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The Neuropeptide Regulation of Host-Seeking Behavior in Aedes Aegypti Mosquitoes

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THE NEUROPEPTIDE REGULATION OF HOST-SEEKING BEHAVIOR IN *AEDES AEGYPTI* MOSQUITOES

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Aedes aegypti mosquitoes are the principal vectors for several human diseases including Dengue Fever, which causes ~400 million cases and ~24,000 deaths per year (Bhatt et al., 2013; WHO, 2002). Novel strategies to combat mosquito-borne diseases are needed for *A. aegypti* and other mosquitoes such as the malaria vector *Anopheles gambiae*. Our goal was to discover new ways to interfere with the ability of a mosquito to locate a human host for a blood meal. Currently, the mechanistic basis of host-seeking and its regulation remain incompletely understood. Although it is known that mosquitoes require human odor cues to locate a human host, the critical odor components and associated olfactory receptors have not been identified (Klowden, 1995; Takken and Knols, 1999). Previous work showed that mosquito host-seeking behavior is inhibited by a hemolymph-borne humoral factor for three days following a blood meal. Subsequent studies identified Head Peptide-I as a candidate neuropeptide modulating this suppression in host-seeking behavior. This conclusion was strengthened by the observation that Head Peptide-I injection into non-blood-fed females triggered the inhibition of host-seeking. The mechanism by which this important peptide alters mosquito behavior and the receptor through which it signals are unknown (Brown et al., 1994).

We used a cell-based calcium-imaging screen to identify the G-protein coupled receptor NPY-Like Receptor-1 (NPYLR1) as a candidate Head Peptide-I

receptor. We found that multiple NPYLR1 agonists, including the feeding-related Short-Neuropeptide-3 (sNPF3), are capable of inhibiting host-seeking behavior when injected into non-blood-fed females. To investigate whether NPYLR1 is required for Head Peptide-I inhibition, we pioneered targeted mutagenesis with zinc-finger nucleases to create multiple NPYLR1 null-mutant mosquito lines. We predicted that these mutants would no longer show inhibition of host-seeking behavior after a blood meal. While we can say with certainty that NPYLR1 is a receptor for Head Peptide-I, we found no behavioral effects for NPYLR1 mutants in locomotion, egg-laying, sugar feeding, blood feeding, or host-seeking behavior. Our results suggest that NPYLR1 is not required *in vivo* for Head Peptide-I action and that a redundant signaling mechanism for behavioral inhibition exists. Future work will determine the necessity of Head Peptide-I during host-seeking inhibition and attempt to identify additional Head Peptide-I and sNPF receptors. This research will clarify the mechanism of Head Peptide-I inhibition and could form the basis for novel strategies to control mosquito host-seeking behavior.

This work is dedicated to:

My mother, Janice Liesch.

My father, Jerrold Liesch.

My undergraduate mentor, Dr. Caren Chang.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
BUFFET	Caged Feeding Assay
CAFE	Capillary Feeder Assay
cAMP	3'-5'-cyclic adenosine monophosphate
CDC	Center for Disease Control
cDNA	Complementary DNA
CO2	Carbon Dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DEET	N,N-dimethyl-meta-toluamide
DNA	Deoxyribonucleic Acid
EC50	Half maximal effective concentration
ECFP	Enhanced Cyan Fluorescent Protein
GPCR	G-Protein Coupled Receptor
HEG	Homing Endonuclease Genes
HEK	Human Embryonic Kidney 293 cells
HP-I	Head Peptide-I
HP-I[Cys10]	Head Peptide-I with a C-terminal mutation to Cysteine
HR	Homologous Recombination
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
MAG	Male Accessory Gland
mRNA	Messenger RNA
NHEJ	Non-homologous End Joining
NPF	Neuropeptide-F
NPFR	Neuropeptide-F receptor
NPYLR	NPY-Like Receptor
NPY	Neuropeptide-Y
OSN	Olfactory sensory neuron
PCR	Polymerase Chain Reaction
PYY	Peptide-YY
qPCR	Qualitative Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase PCR
SIT	Sterile Insect Techniques
sNPF	Short-Neuropeptide-F
SPR	Sex Peptide Receptor
TALEN	Transcription activator-like effector nuclease
WHO	World Health Organization
ZFN	Zinc-Finger Nuclease

