are color-coded in relation to sex, day, and mating status, respectively.

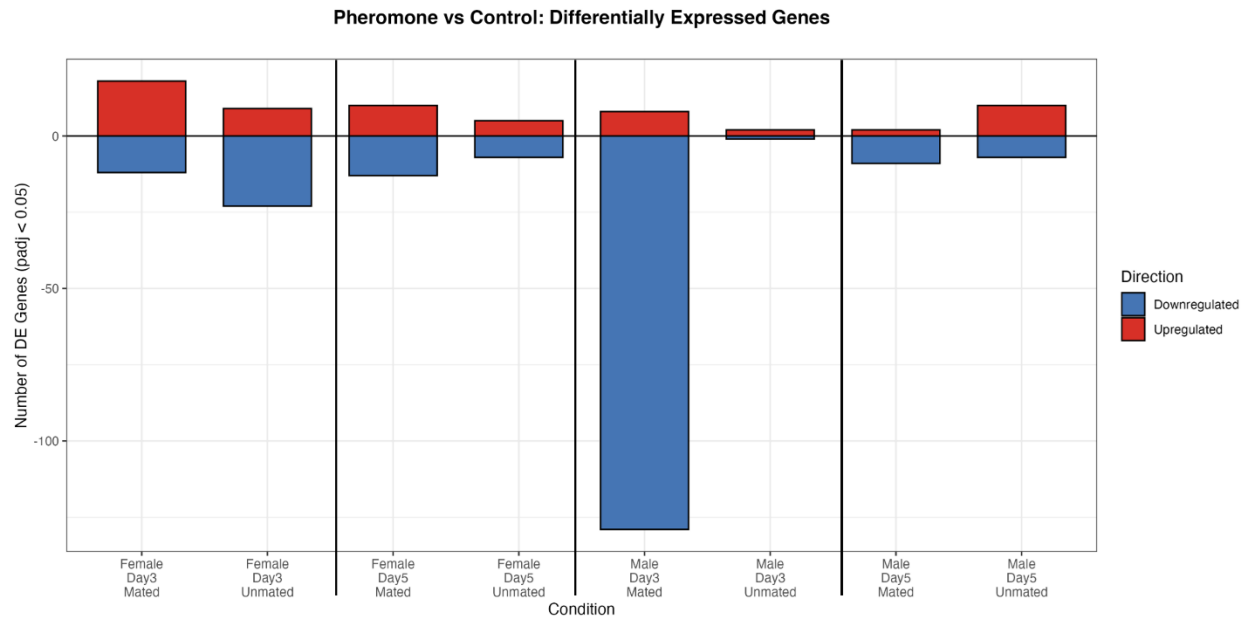


Figure 5.3. Number of differentially expressed genes (DEGs) across pairwise comparisons of sex, mating status, and age (day). Bars represent the count of genes significantly up-regulated (red) or down-regulated (blue) in stimulus groups of ZETA pheromone-exposed individuals relative to control (air). Significance was determined using an adjusted p -value threshold of <0.05 .

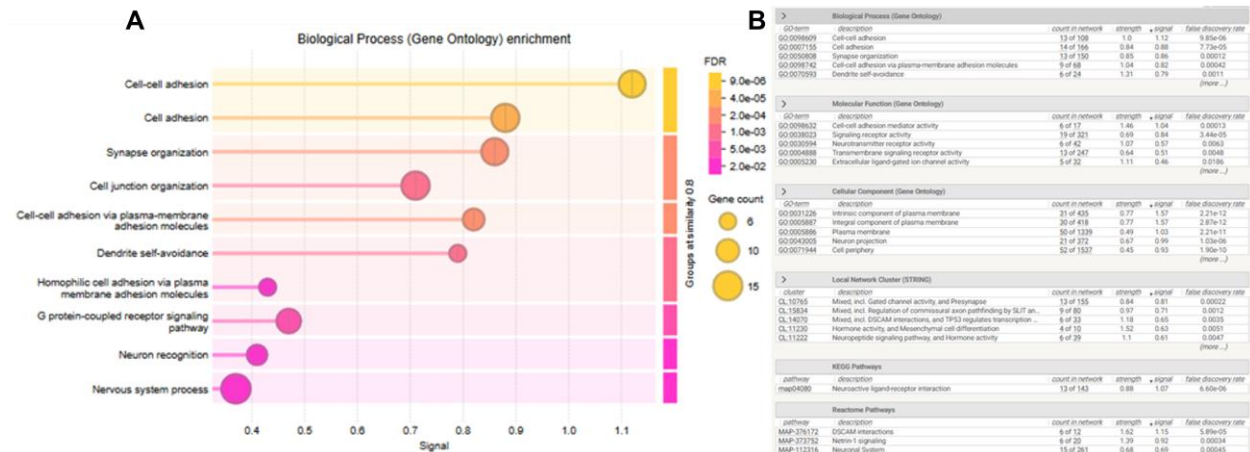


Figure 5.4. Functional enrichment of DEGs of the day 3 mated male pheromone-treated relative to its age $\times$ status-matched control. A) Bubble plot of gene ontology (GO) biological process terms plotted by enrichment signal and statistical significance. Circle size represents the number of genes identified in each category. B) Tabulated enrichment results across multiple databases (GO, KEGG, Reactome), demonstrating overrepresentation of pathways related to cell-cell adhesion (false discovery rate [FDR] = 5.78×10^{-6}), neuroactive ligand-receptor interaction (FDR = 6.60×10^{-6}), and G protein-coupled receptor (GPCR) signaling (FDR = 0.0016).

Table 5.1. Summary of experimental samples. Samples marked in bold were identified as outliers (Pearson $r \leq 0.85$; PCA non-clustering) and were excluded from downstream differential expression analysis.

Sample Name	Sex	Mating Status	Stimulus	Age (Day)	Biological Replicate
FMC3R1	Female	Mated	Control	3	R1
FMC3R2	Female	Mated	Control	3	R2
FMC3R3	Female	Mated	Control	3	R3
FMC3R4	Female	Mated	Control	3	R4
FMC3R5	Female	Mated	Control	3	R5
FMP3R1	Female	Mated	Pheromone	3	R1
FMP3R2	Female	Mated	Pheromone	3	R2
FMP3R3	Female	Mated	Pheromone	3	R3
FMP3R4	Female	Mated	Pheromone	3	R4
FMP3R5	Female	Mated	Pheromone	3	R5
MMC3R1	Male	Mated	Control	3	R1
MMC3R2	Male	Mated	Control	3	R2
MMC3R3	Male	Mated	Control	3	R3
MMC3R4	Male	Mated	Control	3	R4
MMC3R5	Male	Mated	Control	3	R5
MMP3R1	Male	Mated	Pheromone	3	R1
MMP3R2	Male	Mated	Pheromone	3	R2
MMP3R3	Male	Mated	Pheromone	3	R3
MMP3R4	Male	Mated	Pheromone	3	R4
MMP3R5	Male	Mated	Pheromone	3	R5
FMC5R1	Female	Mated	Control	5	R1
FMC5R2	Female	Mated	Control	5	R2
FMC5R3	Female	Mated	Control	5	R3
FMC5R4	Female	Mated	Control	5	R4
FMC5R5	Female	Mated	Control	5	R5
FMP5R1	Female	Mated	Pheromone	5	R1
FMP5R2	Female	Mated	Pheromone	5	R2
FMP5R3	Female	Mated	Pheromone	5	R3
FMP5R4	Female	Mated	Pheromone	5	R4
FMP5R5	Female	Mated	Pheromone	5	R5
MMC5R1	Male	Mated	Control	5	R1
MMC5R2	Male	Mated	Control	5	R2
MMC5R3	Male	Mated	Control	5	R3
MMC5R4	Male	Mated	Control	5	R4
MMC5R5	Male	Mated	Control	5	R5
MMP5R1	Male	Mated	Pheromone	5	R1
MMP5R2	Male	Mated	Pheromone	5	R2
MMP5R3	Male	Mated	Pheromone	5	R3
MMP5R4	Male	Mated	Pheromone	5	R4
MMP5R5	Male	Mated	Pheromone	5	R5
FUC3R1	Female	Unmated	Control	3	R1
FUC3R2	Female	Unmated	Control	3	R2

Sample Name	Sex	Mating Status	Stimulus	Age (Day)	Biological Replicate
FUC3R3	Female	Unmated	Control	3	R3
FUC3R4	Female	Unmated	Control	3	R4
FUC3R5	Female	Unmated	Control	3	R5
FUP3R1	Female	Unmated	Pheromone	3	R1
FUP3R2	Female	Unmated	Pheromone	3	R2
FUP3R3	Female	Unmated	Pheromone	3	R3
FUP3R4	Female	Unmated	Pheromone	3	R4
FUP3R5	Female	Unmated	Pheromone	3	R5
MUC3R1	Male	Unmated	Control	3	R1
MUC3R2	Male	Unmated	Control	3	R2
MUC3R3	Male	Unmated	Control	3	R3
MUC3R4	Male	Unmated	Control	3	R4
MUC3R5	Male	Unmated	Control	3	R5
MUP3R1	Male	Unmated	Pheromone	3	R1
MUP3R2	Male	Unmated	Pheromone	3	R2
MUP3R3	Male	Unmated	Pheromone	3	R3
MUP3R4	Male	Unmated	Pheromone	3	R4
MUP3R5	Male	Unmated	Pheromone	3	R5
FUC5R1	Female	Unmated	Control	5	R1
FUC5R2	Female	Unmated	Control	5	R2
FUC5R3	Female	Unmated	Control	5	R3
FUC5R4	Female	Unmated	Control	5	R4
FUC5R5	Female	Unmated	Control	5	R5
FUP5R1	Female	Unmated	Pheromone	5	R1
FUP5R2	Female	Unmated	Pheromone	5	R2
FUP5R3	Female	Unmated	Pheromone	5	R3
FUP5R4	Female	Unmated	Pheromone	5	R4
FUP5R5	Female	Unmated	Pheromone	5	R5
MUC5R1	Male	Unmated	Control	5	R1
MUC5R2	Male	Unmated	Control	5	R2
MUC5R3	Male	Unmated	Control	5	R3
MUC5R4	Male	Unmated	Control	5	R4
MUC5R5	Male	Unmated	Control	5	R5
MUP5R1	Male	Unmated	Pheromone	5	R1
MUP5R2	Male	Unmated	Pheromone	5	R2
MUP5R3	Male	Unmated	Pheromone	5	R3
MUP5R4	Male	Unmated	Pheromone	5	R4
MUP5R5	Male	Unmated	Pheromone	5	R5

