ABSTRACT

Title of Thesis: STUDIES ON HOST-SEEKING BEHAVIOR AND DIFFERENTIAL CHEMOSENSORY GENE EXPRESSION IN ABOVE- AND BELOW-GROUND CULEX PIPIENS

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Culex pipiens is the primary vector of WNV. It exists as two bioforms which can hybridize in nature. I characterized the behavioral and genetic variation across eight populations collected from above- and below-ground habitats. Three of the five above-ground populations had hybrid ancestry in our single locus assay, whereas the below-ground populations did not. In choice tests, four above-ground populations were avian-seeking, while the fifth showed no clear preference. All three below-ground populations predominately sought the human host. Genetic ancestry was not correlated with host preference. Chemosensation drives host-seeking behaviors in female mosquitoes, which led me to quantify the expression of two chemosensory genes: odorant binding proteins (OBPs) 2 and 12. Both OBPs were more highly expressed in the heads of human-preferring females. While the patterns of OBP2

expression indicate that it may contribute to human host detection, OBP12 expression patterns are more consistent with odor-guided oviposition.

STUDIES ON HOST-SEEKING BEHAVIOR AND DIFFERENTIAL CHEMOSENSORY GENE EXPRESSION IN ABOVE- AND BELOW-GROUND *CULEX PIPIENS*

by

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Dedication

For my grandfather, Edward Keppler.

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Chapter 2

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List of Abbreviations

ACE-2	Acetylcholinesterase-2 gene
AG	Above-ground
BG	Below-ground
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
EIP	Extrinsic Incubation Period
ELISA	Enzyme-Linked Immunosorbent Assay
GR	Gustatory Receptor
HLC	Human Landing Catches
IACUC	Institutional Animal Care and Use Committee
IR	Ionotropic Receptor
OBP	Odorant Binding Protein
ODE	Odor Degrading Enzyme
OR	Olfactory Receptor
ORN	Olfactory Receptor Neuron
PCR	Polymerase Chain Reaction
UMD	University of Maryland
WNV	West Nile Virus

Chapter 1: A Review of the Physiological Mechanisms Underlying Mosquito Host-Seeking and Preference and their Implications for Vector-Borne Disease Transmission

Introduction

Female mosquitoes feed on the blood of vertebrates in order to obtain nutrients necessary for egg production (Mullen et al. 2009; Takken et al. 2013). In doing so, they have the potential to transmit diseases between their hosts. A mosquito's ability to find and feed upon vertebrate animals has significant implications for the epidemiology of vector-borne disease, including transmission to humans. In this review, I will discuss physiological adaptations for blood-feeding, host detection and selection, and mosquito host preference. In addition, I will examine how these factors influence vector-borne disease transmission, and how *Culex* mosquitoes contribute to West Nile virus (WNV) transmission in North America. Finally, I will conclude with exciting possibilities for future research avenues.

Morphological and Physiological Adaptations for Blood-feeding

Vertebrate blood is nutrient and protein-rich, and some necessary nutrients, such as iron, can only be obtained through blood-feeding (Harbach et al. 1988; Telang et al. 2004; Resh et al. 2009). Therefore, mosquitoes possess many adaptations that make them successful blood-feeders. Piercing and sucking

mouthparts allow female mosquitoes to access blood vessels and capillaries in host tissue (Hudson et al. 1970; Kong et al. 2010). Flexible intersegmental membranes in the abdomen allow mosquitoes to imbibe volumes of blood many times their body weight during a single feeding event (Owen et al. 1980; Mullen et al. 2009). Special proteins in mosquito saliva are secreted during blood feeding (i.e. anticoagulants, vasodilators, anti-inflammatory proteins, and analgesics) that suppress vertebrate host immunological and pain responses, thereby reducing the possibility of host detection and interruption of blood-feeding (Ribeiro et al. 2003; Arcà et al. 2005; Lehane et al. 2005; King et al. 2011).

Vertebrate blood is metabolically costly to digest (Lyimo et al. 2009). However, mosquitoes have evolved physiological mechanisms to counteract the cytotoxic effects of heme, including: heme aggregation and digestion in the midgut, neutralization through heme-binding proteins in the hemocoel, and heme excretion (Coluzzi et al. 1982; Pascoa et al. 2002; Zhou et al. 2007). These morphological and physiological adaptations give mosquitoes the stereotypical characteristics of blood-feeding parasites (Lehane et al. 2005). Yet unlike other blood-feeding arthropods, mosquitoes do not live on their vertebrate hosts. Mosquitoes must find their hosts in the landscape for these brief but reproductively important encounters (Marshall et al. 1981).

Host Detection and Selection

Behavioral and electrophysiological work has demonstrated that mosquitoes detect and respond to a number of host cues that allow them to identify their hosts in

the landscape. Such cues include: carbon dioxide (CO_2) emissions, visual appearance, local increases in temperature and humidity, and odorants released from skin and breath (Friend et al. 1977; Takken et al. 1991; Bowen et al. 1991; Bruce et al. 2005; van Breugel et al. 2015; Carde et al. 2015). Mosquitoes must integrate multiple sensory modalities as they transition from long-range to short-range detection of cues during host-seeking (Carde et al. 2010; Cummins et al. 2012; Carde et al. 2015; van Breugel et al. 2015). At long range, CO_2 receptors on the maxillary palps and photoreceptors expressed in the compound eye are engaged as the mosquito actively searches for and navigates toward a host (Kennedy et al. 1940, Reeves et al. 1953; Gillies et al. 1980; Smallegange et al. 2010; McMeniman et al 2014). CO_2 activates host-seeking behaviors, allowing mosquitoes to orient and locate a host from a great distance, and heightens responses to host-associated visual stimuli (Gillies et al. 1980; Hawkes et al. 2016). At short range, CO_2 lowers the threshold potential of the olfactory system to skin odorants, and increases the sensitivity of the peripheral chemosensory system (Dekker et al. 2011). Detection of host body heat and humidity guide landing (Takken et al. 1997), and skin odors are thought to ultimately determine host acceptance (DeGennaro et al. 2013; Cardé et al. 2015). Odor detection relies upon receptors along dendrites of olfactory receptor neurons (ORNs), which are housed in porous sensory hairs covering the antennae, palps, and labellum (Suh et al. 2014).

Insect Chemosensation

Chemoreceptors expressed on the dendrites of ORNs include Odorant Receptors (ORs), co-expressed with an essential co-receptor known as *orco*, and Ionotropic Receptors (IRs), both of which detect volatiles in the air (DeGennaro et al. 2013; Leal et al. 2013). Taste and contact sensation are mediated by Gustatory Receptors (GRs), which are also expressed on ORNs and other non-ORN neurons (Silbering et al. 2011; Suh et al. 2014). The patterning and expression of these chemoreceptors is driven by transcription factors in adult insects (Clyne et al. 1999; Tichy et al. 2008; Guo et al. 2018). Upon entering pores in the sensory hairs, some host volatiles become bound to odor-binding proteins (OBPs), which either chaperone the semiochemicals to their respective receptors or to odor degradation enzymes (Zwiebel et al. 2004; Pelletier et al. 2009). Expression of chemosensory genes is regulated by the circadian clock (Rund et al. 2011, 2013a, 2013b), and such rhythmic expression may regulate diel foraging and host receptivity rhythms described across mosquito taxa (Eilerts et al. 2018). Chemosensation is thought to be the main driver of host localization, but may also play a significant role in certain species' abilities to discriminate between preferred and undesirable hosts (DeGennaro et al. 2013; Raji et al. 2019).

Methods to Assess Mosquito Host Preference

Each mosquito species has its own unique complement of preferred vertebrate hosts which varies in space and time, and is likely driven by variation in sequence and expression of chemosensory genes. Research to elucidate patterns of host use across

mosquito species can inform and improve understanding of species' life-history traits, as host preference can directly influence the survival, reproduction, and disease transmission of a mosquito population (Mullen et al. 2009; Takken et al. 2013). Additionally, epidemiological surveillance and the successful implementation of new and targeted vector control tools requires detailed understanding of mosquito feeding behaviors. Targeted control of species with known capacities to transmit disease can greatly reduce the resources and labor required for effective vector control and minimize widespread application of broad-spectrum pesticides in the environment (WHO et al. 1999; Bhatt et al. 2015).

Broadly speaking, vector biologists use two types of approaches to quantify the host preference of a vector population: blood meal analysis and behavioral observation (Washino et al. 1983; Silver et al. 2008; Takken et al. 2013; Brugman et al. 2017). Blood meal analysis represents the most widely used method to assess host preference in mosquitoes (Day et al. 2005). It is used to identify the vertebrate hosts of wild-caught females, and is often used as a direct measure of the feeding capacity of a vector population in the field (i.e. types/range of vertebrate hosts a population will actively feed upon in nature) (Silver et al. 2008; Takken et al. 2013). Approaches to identify the origins of mosquito blood meals include serological techniques, such as precipitin tests and Enzyme Linked Immunosorbent Assays (ELISA), and/or molecular methods including multiplex PCR and DNA sequencing of blood meal contents (Gomes et al. 2001; Molaei et al. 2007; Hamer et al. 2008; Molaei et al. 2008; Pitzer et al. 2014). Depending on which sampling methodologies are used to collect blood-fed mosquitoes, results from blood meal analyses can be biased. Often,

these assays indicate that wild caught females favor the most abundant host species in the landscape, which may not accurately reflect the populations' most preferred host (Washino et al. 1983; Thiemann et al. 2012).

Behavioral observation, or direct observation of mosquito host use, can be performed under natural conditions in the field, or in laboratory settings (Chaisson et al. 2012; Takken et al. 2013). In the field, traps can be baited with host-specific odorants or odor bouquets to attract mosquitoes (Nelson et al. 1976; Jawara et al. 2011; Sukumaran et al. 2016). Additionally, vertebrate or human landing catches (HLC) can be used to collect mosquitoes attempting to directly feed on study subjects or animals placed in the environment (Reddy et al 2011; Gimnig et al. 2013). Under laboratory conditions, behavioral observations are made using dual-choice olfactometers, wind tunnels, and choice chambers (Constantini et al. 1998; Zwiebel et al. 2004; Smallegange et al. 2010). These devices generally expose mosquitoes to two or more odors simultaneously to uncover specific cues that may drive host preference (Syed et al. 2007; Takken et al. 2013). Host choice tests with live hosts may be a more natural approach for assessing the true host preference of a species, incorporating more of the complexity of host cues (other than olfactory cues) that influence selection of a host (Takken et al. 2013; Fritz et al. 2015). Integration of data from both methods can help vector biologists understand how regionally-divergent populations' feeding behaviors can affect a species' ability to transmit vector-borne diseases.

Host Preference across Culicidae

When searching for a host, some mosquitoes display preferential attraction to a specific vertebrate class or species (Mullen et al. 2009). Specialized feeding behavior can result in efficient transmission of disease between individuals of the preferred host (Costantini et al. 1999; Cohuet et al. 2010; Scott et al. 2012). For example, in sub-Saharan Africa, human malaria (*Plasmodium falciparum*) is most efficiently vectored by the specialized, highly anthropophilic species, *Anopheles gambiae sensu stricto*. (Lindsey et al. 1993; Knols et al. 1995; Costantini et al. 1998; Qui et al. 2006; Mullen et al. 2009). However, even for highly specialized species like *An. gambiae s.s.,* variation in feeding behavior has been observed; where some studies report that over half of all wild-caught females imbibed blood meals from hosts other than humans (White et al. 1973; Bøgh et al. 1998; Lefèvre et al. 2009). The species *Aedes aegypti*, the vector of Yellow Fever Virus, Dengue, and Zika, is also classically defined as a highly anthropophilic species (Heisch et al. 1959; Tempelis et al. 1975; Scott et al. 2000; Ponlawat et al. 2006; Liu-Helmersson et al. 2014). However, *Ae. aegypti* populations sampled in Hawaii, Texas, and Thailand were found to feed indiscriminately on human and non-human hosts (Tempelis et al. 1970; Scott et al. 1993; Takken et al. 2013; Olson et al. 2020). These results indicate that even in species traditionally considered as highly specialized, certain regional populations are capable of feeding on a variety of vertebrate hosts. Thus, the variation observed in blood feeding behavior is likely a function of host availability, environmental factors, and/or underlying genetic differences among regional populations (Takken et al. 2013).

The majority of mosquito species are more opportunistic, feeding indiscriminately on available hosts in the environment (Day et al. 2005; Takken et al. 2013). Mosquitoes displaying generalist feeding behaviors have the potential to transmit zoonotic diseases to humans, obtained from previous non-human blood hosts (Hamer et al. 2008; Farajollahi et al. 2011). For example, the *Culiseta melanura* mosquito can circulate Eastern Equine Encephalitis (EEE) amongst passerine birds (the amplifying host of the virus) within swamp habitats. However, when humans and large mammals move in close proximity to these environments, *Cs. melanura* mosquitoes readily feed on these newly available hosts, a behavior that can facilitate the epizootic transmission of EEE (Soghigian et al. 2018). Blood meal analysis of wild caught females demonstrated *Cx. quinquefasciatus* displays indiscriminate host feeding behaviors in nature, feeding on a variety of vertebrate hosts, including a wide range of avian and mammalian species, and even humans (Heisch et al. 1959; Reisen et al. 1979; Kumar et al. 2002; Zinser et al. 2004; Muturi et al. 2008, Janssen et al. 2015). However, depending on the regional populations analyzed, the species does appear to more readily feed upon avian hosts (Tempelis et al. 1975; Kay et al. 1985; Irby et al. 1988; Molaei et al. 2007; Muturi et al. 2008; Farajollahi et al. 2011). Because of this species' propensity to feed opportunistically, it is not surprising *Cx. quinquefasciatus* is a major vector of many zoonotic diseases, such as WNV and St. Louis Encephalitis virus in North America, and Rift Valley fever virus in Africa (Day et al. 2000; Mullen et al. 2009). In opportunistic species, it appears successful host location and selection may depend on the relative abundance of individual hosts (Day et al. 2005). Abundant hosts will be encountered and fed on more frequently than rare

hosts, possibly accounting for the significant variation in feeding observed in a single species in different geographic regions (Hess et al. 1968).

Vector-Mediated Biological Pathogen Transmission and Vectorial Capacity

Disease-causing organisms (viruses, bacteria, protozoa, helminths, and other arthropods) transmitted by mosquitoes are acquired with a blood meal (Mullen et al. 2009). A single female mosquito can take several blood-meals in her lifetime. The number of blood meals taken is greatly influenced by the physiological characteristics of the individual mosquito species, such as average lifespan and gonadotropic cycle length, host availability, and ambient temperature (which can affect follicular development rate) (Hardy et al. 1983; Lehane et al. 2005; Mullen et al. 2009). Research on *Anopheles* mosquitoes found a small number of females capable of completing as many as eight separate gonotrophic cycles, each of which follow the successful acquisition of a blood-meal (Lines et al. 1991). When mosquitoes feed on humans, they have the potential to transmit diseases from the blood of previous hosts. Successful biological transmission of pathogens by an arthropod vector is dependent on: 1) the pathogens' ability to amplify in the tissues of the invertebrate vector and a vertebrate host, 2) the arthropod vector's contact with infectious vertebrate hosts (or amplifying hosts, which have high enough titers of the pathogen in their blood to be infectious), 3) the infected arthropod vectors' ability to find and successfully transmit the pathogen to a new, uninfected vertebrate host (Weaver et al. 2004; Mullen et al. 2009; Weaver et al. 2010).