1 Introduction

1.1 Arthropods are Major Vectors for Human Disease

Blood-sucking arthropods, such as mosquitoes and ticks, are major vectors of human disease. *Aedes aegypti* is a vector for Dengue Fever, a viral disease that is spread to human hosts during blood-feeding of female mosquitoes, which is estimated to infect 400 million worldwide and cause ~24,000 deaths per year (Bhatt et al., 2013; WHO 2002). The malaria mosquito vector, *Anopheles gambiae,* is described as "the world's deadliest animal," causing nearly 1 million deaths per year and placing an estimated 2.57 billion people at risk of the protozoan parasite *Plasmodium falciparum* (Gething et al., 2011). In North America and Europe, Lyme disease - caused by transmission of the bacteria *Borrelia burgdorferi* through the bites of *Ixodes scapularis* ticks - is the most prevalent vector-borne disease and one of the fastest-growing infectious diseases in the United States (Barbour and Fish, 1993; Levi et al., 2012; CDC 2011).

Although antibiotics are available for treating Lyme disease, no effective treatment or vaccine exists for dengue fever. Despite many decades of research, there is also no highly effective vaccine for malaria (Bejon et al., 2008) and the parasites have developed rapid resistance to anti-malarial drugs (Dondorp et al., 2009; Sá et al., 2011; Vathsala et al., 2004). Once contracted, mosquito-borne diseases lead not only to severe effects on human health but also to socio-economic disruption (Gallup and Sachs, 2001). Because we lack effective treatments, efforts to prevent transmission of vector-borne diseases concentrate on vector control using a combination of chemical and biological targeting and management of breeding sites.

1.2 Strategies to Reduce the Transmission of Vector-Borne Diseases

Current methods to reduce disease transmission from mosquitoes include the use of physical barriers such as insecticide-treated bed nets, interior residual spraying of insecticides, anti-malarial drugs, prophylactic drugs for travelers, vector population control, and chemical insect repellents. Bed nets impregnated with insecticides have been extremely effective when used appropriately (Mutuku et al., 2011). However, distribution problems, maintenance, incorrect usage, and increasing resistance to insecticides are a continual challenge and therefore complementary strategies are needed. Anti-malarial drugs, such as chloroquine and artemisinin derivatives, are broadly used today, although the emergence of parasite resistance to one or both class of drugs is common (Castelli et al., 2012; Vathsala et al., 2004). Administration of prophylactic drugs to prevent malaria infection, such as mefloquine (Lariam), are also commonly prescribed to travelers despite reports of severe mental side-effects (AlKadi, 2007). Population control through the introduction of sterile insects generated by either irradiation or genetic engineering is a promising new strategy, but complicated population genetics of *A. gambiae* mosquito populations in Africa may make their application problematic (Harris et al., 2012; Hoffmann et al., 2011; Wilke and Marrelli, 2012). Lastly, in the developed world the most effective chemical repellent for a broad range of arthropods is DEET (N,N-diethyl-meta-toluamide), but this is not widely used in disease endemic areas because of the need to reapply topically to all areas of exposed skin at frequent intervals. Unfortunately, these current efforts are failing to stem the increasing incidence of vector-borne diseases and are creating a high demand for novel prevention strategies (Gething et al., 2011; Gubler, 1998) (CDC, 2011).

1.3 Novel Prevention Strategies Exploit Genetic Approaches

In response to the shortcomings of current prevention strategies, researchers are beginning to move beyond primitive tactics of physical barriers and drug treatments to more sophisticated approaches based on molecular genetics. With the recent publication of several mosquito genomes including *A. gambiae* (Holt et al., 2002), *A. aegypti* (Nene et al., 2007), and the West Nile vector *Culex quinquefasciatus* (Arensburger et al., 2010), along with successful generation of transgenic mosquitoes, researchers are developing genetically based methods for population disruption (Jasinskiene et al., 1998; Nimmo et al., 2006).

One genetic strategy employs the expression of naturally selfish homing endonuclease genes (HEG), whose nuclease activity triggers DNA repair systems to replicate the HEG between chromosomes and ultimately cause the rapid spread of itself through naïve mosquito populations (Windbichler et al., 2012). Using this strategy, researchers believe they can engineer HEGs for targeted disruption of important genes for vector capacity or to introduce novel genes that impair vector competence. Recent reports indicate that HEGs introduced into *A. gambiae* by transgenesis are effective at spreading through laboratory populations (Windbichler et al., 2012). However, progress has not been reported for engineering HEGs to recognize DNA sequences of interest, which is a major obstacle to further development.