Table 5.2. Number of differentially expressed genes (DEGs) from the multifactorial model analyzing the main effects and interactions of sex, mating status, pheromone stimulus, and age (day). The total significance (total sig; bolded and shaded grey) indicates genes with adjusted p -values ≤ 0.05 ; whereas the up and down columns indicate the number of transcripts that were up- or down-regulated, respectively, for each of the first-named factors in the pairwise coefficient column.

<i>Description</i>	<i>Coefficient</i>	Total Sig	Up	Down
Main Effects				
<i>Sex (Male vs Female)</i>	Sex: M vs F	2428	1145	1283
<i>Mating (Mated vs Unmated)</i>	Mating: Mated vs Unmated	1397	788	609
<i>Stimulus (Pheromone vs Control)</i>	Stimulus: Pheromone vs Control	49	16	33
<i>Day (Day5 vs Day3)</i>	Day: Day5 vs Day3	1386	778	608
Two-way Interactions				
<i>Sex × Mating</i>	Sex: M. × Mating: Mated	191	108	83
<i>Sex × Pheromone</i>	Sex: M × Stimulus: Pheromone	2	0	2
<i>Sex × Day</i>	Sex: M × Day: Day5	383	247	136
<i>Mating × Pheromone</i>	Mating: Mated × Stimulus: Pheromone	4	2	2
<i>Mating × Day</i>	Mating: Mated × Day: Day5	1227	589	638
<i>Day × Pheromone</i>	Stimulus: Pheromone × Day: Day5	3	1	2
Three-way Interactions				
<i>Sex × Mating × Pheromone</i>	Sex: M × Mating: Mated × Stimulus: Pheromone	15	1	14
<i>Sex × Mating × Day</i>	Sex: M × Mating: Mated × Day: Day5	63	30	33
<i>Sex × Pheromone × Day</i>	Sex: M × Stimulus: Pheromone × Day: Day5	7	6	1
<i>Mating × Pheromone × Day</i>	Mating: Mated × Stimulus: Pheromone × Day: Day5	0	0	0
Four-way Interaction				
<i>Sex × Mating × Pheromone × Day</i>	Sex: M × Mating: Mated × Stimulus: Pheromone × Day: Day5	1	0	1

Table 5.3. Number of differentially expressed genes (DEGs) in response to pheromone treatment within sex, mating status, and age (day) combinations conducted by pairwise comparisons with respect to interaction-matched individuals. The total significance (total sig; bolded and shaded grey) indicates genes with adjusted p -values ≤ 0.05 ; whereas, the upregulation (up) and downregulation (down) columns indicate the number of transcripts significantly more or less abundant, respectively, in pheromone-stimulated moths.

Sex	Mating	Day	Total Sig	Up	Down
Female	Unmated	Day3	32	9	23
Female	Unmated	Day5	12	5	7
Female	Mated	Day3	30	18	12
Female	Mated	Day5	23	10	13
Male	Unmated	Day3	3	2	1
Male	Unmated	Day5	17	10	7
Male	Mated	Day3	137	8	129
Male	Mated	Day5	11	2	9

Table 5.4. Summary of differentially expressed chemosensory genes (DEGs) identified through multifactorial analysis and pairwise comparison contrasts. Data shows the count of significantly different genes for each main effect, interaction, and specific pheromone-response contrasts across sex, mating status, and age (day) at an adjusted p -value ≤ 0.05 . Gene families in the columns include odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), odorant binding proteins (OBPs), sensory neuron membrane proteins (SNMPs), and chemosensory proteins (CSPs).

Source	Comparison	CGs	ORs	GRs	IRs	OBPs	SNMPs	CSPs
<i>multifactor</i>	Sex	44	25	3	3	12	0	1
<i>multifactor</i>	Mating	5	0	2	0	2	0	1
<i>multifactor</i>	Pheromone	0	0	0	0	0	0	0
<i>multifactor</i>	Day	6	2	0	0	4	0	0
<i>multifactor</i>	Sex $\times$ Mating	3	1	1	0	0	0	1
<i>multifactor</i>	Sex $\times$ Pheromone	0	0	0	0	0	0	0
<i>multifactor</i>	Sex $\times$ Day	1	0	0	1	0	0	0
<i>multifactor</i>	Mating $\times$ Pheromone	0	0	0	0	0	0	0
<i>multifactor</i>	Mating $\times$ Day	12	3	1	0	7	0	1
<i>multifactor</i>	Day $\times$ Pheromone	0	0	0	0	0	0	0
<i>multifactor</i>	Sex $\times$ Mating $\times$ Pheromone	0	0	0	0	0	0	0
<i>multifactor</i>	Sex $\times$ Mating $\times$ Day	3	2	0	0	1	0	0
<i>multifactor</i>	Sex $\times$ Pheromone $\times$ Day	0	0	0	0	0	0	0
<i>multifactor</i>	Mating $\times$ Pheromone $\times$ Day	0	0	0	0	0	0	0
<i>multifactor</i>	Sex $\times$ Mating $\times$ Pheromone $\times$ Day	0	0	0	0	0	0	0
Female								
<u>Unmated</u>								
<i>contrasts</i>	Pheromone Day3 vs Control Day3	0	0	0	0	0	0	0
<i>contrasts</i>	Pheromone Day5 vs Control Day5	0	0	0	0	0	0	0
<u>Mated</u>								
<i>contrasts</i>	Pheromone Day3 vs Control Day3	0	0	0	0	0	0	0
<i>contrasts</i>	Pheromone Day5 vs Control Day5	4	2	1	0	1	0	0
Male								
<u>Unmated</u>								
<i>contrasts</i>	Pheromone Day3 vs Control Day3	0	0	0	0	0	0	0
<i>contrasts</i>	Pheromone Day5 vs Control Day5	0	0	0	0	0	0	0
<u>Mated</u>								
<i>contrasts</i>	Pheromone Day3 vs Control Day3	2	0	0	1	1	0	0
<i>contrasts</i>	Pheromone Day5 vs Control Day5	0	0	0	0	0	0	0

Chapter 6 - How are *P. interpunctella* chemosensory receptors related to close relatives?

Introduction

The Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), is a pervasive global pest whose management relies largely on semiochemical-based behavioral monitoring and control. For this species, adult life is a high-stakes sensory race against time. Upon emergence, adults do not feed and must navigate vast, complex post-harvest environments to locate mates and suitable oviposition sites using limited energy reserves (Imura and Sinha 1986, Subramanyam and Cutkomp 2017). Within their limited time, individuals can fly up to 22.7 km in 24 h; however, this dispersal capacity is dynamic and inferenced by physiological conditions and the surrounding semiochemical environments (Maille, Reed, et al. 2026 [Chapter 2]). The sensitivity of *P. interpunctella* olfaction begins early in development: larvae can infest commodities by detecting trace volatile semiochemicals through packaging defects as small as a pinhole (Scheff et al. 2018).

The development of novel or improved management strategies, as well as a deeper understanding of lepidopteran chemosensory evolution, requires a comprehensive characterization of the chemosensory repertoire underlying *P. interpunctella* perception. Previous studies using transcriptomic data have identified chemosensory genes in *P. interpunctella*, revealing sex-biased expression, and identifying specific receptors tuned to olfactory cues (Jia et al. 2018, H. Li et al. 2023, Chen et al. 2024, [Chapter 5]). Structural gene models and copy number estimates for chemosensory receptor families, including olfactory receptors (ORs) ionotropic receptors (IRs), and gustatory receptors (GRs) have varied across studies, largely because fragmented transcripts assembled from short read data make it difficult

to distinguish between true orthologs and species-specific gene expansions, as was recently observed by comparing the gene models generated from a short read transcriptome of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) to gene models generated from a whole genome assembly generated with high quality PacBio long reads (Oppert et al. 2022). Unfortunately, the lack of genomic context, such as synteny, limits the ability to resolve phylogenetic relationships, identify orthologs, and determine conserved clades (Elsik et al. 2014, Childers et al. 2021).

The recent release of the chromosome-scale *P. interpunctella* genome assembly (ilPloInte3.2) from by USDA-ARS Ag100Pest initiative (Childers et al. 2021) now provides an opportunity to improve and develop the characterization of chemosensory receptors that have been previously identified in this species (Jia et al. 2018, H. Li et al. 2023, Chen et al. 2024). In this study, we manually annotated the *P. interpunctella* chemosensory repertoire (focusing on gustatory, olfactory and chemosensory gene models) and examined the phylogenetic relationships among three lepidopteran species: *P. interpunctella* (Pyralidae), *Plutella xylostella* (Plutellidae), and *Bombyx mori* (Bombycidae).