Vectorial capacity is a metric used to calculate the disease transmission efficiency of a particular mosquito population (Hardy et al. 1983; Mullen et al. 2009).

$$Vectorial\ Capacity\ (C) = ma^2VP^n / log_eP$$

Many factors can influence the Vectorial Capacity (C) of a mosquito population including: mosquito density (m), vector competence (V) (*i.e.* ability of the mosquito to acquire, maintain, and transmit the pathogen), the lifespan of the mosquito (P), the extrinsic incubation period (EIP) of the parasite (n) (i.e. the time between the initial ingestion of the pathogen by the mosquito, the pathogens' proliferation and dissemination through the body, and finally occupation of the salivary glands making the vector now infectious during future feedings), and finally, mosquito host preference (a) (MacDonald et al. 1961; MacDonald et al. 1968; Hardy et al. 1983; Mullen et al. 2009; Kramer et al. 2015).

Mosquitoes that feed on both non-human and human hosts are referred to as bridge vectors for zoonotic diseases because they act as a "bridge" between an infected reservoir (non-human host) and incidental hosts (often humans, in the case of zoonoses) (Kilpatrick et al. 2005; Medlock et al. 2005). Specialized mosquitoes play an important role in maintaining and amplifying transmission among reservoir hosts but typically do not play a role in epizootic transmission of zoonoses humans. Thus, to better understand the spread of vector-borne disease to humans, it is vital to characterize the feeding behaviors of vector species and elucidate which molecular and physiological mechanisms contribute to a vector's detection and eventual selection of a host.

Culex pipiens *as a Model to study Host Preference and its Epidemiological Consequences*

Cx. pipiens is a member of the broader taxonomic group known as the Pipiens Assemblage, which includes four distinct species: *Cx. pipiens, Cx. quinquefasciatus, Cx. australicus, and Cx. pallens* (Smith et al. 2004; Harbach et al. 2012). More recently within the United States, members of this complex have emerged as important vectors of St. Louis-, North American- and Eastern Equine- Encephalitis, and WNV (Smith et al. 2004; Thiemann et al. 2012). While *Cx. australicus* and *Cx. pallens* are found in Southern Australia, and North-Eastern Asia, respectively, *Cx. pipiens* and *Cx. quinquefasciatus* are invasive, globally-distributed species, owing their ubiquitous distributions to global human transportation and commerce networks (Farajollahi et al. 2011; Ciota et al. 2013).

Although *Cx. pipiens* is generally a more northerly distributed species and *Cx. quinquefasciatus* a more southerly distributed species, it is well documented that the two will readily hybridize in mid-latitude regions where their geographical distributions overlap (Urbanelli et al 1997; Humeres et al. 1998; Smith et al. 2004; Kilpatrick et al. 2005; Silberbush et al. 2014; Cardo et al. 2016). Both species are synanthropic, benefiting from an association with human beings and the artificial habitats and ovipositional resources they create (Reisen et al. 2012). The success of these synanthropic species can be attributed to their close evolution with human urbanization and ability to extract nutrients from standing water generated by humans and livestock (Byrne et al. 1999; Farajollahi et al. 2011). Unlike many other mosquito species, members of Pipiens Assemblage thrive in minimal amounts of water, such as

aquatic habitats created by transient puddles or containers containing 200 - 300 mL of water, with high organic content (Vinogradova et al. 2000; Mullen et al. 2009).

The single species *Cx. pipiens* exists as two unique bioforms: *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* (Spielman et al. 1957; Harbach et al. 2012; Savage et al. 2012). Although they are morphologically indistinguishable, the two bioforms are physiologically, genetically, and behaviorally divergent (Huang et al. 2009; Harbach et al. 2012; Fritz et al. 2014; 2015). While *Cx. pipiens* form *pipiens* are diapausing, anautogenous (requiring a blood meal for egg development - Figure 1), and eurygamous (mating in above ground swarms); *Cx. pipiens* form *molestus* cannot diapause, are autogenous (laying their first egg raft without a blood meal - Figure 1), and stenogomous (preferring to breed in confined, subterranean spaces) (Byrne et al. 1999; Vinogradova et al. 2000; Harbach et al. 2012; Reisen et al. 2012; Kothera et al. 2013)

Cx. pipiens are reportedly avian-seeking, while *Cx. molestus* are considered to be mammalian and human-seeking (Kent et al. 2007; Simpson et al. 2009; Harbach et al. 2012; Fritz et al. 2015). The two forms readily hybridize in nature (Spielman et al. 1957; Bahnck et al. 2006; Huang et al. 2008; Kothera et al. 2010; Farajollahi et al. 2011). Progeny of matings between the above- and below-ground forms indiscriminately feed on mammalian and avian hosts, a behavior thought to contribute to the epizootic transmission of WNV to humans in North America (Fonseca et al. 2004; Kilpatrick et al. 2007; Huang et al. 2009; Fritz et al. 2015).

West Nile virus and Epizootic Transmission by Cx. pipiens

Since its initial isolation in Uganda in 1937 by Smithburn et al., WNV (family *Flaviviridae*) has become an important cause of human and animal disease worldwide. Historically the virus was isolated to the Eastern Hemisphere, where it was well documented as causing sporadic episodes or outbreaks of illness (Zeller et al. 2004; Sambri et al. 2013). The emergence of WNV in North America is a relatively recent development; the first cases of the virus were reported in 1999 in the metropolitan area of New York City, New York (Laniciotti et al. 1999). Within three years of its emergence, WNV had spread to most of the contiguous United States, as well as Canada and Mexico (Roehrig et al. 2013). Various environmental and human-mediated conditions have facilitated the recurring, endemic, and seasonal intensification of WNV activity within the United States (Andreadis et al. 2012).

Epidemiology and the WNV Transmission Cycle in North America

Emergence of WNV occurs when and where the natural reservoir hosts, populations of the arthropod vector, and humans are all in close proximity. In North America this occurs most frequently in suburban environments (Rochlin et al. 2011). Suburban landscapes (defined as geographic regions containing a large number of single-family homes that often have a yard in close proximity to wooded or natural areas) are a preferred environment for many passerine bird species, which act as natural reservoirs of WNV (McLean et al. 2001; Reisen et al. 2005; Simpson et al. 2011; Petersen et al. 2013). One such species, the American robin (*Turdus migratorius*), is considered to be one of the most important amplifying hosts of WNV

(Hamer et al. 2009). The American robin acts as a super-spreader of the virus, disproportionately infecting more secondary contacts/mosquito-vectors than other avian hosts (Komar et al. 2003; Kilpatrick et al. 2006; Petersen et al. 2013). Additionally, unlike other passerine species, American robins do not display defensive behaviors against mosquito feeding (Darbro et al. 2007). Multiple studies analyzing the blood meal contents of wild caught North American female *Culex* mosquitoes revealed the preferred avian host of the species to be American Robins (Molaei et al. 2006; Hamer et al. 2009; Simpson et al. 2009).

WNV is naturally maintained in an enzootic cycle between birds and ornithophilic mosquitoes (those that have specialized host preference for avian species), such as *Cx. pipiens* form *pipiens* mosquitoes (Turell et al. 2001; Hamer et al. 2009). However, epizootic disease transmission (i.e. WNV spillover into human populations from non-human hosts) is thought to be facilitated by the hybrid progeny of the two *Cx. pipiens* forms (Kothera et al. 2013). Hybrids of the two *Cx. pipiens* bioforms are thought to lose their specialized preferences and are then capable and willing to bite avian and human hosts (Kilpatrick et al. 2006). Considering the endemic transmission of WNV, it is crucial to have a better understanding of the behaviors and population dynamics of vectors like *Cx. pipiens* in North-Eastern America.

Future Prospects for Vector-Borne Disease Management

Research on arthropod vectors is crucial for development of control measures that target transmission by vector species (Klempner et al. 2007; Leitner et al. 2015).

Additionally, as the rate of insecticidal resistance increases in vector species (Brogdon et al. 1998), new intervention strategies must be developed. Potentially fruitful areas of research within this system include the development of novel tools for genetic manipulation of arthropod vectors and other biological control mechanisms that could reduce vector-borne pathogen transmission (James et al. 2005; Hoffman et al. 2011; Kyrou et al. 2018). As a preventative measure, empirically-based models capable of accurately predicting future outbreaks in human populations should be developed and refined.

As chemosensation is the primary means by which female mosquitoes locate human hosts and transmit disease, identification of natural or synthetic odorants that could modulate mosquito chemosensory function could provide a novel approach to preventing vector borne disease transmission (Chen et al. 2012; Jones et al. 2012). This can be accomplished by identifying compounds capable of activating olfactory pathways associated with repellency and/ or inhibiting those responsible for attraction. Ultimately, this work could be used to develop new repellents or better odor baits for traps used to reduce mosquito populations in the wild. To develop these tools, elucidating the physiological mechanisms and molecular basis underlying mosquito host preference chemosensation is essential.

Figures: Chapter 1

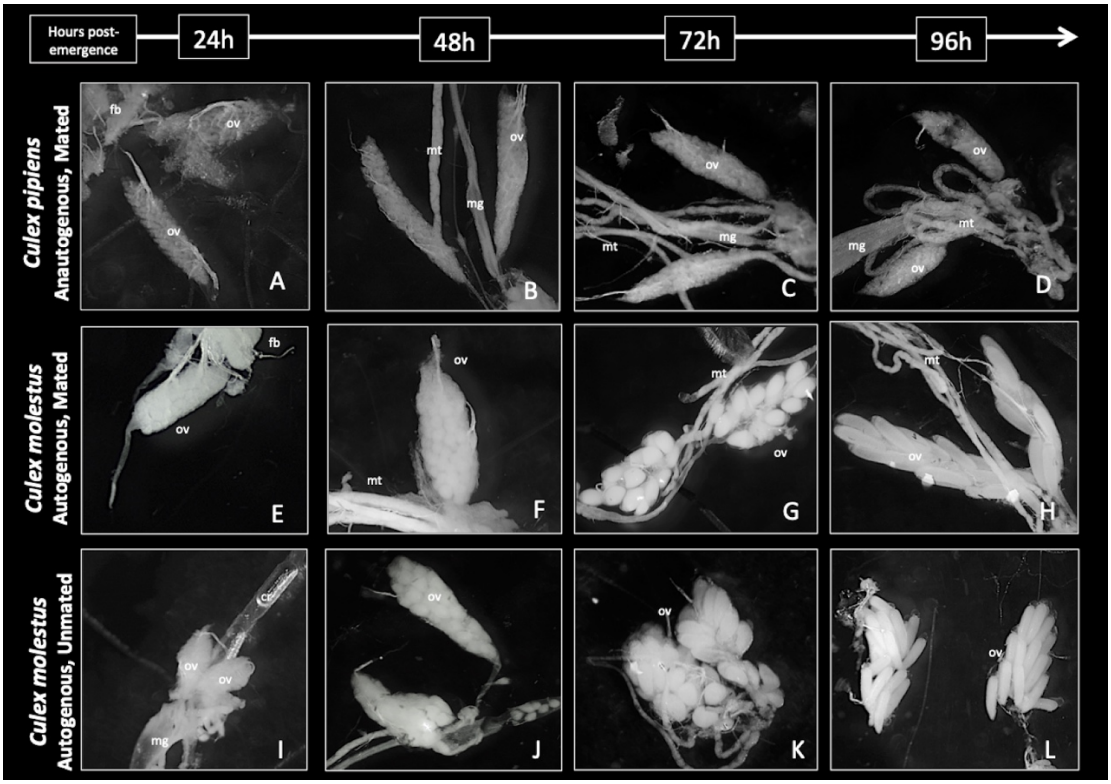

Figure 1: Ovarian development one to four days post adult emergence in anautogenous and autogenous *Cx. pipiens* females. Panels A-D depict ovarian development in anautogenous females (form *pipiens*) in which follicles remain in resting stage (Christopher's Stages - IIb, Mullen et al. 2019) until a blood meal is taken. Panels E-H depict autogenous ovarian development (form *molestus*). Autogenous females utilize metabolic reserves obtained during larval development to provision an initial egg raft in the absence of a blood meal. Panels I-L demonstrate ovarian development in autogenous females is occurs in the absence of mating where eggs produced would be non-viable.

Chapter 2: Genetic Ancestry and Host Preference in Behaviorally-Divergent, North American *Culex pipiens* Populations

Abstract

Culex pipiens (Diptera: Culicidae) is a mosquito species with two morphologically identical, but behaviorally divergent forms. Progeny of these forms can hybridize in nature, and the host-seeking behaviors of parents and hybrid progeny have the potential to impact WNV transmission. Genetic approaches to distinguish between identical parents and progeny are often used to predict host preference. Therefore, I characterized behavioral and genetic variation across eight *Cx. pipiens* populations collected from either above-ground (AG) or below-ground (BG) breeding sites throughout the USA. For each population, I examined the *molestus*, *pipiens*, and heterozygous genotype frequencies at the CQ11 locus. Host choice landing assays were used to examine variation in host preference (towards human and avian hosts) for these same eight populations. Three of the five AG populations had hybrid ancestry, whereas BG populations did not. Host choice landing assays confirmed that four AG *Cx. pipiens* populations were predominantly avian-seeking, while the fifth showed no clear preference for either host. All three BG populations tested were biased toward mammal feeding, but the strength of the bias varied by population. Because of the extensive use of these rapid molecular assays to characterize *Cx. populations* as a means of inferring populations feeding behaviors in the field, I tested whether results of these assays could predict the host preference. Genetic ancestry as

determined by the CQ11 locus was not predictive of a populations host preference, as determined by our behavioral assay.

Introduction

Morphologically identical vector species pose significant challenges for management when members are of differing epidemiologic importance (Besansky et al. 1999). The ability to distinguish between morphologically identical species allows for elucidation of the unique behavior and ecology of each vector species, as well as complex interactions among species. Furthermore, accurate measurement of vectorial capacity and development of targeted control measures rely upon the ability to discriminate one vector species from another. The *Anopheles gambiae* complex, with eight morphologically indistinguishable members all capable of transmitting human malaria, highlight the importance of species discrimination (White et al. 2011; Coetzee et al. 2013). Only *An. gambiae (s.s)* and *An. arabiensis* are considered the primary vectors of malaria; in contrast to other members of this complex, both species are anthropophilic and capable of foraging (and resting) in indoor environments (Coluzzi et al. 1979; Coetzee et al. 2004). Consequently, historic malaria intervention focused vector control efforts inside homes (through use of pyrethroid-coated bed nets and indoor pesticide sprays), specifically targeting the endophagic behavior of these primary vector species (WHO et al. 1999; Bhatt et al. 2015). Over the past three and a half decades, molecular approaches have been developed to identify cryptic species and forms present in a number of morphologically identical vector

species complexes (Hillis et al. 1987; Collins et al. 1987, 1988; Walton et al. 1999; Talbalaghi et al. 2006, Müller et al. 2013).

The Pipiens Assemblage is composed of several globally important vector species capable of transmitting pathogens, including helminths that cause lymphatic filariasis and dirofilaria, as well as St. Louis-, North American- and Eastern Equine-Encephalitis, and West Nile Virus (WNV) (Smith et al. 2004; Farajollahi et al. 2011; Vezzani et al. 2011; Thiemann et al. 2012). Members of the Pipiens Assemblage are morphologically identical, but each possesses distinct physiological traits, ecologies, (such as the ability to diapause, autogeny, habitat selection, seasonality, and host preference) and vectorial capacities (Vinogradova et al. 2003; Ciota et al. 2013). Within the United States, the Pipiens Assemblage is composed of the two species: *Cx. pipiens* (with two bioforms, form *pipiens* and form *molestus*) and *Cx. quinquefasciatus*. Specifically, these two species are implicated in the 'newly' endemic transmission of WNV in the continental United States (Laniciotti et al. 1999; Kilpatrick et al. 2006; Farajollahi et al. 2011). WNV, in contrast to malaria, is enzootically transmitted among passerine birds, with humans serving as incidental hosts (McLean et al. 2001; Simpson et al. 2011; Petersen et al. 2013). Therefore, epizootic transmission of WNV to a human host requires mosquito vectors to be capable and willing to feed on multiple vertebrate hosts.