A second strategy aims to use genetics to improve the generation of mosquitoes that are infertile or unable to mate for use in Sterile Insect Techniques (SIT). Historically, SIT programs have been effective at population disruption by introducing large numbers of sterile insects produced by irradiation that compete with wild-type individuals for breeding opportunities.

Researchers are modernizing the approach for mosquito application by replacing irradiation with transgenic male mosquitoes carrying lethal genes that prevent the development of offspring (Wise de Valdez et al., 2011). A complementary strategy was recently reported that created female-specific flightless transgenic mosquitoes to remove logistical delays from manually separating males prior to release (Fu et al., 2010).

A last strategy of note, though note technically genetic, is the introduction of the life-shortening bacterial symbiont, *Wolbachia*, into *A. aegypti* to reduce opportunities for disease transmission (Mcmeniman et al., 2009). Interestingly, *Wolbachia* possesses an inherent drive to spread through populations using cytoplasmic incompatibility, a type of embryonic lethality that prevents development of offspring that do not carry the bacteria. Recent reports describe field trials where *Wolbachia* successfully invaded two natural populations of *A. aegypti* in Australia within a few months (Hoffmann et al., 2011). Larger field trials are currently being administered in South East Asian countries.

Despite promising results, we were surprised to find that none of these approaches applied genetics to understand the mechanisms by which female mosquitoes locate a human host for blood-feeding. Further understanding of the molecular basis of mosquito host-seeking behavior could uncover new approaches for interfering with host location to prevent disease transmission.

1.4 The Importance of Olfaction in Host-Seeking Behavior

Host-seeking behavior can more broadly be understood as a set of behaviors that an organism undertakes to locate food. For blood-feeding arthropods, the food is blood, which is contained within a living host. For other organisms such

as the fly (*Drosophila melanogaster*) the food source is yeast growing on rotting fruit and plant materials (Dethier, 1976). Typically, food-search and host-seeking behaviors in arthropods utilize long-distance odor cues for guidance to a food source (Vosshall and Stocker, 2007). *A. aegypti* and *A. gambiae* are anthropophilic - specialists that feed nearly exclusively on humans (Pates et al., 2007; Ponlawat and Harrington, 2005; Scott and Takken, 2012) - whereas other mosquitoes feed on other mammals, birds, and even reptiles (Besansky et al., 2004; Loaiza et al., 2012; Takken and Knols, 1999). Therefore, mosquitoes also use their sense of smell to identify a compatible host within a complex and often dangerous environment. When successful, additional senses including taste, vision, and heat are incorporated at shorter distances to evaluate the quality of the host for feeding. Notably, the dependency on olfaction for host location presents an opportunistic target for disrupting the ability of mosquitoes to spread disease, as exemplified by the long-distance effectiveness of DEET (Paluch et al., 2010).

In the laboratory, *A. aegypti* host-seeking behavior can be modeled as an olfactory-dependent process that orients mosquitoes to human odor cues and guides them to the proximity of host stimuli. Using a uniport olfactometer, based on a design by Dr. Marc J. Klowden (Klowden and Lea, 1979a), we have confirmed the attraction of female mosquitoes to various stimuli (**Figure 1.1A**). In the absence of any human odor cues, mosquitoes display very low levels of attraction to ambient air (**Figure 1.1B**). In contrast, when a human hand or forearm is supplied as an odor source, mosquitoes express robust attraction. There is a synergistic increase in attraction when human hand odor is combined with carbon dioxide (CO_2) at comparable levels to human breath (4%) (Smith et al., 2009). The uniport olfactometer provides a functional behavioral assay to