Materials and Methods

Genome assembly and quality control

The genome assembly of *P. interpunctella* (ilPloInte3.2, GCF_027563975.2) was utilized for this study. This assembly consisted of 45 scaffolds generated by the USDA-ARS Ag100Pest pipeline. The genomic data and assembly metadata were accessed through the National Center for Biotechnology Information (NCBI) database.

Chemosensory receptor gene annotation

Gene models for this assembly were predicted using NCBI's Eukaryotic Genome Annotation Pipeline (EGAP)(Thibaud-Nissen et al. 2013); however, gene model predictions for chemosensory genes are difficult due to their high sequence divergence, even between closely related taxa, high copy numbers, the presence of pseudogenes, low gene expression levels, and rapid evolution (Vieira et al. 2007, Sánchez-Gracia et al. 2009, Eyun et al. 2017, Robertson 2019). Therefore, we manually curated gene models of three key receptor families that are important for olfaction and taste: gustatory receptors (GRs), odorant receptors (ORs), and ionotropic receptors (IRs) using WebApollo software (Lee et al. 2013) hosted at the i5k workspace (<https://i5k.nal.usda.gov/>). To ensure model accuracy, gene boundaries and splice sites were validated using RNA-seq reads from male and female antennae (Jia et al. 2018) that were mapped separately to the assembly using hisat2 (Kim et al. 2019) and recursive tblastn searches using complete gene models as queries to identify gene models or exons that were missed during gene prediction. To verify the structural characteristics and completeness of the annotated gene models, the presence and number of transmembrane domains were predicted using DeepTMHMM 1.0. (Hallgren et al. 2022). Only models exhibiting the characteristics of 5–7 transmembrane domains topology typical of insect chemosensory receptors were utilized for multisequence alignment and comparative analysis. Candidate gene models were consolidated and improved by the prior transcriptomic and genomic studies (Jia et al. 2018, Li et al. 2023, Chen et al. 2024).

Sequence alignment and comparative analysis

For comparative analysis, chemosensory gene models within each family were separately compiled from our *P. interpunctella* models, *P. xylostella* (Yang et al. 2017), and *B. mori*

(references used in (Li et al. 2023)). Sequences from *P. interpunctella* containing 292–467, 300–483, or 383 –1470 amino acid residues were retained for downstream analysis of GRs, ORs, and IRs, respectively. However, no restrictions were placed on the comparative species gene models. Multiple sequence alignments were performed on translated amino sequences using the Muscle 5.1 (Edgar 2004) plugin for Geneious Prime 2026.0.2 software suite (<https://www.geneious.com>).

Phylogenetic relationships were inferred using Maximum Likelihood method implemented using the FastTree v.2.1 plugin in Geneious Prime. We employed the Jones-Taylor-Thronton model of amino acid evolution with CAT approximation. Branch support was assessed using the Shimodaira-Hasegawa (SH)-like local support test with 1,000 resamples. Final phylogenetic trees for GRs, Ors, and IRs were rooted in gustatory receptor 28b (GR28b), odorant receptor co-receptor (ORCO), and ionotropic 25a (IR25a), respectively. The resulting trees were visualized and formatted within the Geneious environment.

Results

Identification and chromosomal distribution of chemosensory receptor genes

A total of 132 candidate chemosensory gene models for *P. interpunctella* were identified and manually curated, including 18, 67, and 26 GR, OR, and IR genes, respectively. Using manual curation increased the number of chemosensory gene models compared to what was generated by EGAP by 1.2, 1.4, and 2.3-fold, as in automatic annotations only 15, 47, and 11 were captured for GRs, ORs, and IRs, respectively (Table 6.1). Additionally, chemosensory genes were distributed across all chromosomes with an abundance of GRs, ORs, and IRs on chromosomes 5, 7, and 8, respectively (Figure 6.1).

Gustatory receptors

Phylogenetic analysis of the GR family identified several well-supported clades corresponding to known functional groups in Lepidoptera, including CO₂ receptors, sugar receptors, and a large, divergent collection of candidate bitter receptors. A total of 91 amino acid translations were utilized in maximum likelihood phylogenetic analysis of the GR repertoires from *P. interpunctella* (18), *B. mori* (66), and *P. xylostella* (7) (Figure 6.2).

The most supported lineage (Shimodaira-Hasegawa-like local support value = 1.0) was the sugar and CO₂-sensing clade. PintGR1, 2, and 3 were orthologous to BmorGR1, 2, and 3 in the CO₂ receptor lineage. Additionally, PintGR 4, 5, 6, 7, and 8 clustered with the sugar detecting receptor family of *B. mori* (BmorGR4, 5, 6, 7, and 8). There was an expansion of *B. mori* GRs containing BmorGR12–65. Several of the novel *P. interpunctella* candidate GRs (PintGR11, 13, and 14 (Table 6.1)) are clustered into smaller divergent groups. The *B. mori* bitter receptors (BmorGR28, 64) clustered with PintGR16a/b and PintGR18 with low nodal support (Shimodaira-Hasegawa-like support value = 0.18).

Olfactory receptors

A total of 238 amino acid translations were utilized in maximum likelihood phylogenetic analysis of the OR repertoires from *P. interpunctella* (72), *B. mori* (71), and *P. xylostella* (95), which revealed a divergent gene family characterized by several expansions and highly conserved orthologous groups (Figure 6.3). As expected, a single highly conserved clade containing the odorant receptor co-receptor (Orco) was identified with maximal node support (local support value = 1.0), establishing a well-supported root for the remaining divergent OR subfamilies. A distinct, well-supported lepidopteran pheromone receptor clade was identified with a notable expansion of *P. xylostella* ORs, which formed a sister group to the arrangement of

P. interpunctella and *B. mori* receptors. PintOR6, PintOR59, and PintOR64 clustered with the known pheromone receptors of *B. mori* (BmorOR1, BmorOR19, and BmorOR3). There was also a conserved ortholog shared between the clade containing PintOR27, BmorOR51, and PxylOR55. There was also a possible expansion for *P. interpunctella* containing PintOR10, PintOR61, PintOR62, PintOR63, and PintOR95.

Ionotropic receptors

A total of 151 amino acid translations were utilized in maximum likelihood phylogenetic analysis of the IR repertoires from *P. interpunctella* (38), *B. mori* (18), and *P. xylostella* (95) (Figure 6.4). The IR family in *P. interpunctella* exhibited a split between the conserved IRs and the expansive divergent IRs. The IR co-receptors (BmorIR8a, PxylIR8a, PintIR11) and (BmorIR76b, PxylIR76b, PintIR3) demonstrated orthology across all three species (Shimodaira-Hasegawa-like support value = 1.0). Additionally, PintIR3 and PintIR17 showed orthology to IR64a and 76b, which detect acids and amines. There was an expansion (node support = 0.63) of PintIR receptors (PintIR19a–e, PintIR21, 22, 23, 25a/b, and 26a–c).

Discussion

Despite the importance of olfaction and gustation in the life history of *P. interpunctella*, our understanding of the underlying molecular mechanisms has remained limited. Prior transcriptomic studies provided a glimpse into *P. interpunctella* chemosensation; however, these studies were inherently restricted by the absence of a high-quality genomic assembly and low expression values of some chemosensory genes. By manually annotating the chromosome-scale ilPloInte 3.2 assembly, we were able to examine the genomic architecture of these gene families and more accurately resolve the resolution on complex orthologous relationships.

By leveraging the genome, we improved the chemosensory repertoire by increasing confidence and copy number predictions across all receptor genes, but distinctly in IRs. The automatic annotation and prior transcriptomic efforts (Jia et al. 2018, Li et al. 2023, Chen et al. 2024) underestimated the diversity of *P. interpunctella* families due to the lack of genomic resources and likely due to low expression or fragmented assemblies (Childers et al. 2021). Chromosomes 5, 7, and 8 are abundant with chemosensory receptor genes and indicate that tandem duplications are likely present, as seen across other lepidopterans (Engsontia et al. 2014).

Our phylogenetic analysis showed that there was a narrow number of gustatory receptors (GRs) present in *P. interpunctella*, which was unexpected. Since *P. interpunctella* is a polyphagous pest infesting over 161 different commodities (Hagstrum et al. 2017), we hypothesized that it would have an expansion of GRs as seen in a distantly related polyphagous moth, *Spodoptera littorals* (Meslin et al. 2022) or other stored product insects like *Tribolium castaneum* (Engsontia et al. 2008) and *Rhyzopertha dominica* (Oppert et al. 2022).

The conservation of the pheromone receptor clade (PintOR6, 59, 64) with *B. mori* orthologs suggests that detecting specialized pheromone blend use is well conserved in Lepidoptera for mate finding, even though these two species utilize different sex pheromones. This stability in mate finding is particularly important for *P. interpunctella* because they have a relatively short reproductive window, and they must navigate across diverse storage landscapes to distinguish potential mates from high levels of background volatile organic compounds emitting from commodities (Maille, Dryer, et al. 2026 [Chapter 4], Maille, Reed, et al. 2026 [Chapter 2]).

There was a species-specific expansion of divergent IRs clustered on chromosome 8. The IRs that detect humidity (PintIR3, 17), and amino acids, N-methyl-D-aspartate (PintIR16, 20),

and kainite (Pint22,25a and b, 26a–c). For a pest that must locate stable microclimates within grain masses or warehouses, this expansion of IRs may provide an advantage in navigating high CO₂ and low moisture environments typical of post-harvest storage, especially in packaged products (Scheff et al. 2018).