Within the species *Cx. pipiens*, form *pipiens* are primarily avian-seeking, while form *molestus* are primarily mammalian and human-seeking (Spielman et al. 1967; Kent et al. 2007; Simpson et al. 2009; Harbach et al. 2012; Ciota et al. 2015; Fritz et al. 2015). The two bioforms readily interbreed with many stable hybrid

populations documented around major human urban centers, such as Boston, MA, New York City, NY, and Chicago, IL (Spielman et al. 1957; Huang et al. 2008; Mutebi et al 2009; Kothera et al. 2010; Fritz et al. 2015). Progeny of matings between the *pipiens* and *molestus* bioforms indiscriminately feed on mammalian and avian hosts, a behavior thought to contribute to the epizootic transmission of WNV to humans in North America (Fonseca et al. 2004; Kilpatrick et al. 2007; Huang et al. 2009; Kothera et al. 2010; Farajollahi et al. 2011; Fritz et al. 2015). *Cx. pipiens* will also hybridize with *Cx. quinquefasciatus*, a species with relatively indiscriminate blood feeding preferences, where their geographical distributions overlap (Smith et al. 2004; Kilpatrick et al. 2005; Kothera et al. 2009; Harbach et al. 2012; Ciota et al. 2013; Cardo et al. 2016). However, it is the ongoing hybridization between the two bioforms (rather than between *Cx. pipiens* and *Cx. quinquefasciatus*) that is thought to facilitate the endemic occurrence of WNV outbreaks unique to this continent (Kothera et al. 2010). Thus, it is imperative that *Culex* populations with *pipiens-molestus* hybrid ancestry are accurately identified and targeted for control efforts, as they have the potential to increase the epizootic transmission of diseases like WNV to humans.

Limited morphological divergence in the Pipiens Assemblage led to development of a pair of molecular assays capable of distinguishing between members (Smith et al. 2004, Bahnck et al. 2006). The first is a polymerase chain reaction (PCR) based assay that exploits *Culex* species-specific polymorphisms in the second nuclear intron of the acetylcholinesterase-2 (ACE-2) gene. Member species are identified by their unique amplicon size when viewed on an agarose gel (Aspen et

al. 2003; Smith et al. 2004; Savage et al. 2007; Farajollahi et al. 2011; Rudolf et al. 2013). To improve the identification of the two *Cx. pipiens* bioforms, Bahnck et al. sequenced eight microsatellite loci of 18 populations of North American and European *Cx. pipiens* and found that the CQ11 locus was suitable for differentiation of the two forms (Bahnck et al. 2006). Thus, this second PCR-based assay, which exploits indels in the flanking region of a microsatellite locus, called CQ11, is used to discriminate between the two forms of *Cx. pipiens* (Bahnck et al. 2006; Kent et al. 2007; Gomes et al. 2009; Reusken et al. 2010; Farajollahi et al. 2011; Rudolf et al. 2013; Gomes et al. 2013; Osorio et al. 2013; Di Luca et al. 2016). These assays are often used to make assumptions about how mosquito populations will behave in the environment and what vertebrate hosts they are most likely to seek (Gomes et al. 2013; Osorio et al. 2013; Di Luca et al. 2016).

Here, I determined the genetic ancestry of eight North American populations of above- and below-ground collected *Cx. pipiens* using the two PCR-based assays targeting the ACE-2 gene and CQ11 locus. I hypothesized that our AG collected *Cx. pipiens* would, at the population level, display a higher frequency of form *pipiens* alleles, while those *Cx. pipiens* populations initiated from BG habitats would have a higher frequency of form *molestus* alleles. I then quantified the behavioral responses of individual females from each of the genotyped *Cx. pipiens* populations in response to human and avian hosts in multi-day host choice assays. Considering the epidemiological significance of non-specialized feeding behaviors and epizootic disease transmission, I also assessed the propensity for individual mosquitoes to switch between either human or avian hosts over multi-day testing. I hypothesized

that populations with higher frequency of *pipiens* alleles at the CQ11 locus would more readily seek avian hosts, while populations with higher frequency of *molestus* alleles would seek human/mammalian hosts. Lastly, I assessed whether genetic ancestry, as determined by the CQ11 assay, is predictive of these populations' host preference, as determined by our behavioral assay.

Materials and Methods

Cx. pipiens - Test populations: Eight North American *Cx. pipiens* populations derived from either above-ground (AG) or below-ground (BG) breeding sites were used. Five populations were collected from AG habitats and were initiated from diapausing adults collected from AG hibernacula or as egg rafts collected from AG breeding sites. These AG populations were collected from three different North American metropolitan areas: Chicago, IL (n = 3), Laurel, MD (n = 1), and New York City, NY (n = 1).

Three populations were collected from BG breeding sites. A single BG population from California was derived from wild-caught females that were then blood-fed to initiate a laboratory colony. The remaining two BG populations were derived from eggs, larvae, and adults captured in a single collection event at a BG breeding site in Calumet, IL (Mutebi et al. 2009). Progeny from that single collection were either: 1) reared at Michigan State University and were historically offered a blood meal (see Fritz et al. 2015, hereafter CAL1), or 2) maintained at the Centers for Disease Control in Fort Collins, CO and reared without blood-feeding (hereafter CAL2). All mosquito populations were reared identically apart from their blood-

feeding regime; AG populations were anautogenous and required blood-feeding for egg production. Therefore, they were fed 9 parts Na-heparinated goose blood sweetened with 1 part 50% sucrose solution twice per generation to support egg production. BG populations were autogenous and did not require blood-feeding for egg production, CAL1 has not been offered a blood meal since 2016. BG colonies were maintained using autogenous egg rafts produced in the first gonotrophic cycle.

Determination of Genetic Ancestry: Thirty 1-3 week old females from each population were randomly selected from colony cages for genotyping. Mosquitoes were euthanized in a -80^{o}C freezer for five minutes. Dissection and removal of the abdomen took place under an Olympus stereoscopic microscope (model SZ61) on a small petri dish filled with dry ice to prevent DNA degradation. A Zymo ZR Genomic DNA Tissue Miniprep Kit (Zymo Research, Irvine, CA) was then used to isolate genomic DNA from the head and thorax only. The abdomens of all mosquitoes were removed to prevent genomic DNA from sperm, stored in mated females' spermatheca, from being detected by these molecular assays (Tripet et al. 2001). To determine *pipiens/quinquefasciatus* species ancestry, amplification of the second intron of the acetylcholinesterase-2 (ACE-2) gene was accomplished using B1246Rs, ACEpip, and ACEquin primers (Table 2) (Smith et al. 2004). Amplification of the microsatellite CQ11, to determine *pipiens/molestus* form ancestry, was carried out using CQ11F2, pipCQ11R, and molCQ11R primers (Table 2) (Bahnck et al. 2006). Following PCR, the ACE-2 amplicons were electrophoresed on a 2% agarose gel for 45 to 55 minutes at 90V. For the CQ11 assay, amplicons were run on a 3.5% agarose gel for 65 minutes at 90V, where the higher agarose

concentration was selected for better resolution between the two CQ11 bioform amplicons of relatively similar size (18-26 bp difference).

Both reactions followed the original protocols (Smith et al. 2004; Bahnck et al. 2006) with the following exceptions: GoTaq (ProMega Corp., Madison, WI, U.S.A.) was used for amplification and no bovine serum albumin (BSA) was added to the reaction. All samples were run alongside BenchTop 1kb DNA Ladder (Promega). Two controls were included with each run: 1) a negative control from the extraction process, in which DNA isolation was run in the absence of a mosquito carcass; and 2) a PCR control, in which no sample DNA was included.

Genetic Ancestry Data Analysis: I calculated the species- and form-specific allele frequencies at each locus for eight *Cx. pipiens* populations. Additionally, the mean allele frequency for all five AG populations was compared with the mean allele frequency of all three BG populations using a Fisher's exact test (McDonald et al. 2009; Ostrowski et al. 2016). To quantify change in allele frequency over time, I analyzed the three AG populations our lab had initiated (N-IL, E-IL, MD), for which we had DNA isolated from individuals from G0 (Table 4). The number of individuals assessed from G0 was limited by the number of samples successfully recovered at the time of analysis. The total number of individuals for which DNA was available from the original G0 populations were as follows: N-IL (n=28), E-IL (n=20), and MD (n=7) (unpublished data - Josh Yeroshefsky, 2017). I used a Fisher's Exact Test to compare observed allele frequencies during the time of assessment (G20 for N-IL, G19 for E-IL, and G8 for MD) with those found at the populations' initiation (G0).

Determination of Host Preference: After determining the genetic ancestries of the eight AG and BG collected *Cx. pipiens* populations at the ACE-2 and CQ11 loci, I assessed the behavioral responses of individual females to either an avian or human host. Females from AG populations were allowed to mate and age for 2 weeks, while BG populations were aged for 3 weeks prior to testing. The BG populations were autogenous, and postponed blood-feeding until the completion of a first bout of autogenous egg laying at 4–7 days post-eclosion (Spielman et al. 1971, Klowden et al. 1979; Fritz et al. 2015). Thus, the timing of physiological readiness for blood-feeding was similar in the AG and BG populations despite their difference in age. Mosquitoes were offered a 10% sucrose solution and an ovipositional resource *ad libitum*, until they were collected, no more than 1 hour prior to testing.

The use of animal subjects was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland (UMD) in June 2018 (IACUC permit #1094335). In accordance with the UMD IACUC, as well as the UMD Animal Research Facility policies, mosquitoes were reared in the UMD Entomology Department and transported to the Animal Research Facility for behavioral testing. Additionally, to address institutional biosafety concerns, mosquitoes were not permitted to blood-feed upon human or avian hosts.

Host Landing Assay: To quantify the extent of variation in host preference across multiple AG and BG populations of *Cx. pipiens*, I used a multi-day landing assay described by Fritz et al. (2015). The behavioral arena contained two white cylindrical platforms placed on opposite sides (Figure 1). An unrestrained 1-3 week old chicken (*Gallus domesticus*) rested on one platform, while the unwashed hand of

a 24-year-old white, female investigator rested on the other. Host were selected as they are representative of the two most commonly fed upon vertebrate host classes (avian and mammalian) for *Cx. pipiens* (Spielman et al. 1957; Harbach et al. 2012; Savage et al. 2012). White leghorn chicks were chosen because 1) as the breed is considered docile, 2) chicks (aged 1-3 weeks) are size-matched to the investigator's hand, 3) rested quietly and even fell asleep during testing, such that they never required restrained or anesthesia, and 4) there is past precedent of their use in both behavioral studies using *Culex* sp. and epidemiological surveillance work (Darbro et al. 2007; Fritz et al. 2015).

A 50g block of dry ice was placed under each platform, which had a small hole (d=1cm) at the top, and the investigator's breath was piped away from the arena. This allowed CO_2 to be released into the arena at a mean hourly release rate of *ca.* 258 mL/min, which falls within the acceptable range for host attraction for both anthropophilic and ornithophilic mosquito species (Reeves et al. 1953; Fritz et al. 2015). Host positions in the cage were alternated between testing days, and the investigator also alternated the hand (*i.e.* right or left) offered each day. Multiple chicks (n = 62) were used throughout the course of the experiment, but individual mosquitoes were always exposed to the same chick across testing days.

At the time of assessment, five females of a single population were collected in 20 mL glass scintillation vials by a gloved hand, and held for no more than one hour. Upon release from their scintillation vials into the arena, females were monitored by at least two observers for 15 min or until all landed and tapped a host with the labellum. After landing but prior to blood-feeding, female mosquitoes were

removed from the arena by mouth aspirator. Responders were held individually in scintillation vials, where they were provided a 10% sucrose solution, and tested twice more over the course of the three-day period.

Host Preference Data Analysis: Data from the multi-day host choice assay were analyzed in R (A language and environment for statistical Computing; R Foundation for Statistical Computing, Vienna, Austria) Version 3.3.2 (2016-10-31). To investigate variation in host preference across our eight AG and BG pipiens populations, a mixed logistic regression model with a binomial error structure was used. We assessed if three types of host response varied according to mosquito population: 1.) acceptance of either host during day 1, and 2.) selection/preference for a human host, and 3.) probability to alternate host across multi-day testing.

The model was constructed using the R package lme4 (v. 1.1-14; Bates et al. 2015) and was fit to our multi-day, host choice dataset. In order to find which response variables significantly impacted the construction of the model, minimal adequate models were fit by sequentially eliminating model terms using likelihood ratio tests conducted using the lmtest package (v. 0.9-35; Zeileis et al. 2002). Two fixed effects were initially considered: the 'population' from which the mosquito originated, and the 'day' corresponding to days 1-3 of multi-day testing. A single random effect of the 'chick host' was also included to account for interindividual variation in attractiveness across avian hosts used throughout the assay (i.e. some chickens more attractive than others) (Lindsay et al. 1993; Qui et al. 2006; Molaei et al. 2006; Simpson et al. 2009; Hamer et al. 2009). When the two fixed effects were independently considered, only 'population,' not the 'day' of assessment,

significantly impacted all three measured host response rates. Our full model examined the response by the i^{th} mosquito to the investigator's hand and/or the j^{th} chick:

Full model to examine individual (i) host response:

$$\Pr[y_i=1] = \text{logit}^{-1}(\beta_{0ij} + \beta_1 \text{Population}_{ij} + u_{0ij})$$

For $1 \leq i \leq n$ and $1 \leq j \leq m$

where $u_0 \sim N(0, \sigma^2_j)$ represents the random effect of chick.

This model was fit to three datasets, independently. 1) To assess the overall response rate of the populations to either host (i.e. human or avian) on the first day of our assessment, females that selected a host were assigned a value of 1, while 'no response' was given a value of 0. 2) To assess variation in human host preference, data for individuals that failed to respond on multiple days were removed from the dataset, and host choice (i.e. chick vs. human) was examined for individuals across test dates. For this model, a host selection of the 'human' host was given a value of 1, and selection of a 'chick' host a 0. Additionally, because host response was measured across the same individuals over the multi-day testing period, 'day' was also included as a second random effect in this model. 3) To investigate the probability that a mosquito would change its host selection across test dates (*i.e.* deviate from the initial host choice made on day 1), individuals that accepted both hosts during the 3-day test period were scored as 1, while mosquitoes that accepted the same host type across test dates were scored as 0. Additionally, for each population, the observed probability of each of the three measured responses was calculated, as well as the corresponding non-parametric bootstrapped 95% CIs (n = 5000) (Fritz et al 2015).

Assessing if genetic ancestry is predictive of host preference: To measure the strength of association between genetic ancestry (specifically, frequency of *molestus* alleles at the population level) and observed host preference (frequency of females seeking a human host) across all eight populations a Kendall's Tau correlation test was used (Hipel et al. 1994).

Results

Determination of Genetic Ancestry: There was no evidence of *Cx. quinquefasciatus* ancestry in any of the eight populations tested. Every individual produced amplicons during gel electrophoresis and was homozygous for the *pipiens* allele at the ACE-2 locus, indicating all eight populations were *Cx. pipiens*, rather than *pipiens* - *quinquefasciatus* hybrids (data not shown).

At the time of individual colony initiation, the three AG populations initiated by our lab, E-IL, N-IL and MD, were independently found to be fixed for the *pipiens* allele at the ACE-2 locus. However, a single BG population, CAL2, which had been previously maintained at the CDC before our lab acquired the population, showed evidence of *Cx. quinquefasciatus* introgression in 2017 in the first generation reared by our lab (Table 3). One individual was found to be homozygous for the *quinquefasciatus* allele, one individual was heterozygous, while the other five individuals were homozygous for the *pipiens* allele. A single individual did not produce an amplicon using ACE-2 primers. However, when CAL2 individuals were genotyped for this work, after ~ 1.5 years later in colony, all 30 individuals tested were fixed for the species *pipiens* allele at the ACE2 locus.