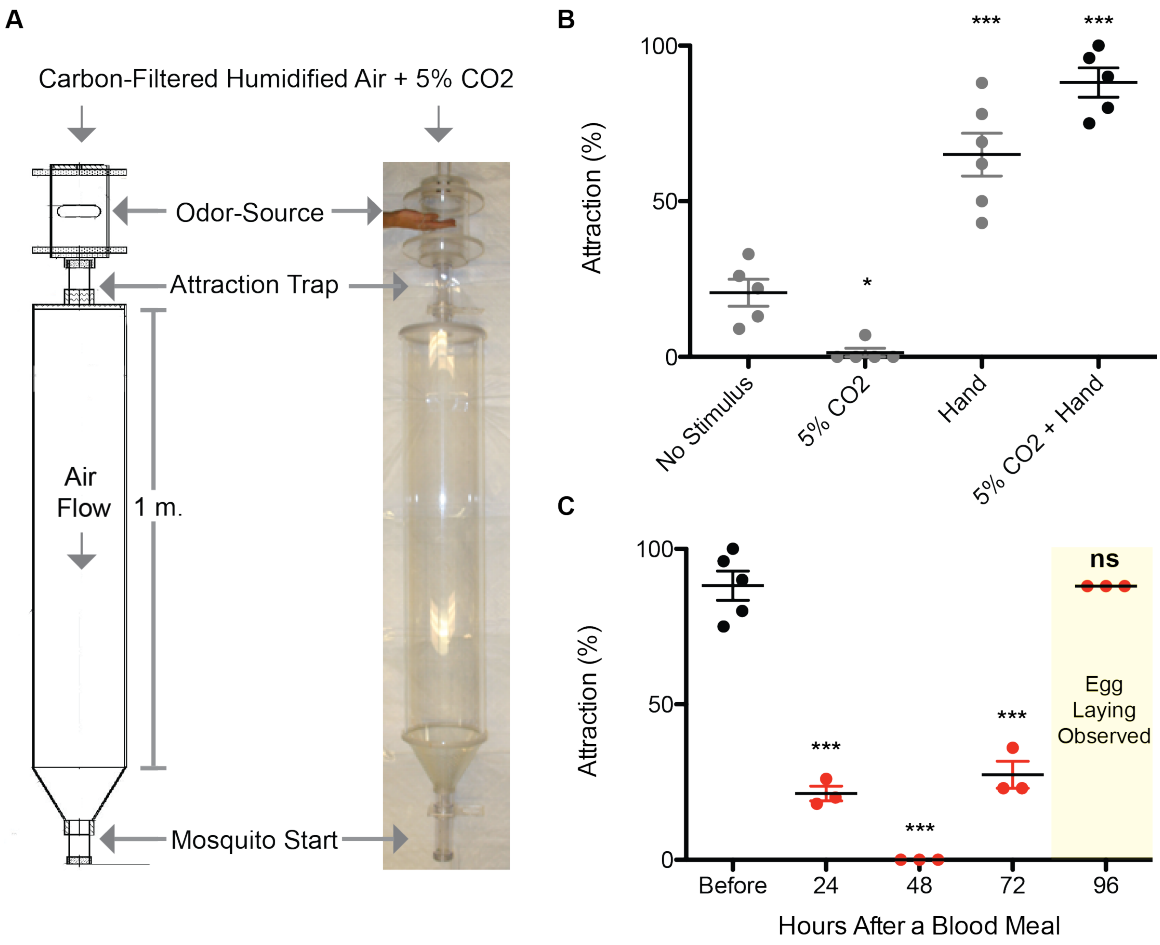


Figure 1.1 Host-seeking Behavior is Olfactory-Dependent and Inhibited by Blood-Feeding. (A) Diagram of the uniport olfactometer. **(B)** Percent attraction of female mosquitoes to various stimuli (n=5-6; ~20 mosquitoes per trial)**. (C)** Percent attraction of females to human hand odor before and after a blood meal (n=3-5; ~20 mosquitoes per trial). Access to egg laying locations was permitted after 72h and observed at 96h. In B-C, data are plotted as mean ± SEM. ANOVA with Dunnett's Correction for Multiple Comparison; * = $p<0.05$, *** = $p<0.001$, ns = not significant.

address questions related to the molecular basis of olfactory-dependent host-seeking behavior in *A. aegypti*.

1.5 Internal Regulation of Mosquito Host-Seeking Behavior

Early behavioral studies of *A. aegypti* identified a quiescent period after blood-feeding where female mosquitoes are no longer attracted to host odor cues (**Figure 1.1C**). Recovery to host stimuli occurs following egg-laying, starting a new cycle of host-seeking behavior, quiescence, and egg-laying, referred to collectively as the gonotrophic cycle. Pioneering researchers of mosquito behavior including Dr. Marc J. Klowden, Dr. Marc R. Brown, and Dr. Arden O. Lea realized that naturally evolved mechanisms for regulating host-seeking behavior were hidden within the gonotrophic cycle, and if understood, may lead to the discovery of a universal strategy to stop the spread of disease (Klowden, 1995).