Our findings remain constrained by the unequal quality of available chemosensory annotations across Lepidoptera. While our study utilized a chromosome-level assembly and rigorous manual curation, the chemosensory genes utilized for comparison in our phylogenetic trees often lacked structural characteristics expected of functional receptor types and were often missing transmembrane domains. This discrepancy highlights a limitation in the field in that manual curation of chemosensory gene families is not being consistently integrated into genomic or transcriptomic pipelines. The iterative nature of refining gene models is time-intensive and requires specialized training, often acting as a bottleneck for high-throughput studies. Furthermore, the lack of a centralized repository for curated chemosensory genes presents a significant challenge. Curated sets are frequently shared in supplemental materials or maintained in private personal databases, making it challenging to ensure that the most current or high-quality models are being utilized for evolutionary comparisons. However, initiatives like the i5k and USDA-ARS AgPest100 projects are combating these challenges directly. They foster collaborative environments, provide specialized manual curation training, and standardize resource sharing across research groups, which establishes necessary infrastructure for more robust and reproducible chemosensory research (Childers et al. 2019).

Though our findings have limitations due to the need to improve gene models for other lepidopteran species, our results provide insights into next-generation behavioral monitoring. For example, reverse chemical ecology studies have been successful in *Grapholita molesta* by

utilizing the sex pheromone and pheromone binding proteins to recommend an improved pheromone synergist formulation that improved trap capture by 5–6x compared to the commercial sex attractant (Liu et al. 2022). To this end, highly accurate annotations of the entire chemosensory repertoire can allow researchers to leverage reverse chemical ecology approaches to identify high-affinity ligands to create or improve semiochemical lures for the detection and management of *P. interpunctella*. Additionally, this work identifies receptors within the pheromone, sugar, amino acid, and CO₂ sensing clades. Future studies could functionally validate receptors to known semiochemicals. Ultimately, our work provides an improved genomic-based chemosensory foundation for enhanced studies regarding *P. interpunctella*.

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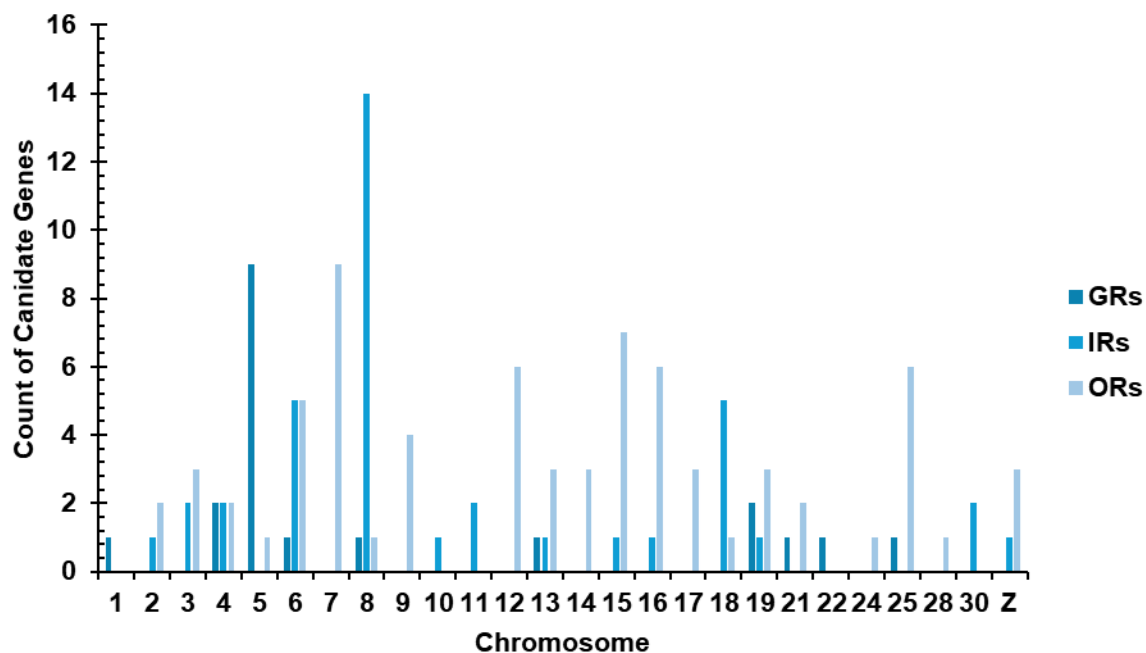


Figure 6.1. Chromosomal distribution of the chemosensory gene families in *P. interpunctella*. The total count of candidate gustatory (GRs), ionotropic (IRs), and odorant (ORs) receptors across the 30 identified autosomal chromosomes and the sex chromosome (Z).

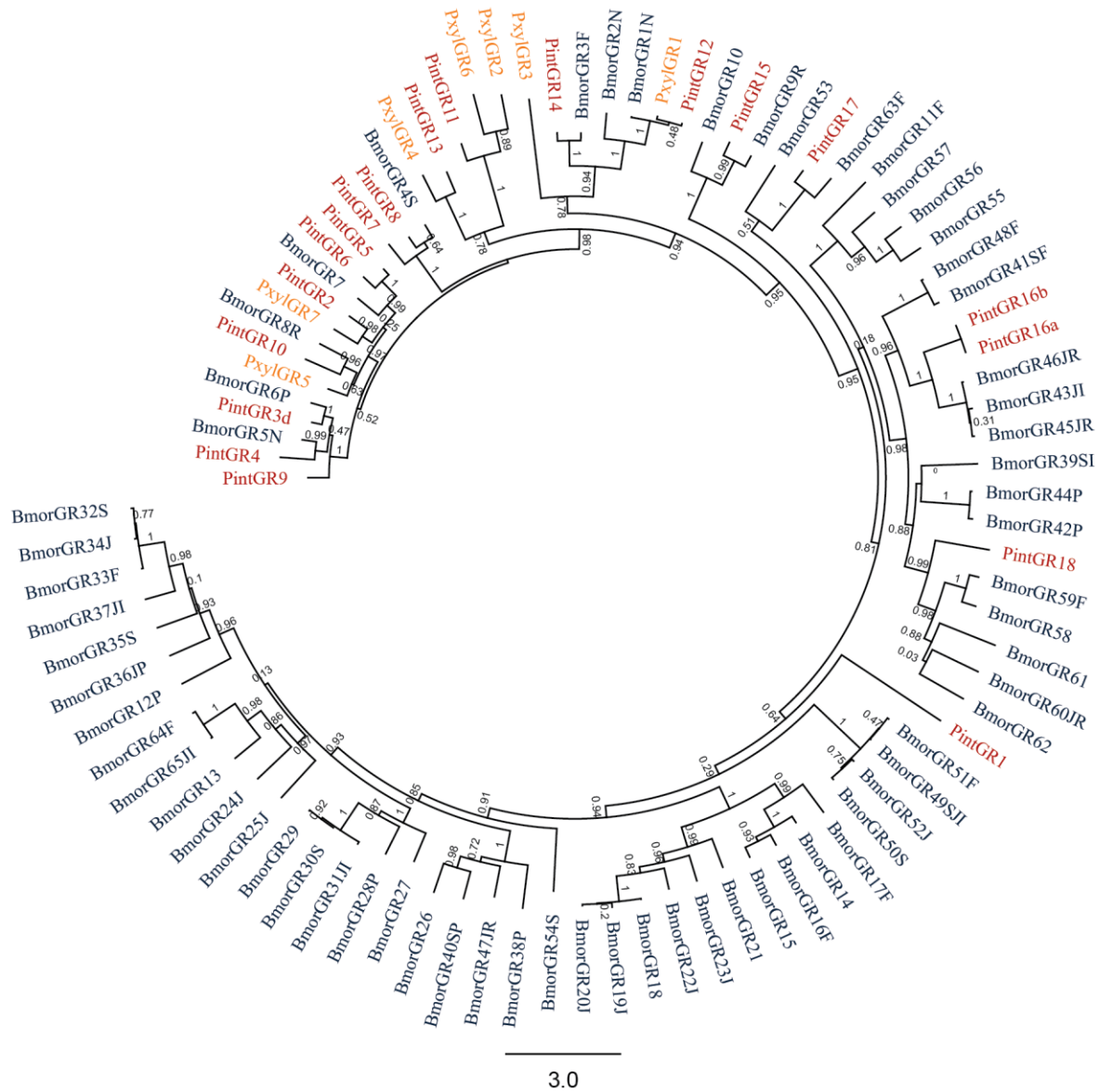


Figure 6.2. Maximum likelihood phylogeny of *P. interpunctella* gustatory receptor (GR) repertoire. The circular dendrogram demonstrates the relationship between candidate GRs of *P. interpunctella* (red), *B. mori* (black), and *P. xylostella* (orange). The scale bar represents 3.0 substitutions per residue site. Local support values are indicated at the branch nodes.