Next, I assessed genetic ancestry at the CQ11 locus. Genotype calls were made for individual females of each population and allele frequencies were calculated to determine the extent our populations (if any) were the product of hybridization between the *pipiens* and *molestus* forms (Table 3). Interestingly, out of all populations tested, only a single individual from the AG population initiated from Northfield, IL was heterozygous at the CQ11 locus (Table 4). All other individuals were homozygous at the CQ11 locus, regardless of their determined genetic ancestry.

Although DNA was isolated for 30 individuals from each population, not all individuals produced amplicons at the CQ11 locus. The n-values (Table 4) indicate the number of females out of the 30 tested that had successful DNA amplification using CQ11 primers. Overall amplification at the CQ11 locus as a whole was observed to be lower across the five AG populations (119/150 = 79%), than in their BG counterparts (87/90 = 97%).

Genetic Ancestry Data Analysis: Allele frequencies for the eight AG and BG populations is depicted in Figure 2. For all three BG populations, the form *molestus* allele was fixed at the CQ11 locus. In contrast, there was significant variation in genetic ancestry across the five AG populations (d.f. = 4; $\chi 2 = 62.16$; p = 1.02e-12). Only a single AG population from New York was fixed for the form *pipiens* allele. Three of the five AG collected populations (E-IL, N-IL, MD) showed evidence of hybrid ancestry, where the frequencies of the form *molestus* allele ranged from 0.61 - 0.93 (Table 3). Additionally, a single AG population from Chicago, IL was fixed for the form *molestus* allele. This is a surprising result considering the population's historical collection from an AG breeding-habitats and anautogeny observed in

colony; autogenous egg rafts have never been observed while this population has been cultured at the University of Maryland. Genetic ancestry, characterized at the CQ11 locus, differed significantly between our AG and BG populations ($p < 2.2e-16$).

Because of the unexpectedly high form *molestus* ancestry observed in the AG populations, I wanted to ensure no cross contamination had occurred between the AG and BG populations during their time in culture at the University of Maryland (Table 5). No significant change in allele frequency over time was observed for the N-IL and E-IL populations ($p = 0.6529$ and $p = 0.5152$, respectively). In contrast, a significant change in allele frequency was observed in the MD population ($p < 6.994e-05$) where the frequency of *molestus* alleles increased over time (Table 5). However, the small G0 sample size likely did not capture genetic variation at the population level at this time point, and thus may not accurately reflect their initial allele frequency.

Host Preference Data Analysis: In total, 686 AG and 377 BG individuals were assessed on the initial day of testing. 348 AG (51%) and 221 BG (59%) individuals accepted a host on day 1 (either human or avian) and were maintained for multi-day testing. Mean overall response rate (2.5%, 97.5% CI; n=5000) to any host on day 1 varied according to population (d.f. = 8; $\chi 2 = 33.57$; $p < 4.7e-5$; Figure 3A).

For seven of the eight populations, at least half of the individuals responded to a host on day 1 within the 15 minute trial period. In the three BG populations, 50% (39%, 61%), 57% (48%, 65%), and 65% (58%, 72%) of females from CA, CAL2, and CAL1 selected a vertebrate host on day 1. In four of the AG populations, N-IL, E-IL, C-IL, and NY, individuals accepted a host 49% (40%, 58%), 58% (49%, 67%),

73% (67%, 81%), and 76% (67%, 84%) of the time, respectively. Individuals belonging to the AG population from Maryland displayed the lowest host acceptance in our assay, with only 28% (22%, 34%) of those tested selecting a host on day 1.

Host preference varied significantly among our AG and BG populations (d.f. = 8; $\chi 2 = 147.3$; $p < 2.2e\text{-}16$) (Figure 3, Panel B). All three BG populations were biased toward mammal feeding, but the strength of the bias varied by population. BG populations accepted a human host 68% (55%, 81%), 69% (57%, 80%), and 84% (77%, 91%) of the time, for populations CA, CAL2, and CAL1. Four of the five AG *Cx. pipiens* populations were predominantly avian-seeking with 85% or more of responding females selecting the chicken host. Avian response rates were 86% (78%, 93%), 86% (77%, 93%), 88% (80%, 95%), and 88% (81%, 96%) for AG populations E-IL, C-IL, MD, and N-IL, respectively. The strength of the bias did not significantly differ between these four populations (d.f. = 3; $\chi 2 = 3.9274$; $p = 0.2694$). Individuals belonging to the AG population from New York showed no clear host preference, however, selecting an avian host only 43% (32%, 54%) of the time.

The probability of alternating hosts at least once during multi-day assessment differed significantly by population (d.f. = 8; $\chi 2 = 28.6$; $p < 0.00038$). In the three human-seeking BG populations, the probabilities (2.5%, 97.5% CIs) to switch hosts were 0.217 (0.117, 0.317), 0.3 (0.0, 0.6), 0.452 (0.258, 0.613) for CAL1, CA, and CAL2. In the four avian-preferring AG populations (MD, N-IL, E-IL, C-IL - see above) observed probabilities to switch hosts (in this case, to the human host) were significantly lower: 0.0 (0.0, 0.0), 0.056 (0.0, 0.167), 0.125 (0.031, 0.250) (Figure 4). The single AG population from NY with indiscriminate host-acceptance

behaviors had the highest observed probability of switching hosts across test days, 0.514 (0.351, 0.676).

Assessing if genetic ancestry is predictive of host preference: Despite the widespread use of the CQ11 locus to characterize populations of *Cx. pipiens* (Bahnck et al. 2006; Di Luca et al. 2016; Kent et al. 2007; Rudolf et al. 2013; Gomes et al. 2009; Gomes et al. 2013; Osorio et al. 2013; Reusken et al. 2010; Farajollahi et al. 2011), genetic ancestry as determined by this assay was not predictive of these populations' host preference (tau = 0.4029115, p = 0.184) (Figure 5).

Discussion

In our molecular assays to determine genetic ancestry, we observed a varying degree of amplification among populations at the CQ11 locus. A single AG population from NY only had 14 individuals that produced amplicons at the CQ11 locus, while DNA from the same 30 individuals with the ACE-2 primers produced amplicons for every individual. This indicates that high quality DNA was used for the CQ11 PCR and poor DNA quality was unlikely to cause the low observed amplification. Perhaps some individuals from the NY population have a mutation within the region of the microsatellite where the CQ11 primers bind. Mutations in the primer region would inhibit PCR amplification of this gene marker, making it appear as though an individual has lost diagnostic bands. It is also possible that backcrossing events resulted in recombination and independent assortment of this gene marker in hybrid individuals, which could also cause the loss of diagnostic bands at the CQ11 locus. Because of these challenges, I would propose examining a larger number of

loci when characterizing *Cx. pipiens* populations in the future, especially when genotyping *Cx. pipiens* populations collected from regions with historically-documented hybridization between the forms.

According to their genotypes at the CQ11 locus, all three BG populations assessed were fixed for the form *molestus* allele. Therefore, it does not appear our BG populations experienced historical hybridization with local AG populations prior to colony establishment. In contrast, we observed a significant amount of genetic variation at the CQ11 locus across the five AG populations. Only a single AG population was fixed for the form *pipiens* allele, three populations showed evidence of hybrid ancestry, and one AG population was fixed for the form *molestus* allele. Thus, from the results of this single gene marker, it is likely that four out of five of the AG populations experienced historical hybridization with local below-ground populations before they were established in laboratory culture. Similar patterns of genetic admixture have been observed in above-ground, field-collected North American populations of *Cx. pipiens* (Kent et al. 2007; Huang et al. 2009; Gomes et al. 2009).

Natural mating barriers between the forms could influence the direction of gene flow between them (Spielman et al. 1967; Fonseca et al. 2004). The significant amount of *molestus* ancestry observed in the AG populations may be explained by the way AG and BG populations encounter one another under natural conditions. Form *molestus* mosquitoes are often found as populations occupying subway/metro systems or underground sumps (Bryne et al. 1999; Mutebi et al. 2009). The isolated occurrence of BG populations may inhibit AG individuals from locating them under

natural conditions. It is more likely that BG individuals will escape their subterranean habitats and mate with AG conspecifics. Even if form *pipiens* mosquitoes are able to locate underground populations of form *molestus* mosquitoes, form *pipiens* males prefer to mate in large above-ground swarms and would likely be unwilling to breed in the confined environments where form *molestus* females would be found (Mullen et al. 2009; Harbach et al. 2012). Thus, we may conclude that gene flow is relatively unidirectional between the two forms, a conclusion supported by others studying North American *Cx. pipiens* populations (Yurchenko et al. 2019). It is challenging to make any meaningful conclusions from a single gene marker, however.

Rearing practices may contribute to the increased frequency of *molestus* ancestry in the AG populations. Form *molestus* populations adapt quickly to laboratory conditions, given that they are naturally found in confined, underground habitats (Harbach et al. 2012). True form *pipiens* populations are more difficult to maintain as laboratory colonies, due to the eurogamous mating preference of males (Spielman et al. 1967). Thus, it is possible that by maintaining AG populations in confined laboratory cages we caused a genetic bottleneck in the population, unknowingly selecting for genes associated with living in confined spaces (like their form *molestus* counterparts). If these genes are closely associated with the CQ11 locus, we may expect to see an increase in the frequency of form *molestus* alleles for these AG populations as they adapt to laboratory conditions.

The mosquito populations were found to significantly differ from one another in their willingness to respond to a vertebrate host during behavioral testing. This result was expected as willingness to engage in host-seeking behaviors in an indoor

setting is considered a highly variable trait in mosquitoes (Takken et al. 2013). The lowest initial response rates were found in the AG populations. In similar host choice assays, Fritz et al. also reported the lowest overall response rates were observed in AG collected *Cx. pipiens* populations (Fritz et al. 2015). The most recently established population from Maryland had the lowest response rate, where only 28% of females tested selected a host during preference assessment. It is possible that time in culture and adaptation to host-seeking in an indoor environment can influence willingness to participate in laboratory assays.

BG females readily accepted the human host in the host choice test. Response rates for the three BG populations to the human host were three to six-fold greater than response rates to the chick (Figure 3). Although allele frequencies at the CQ11 locus revealed mixed ancestry, the majority of the AG populations' host preferences were consistent with those of the *pipiens* form described elsewhere (Harbach et al. 1984; Savage et al. 2007). AG females tended toward avian acceptance; four of the five AG populations were predominantly avian-seeking with 85% or more of responding females selecting the chicken host. Similar avian feeding rates in field AG populations have been reported (Hamer et al., 2009). Only a single AG population from New York was found to indiscriminately feed on the two available hosts. Populations that exhibit non-specialized feeding behaviors in combination with the propensity for these mosquitoes to feed on multiple hosts during their lifetime would provide the conditions necessary for epizootic transmission and WNV spillover into human populations in nature.

Figure 5 represents a composite figure where allele frequency data (from Figure 2) has been overlaid on the results of our host-preference assay (Figure 3B). If we expect genetic ancestry to be predictive of host preference we would expect the bars of the overlaid figures to face the same direction. This is the case with our three BG populations, all three of which are fixed for the form *molestus* allele and display strong preference for human hosts. However, genetic ancestry at the CQ11 locus was not predictive of host preference in the AG populations. Although four of the five AG populations demonstrated a strong preference for avian hosts, frequency of form *pipiens* alleles varied greatly across the populations collected from above-ground habitats. Additionally, the single AG population from New York that was fixed for form *pipiens* allele at the CQ11 locus was the only population to display indiscriminate feeding behaviors. Although this single gene marker is not predictive of a population's host preference, the assay can still be used to assess whether a *Cx. pipiens* population has experienced historical hybridization.

In conclusion, understanding the population dynamics of *Cx. pipiens* is essential for surveillance and control of WNV transmission. Because the two bioforms of *Cx. pipiens* and hybridized populations have different vectorial capacities and epidemiologic importance but are morphologically indistinguishable, it is essential to develop molecular approaches that can be used to identify them. Genetic ancestry as determined by the CQ11 locus was not found to be predictive of observed host preference. Thus, future work should be aimed at the identification of the genetic basis of host preference for the development of new molecular markers that target genes directly affecting phenotypic host preference.

Tables: Chapter 2

Table 1: Collection information for eight study populations of above- and below-ground collected *Cx. pipiens,* including year of population initiation, location of collection, and the size of G0. CAL1 and CAL2 were originally collected by Mutebi et al. 2009.

Population ID **Breeding site**	**Collection Year**	**Collection Site** **GPS Coordinates**	**Size of G0**
CAL1 (below-ground)	2009	41.6502247, -87.600140 Calumet Water Reclamation Plant; Chicago, IL	7000 adults and larvae
CAL2 (below-ground)	2009	41.6502247, -87.600140 Calumet Water Reclamation Plant; Chicago, IL	7000 adults and larvae
CA (below-ground)	2013	37.904445, -122.653184 Stinson Beach, California	20 females (wild caught then blood-fed in lab)
C-IL (above-ground)	2010	*GPS coordinates unknown* Oak Lawn, IL	200 diapausing adults
E-IL (above-ground)	2016	42.029246, -87.70564 Evanston, IL	28 egg rafts
N-IL (above-ground)	2016	42.094783, -87.770168 Northfield, IL	31 egg rafts
MD (above-ground)	2017	39.111413, -76.903376 Laurel, MD	80 egg rafts
NY (above-ground)	2008	40.65874, -73.9862; 40.7900, -73.7808 New York City, NY	300 diapausing adults

Table 2: CQ11/ACE-2 PCR Assays. Included are the forward and reverse primer sequences and expected amplicon length in base pairs. CQ11 is a microsatellite locus and contains a varying number of TG repeats across the two forms. During the development of the assay, Bahnck et al. reported form *molestus* mosquitoes had a single allele size at the CQ11 locus (fixed at 4 TG repeats). In contrast, form *pipiens* mosquitoes were found to have between 6 and 10 TG repeats in the CQ11 microsatellite, resulting in the 258-256 bp range in expected amplicon size. However, CQ11 assay discriminates between the forms by exploiting differences in insertions and deletions around the microsatellite (not the TG repeats in the microsatellite itself).

Primer	**Primer Sequence 5' - 3'**	**PCR fragment length**
Reverse - B1246	TGGAGCCTCCTCTTCACGGC	-
Forward - ACEpip	CTTTCTTGAATGGCTGTGGCA	274 bp
Forward - ACEquin	GGAAACAACGACGTATGTACT	610 bp
Forward - CQ11F2	GATCCTAGCAAGCGAGAAC	-
Reverse - pipCQ11R	CCCTCCAGTAAGGTATCAAC	258 - 266 bp
Reverse - molCQ11R	CATGTTGAGCTTCGGTGAA	284 bp

Table 3: Genetic ancestry at the ACE-2 and CQ11 loci for the BG, CAL2 population at the time of colony acquisition. DNA was isolated on 4/27/2017.

Sample ID	Alleles at CQ11	Alleles at ACE-2
CAL2_1	mol/mol	pip/pip
CAL2_2	mol/mol	pip/pip
CAL2_3	**mol/mol**	**quinq/pip**
CAL2_4	mol/mol	pip/pip
CAL2_5	**mol/mol**	**quinq/quinq**
CAL2_6	mol/mol	pip/pip
CAL2_7	mol/mol	BLANK
CAL2_8	mol/mol	pip/pip

Table 4: Genetic Ancestry at the CQ11 locus for eight study populations collected from AG and BG habitats. For those populations initiated by our lab (not acquired from collaborators) generation number at the time of genotyping was recorded (N-IL, E-IL, MD). Generation number was not recorded for the other five populations (C-IL, NY, CA, CAL1, CAL2) as these populations had long been in colony before they were acquired from collaborators. (n) indicates the number of individuals of the 30 originally included where successful amplification was observed. Genotype calls were made for each individual, as either homozygous for the *pipiens* allele, heterozygous, or homozygous for the *molestus* allele. Genotype calls were used to calculate allele frequency at the population level.