1.5.1 The Gonotrophic Cycle – Hidden Mechanisms for Regulation

Approximately 3 to 4 days after adult emergence, female *A. aegypti* mosquitoes achieve a fully developed olfactory system as indicated by reproducible sensitivity to the host odor lactic acid (Davis, 1984). To obtain a protein-rich blood meal for egg development, robust host-seeking behavior occurs until the act of blood-feeding is initiated on a human host. The female will then continue to feed until either physically startled or the anterior midgut senses maximal distension of approximately 3-5 μl (Klowden and Lea, 1978).

Once fully engorged, abdominal distension inhibits attraction to hosts by an unknown mechanism, permitting females to retreat to safer resting locations to

begin digestion of the blood meal (Klowden and Lea, 1979b). ~24 hours after blood-feeding, abdominal distension is relieved and mosquitoes evaluate the volume and quality of the meal to determine if egg development is permissible (Klowden and Lea, 1978). Inadequate amounts of blood trigger an immediate return to host-seeking behavior to obtain a supplementary meal from the same or a different host. Adequate blood meals proceed into egg development where inhibition of host-seeking behavior continues from approximately 24 to 72 hours due to a putative humoral factor that circulates in the hemolymph of the mosquito (Klowden and Lea, 1979a). This hypothesis was developed from the observation that when hemolymph from a female *A. aegypti* mosquito, 48 hours after a blood meal, was injected into non-blood-fed females, a behavioral switch to host-seeking inhibition was induced in the recipients (Klowden and Lea, 1979a). Further examination identified that release of the humoral factor was dependent on an unknown signal originating in the ovaries roughly 6-10 hours post-blood meal (Klowden, 1981).

Egg maturation completes ~72-96 hours after blood-feeding, which coincides with gravid females exhibiting attraction to odor cues from egg laying sites, such as 0.2% methyl propionate, rather than human odors (Klowden and Blackmer, 1987). Once eggs are laid, female mosquitoes quickly recover host-seeking behavior and the gonotrophic cycle begins again, leading to a second blood meal (Klowden, 1981). Female *A. aegypti* mosquitoes will complete several gonotrophic cycles throughout their life, providing efficient means for transmitting diseases to human hosts.

In all, these observations suggested that a humoral factor is released into the hemolymph of females somewhere between 6-72 hours after a blood meal that

triggers host-seeking inhibition. Using techniques available at the time, those working in the field attempted to isolate this candidate humoral factor. The overall goal of this work was to determine, at the molecular level, how mosquitoes internally regulate their attraction to human hosts.

1.5.2 Identification of Head Peptide-I

A biochemical screen by Matsumoto et al. (1989) led to the purification of several *A. aegypti* hemolymph-borne FMRFamide-like neuropeptides, which provided candidates for the humoral regulator of host-seeking behavior. The neuropeptides were tested in a functional assay for the ability to inhibit host-seeking behavior when injected into non-blood-fed females. A single candidate neuropeptide, Head Peptide-I (HP-I, pERPhPSLKTRFa, pE = pyroglutamic acid, hP = hydroxyproline, a = c-terminal amidation) was found to be capable of inhibiting behavior (Brown et al., 1994). Control experiments showed that a synthetic peptide containing a single mutation of the terminal amino acid (HP-I [Cys10], pERPhPSLKTR**C**) was inactive in modulating host-seeking behavior. Subsequent radioimmunoassays determined that Head Peptide-I titers in female *A. aegypti* hemolymph increased and sustained at 5-fold regular levels between 24-36h, correlating well with behavioral inhibition (**Figure 1.2A and B**). This sustained increase in Head Peptide-I may be necessary for long-term suppression of host-seeking, because after a single injection of synthetic Head Peptide-I, behavior was recovered within an hour (Brown et al., 1994).