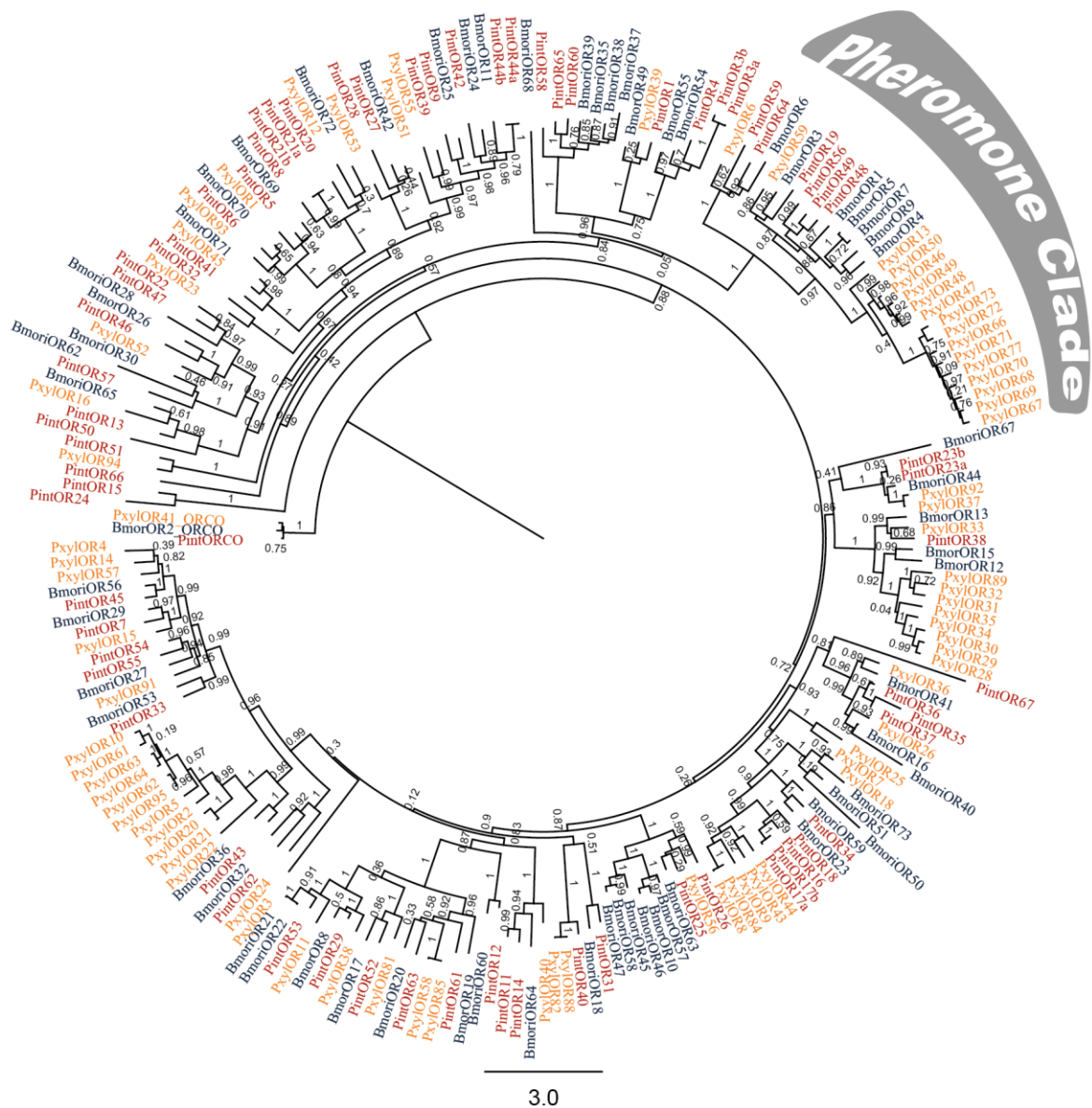


Figure 6.3. Maximum likelihood phylogeny of *P. interpunctella* odorant receptor (OR) repertoire. The circular dendrogram demonstrates the relationship between candidate GRs of *P. interpunctella* (red), *B. mori* (black), and *P. xylostella* (orange). The scale bar represents 3.0 substitutions per residue site. Local support values are indicated at the branch nodes.

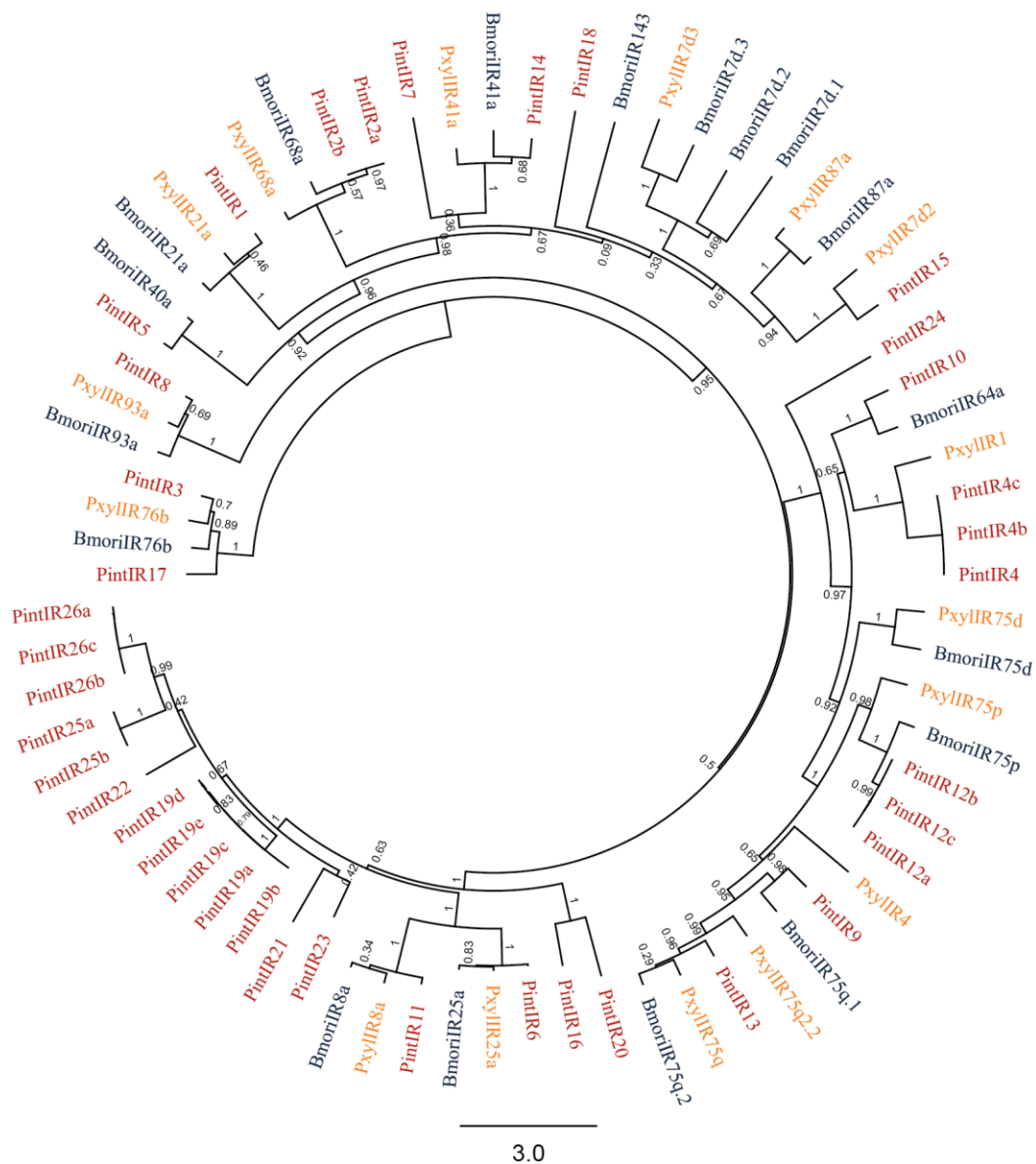


Figure 6.4. Maximum likelihood phylogeny of *P. interpunctella* ionotropic receptor (IR) repertoire. The circular dendrogram demonstrates the relationship between candidate GRs of *P. interpunctella* (red), *B. mori* (black), and *P. xylostella* (orange). The scale bar represents 3.0 substitutions per residue site. Local support values are indicated at the branch nodes.

Table 6.1. Characteristics and manual curation of *P. interpunctella* chemosensory repertoire. Summary of identified gustatory (GRs), odorant (ORs), and ionotropic (IRs) receptors. Gene model # refers to the manually assigned numeric identifier used throughout this chapter; LOC indicates the NCBI locus identifier. Chromosome and location refers to NCBI accessions and genome coordinates. Model structural data includes the number of exons, amino acid residue length (AA #), and predicted transmembrane domains (TMs). Transcriptomic evidence is categorized under RNAseq support (F: Female, M: Male, O: Overall, or MFO: all three categories). Blastp support (1=Yes, 2=Partial) is the protein blast search evidence and support by previously published studies (Jia et al. 2018, Li et al. 2023, Chen et al. 2024). Red text indicates gene models that have been manually curated, italicized text refers to novel candidate genes identified in this study, and bold text highlights receptors that are pheromone-related based on phylogenetic clustering.