Population Information			**Genotype calls for Individuals**			**Allele Frequencies at the CQ11 locus**	
Population	**Gen.**	**n**	**pip/pip**	**pip/mol**	**mol/mol**	**freq. *pipiens***	**freq. *molestus***
N-IL (AG)	(G20)	24	8	1	15	0.35	0.65
C-IL (AG)	NA	23	0	0	23	0.00	1.00
E-IL (AG)	(G19)	28	11	0	17	0.39	0.61
MD (AG)	(G8)	30	2	0	28	0.07	0.93
NY (AG)	NA	14	14	0	0	1.00	0.00
CA (BG)	NA	28	0	0	28	0.00	1.00
CAL2 (BG)	NA	29	0	0	29	0.00	1.00
CAL1 (BG)	NA	30	0	0	30	0.00	1.00

Table 5: Genetic ancestry of three AG populations of *Cx. pipiens* at the CQ11 locus, assessed at the time of colony initiation and after 1.5 years in culture. Generation number at the time of genotyping was recorded. (n) is the number of individuals for which we observed successful amplification at this locus. Genotype calls were made for each individual, as either homozygous for the form *pipiens* allele, heterozygous, or homozygous for the form *molestus* allele. Genotype calls were again used to calculate allele frequency at the population level.

Population Information			**Genotype calls for Individuals**			**Allele Frequencies at the CQ11 locus**	
Population	**Gen.**	**n**	**pip/pip**	**pip/mol**	**mol/mol**	**freq. *pipiens***	**freq. *molestus***
N-IL (AG)	(G0)	20	4	4	12	0.30	0.70
N-IL (AG)	(G20)	24	8	1	15	0.35	0.65
E-IL (AG)	(G0)	19	4	4	11	0.32	0.68
E-IL (AG)	(G19)	28	11	0	17	0.39	0.61
MD (AG)	(G0)	7	4	0	3	0.57	0.43
MD (AG)	(G8)	30	2	0	28	0.07	0.93

Figures: Chapter 2

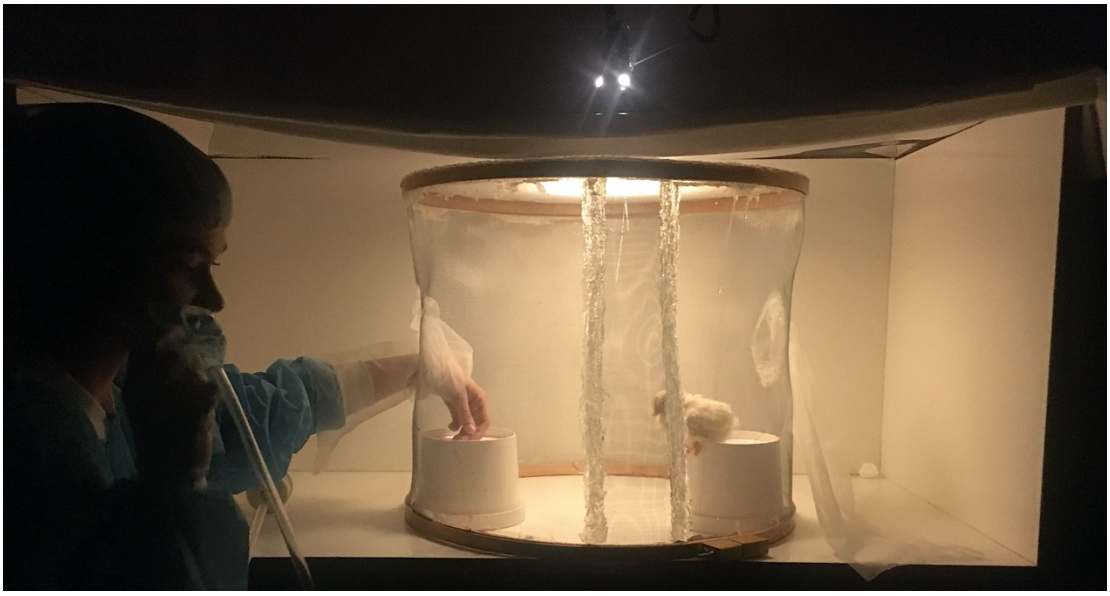

Figure 1: The behavioral arena used to assess host preferences *Cx. pipiens* females. The arena was constructed as a large, enclosed mesh structure with two platforms on opposing sides, one with a 1-3-week old, acclimatized chick and the other supporting the arm of the investigator. Two circular ports (12 cm in diameter) were cut into the sides of the arena to allow the insertion of hosts. Ports were covered in mesh sleeves to prevent mosquito escape. To control for the potentially confounding effects of CO_2, a 50g block of dry ice was placed under each platform. Platforms had a small hole (d=1cm) at the top to allow for a steady stream of CO_2 throughout the trials. During testing, the investigator's breath was piped away from the arena, and dry ice was replaced hourly.

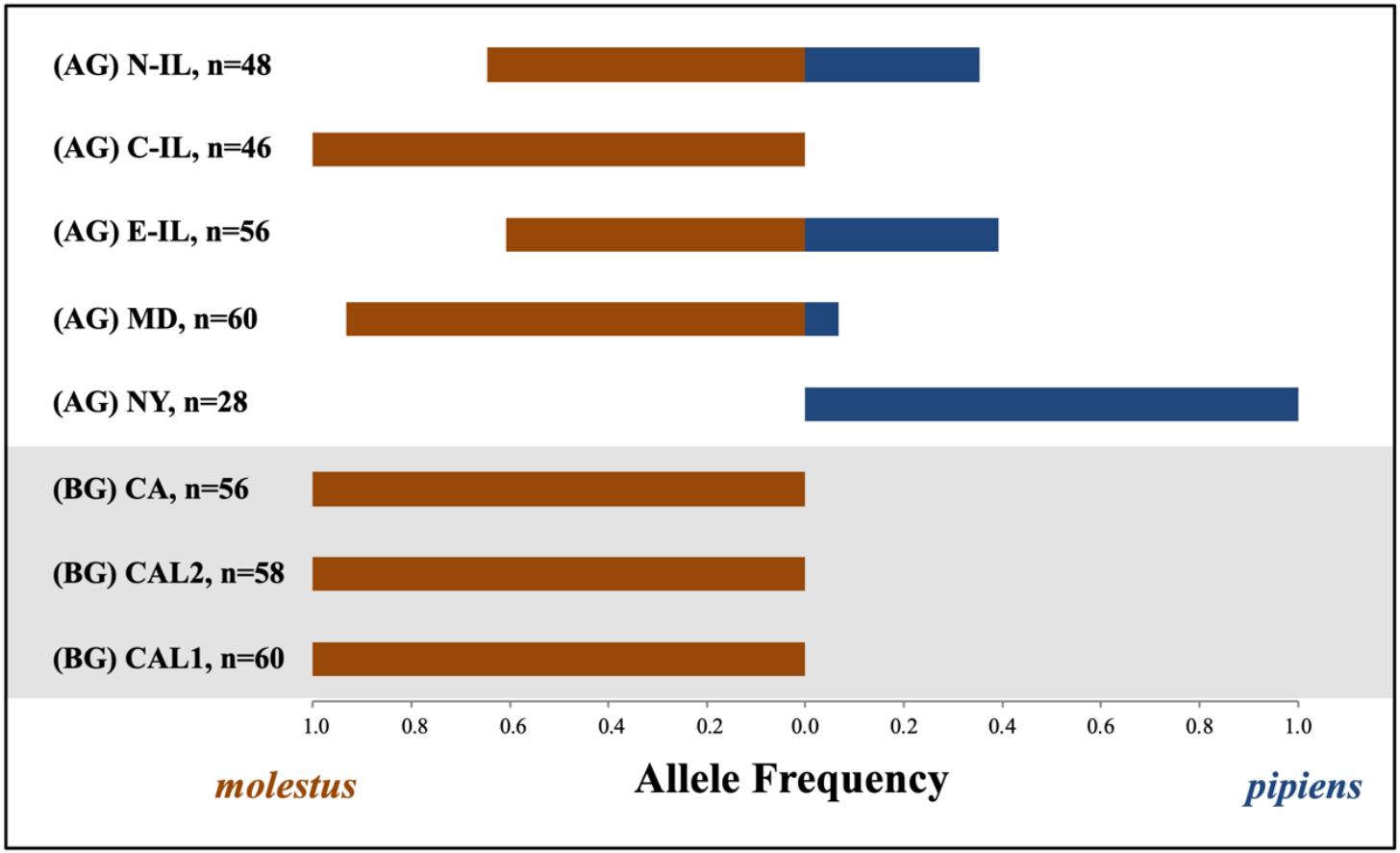

Figure 2: Allele Frequency at the CQ11 locus for AG and BG populations of *Cx. pipiens.* The n-values indicate the number of alleles (2 per individual) that were successfully amplified and used to make genotype calls.

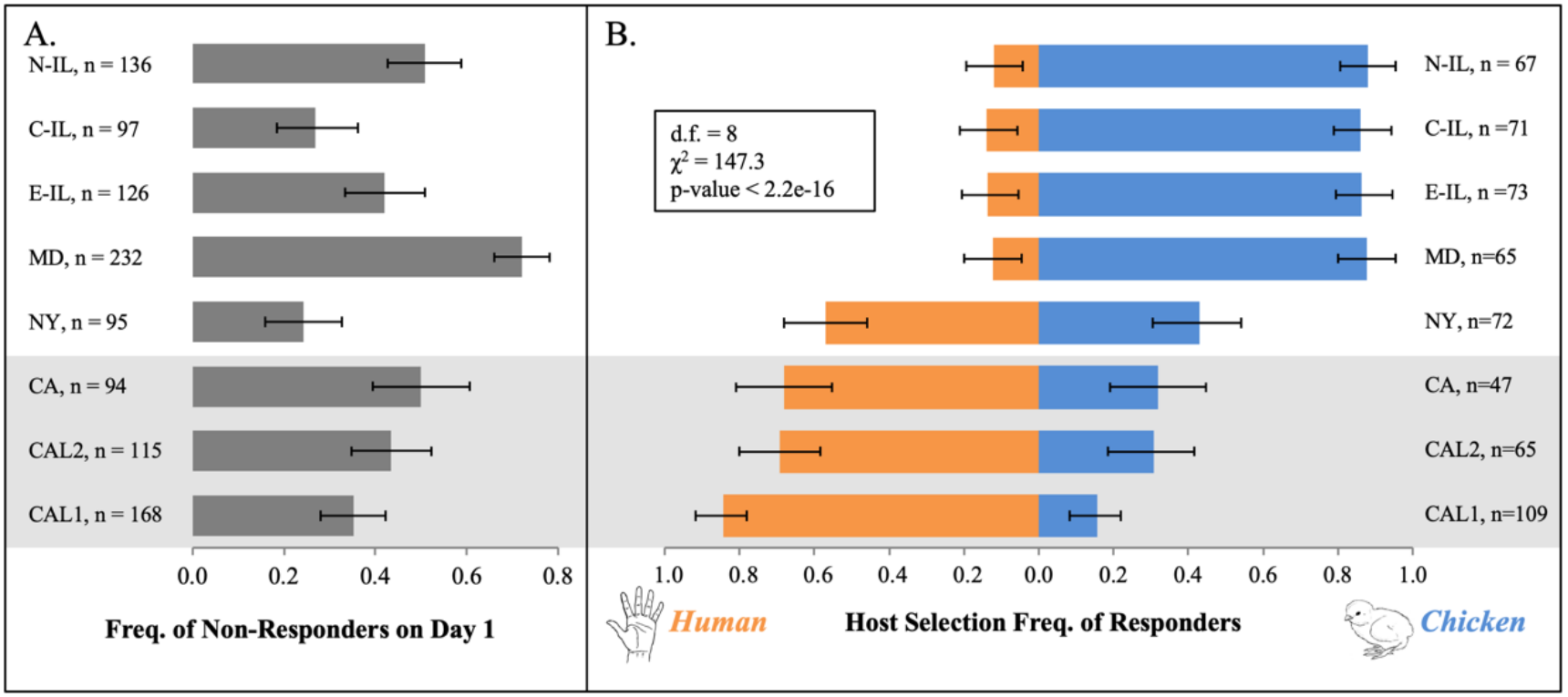

Figure 3: Observed host response rates with 95% confidence intervals (CIs). Panel A depicts the frequency of non-responders on day 1, where n = total number of mosquitoes assessed. Panel B depicts the frequency of responders selecting either the human or avian host over the multiday testing period, n = number of mosquitoes that selected a host on day 1.

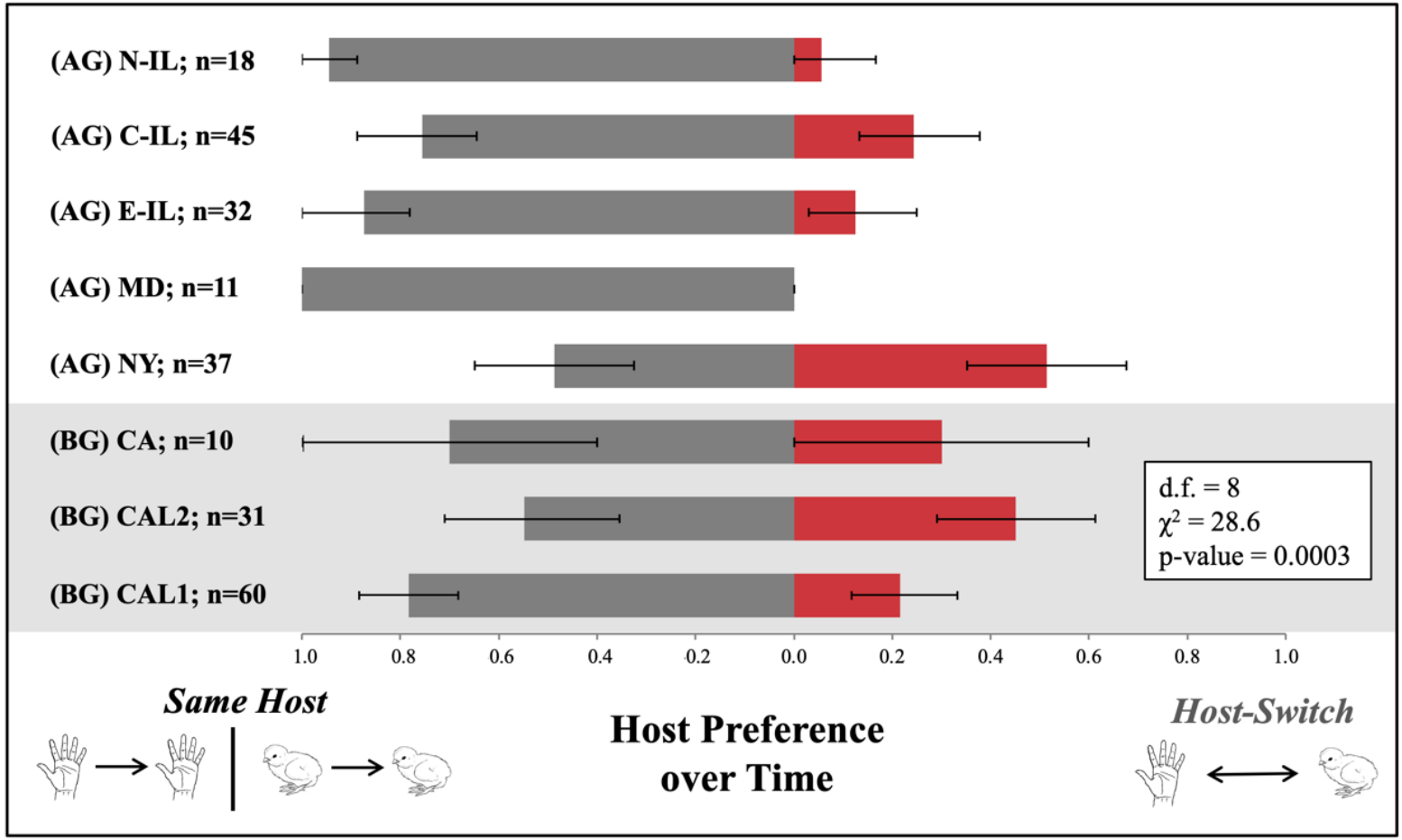

Figure 4: Probability of alternating hosts with 95% confidence intervals (CIs); n = number of mosquitoes that selected either host at least two times during multiday testing. Grey bars facing the left indicate individuals selecting the same host across multi-day testing, while red bars facing the right indicate individuals that selected both hosts across multi-day testing.