Receptor Type	Gene Model #	LOC	Chromosome	NCBI Gene Accession	NCBI Protein Accession	Isoform	Exon #	AA #	T M	Location	RNAseq Support	Blastp Support	Jia et al. 2018 Receptor Name	Chen et al. 2024 Receptor Name	Li et al. 2023 Receptor Name
GR	1	128683106	1	XM_064435923.1	XP_064291993.1	1	8	467	7	NC_071294.1:6077351..6092580 (15.23 Kb)	FO	1			
GR	2	128670287	5	XM_064435940.1	XP_064292010.1	1	10	435	7	NC_071298.1:2775824..2784891 (9.07 Kb)	O	1		PintGR64e	
GR	3	128670286	5	XM_053745844.2	XP_053601819.1	4	10	357	6	NC_071298.1:2783865..2794104 (10.24 Kb)	MFO	1	PintGR6	PintGR64a	PintGR10
GR	4	128669703	5	XM_064435945.1	XP_064292015.1	1	11	391	5	NC_071298.1:2798961..2829420 (30.46 Kb)	N	1			
GR	5	128669704	5	XM_053744756.1	XP_053600731.1	1	11	392	7	NC_071298.1:2816781..2838450 (21.67 Kb)	O	1			
GR	6	128669705	5	XM_053744758.1	XP_053600733.1	1	9	336	5	NC_071298.1:2819821..2850280 (30.46 Kb)	N	2			
GR	7	128669707	5	XM_053744760.1	XP_053600735.1	1	9	406	6	NC_071298.1:2852381..2882840 (30.46 Kb)	MFO	1	PintGR2		PintGR4
GR	8	128669708	5	XM_053744761.1	XP_053600736.1	1	9	419	6	NC_071298.1:2862161..2892620 (30.46 Kb)	N	1			
GR	9	128670290	5	XM_053745851.1	XP_053601826.1	1	12	458	7	NC_071298.1:2871361..2901820 (30.46 Kb)	MO	1		PintGR64c/d	PintGR9
GR	10	128669709	5	XM_053744762.1	XP_053600737.1	1	7	292	4	NC_071298.1:2996116..3003730 (7.62 Kb)	O	1		PintGR64f	PintGR2
GR	11	128670690	6	XM_053746554.1	XP_053602529.1	1	4	418	5	NC_071299.1:6436546..6444160 (7.62 Kb)	N	1			
GR	12	128672050	8	XM_053748942.2	XP_053604917.1	1	9	462	6	NC_071301.1:3293541..3324000 (30.46 Kb)	MFO	1	PintGR3		
GR	13	128674792	13	XM_053753687.1	XP_053609662.1	1	4	399	7	NC_071306.1:3927432..3929798 (2.37 Kb)	N	1		PintGR6	
GR	14a	128678224	19	XM_053759620.2	XP_053615595.1	1	5	515	8	NC_071312.1:4279461..4291378 (11.92 Kb)	MFO	1		PintGR3	PintGR12
GR	15	128679164	21	XM_053761218.1	XP_053617193.1	1	10	459	7	NC_071314.1:6372401..6448550 (76.15 Kb)	O	1		PintGR43a1/a2	PintGR3
GR	16a	128669627	4	XM_053744557	XP_053600532.1	1	4	395	6	NC_071297.1:7209701..7225630 (15.93 Kb)	MFO	1	PintGR4		
GR	17	128680701	25	XM_053764022	XP_053619997	1	4	416	7	NC_071318.1:5934281..5950210 (15.93 Kb)	MFO	1	PintGR5	PintGR8	
GR	18	128679825	22	XM_053762282	XP_053618257.1	1	5	435	6	NC_071315.1:6157601..6173530 (15.93 Kb)	N	1			PintGR5
IR	1	128680133	2	XM_053762975.2	XP_053618950.1	1	9	837	3	NC_071295.1:9420521..9435750 (15.23 Kb)	MFO	1	PintIR2	PintIR21a/68a	PintIR8
IR	2	128683565	3	XM_053769319.1	XP_053625294.1	1	9	702	4	NC_071296.1:1210125..1216018 (5.89 Kb)	MFO	1	PintIR3		
IR	2	128683565	3	XM_053769320.1	XP_053625295.1	2	8	618	4	NC_071296.1:1210125..1216018 (5.89 Kb)	MFO	1	PintIR3		
IR	3	128669087	4	XM_053743555.1	XP_053599530.1	1	10	549	3	NC_071297.1:8387001..8402230 (15.23 Kb)	MFO	1	PintIR6		PintIR7
IR	4a	128670652	6	XM_053746482.1	XP_053602457.1	1	15	620	3	NC_071299.1:194991..207355 (12.37 Kb)	MFO	1		PintIR64a.1	PintIR1/PintIR21
IR	4b	128670652	6	XM_053746484.1	XP_053602459.1	2	15	620	3	NC_071299.1:194991..207355 (12.37 Kb)	MFO	1		PintIR64a.1	PintIR1/PintIR21
IR	4c	128670652	6	XM_053746485.1	XP_053602460.1	3	15	620	3	NC_071299.1:194991..207355 (12.37 Kb)	MFO	1		PintIR64a.1	PintIR1/PintIR21

Receptor Type	Gene Model #	LOC	Chromosome	NCBI Gene Accession	NCBI Protein Accession	Isoform	Exon #	AA #	T M	Location	RNAseq Support	Blastp Support	Jia et al. 2018 Receptor Name	Chen et al. 2024 Receptor Name	Li et al. 2023 Receptor Name
IR	5	128670875	6	XM_053746890.1	XP_053602865.1	1	15	708	3	NC_071299.1:8980801..9056950 (76.15 Kb)	MFO	1		PintlR40a	PintlR16
IR	6	128671947	8	XM_053748794.2	XP_053604769.1	1	13	932	3	NC_071301.1:2754251..2830400 (76.15 Kb)	MFO	1	PintlR5	PintlR21a.1/a.2	PintlR4/22
IR	7	135309758	8	XM_064436091.1	XP_064292161.1	1	2	622	3	NC_071301.1:9340251..9415850 (75.6 Kb)	MFO	1			
IR	8	128672070	8	XM_053748974.1	XP_053604949.1	1	19	883	3	NC_071301.1:9437441..9467900 (30.46 Kb)	MFO	1	PintlR14	PintlR93a.1/a.2/a.3	PintlR12/13
IR	9	128673397	11	XM_064436243.1	XP_064292313.1	1	8	441	3	NC_071304.1:6014341..6044800 (30.46 Kb)	MFO	1			PintlR18
IR	10	128677409	18	XM_053758213.1	XP_053614188.1	1	6	595	1	NC_071311.1:7156771..7172000 (15.23 Kb)	MFO	1			PintlR14/15
IR	11	128682487	30	XM_053767190.2	XP_053623165.1	1	19	901	4	NC_071323.1:3274766..3282325 (7.56 Kb)	MFO	1	PintlR1	PintlR8a.1	PintlR2/31
IR	12a	128677453	18	XM_053758315.2	XP_053614290.1	1	12	645	3	NC_071311.1:7759559..7768508 (8.95 Kb)	MFO	1	PintlR4		PintlR3
IR	12b	128677453	18	XM_053758316.2	XP_053614291.1	2	11	620	3	NC_071311.1:7759559..7768508 (8.95 Kb)	MFO	1	PintlR4		
IR	12c	128677453	18	XM_053758317.2	XP_053614292.1	3	11	567	3	NC_071311.1:7759559..7768508 (8.95 Kb)	MFO	1	PintlR4		
IR	13	128673874	11	XM_064436221.1	XP_064292291.1	1	19	1470	4	NC_071304.1:6026581..6058440	MFO	1	PintlR7	Pint75q	PintlR19
IR	14	128670906	6	XM_064436030.1	XP_064292100.1	1	14	822	4	NC_071299.1:5120594..5131451 (10.86 Kb)	MFO	1	PintlR8		PintlR17
IR	15	128675743	15	XM_053755353.1	XP_053611328.1	1	1	664	3	NC_071308.1:6486621..6494585 (7.96 Kb)	MFO	1	PintlR11		
IR	16	128678142	19	XM_053759484.1	XP_053615459.1	1	16	927	3	NC_071312.1:4252551..4263148 (10.6 Kb)	MFO	1		PintlR8a.2	PintlR27/45/51
IR	17	128669086	4	XM_053743554.1	XP_053599529.1	3	8	382	3	NC_071297.1:8380245..8390336 (10.09 Kb)	O	1			PintlR5
IR	18	128674641	13	XM_053753352.1	XP_053609327.1	1	2	690	3	NC_071306.1:7900086..7908050 (7.96 Kb)	MO	1			PintlR11
IR	19a	128672021	8	XM_053748905.2	XP_053604880.1	3	18	900	3	NC_071301.1:5398125..5414668 (16.54 Kb)	MFO	1			PintlR20/28
IR	19b	128672021	8	XM_053748908.2	XP_053604883.1	5	18	853	3	NC_071301.1:5398125..5414668 (16.54 Kb)	MFO	1			PintlR20/28
IR	19c	128672021	8	XM_053748903.2	XP_053604878.1	1	17	903	3	NC_071301.1:5398125..5414668 (16.54 Kb)	MFO	1			PintlR20/28
IR	19d	128672021	8	XM_053748907.2	XP_053604882.1	4	17	898	3	NC_071301.1:5398125..5414668 (16.54 Kb)	MFO	1			PintlR20/28
IR	19e	128672021	8	XM_053748904.2	XP_053604879.1	2	17	901	3	NC_071301.1:5398125..5414668 (16.54 Kb)	MFO	1			PintlR20/28
IR	20	128672955	10	XM_053750491.2	XP_053606466.1	1	15	1062	4	NC_071303.1:5841601..5857530 (15.93 Kb)	MFO	1			PintlR25/36/43
IR	21	128682762	Z	XM_053767580.1	XP_053623555.1	1	17	912	3	NC_071324.2:8386581..8418440 (31.86 Kb)	O	1			PintlR34
IR	22	128671805	8	XM_053748574.1	XP_053604549.1	1	18	931	3	NC_071301.1:155872..170289 (14.42 Kb)	MFO	1			PintlR39/47
IR	23	128682488	30	XM_053767191.2	XP_053623166.1	1	19	882	4	NC_071323.1:3166951..3246600 (79.65 Kb)	MFO	1			PintlR40
IR	24	128676810	16	XM_053757177.1	XP_053613152.1	1	11	748	3	NC_071309.1:6077457..6089958 (12.5 Kb)	O	1			PintlR41
IR	25a	128671740	8	XM_053748471.2	XP_053604446.1	1	16	905	4	NC_071301.1:5348629..5357187 (8.56 Kb)	O	1			PintlR42
IR	25b	128671740	8	XM_053748472.1	XP_053604447.1	2	16	919	4	NC_071301.1:5348629..5357187 (8.56 Kb)	O	1			PintlR42
IR	26a	128671741	8	XM_053748473.2	XP_053604448.1	1	16	916	3	NC_071301.1:5355074..5373659 (18.59 Kb)	O	1			PintlR46/50
IR	26b	128671741	8	XM_053748474.2	XP_053604449.1	2	16	914	3	NC_071301.1:5355074..5373659 (18.59 Kb)	O	1			PintlR46/50
IR	26c	128671741	8	XM_053748475.2	XP_053604450.1	3	16	903	3	NC_071301.1:5355074..5373659 (18.59 Kb)	O	1		PintlR46/50	
OR	1	128677890	2	XM_053759055.2	XP_053615030.2	1	4	467	5	NC_071311.1:4673091..4680260 (7.17 Kb)	N	1			PintOR49
OR	2	128679334	2	XM_053761481.1	XP_053617456.1	1	10	473	7	NC_071314.1:2369151..2383490 (14.34 Kb)	MFO	1	PintOR1	PintORCO	PintoOR9/42
OR	3a	128683451	3	XM_053769120.1	XP_053625095.1	2	4	387	7	NC_071296.1:11944861..11971020 (26.16 Kb)	FM	1		PintOR5	

Receptor Type	Gene Model #	LOC	Chromosome	NCBI Gene Accession	NCBI Protein Accession	Isoform	Exon #	AA #	T M	Location	RNaseq Support	Blastp Support	Jia et al. 2018 Receptor Name	Chen et al. 2024 Receptor Name	Li et al. 2023 Receptor Name
OR	3b	128683451	3	XM_053769121.1	XP_053625096.1	3	4	415	7	NC_071296.1:11944861..11971020 (26.16 Kb)	FM	1		PintOR5	PintOR41
OR	4	128683838	3	XM_053769828.1	XP_053625803.1	1	4	380	6	NC_071296.1:3613406..3620575 (7.17 Kb)	MF	1	PintOR18	PintOR4	PintOR7
OR	5	128669421	4	XM_053744244.1	XP_053600219.1	1	9	405	6	NC_071297.1:7235821..7250160 (14.34 Kb)	MFO	1			PintOR12
OR	6	128669722	4	XM_053744775.1	XP_053600750.1	1	9	412	6	NC_071298.1:6551541..6565880 (14.34 Kb)	MF	1	PintOR24	PintOR18	PintOR28
OR	7	128670610	6			1	7	370	6	NC_071299.1:11464881..11479220 (14.34 Kb)	MFO				
OR	8	128670927	5	XM_053746952.1	XP_053602927.1	1	9	421	6	NC_071299.1:10872451..10886790 (14.34 Kb)	N	2			PintOR37
OR	9	128669305	6	XM_053744047.1	XP_053600022.1	1	7	411	6	NC_071297.1:6783491..6790660 (7.17 Kb)	N	1			
OR	10	128670672	6	XM_053746520.1	XP_053602495.1	1	8	391	6	NC_071299.1:10488021..10514180 (26.16 Kb)	FMO	2		PintOR28	PintOR24
OR	11	128671183	6	XM_053747455.1	XP_053603430.1	1	9	428	6	NC_071300.1:7682541..7696880 (14.34 Kb)	MFO	1		PintOR8/PintOR43	PintOR19/22
OR	12	128671379	6	XM_053747810.1	XP_053603785.1	1	5	445	7	NC_071300.1:3019061..3033400 (14.34 Kb)	FMO	1	PintOR3	PintOR16	PintOR3
OR	13	128671324	7	XM_053747722.1	XP_053603697.1	1	8	432	6	NC_071300.1:7449711..7464050 (14.34 Kb)	MF	1			PintOR29
OR	14	128671420	7	XM_053747870.1	XP_053603845.1	1	10	430	4	NC_071300.1:7691141..7705480 (14.34 Kb)	O	1			
OR	15	128672050	7	XM_053748942.1	XP_053604917.1	1	9	462	7	NC_071301.1:3294441..3323120 (28.68 Kb)	MFO	1			
OR	16	128672399	8	XM_053749527.1	XP_053605502.1	1	9	394	6	NC_071302.1:5345341..5359680 (14.34 Kb)	N	1			PintOR39
OR	17a	128672382	9	XM_053749501.1	XP_053605476.1	1	8	400	6	NC_071302.1:5348771..5363000 (14.23 Kb)	MF	1	PintOR26		
OR	17b	128672382	9	XM_053749502.1	XP_053605477.1	2	9	395	6	NC_071302.1:5348771..5363000 (14.23 Kb)	MF	1			
OR	18	128672400	9	XM_053749528.1	XP_053605503.1	1	9	391	6	NC_071302.1:5341491..5348660 (7.17 Kb)	MF	1	PintOR29		PintOR10
OR	19	128674429	9	XM_053752942.1	XP_053608917.1	1	8	416	6	NC_071305.1:2894431..2908770 (14.34 Kb)	F	2	PintOR7		
OR	20	128674254	12	XM_053752726.1	XP_053608701.1	1	9	411	6	NC_071305.1:2125841..2152000 (26.16 Kb)	FM	1	PintOR23		
OR	21a	128674255	12	XM_053752729.1	XP_053608704.1	1	10	464	6	NC_071305.1:2107241..2133400 (26.16 Kb)	FM	1		PintOR13	
OR	21b	128674255	12	XM_053752730.1	XP_053608705.1	2	9	408	6	NC_071305.1:2107241..2133400 (26.16 Kb)	FM	1	PintOR9	PintOR13	
OR	22	128674652	12	XM_053753373.1	XP_053609348.1	1	2	398	7	NC_071306.1:217826..224995 (7.17 Kb)	MF	1	PintOR35	PintOR9	
OR	23a	128675732	12	XM_053755322.1	XP_053611297.1	1	9	429	6	NC_071308.1:4264881..4279220 (14.34 Kb)	MF	1	PintOR4	PintOR23	
OR	23b	128675732	12	XM_053755323.1	XP_053611298.1	2	9	412	6	NC_071308.1:4264881..4279220 (14.34 Kb)	MF	1		PintOR23	
OR	24	128674726	13	XM_053753558.1	XP_053609533.1	2	9	433	7	NC_071306.1:4248361..4261440 (13.08 Kb)	O	1	PintGR8		PintGR8
OR	25	128674881	13	XM_053753862.1	XP_053609837.1	1	8	392	6	NC_071306.1:975501..1004180 (28.68 Kb)	MFO	2	PintOR17/40/R47	PintOR25	PintOR14
OR	26	128674881	13	XM_053753862.2	XP_053609837.2	1	9	389	6	NC_071306.1:975501..1004180 (28.68 Kb)	MFO	2	PintOR17/40/48	PintOR26	PintOR15
OR	27	128675801	15	XM_053755483.1	XP_053611458.1	1	7	385	4	NC_071308.1:6721051..6786450 (65.4 Kb)	FM	1	PintOR31		PintOR23
OR	28	128675805	15	XM_053755488.1	XP_053611463.1	1	7	325	4	NC_071308.1:6743211..6756290 (13.08 Kb)	FM	1			PintOR11
OR	29	128675848	15	XM_053755554.1	XP_053611529.1	1	6	414	6	NC_071308.1:4232171..4246510 (14.34 Kb)	N	1			PintOR51
OR	30	128676381	15	XM_053756469.1	XP_053612444.1	1	6	184	2	NC_071309.1:4406311..4420650 (14.34 Kb)	MFO	1			PintOR27
OR	31	128676495	15	XM_053756630.1	XP_053612605.1	1	5	379	7	NC_071309.1:8702931..8717270 (14.34 Kb)	MFO	1	PintOR21		PintOR45/46
OR	32	128676535	16	XM_053756688.1	XP_053612663.1	1	5	408	6	NC_071309.1:3566606..3573775 (7.17 Kb)	F	1	PintOR11		
OR	33	128676766	16	XM_053757085.1	XP_053613060.1	1	10	399	6	NC_071295.1:60221..74560 (14.34 Kb)	MF	1	PintOR27	PintOR26	