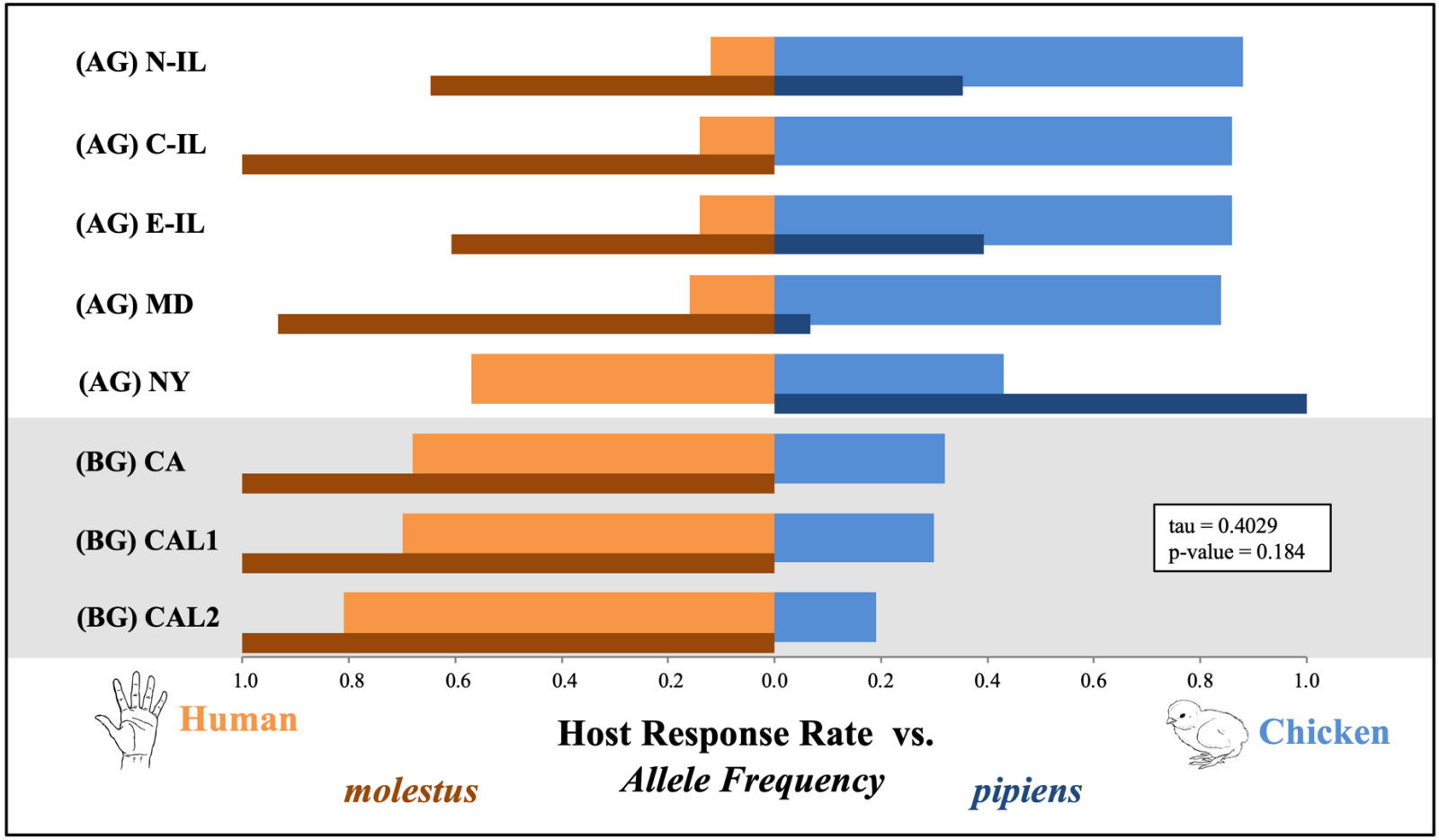

Figure 5: Genetic ancestry, as determined by the CQ11 locus, is not predictive of observed host preference across *Cx. pipiens* bioforms. Genotypic data is depicted by the lower, thinner and darker bands, while the host preference data are shown with the thicker lighter bands.

Chapter 3: Expression Patterns of Odorant-Binding Proteins 2 and 12 in Behaviorally-Divergent *Culex pipiens*

Abstract

The spacial and temporal patterning of chemosensory genes has the potential to influence odor-mediated behaviors in mosquitoes. I characterized differential gene expression patterns for two behaviorally divergent above-ground (AG) and below-ground (BG) *Cx. pipiens* populations, which sought avian and human hosts in previous laboratory studies. Based on preliminary RNA-sequencing data, odorant binding proteins (OBPs), OBP2 and OBP12, expression patterns were characterized to investigate their contributions to the observed differences in host preference between populations. Expression was quantified at three time points: 24 hours, 96 hours, and 168 hours (7 days) post adult eclosion. OBP2 had higher levels of expression in the heads of BG females relative to AG females at both 96 and 168 hours post adult eclosion. OBP12 was more highly expressed in the heads of BG females at all three time-points and was upregulated to a greater degree at the 24hr and 168hr timepoints. Considering the unique expression patterns of these OBPs over time, it is likely OBP2 contributes to the detection of host volatiles in the human-preferring, BG population, while OBP12 may instead contribute to odor-mediated ovipositional behaviors.

Introduction

Female mosquitoes perceive the environment through semiochemicals, which they utilize to detect nectar resources, oviposition sites, and vertebrate hosts for a blood meal (DeGennaro et al. 2013; Suh et al. 2014; Raji et al. 2019). To influence mosquito behavior, volatiles must come in contact with specialized olfactory receptors (ORs) located on the dendrites of olfactory receptor neurons present in the chemosensory appendages located on the head (Leal et al. 2013). The transport of these molecules is assisted by odorant-binding proteins (OBPs), which solubilize volatiles and transport them across sensillar lymph to either their designated ORs or odor degradation enzymes (Vogt et al. 1981; Xu et al. 2010). OBPs are thought to undergo pH dependent conformational change, releasing chaperoned odorants when in the presence of negatively charged membranes of ORNs (Wojtasek et al. 1999).

The first insect OBP was discovered in 1981 as a small pheromone binding protein found at high abundance in the antennae of male silkmoths (*Antheraea polyphemus*) (Vogt et al. 1981). Since then, OBPs have been identified and characterized in a number of insect taxa, including members of Culicidae. Within many insect genomes, OBPs are thought to exist as large, multigenic families (Vieira et al. 2011; Leal et al. 2013); the genome of *Drosophila melanogaster* contains 57 OBPs (Galindo et al. 2002), *Aedes aegypti* contains 66 OBPs (Zhou et al. 2008), *Anopheles gambiae* contains 69 OBPs (Vieira et al. 2011), and *Culex quinquefasciatus* contains 109 OBP genes (Pelletier et al. 2009). Although other subgroups of OBPs have been described (Hekmat-Scafe et al. 2002), Classical OBPs are defined as having 3 primary criteria: small molecular size (molecular weight ~14

kDa), an N-terminal peptide sequence, and a 'classic' motif of six highly conserved cysteine residues (Vogt et al. 1981; Pelletier et al. 2011, Leal et al. 2005).

OBPs play an important role in host specialization for other insect species. A single member of the *Drosophila melanogaster* species complex (DMSC), *D. sechellia*, has evolved distinct physiological and behavioral adaptations to its preferred host plant, *Morinda citrifolia* (Matsuo et al. 2007). *D. sechellia* uses chemosensation to detect specific odorant volatiles (hexanoic and octanoic acid) generated by the plant's ripe fruit. While *D. sechellia* is attracted to these compounds, other closely related species in the DMSC are repelled (Matsuo et al. 2007). In 2007, Matsuo et al. demonstrated that two genes encoding OBPs are directly involved in behavioral differences between species. Using P-element mediated targeted mutagenesis, Matsuo et al. (2007) generated OBP57d/e knockout *D. melanogaster,* which displayed altered behavioral responses to hexanoic and octanoic acids. In oviposition-site preference assays, *D. melanogaster* Obp57d/eKO flies showed a stronger preference for the acids than their wild-type *D. melanogaster* counterparts (Harada et al. 2008). These findings indicated that the normal function of these OBP genes is to suppress attraction/behavioral preference for the compounds (Harada et al. 2008). Additionally, when Obp57d and Obp57e from *D. sechellia* were introduced into *D. melanogaster* Obp57d/eKO flies, the *D. melanogaster* Obp57d/eKO flies shifted their oviposition site preference to favor higher concentrations of octanoic acid as does *D. sechellia*, confirming the contribution of these genes to specialization on *M. citrifolia* (Matsuo et al. 2007). I postulate that OBPs also play an essential role

in the sensitivity of the mosquito chemosensory system and could contribute to specialized host-preference.

The first mosquito OBP was isolated from the antennae of a female *Culex quinquefasciatus* and identified as CquiOBP1 (Ishida et al. 2002). CquiOBP1 was demonstrated to bind to mosquito oviposition pheromone (MOP), indole, and 3-methylindole, shown previously to influence behavior associated with oviposition (Ishida et al. 2002; Pelletier et al. 2010). In electroantennography studies, knockdown of CquiOBP1, using RNA interference, resulted in decreased antennal sensitivity to MOP but not other experimentally-tested volatiles, which suggests CquiOBP1 is vital for the reception of specific odorants (Pelletier et al. 2010). Additionally, when the OBP1 ortholog in *Anopheles gambiae*, AgamOBP1, was knocked down, antennal sensitivity to indole and 3-methylindole decreased significantly (Biessmann et al. 2010). Together, these findings demonstrate that OBPs are critical for selectivity and sensitivity of the mosquito olfactory system, and may be instrumental in detecting host-specific volatiles to influence host-seeking behavior.

Preliminary RNAseq data from our lab revealed 3179 genes were differentially expressed in the heads of two behaviorally-divergent populations of Cx. pipiens: an avian-seeking, AG collected populations and human-seeking, BG collected population. These included genes with known chemosensory function, including odorant receptors, ionotropic receptors, gustatory receptors and OBPs. OBPs were most frequently detected as differentially expressed between the populations. Seventy-two of the 109 Culex OBPs were reliably detected in the heads of both populations, where 16 OBPs had increased expression in the BG population,

while 14 OBPs were more highly expressed in the AG population. Two of these differentially expressed OBP genes, OBP2 and OBP12, were selected for further examination (Table 1).

Expression of many OBPs is regulated by the circadian clock (Rund et al. 2011, 2013a, 2013b), and shifts in chemosensory gene expression appear to correlate with the timing of odor-mediated behaviors, like foraging and host receptivity (Eilerts et al. 2018). Physiological state also plays an important role in fluctuation of chemosensory gene expression in female mosquitoes. For example, transcriptome analyses in *Ae. aegypti*, *An. gambiae,* and *Cx. quinquefasciatus* reveal changes in overall chemosensory gene expression, including OBPs, pre- and post- a blood meal (Fox et al. 2001; Rinker et al. 2013; Matthews et al. 2016; Taparia et al. 2017; Hill et al. 2019). After successful acquisition of a blood meal and entry into the gonadotropic cycle, an overall reduction in chemosensory gene transcripts and odor sensitivity has been observed (Rinker et al. 2013; Matthews et al. 2016; Taparia et al. 2017). During this period, females also undergo a remarkable change in behavior. High activity and foraging behaviors associated with host-seeking behaviors are repressed, and gravid females seek refugia, reducing activity to facilitate egg maturation (Speilman et al. 1967; Klowden et al. 1981). Therefore, odor-mediated behaviors that occur during specific times in the female life-cycle are likely to correspond to peaks in expression of their underlying chemosensory genes. Here, I examine expression levels of two OBPs, and quantify their changes in expression-level throughout the first 7-9 days of adulthood in females.

Studies that examine the expression profiles of putative OBPs represent an important step toward determining their role in odor perception. Previous work demonstrated that OBPs with chemosensory function are exclusively expressed in olfactory tissues (Ishida et al. 2002; Pelletier et al. 2010; 2011). Although other OBPs and OBP-like proteins have been found expressed in other non-chemosensory tissues, their olfactory functions have never been explicitly demonstrated (Leal et al. 2005). Since in close sibling species *Cx. quinquefasciatu*s, orthologues of OBP2 and OBP12, CquiOBP2 and CquiOBP12, were found to be specifically expressed in the antennae (Pelletier et al. 2009; 2011), I hypothesize OBP2 and OBP12 will also have chemosensory-tissue specific expression and olfactory function in *Cx. pipiens.* Additionally, since RNA-sequencing work demonstrated these OBPs are differentially expressed between our behaviorally divergent *Cx. pipiens* populations - I hypothesize they may contribute to the differences in host preference observed between our two mosquito populations. In particular, rather than seeing large expression differences between populations immediately after adult eclosion, we may expect to see differential expression of both OBPs increase significantly when these females would naturally engage in host-seeking and blood-feeding behaviors (around 7-9 days post eclosion).

Materials and Methods

Mosquitoes: The two populations used for qPCR analysis were an anautogenous, avian-seeking, AG population from Evanston, IL and an autogenous, mammalian-seeking, BG population (CAL1) from Calumet, IL. Both populations

were used in our preliminary RNA-sequencing study. OBP expression was examined at three timepoints post adult eclosion: 0-24 hours, 72-96 hours, and 168-216h (7-9 days). Three biological replicates were made for each timepoint assessed, per each population. Experimental samples were created by pooling 10x whole heads with females of the same age and population origin. All mosquitoes (regardless of age) were euthanized between 2 - 4 h after the onset of scotophase, when females are most actively engaged in blood-feeding behavior (Fritz et al. 2014). Seven to nine day old autogenous females will not bloodfeed until they have laid their first egg raft. Therefore, I only collected BG females once the number of egg rafts deposited in the provided ovipositional resource equaled the number of females in the cage. Additionally, at the time of tissue collection, 7-9 day old BG females were dissected to ensure ovaries were in the resting stage of ovarian development, as expected after the deposition of the first autogenous egg raft.

RNA isolation and cDNA synthesis: Whole heads were dissected on a 100 mm diameter, clear, polystyrene petri dish (Thermo Fisher Scientific, Waltham, MA) filled with dry ice to prevent RNA degradation and were stored in 300 ul Trizol at -80 degree C until RNA isolation. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with the following exception: the aqueous RNA–containing layer was back-extracted with chloroform twice (one more wash than what was required in the original protocol) to remove trace amounts of TRIzol components. Residual genomic DNA contamination was removed with a Turbo DNA-free kit (Invitrogen, Carlsbad, CA), and final RNA yield was assessed using a Nanodrop Lite spectrophotometer (Thermo Fisher

Scientific, Waltham, MA). First-strand cDNAs were synthesized from an input of 250 ng RNA using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) and a blend of oligo(dT) and random hexamer primers. Thermocycler conditions for cDNA synthesis followed manufacturer's instructions: 5 min at 25 degrees C, 20 min at 46 degrees C, 1 min at 95 degrees C.