Receptor Type	Gene Model #	LOC	Chromosome	NCBI Gene Accession	NCBI Protein Accession	Isoform	Exon #	AA #	T M	Location	RNAseq Support	Blastp Support	Jia et al. 2018 Receptor Name	Chen et al. 2024 Receptor Name	Li et al. 2023 Receptor Name
OR	34	12867819 2	16	XM_053759575.1	XP_053615550.1	1	5	403	6	NC_071312.1:2576481..2590820 (14.34 Kb)	MF	1	PintOR13	PintOR7	
OR	35	12867692 2	17	XM_053757378.1	XP_053613353.1	1	8	391	5	NC_071310.1:2394391..2400930 (6.54 Kb)	FM	1		PintOR20	PintOR18-As whole 12 TM model; this is not real; PintOR36
OR	36	12867692 2	17	XM_053757379.1	XP_053613354.1	2	7	386	7	NC_071310.1:2393646..2400185 (6.54 Kb)	FM	1		PintOR20	PintOR18-As whole 12 TM model; this is not real; PintOR36
OR	37	12867692 2	17	XM_053757381.1	XP_053613356.1	4	8	388	6	NC_071310.1:2392396..2398935 (6.54 Kb)	FM	1		PintOR20	PintOR18-As whole 12 TM model; this is not real; PintOR36
OR	38	12867868 6	18	XM_053760410.1	XP_053616385.1	1	5	388	6	NC_071295.1:7929961..7937130 (7.17 Kb)	MF	1	PintOR19	PintOR24	
OR	39	12868084 6	19	XM_053764290.1	XP_053620265.1	1	7	417	6	NC_071318.1:2032721..2047060 (14.34 Kb)	FMO	1	PintOR12	PintOR17	PintOR31
OR	40	12867951 2	21	XM_053761804.1	XP_053617779.1	1	8	451	6	NC_071314.1:6735431..6749770 (14.34 Kb)	MF	1	PintOR2		PintOR38
OR	41	12868033 4	21	XM_053763401.1	XP_053619376.1	1	10	368	6	NC_071317.1:2975961..2990300 (14.34 Kb)	N	1			PintOR1
OR	42	12868084 8	24	XM_053764298.1	XP_053620273.1	1	7	396	6	NC_071318.1:2045251..2059590 (14.34 Kb)	MFO	1	PintOR39		PintOR8
OR	43	12867101 2	25	XM_053747079.1	XP_053603054.1	1	5	400	4	NC_071299.1:8940831..8955170 (14.34 Kb)	FMO	1			
OR	44a	12868084 7	25	XM_053764293.1	XP_053620268.1	2	6	394	6	NC_071318.1:2049541..2075700 (26.16 Kb)	MO	1	PintOR43		
OR	44b	12868084 7	25	XM_053764294.1	XP_053620269.1	3	7	392	6	NC_071318.1:2049541..2075700 (26.16 Kb)	MO	1	PintOR36		PintOR17
OR	45	12868089 4	25	XM_053764370.1	XP_053620345.1	1	5	401	6	NC_071318.1:3475631..3489970 (14.34 Kb)	FMO	1	PintOR38	PintOR6	PintOR34/PintOR35
OR	46	12868181 4	25	XM_053766016.1	XP_053621991.1	1	9	408	7	NC_071321.1:1347626..1355042 (7.42 Kb)	F	1			
OR	47	12868343 8	28	XM_053769089.1	XP_053625064.1	1	2	403	5	NC_071296.1:3533611..3540725 (7.12 Kb)	MF	1	PintOR14	PintOR10	PintOR40
OR	48	12868318 9	Z	XM_053768511.1	XP_053624486.1	1	8	425	6	NC_071324.2:2887294..2902752	MFO	2	PintOR5	PintOR1	PintOR4/PintOR5
OR	49	12868318 9	Z	XM_053768511.1	XP_053624486.1	1	8	528	5	NC_071324.2:2887294..2902753	MFO	2	PintOR5	PintOR1	PintOR4/PintOR5
OR	50	12867124 8	7	XM_053747576.1	XP_053603551.1	1	8	423	6	NC_071300.1:4157145..4161933	MF	1	PintOR6	PintOR30	
OR	51	12868093 3	25	XM_053764428.1	XP_053620403.1	1	7	410	7	NC_071318.1:6145487..6154493	MF	1	PintOR8	PintOR22	PintOR30
OR	52	12867585 6	15	XM_053755566.1	XP_053611541.1	1	7	399	6	NC_071308.1:3143739..3148186	MF	1	PintOR10	PintOR27	PintOR33
OR	53	12867584 5	15	XM_053755551.1	XP_053611526.1	1	6	399	6	NC_071308.1:4475394..4479193	MF	1	PintOR15	PintOR29	
OR	54	12867848 5	19	XM_053760069.1	XP_053616044.1	1	5	394	6	NC_071312.1:2565668..2572930	MF	2	PintOR16	PintOR14/PintOR15	
OR	55	12867848 5	19	XM_053760069.1	XP_053616044.1	1	5	400	5	NC_071312.1:2565668..2572930	MF	2	PintOR16	PintOR14/PintOR15	
OR	56	12868323 7	Z	XM_053768641.1	XP_053624616.1	1	9	436	6	NC_071324.2:426580..435866	MFO	1	PintOR22		
OR	57	12867124 5	7	XM_053747569.1	XP_053603544.1	1	4	395	4	NC_071300.1:3817125..3819383	F	1	PintOR25		
OR	58	12867538 4	14	XM_053754769.1	XP_053610744.1	1	5	407	4	NC_071307.1:9201976..9205284	MF	1	PintOR28		
OR	59	12867634 5	16	XM_053756408.1	XP_053612383.1	1	8	404	6	NC_071309.1:1297125..1304115	F	1	PintOR30	PintOR3	
OR	60	12867538 3	14	XM_053754768.1	XP_053610743.1	1	4	416	5	NC_071307.1:9209352..9220252	MFO	1	PintOR32	PintOR12	
OR	61	12867114 8	7	XM_053747382.1	XP_053603357.1	1	8	384	6	NC_071300.1:10537470..10541855	MF	1	PintOR33		PintOR50
OR	62	12867682 2	16	XM_053757197.1	XP_053613172.1	1	6	410	6	NC_071309.1:5940742..5947621	MFO	1	PintOR37	PintOR19	PintOR21
OR	63	12867119 0	7	XM_053747476.1	XP_053603451.1	1	7	423	6	NC_071300.1:10561101..10650250	MFO	2	PintOR41		

Receptor Type	Gene Model #	LOC	Chromosome	NCBI Gene Accession	NCBI Protein Accession	Isoform	Exon #	AA #	T M	Location	RNAseq Support	Blastp Support	Jia et al. 2018 Receptor Name	Chen et al. 2024 Receptor Name	Li et al. 2023 Receptor Name
OR	64	128676344	16	XM_053756405.1	XP_053612380.1	1	9	408	6	NC_071309.1:1344191..1362090 (17.9 Kb)	MF	1		PintOR2	
OR	65	128675381	14	XM_053754764.1	XP_053610739.1	1	4	438	6	NC_071307.1:9232581..9241530 (8.95 Kb)	MF	1		PintOR11	
OR	66	128671275	7	XM_053747621.1	XP_053603596.1	1	5	393	6	NC_071300.1:9304896..9313845 (8.95 Kb)	MF	1		PintOR21	
OR	67	128671380	7	XM_053747812.1	XP_053603787.1	1	5	256	3	NC_071300.1:3030316..3035210 (4.89 Kb)	MFO				PintOR2

Chapter 7 - Conclusions and future directions

The integration of genetic, molecular, and behavioral frameworks in this dissertation reveals that chemosensation in *Plodia interpunctella* is a dynamic, internal state-dependent process. By investigating behavior and molecular drivers, we established that *P. interpunctella* possesses a significant active dispersal capacity, with unmated individuals capable of traversing over 22 km in 24 hours. This movement is modulated by the semiochemical environment; specifically, the combination of pheromone and food volatiles which induces significant behavioral arrestment. Chapter 2 findings suggest that management strategies should account for *P. interpunctella* having the capacity for being a landscape-level pest.

Upon arrival at a resource patch, the transition from long-range anemotaxis to successful reproduction is governed by a probabilistic state-transition system. Our Markov chain modeling identified that *P. interpunctella* courtship is characterized by redundant early-stage signals (calling and pursuit) and has a critical late-stage bottleneck: head-to-head (HH) contact. This behavior acts as a gate where female-mediated choice filters males through non-progression rather than explicit refusal serving potentially as mate assessment. Chapter 3 results shifts our understanding of courtship behavior vulnerabilities, suggesting that while early-range courtship signals are resilient and redundant, the tactile and short-range transitions represent underutilized targets for behavioral interference.

In chapter 4, we explored pheromone perception by the antenna. We documented functional antennal lateralization for the first time in this species, noting a right-handed dominance that is impacted by physiological state and reproductive behavior. Furthermore, our findings provide physiological evidence of female autodetection which provides evidence for how females interact in a pheromonal landscape. In chapter 5, we revealed that plasticity is

mirrored at the molecular level, where transcriptomic analysis revealed a possible post-mating shift in *P. interpunctella* through chemosensory receptor down regulation.

The evolutionary relationships for chemosensory receptors were hypothesized in chapter 6 through the manual curation of the ilPloInte 3.2 assembly, which identified species-specific expansions of Ionotropic Receptors (IRs) on Chromosome 8. These receptors, associated with sensing humidity, CO₂, and amino acids. While pheromone receptor clades remain evolutionarily conserved, explaining the continued efficacy of ZETA-based management. This high-quality genomic foundation provides the necessary resolution to move from broad observations to precision molecular interference.

Future research should leverage this curated chemosensory repertoire through reverse chemical ecology to identify high-affinity ligands for the expanded IR clades to identify kairomonal cues that may be developed into multi-component lures for arrestment in both sexes. Furthermore, integrating AI-driven monitoring with multi-modal disruptors, such as combining pheromones with ultrasonic or visual interference could allow for more robust integrated pest management. Ultimately, bridging the gap between fundamental chemosensory etho-neurobiology and applied behaviorally-based management will prove paramount for the sustainability of global food protection and the continued progression of integrated pest management.