Designing Primers for qPCR analysis: In the absence of a published genome for *Cx. pipiens*, cDNA sequences used to design the two OBP primer sets were obtained from the published genome assembly of the close relative, *Cx. quinquefasciatus,* accession number: AAWU00000000 (Arensburger et al. 2010). For both OBP2 and OBP12, *Culex quinquefasciatus* cDNA sequences were aligned to a preliminary, unpublished assembly of the *Culex pipiens* genome (made available by Dr. Megan Fritz and collaborators). The resulting *Cx. pipiens* sequence was then used for primer design. To examine the OBP expression across time the reference gene *Appl* (Vectorbase: CpipJ2_supercont3.523_299265_309049) was selected, as it has been demonstrated to have uniform expression across the two behaviorally divergent *Cx. pipiens* populations (Ling et al. 2011; Alys Jarvela, unpublished).

Primers for OBP2 and OBP12 were designed with the IDT PrimerQuest Tool, according to four criteria: 1.) primers spanned at least one exon-exon junction (to distinguish between genomic DNA and cDNA amplification), 2.) primers were designed using an annealing temperature between 57^{o}C-59^{o}C in order to prevent non-specific amplification, 3.) the last 5 bp of all the primer sequences contained less than three G and/or C bases, and 4.) all qPCR amplicons had a length 90-150 bp, consistent with qPCR primer standards (Buston et al. 2017). Amplicons for the three

primer sets were sequenced to confirm successful amplification and primer specificity (Table 2 and Table 3) (Altschul et al. 1990).

qPCR: qPCR reactions were carried out in a Roche LightCycler 480 Real-Time qPCR machine using the Luna Universal qPCR Master Mix (New England BioLabs) in a final volume of 20 ml. All PCR reactions were carried out using 40 cycles and technically replicated three times. Three qPCR controls were included in each run of the qRT-PCR experiment: 1) a *no template control (NTC)*, which served as a control for nucleic acid cross-contamination of reagents and surfaces and as a control for primer-dimer formation when using sybr green dyes; 2) A *negative reverse transcriptase control (NRT)*, which served as a control for contamination of genomic DNA within the samples, 3) *a no amplification control (NAC)*, which omitted the DNA polymerase from the qPCR reaction and assessed background fluorescence not associated with PCR product. The NRT control was conducted during the cDNA synthesis step immediately prior to qPCR (as cDNAs and not RNAs were used in the final qPCR assay).

Data Analysis: Raw Ct values were analyzed in R (A language and environment for statistical Computing; R Foundation for Statistical Computing, Vienna, Austria) Version 3.3.2 (2016-10-31). To investigate variation in OBP expression across our two behaviorally divergent *pipiens* populations over time, a generalized linear mixed model was used (Steibel et al. 2009). The model was constructed using the R package lme4 (v. 1.1-14; Bates et al. 2015) and was fit to the raw Ct values determined by Roche LightCycler 480 qPCR Software (which generates raw Ct values using the 'fit points' method). Three fixed effects were

considered: the ‘population’ from which the mosquito originated, and the ‘age’ corresponding to 24 hours (1 day), 96 hours (4 days) and 168 hours (7 days) post adult eclosion, and the ‘gene’ being assessed. To control for variation between replicates of the experiment, a single random effect of the ‘biological replicate’ was included. In order to find which response variables significantly impacted the construction of the model, minimal adequate models were fit by sequentially eliminating model terms using likelihood ratio tests conducted using the lmtest package (v. 0.9-35; Zeileis et al. 2002). Two 2-way interactions were found to be significant and were included in the model - the interaction between population*gene and the interaction between age*gene. The model was fit to two datasets, independently: 1) the raw Ct values for OBP2 and *Appl* for both populations and 2) the raw Ct values for OBP12 and *Appl* for both populations. For both generalized linear mixed models, model estimated Ct values for OBP2 and OBP12 were calculated relative to the BG population at 24h for the control gene, *Appl* (Table 4 - 7).

Change in fold expression between populations was analyzed using the ΔΔCt method (Livak et al. 2001). Model-estimated Ct values were used for this analysis, and expression was normalized using *Appl* reference gene. To investigate OBP expression between populations, at each timepoint, fold changes in expression for the BG *Cx. pipiens* (test sample) were calculated relative to the AG *Cx. pipiens* (calibrator sample) at that time point. Data were transformed using the log base 10 of the fold expression change, because the fold changes ranged from (0.24) to (1751.29).

To compare OBP expression over time within a single population, fold changes in expression at each timepoint were calculated relative to the populations' expression at 24 hours. This was conducted for the expression of both OBP2 and OBP12 in both the AG and BG populations.

Results

OBP2 fold expression difference was 0.90 (0.18, 4.69) when calculated for the BG population relative to the AG population at 24 hrs post adult eclosion (Table 8; Figure 1A). This indicated no statistically significant differences in expression-level between the two forms at this time point. In contrast, OBP2 had significantly higher levels of expression in the heads of BG females at both 96 and 168 hrs post adult eclosion, at 7.56 (1.30, 43.81) and 437.42 (75.89, 2521.01) fold higher, respectively (Table 8; Figure 1A). OBP12 was more highly expressed in the heads of BG females relative to the AG females at all three time-points assessed. Interestingly, OBP12 was upregulated to a greater degree in the BG females relative to AG females at the 24hr and 168hr timepoints (Table 9, Figure 1B).

Next, expression was examined over time for each OBP within a single population, where fold changes in expression at each timepoint were calculated relative to that populations' expression at 24 hours post adult eclosion. For OBP2, no change in fold expression over time (either 96 hrs or 168 hrs post eclosion) was observed for the AG, avian-seeking *Cx. pipiens* population (Table 10, Figure 2A). However, change in OBP2 fold expression was found to significantly increase between the 96hr and 168hr time points in the BG, human-seeking *Cx. pipiens*

population, where fold change in expression was 8.36 (1.44, 48.42) and 483.48 (83.88, 2786.40) higher, respectively (Table 10, Figure 2A). For OBP12, no change was observed among time points for the AG population (Table 11, Figure 2B). In the BG population, OBP12 was downregulated at the 96 hr time point, 0.24 (0.05, 1.18), relative to its level 24hr post adult eclosion. Additionally, OBP12 was significantly upregulated 168hr in the BG population, where it was expressed 103.06 (21.30, 497.99) fold higher than at 24hr post eclosion (Table 11, Figure 2B).

Discussion

I used qPCR to investigate the expression of OBP2 and OBP12 over time between two populations of behaviorally-divergent *Cx. pipiens* mosquitoes. Although we do not know which ligand(s) bind these OBPs *in vivo*, we investigated the expression of these genes to better understand their role in localization and selection of a vertebrate host for a blood meal.

BG females had higher OBP2 expression relative to AG females at the 96hr and 168hr time points, with no difference between the two populations at the 24hr time point. In the AG, avian-preferring population, OBP2 was reliably detected in the heads of these females at each time point assessed, but no change in expression was observed over time. Within the BG human-preferring population, OBP2 was most highly expressed at 168hrs (or 7 days) post adult eclosion, when these females would be actively seeking a host for a blood meal. These results support the role of OBP2 in the detection and perception of host volatiles in the human-preferring BG population.

In the BG population, OBP2 was more highly expressed 96hrs post eclosion relative to its expression at 24 hrs. At this time, most BG autogenous females are gravid, have not yet laid their initial egg clutch, and thus would not be engaging in host-seeking behaviors. Most studies citing the repression of chemosensory gene expression during egg maturation only investigate time points immediately (24hrs - 72hrs) post the acquisition of a blood meal (Rinker et al. 2013; Matthews et al. 2016; Taparia et al. 2017). However, autogenous *Cx. pipiens* females begin maturation of ovarian follicles for egg-laying immediately post adult eclosion (Chapter 1: Figure 1) (Spielman et al. 1967). At 96hrs (or 4 days) post eclosion, when ovarian development is likely to be or almost complete (Chapter 1: Figure 1; unpublished data), the overall repression of chemosensory gene expression is likely to be lifted. This makes sense considering these gravid BG females will also be actively seeking out an ovipositional resource, necessitating the detection of semiochemicals like MOP, indole, and 3-methylindole (Ishida et al. 2002; Pelletier et al. 2010). The increased expression of important chemosensory genes associated with the perception of host volatiles is likely initiated prior to the execution of host-seeking, so that females will be capable of host-seeking immediately following deposition of her autogenous egg raft. Additionally, considering the significant and dramatic increase in the relative expression of OBP2 (observed only in the BG population) at the 168-hour time point, it is likely this antennal-specific OBP contributes to the detection of host volatiles in this human-preferring population.

With the exception of a few OBPs, most insect OBPs are thought to bind to multiple odorants with similar molecular structures and functional groups (Vogt et al.

1981; Pelletier et al. 2010; Rollman et al. 2010; Swarup et al. 2011). Perhaps considering the increased expression of OBP2 at 96hrs, in addition to contributing to host detection, OBP2 is also capable of binding odorants that contribute to other mosquito behaviors that would necessitate its upregulation at this earlier time point.

BG females had higher OBP12 expression relative to AG females at all three timepoints assessed. In the AG population, OBP12, like OBP2, had relatively low expression that remained unchanged over time. OBP12 is also most highly expressed in the BG population at the 168hr timepoint when these females are most likely to be engaging in host-seeking behaviors. Unlike OBP2, which was seen to increase in expression over time in the BG population, OBP12 was downregulated at the 96hrs relative to 24hrs post eclosion. In contrast to the anautogenous AG population, a major physiological change occurs in autogenous females from the BG population at the 96hr time point. These females would be gravid and seeking an aquatic environment suitable for egg-laying, which requires the detection of volatiles. At the 168hr, however, autogenous females would have already laid their first egg raft and would likely be host-seeking.

OBP12 could also be associated with host-seeking as it is most highly expressed at the 168hr timepoint, just like OBP2, when BG females are actively host-seeking. If downregulation of OBP12 reduces BG females' chemosensory sensitivity to a host, it's downregulation at 96 hours may be explained by the fact that these females are gravid at 96hrs and would need to deposit their egg raft before engaging in host-seeking behaviors. If the volatile(s) OBP12 binds contribute only to the

detection of a host, it may be highly expressed when BG females are host-seeking and otherwise suppressed when host detection is not behaviorally relevant.

As an intriguing alternative hypothesis, perhaps OBP12 instead contributes to odor-mediated ovipositional behaviors. Rather than chaperoning volatiles directly to their designated ORs for odorant detection and perception, perhaps OBP12 is responsible for transporting ovipositional volatiles to odor degradation enzymes for removal from the sensillar lymph (Zwiebel et al. 2004; Pelletier et al. 2009). Under these circumstances, OBP12 would be down-regulated when gravid females are actively seeking an ovipositional resource. Indeed, this trend is observed in our BG autogenous females, which are gravid at 96 hrs. Following egg deposition, OBP12 would be upregulated to prevent ovipositional volatiles from reaching ORs, as the detection of these semiochemicals would not be behaviorally relevant until they had acquired a successful blood meal and undergone ovarian development and egg maturation. This is what we found in the BG population at 168hrs, where OBP12 was significantly upregulated in comparison to its expression at any other time point.

In conclusion, both OBPs were more highly expressed in the heads of the BG, human-seeking population confirming the results of our preliminary RNA-seq study. The two OBPs differed in their temporal expression patterns in the BG population over the first 7-9 days of adulthood. As both OBPs were most highly expressed at 168hrs when these females would be actively engaging in host-seeking, it may be possible both OBPs are contributing to the BG population's detection of a human host. However, based on the timing of their expression, OBP2 and OBP12 may be binding different host volatiles and thus play slightly different roles in overall odor

perception. Alternatively, the absence of OBP12 expression at 96hrs could indicate it instead is contributing to the suppression of odor-mediated ovipositional behaviors.

Because the roles I am assigning to these OBPs are speculative, future work should address what semiochemical or semiochemicals these OBPs are binding *in vivo* and demonstrate they influence mosquito behavior through functional analyses. Specifically, are the ligands of these OBPs a volatile unique to or highly represented in the odorant bouquets produced by the skin and breath of mammalian vertebrate hosts (in contrast to avian hosts)? To prove the essential role of OBP2 and OBP12 in human host-seeking behaviors, these OBPs could be individually knocked down in the BG population and behavioral testing would reveal whether these females are selecting a human host less frequently than the wild-type females. However, it is likely even if the OBPs contribute to host-seeking behaviors, BG *Cx. pipiens* may still be able to detect and seek out human hosts, as females use a multitude of host volatiles and non-chemosensory cues to locate a vertebrate host.

The ligand or ligands of OBP12 should also be investigated. Future work should address whether this volatile is used by gravid *Culex* females to locate suitable oviposition resources. Perhaps the volatile(s) OBP12 binds are more readily found in the ovipositional resources of underground breeding sites. Expression of OBP12 could be examined in the BG population after blood-feeding when these females would again be gravid. Perhaps, if OBP12 contributes to decreasing the overall sensitivity to ovipositional odorants we might expect to see expression again downregulated at this new time-point. Alternatively, we could artificially upregulate

OBP12 expression in gravid females and see whether this delays or prevents oviposition.

If the ligands of these OBPs and the mechanisms of action can be elucidated, in the distant future, we could develop baits that target human-seeking, BG populations of *Cx. pipiens*, novel repellents that prevent females from detecting hosts in the field, and ovipositional baits that specifically attract BG gravid females for control or vector population monitoring purposes.

Tables: Chapter 3

Table 1: OBP expression differences from preliminary RNA sequencing work. Differential expression and corrected p-values are included from preliminary RNA-seq analysis and represent the difference between 7-9 day old parous below-ground *Cx. pipiens* females and above-ground collected females. Both OBPs 2 and 12 were more highly expressed in parous BG females (*Differential expression value - log2FoldChange*)

Name	Fold Change	Differential Expression Value	Corrected p-value
CquiOBP2/CPIJ007617	1.85	0.89	0.013
CquiOBP12/CPIJ016949	3.61	1.85	2.07E-11

Table 2: qPCR was performed using the following gene specific primers. Amplicon length in base pairs indicates the length of the full cDNA fragment determined during primer development. The sequenced amplicons were a composite of the amplified product of the forward and reverse primer.

Name	Seq. Source	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'	Amplicon Size	Recovered Amplicon Size	Sequenced Amplicon
OBP2 (CPIJ007617)	Unpublished - *Cx. molestus* genome assembly	ACCGAGGC GAGATGCT GAATACC	GAAGATG CCATCAAG CGCTTCAG C	115 bp	115 bp	ACCGAGGCGAGATGCTGAA TACCCTCCACCGGAGTTTTT GGTGAAGATGAAGCCCATG CATGATGAATGTGTTGCAGA AACAGGTGCCTCCGAAGAT GCCATCAAGCGCTTCAGC
OBP12 (CPIJ016949)	Unpublished - *Cx. molestus* genome assembly	CCTGGAAG AGGCAAA GAAAG	GCCTCGTT TGGTAGCT TG	97 bp	94 bp	TCCTGGAAGAGGCAAAGAA AGGAGTGTTCCCAACGAAA AGACTCAAATGCTATGTCAG CTGTCTGCTCGACATGATGC AAGCTACCAAACGAGG
Appl (A. Jarvela, unpublished)	VectorBase	AGGAAGC GGAACCG AAGATG	CGAAGGC CAGCGTA AAGTAC	135 bp	135 bp	GGAAGCGGAACCGAAGATG CAGCTAGGGATGGCCCACG ACATCGGACACGGCGAACC GAGCTACTCGGTCCGGCGGG AGATCTACGGCTCGAGCGGC CACGAGGGCAAGAACGTGT ACTTTACGCTGGCCTTCGA

Table 3: Sanger Sequencing results for forward and reverse primers. Recovered sequence length refers to the number of base pairs recovered with unambiguous base calls for the forward or reverse primer for each gene. The recovered sequences were identified through the National Center for Biotechnology Information "basic local alignment search tool" (BLAST) against the nucleotide database to demonstrate primer specificity and correct amplification of target genes (Altschul et al. 1990).

OBP / Primer	Amplicon Length	Recovered Sequence Length	BLAST RESULTS
OBP2_v2_F	115 bp	61 bp	1. *Culex quinquefasciatus* odorant-binding protein 2 (OBP2) XM_001848887.1 / FJ947084.1 - (98% IM) 2. *Culex pipiens pallens* odorant binding protein 2 (OBP2) KU847963.1 - (96.45% IM)
OBP2_v2_R	115 bp	66 bp	ONLY RESULT: *Culex pipiens pallens* odorant binding protein 2 (OBP2) KU847963.1 (100% IM)
OBP12_v2_F	97 bp	54 bp	ONLY RESULT: *Culex pipiens pallens* odorant binding protein 12 (OBP12) KU847971.1 - (96.36% IM)
OBP12_v2_R	97 bp	52 bp	ONLY RESULT: *Culex pipiens pallens* odorant binding protein 12 (OBP12) KU847971.1 - (94.55% IM)
Appl_F	135 bp	87 bp	ONLY RESULT: *Culex quinquefasciatus* conserved hypothetical protein, mRNA - XM_001864448.1; (98% IM)
Appl_R	135 bp	85 bp	ONLY RESULT: *Culex quinquefasciatus* conserved hypothetical protein, mRNA - XM_001864448.1; (100% IM)

Table 4: Results from generalized linear mixed model with exploring the effect of population, age and gene on Ct value for the expression of OBP2, where Ct ~ population*gene + age*gene + (1| biological replicate). The model intercept was set to the BG population at 24h for the control gene, Appl. Where BG = below-ground *Cx. pipiens* population and AG = above-ground *Cx. pipiens* population.

Term	Estimate	Std. Error	t Value	2.5% CI	97.5% CI
BG-24h Appl (Intercept)	22.0179	0.5908	37.27	20.5038	23.5321
AG-24h Appl	-0.7336	0.2986	-2.46	-1.3242	-0.1429
BG-24h OBP2	2.1223	0.4186	5.07	1.2943	2.9503
BG-96h Appl	1.4398	0.3675	3.92	0.7127	2.1668
BG-168h Appl	1.345	0.3620	3.72	0.6288	2.0612
AG-24h OBP2	1.2443	0.4202	2.96	0.4131	2.0755
BG-96h OBP2	0.4991	0.5159	0.97	-0.5214	1.5197
BG-168h OBP2	-5.45	0.5120	-10.64	-6.4628	-4.4372

Table 5: OBP2 - Mean Ct values and 2.5% and 97.5% confidence intervals calculated from model estimates

Population	Age - Gene	Raw Mean Ct	Mean Ct (from model)	2.5% CI	97.5% CI
Above-ground	24h - OBP2	24.57	23.26	20.92	25.61
	96h - OBP2	27.05	24.70	21.63	27.77
	168h - OBP2	20.17	24.61	21.55	27.67
Below-ground	24h - OBP2	24.22	24.14	21.80	26.48
	96h - OBP2	25.62	22.52	19.98	25.05
	168h - OBP2	20.41	16.57	14.04	19.09
Above-ground	24h - Appl	21.32	21.28	19.18	23.39
	96h - Appl	22.89	22.72	19.89	25.56
	168h - Appl	22.42	22.63	19.81	25.45
Below-ground	24h - Appl	21.98	22.02	20.50	23.53
	96h - Appl	23.26	23.46	21.22	25.70
	168h - Appl	23.57	23.36	21.13	25.59

Table 6: Results from generalized linear mixed model with exploring the effect of population, age and gene on Ct value for the expression of OBP12 where Ct ~ population*gene + age*gene + (1| biological replicate). The model intercept was set to the BG population at 24h for the control gene, Appl. Where BG = below-ground *Cx. pipiens* population and AG = above-ground *Cx. pipiens* population.

Term	Estimate	Std. Error	t Value	2.5% CI	97.5% CI
BG-24h Appl (Intercept)	22.0217	0.4554	48.36	21.0428	23.0024
AG-24h Appl	-0.7412	0.3813	-1.94	-1.4956	0.0135
BG-24h OBP12	0.2929	0.5370	0.55	-0.7695	1.3559
BG-96h Appl	1.4512	0.4693	3.09	0.5223	2.3798
BG-168h Appl	1.345	0.4623	2.91	0.4301	2.2599
AG-24h OBP12	3.6386	0.5464	6.66	2.5567	4.7194
BG-96h OBP12	3.826	0.6773	5.65	2.4853	5.1658
BG-168h OBP12	-5.0494	0.6538	-7.72	-6.3432	-3.7556

Table 7: OBP12 - Mean Ct values and 2.5% and 97.5% Confidence Intervals calculated from model estimates

Population	Age - Gene	Raw Mean Ct	Mean Ct (from model)	2.5% CI	97.5% CI
Above-ground	24h – OBP12	22.89	25.66	23.60	25.08
	96h – OBP12	31.90	27.11	24.12	27.24
	168h – OBP12	20.41	27.01	24.03	27.14
Below-ground	24h – OBP12	23.26	22.31	20.27	25.95
	96h – OBP12	19.71	25.85	23.53	24.52
	168h – OBP12	25.28	16.97	14.70	18.57
Above-ground	24h - Appl	21.32	21.28	19.55	22.86
	96h - Appl	26.56	22.73	20.07	25.03
	168h - Appl	23.57	22.63	19.98	24.92
Below-ground	24h - Appl	21.98	22.02	21.04	23.00
	96h - Appl	22.42	23.47	21.57	25.17
	168h - Appl	22.25	23.37	21.47	25.06

Table 8: ΔΔCt, fold change, and log fold expression of OBP2 in below-ground *Culex pipiens* populations relative to above-ground populations calculated at three timepoints.

Age	ΔΔCt	Fold Change (2.5%, 97.5% CIs)	Log (10) Fold Change (2.5%, 97.5% CIs)
0-24h	0.1444	0.90 (0.18, 4.69)	-0.04 (-0.75, 0.66)
72-96h	-2.9186	7.56 (1.30, 43.81)	0.88 (0.12, 1.64)
168 - 216h (7-9d)	-8.7729	437.42 (75.89, 2521.01)	2.64 (1.88, 3.40)

Table 9: ΔΔCt, fold change, and log fold expression of OBP12 in below-ground *Culex pipiens* populations relative to above-ground populations calculated at three timepoints.

Age	ΔΔCt	Fold Change (2.5%, 97.5% CIs)	Log (10) Fold Change (2.5%, 97.5% CIs)
0-24h	-4.0869	16.99 (1.36, 69.94)	1.23 (0.14, 1.84)
72-96h	-2.005	4.01 (0.80, 20.04)	0.60 (-0.09, 1.30)
168 - 216h (7-9d)	-10.7742	1751.29 (361.99, 8462.67)	3.24 (2.56, 3.93)

Table 10: ΔΔCt, fold change, and log fold expression of OBP2 over time. For each population, $\log_{10}$ fold change expression of OBP2 was determined by ΔΔCt method, normalizing first to housekeeping gene *Appl*, then to OBP2 expression at the 24-hour timepoint.

Population	Gene	Age	ΔΔCt	Fold Change (2.5%, 97.5% CIs)	Log (10) Fold Change (2.5%, 97.5% CIs)
Above-ground	OBP2	0-24h	0.00	1.00 (0.20, 5.08)	0.00 (-0.71, 0.71)
		72-96h	0.00	1.00 (0.12, 8.41)	0.00 (-0.92, 0.92)
		168 - 216h (7-9d)	0.00	1.00 (0.12, 8.35)	0.00 (-0.92, 0.92)
Below-ground	OBP2	0-24h	0.00	1.00 (0.20, 5.07)	0.00 (-0.71, 0.71)
		72-96h	-3.06	8.36 (1.44, 48.42)	0.92 (0.16, 1.96)
		168 - 216h (7-9d)	-8.92	483.48 (83.88, 2786.40)	2.68 (1.92, 3.45)

Table 11: ΔΔCt, fold change, and log fold expression of OBP12 each single population over time. For each population, log fold change expression of OBP12 was determined by ΔΔCt method, normalizing first to housekeeping gene *Appl*, then to OBP12 expression at the 24-hour timepoint.

Population	Gene	Age	ΔΔCt	Fold Change (2.5%, 97.5% CIs)	Log (10) Fold Change (2.5%, 97.5% CIs)
Above-ground	OBP12	0-24h	0.00	1.00 (0.23, 4.17)	0.00 (-0.62, 0.62)
		72-96h	0.00	1.00 (0.13, 7.94)	0.00 (-0.90, 0.90)
		168 - 216h (7-9d)	0.00	1.00 (0.13, 7.87)	0.00 (-0.90, 0.90)
Below-ground	OBP12	0-24h	0.00	1.00 (0.24, 4.12)	0.00 (-0.62, 0.62)
		72-96h	2.08	0.24 (0.05, 1.18)	-0.63 (-1.33, 0.07)
		168 - 216h (7-9d)	-6.69	103.06 (21.30, 497.99)	2.01 (1.33, 2.70)

Figures: Chapter 3

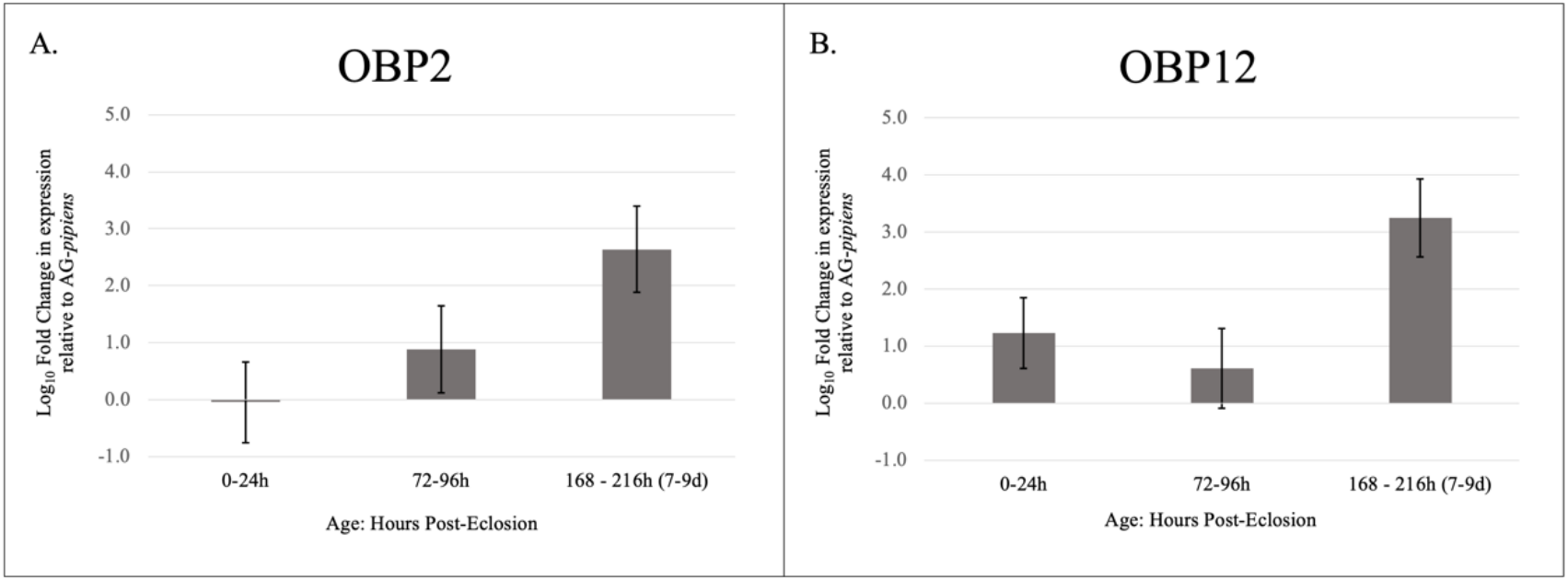

Figure 1: OBP log fold expression change over time in below-ground *Culex pipiens* populations relative to above-ground populations. Log fold change OBP2 (Panel A) and OBP12 (Panel B) expression at each timepoint determined by ΔΔCt method, normalizing first to housekeeping gene *Appl*, then to the AG-population sample. Error bars indicate 95% CIs

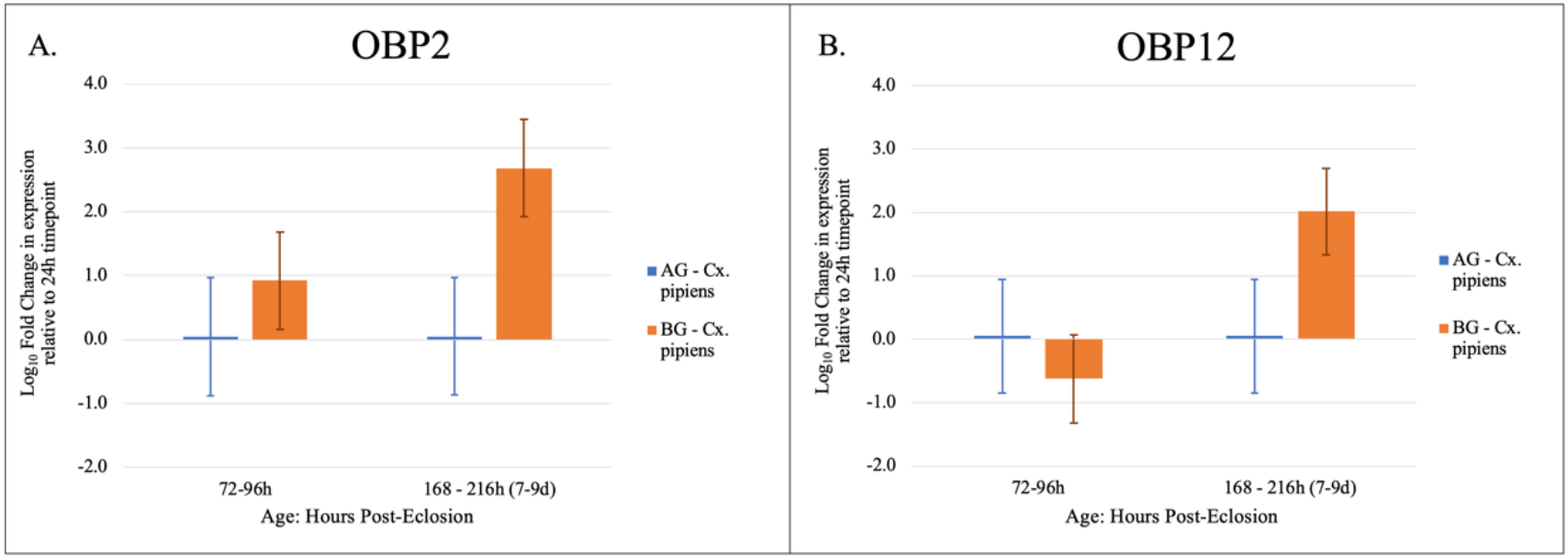

Figure 2: OBP log fold expression over time in above- and below-ground *Culex pipiens* populations. Log_{10} fold change expression of OBP2 (Panel A) and OBP12 (Panel B) determined by ΔΔCt method, normalizing first to housekeeping gene *Appl*, then to the expression at the 24-hour timepoint. Error bars indicate 95% CIs.

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