Interestingly, mosquitoes exhibiting inhibition of host-seeking behavior from Head Peptide-I injections were still observed to blood-feed when placed

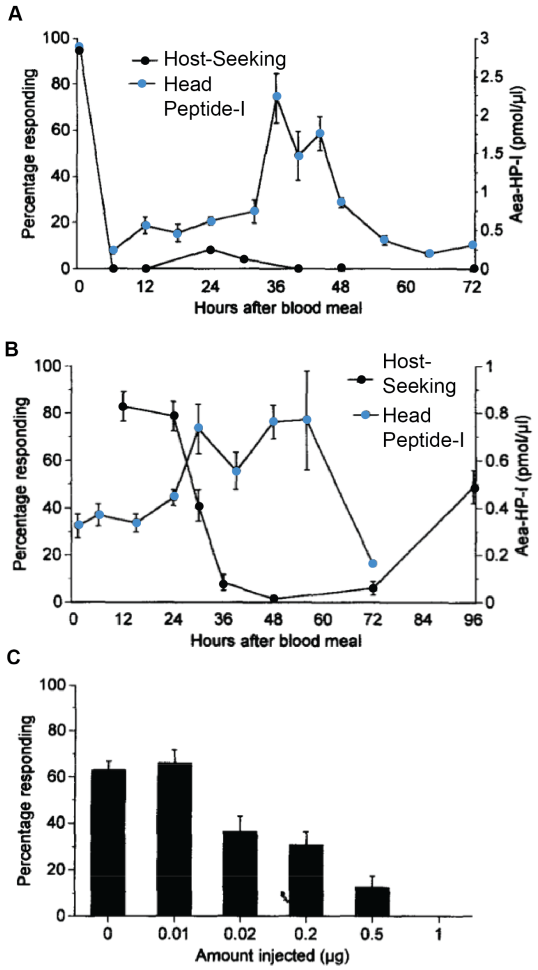


Figure 1.2 Head Peptide-I Inhibits Host-Seeking Behavior. *Figures and text reproduced from Brown et al. 1994 with slight graphical modifications for clarity.* **(A)** Mosquito host-seeking behavior at intervals after a replete blood meal [black circles (data from Klowden, 1990)] compared to the hemolymph titer of Head Peptide-I (blue circles) determined by a radioimmunoassay. **(B)** Mosquito host-seeking behavior at intervals after a ~1μl enema of blood [black circles (data from Klowden and Lea, 1979a)] compared to the hemolymph titer of Head Peptide-I (blue circles) from similarly treated females as determined by radioimmunoassay. **(C)** Effect of injections of synthetic Head Peptide-I on mosquito host-seeking behavior. Host-seeking behavior was measured in an olfactometer within 1h of peptide injection. In A-C, vertical lines represent standard errors.

directly on a human arm (Brown et al., 1994). This finding highlights that Head Peptide-I appears to regulate specific sensory modalities critical to host-seeking behavior, namely olfaction, rather than those needed for feeding such as taste, vision, and heat (Allan et al., 1987; Takken and Knols, 1999).

Eight years after this initial work on Head Peptide-I function, the Head Peptide-I gene was cloned using RT-PCR with degenerate primers guided by the protein sequence of the peptide (Stracker et al., 2002). The gene has an open reading frame encoding a prepropeptide of 128 amino acids, consisting of a 22-residue signal peptide followed by three tandem repeats of the propeptide QRPPSLKTRFG. After post-translational modifications, the propeptide is processed into mature Head Peptide-I protein (pERPhPSLKTRFa)(Stracker et al., 2002). The role of Head Peptide-I post-translational modification is unknown, but may be a point of regulation in Head Peptide-I production or confer protein stability when released into the hemolymph.

With the gene identified, Stracker et al. (2002) performed a combination RT-PCR/DNA blotting technique on RNA isolated from dissected tissue and found expression of Head Peptide-I in the brains of larva, males, females, and in the midgut and terminal ganglion of adult mosquitoes. Further RNA *in situ* hybridization localized expression of Head Peptide-I in adult females to endocrine cells of the posterior midgut as well as neurosecretory cells in the brain and the terminal ganglion, which is housed within the last two abdominal sections of adults. It follows that these tissues, already known to release various neuropeptides, could comprise major sources of Head Peptide-I production and secretion (Nässel and Homberg, 2006; Veenstra et al., 2008).

Over the past 30 years, this body of work has made a substantial case for Head Peptide-I playing an important role in the regulation of *A. aegypti* host-seeking behavior. Further work to determine the mechanism of action for the neuropeptide, and potential involvement in modulating olfactory perception, has been impossible because of a lack of molecular genetic techniques. Only recently has there been genetic access to *A. aegypti,* as well as several other arthropod disease vectors, that permitted our analysis of a potentially conserved role for Head Peptide-I (DeGennaro et al., 2013).

1.5.3 A Conserved Role for Head Peptide-I in Other Organisms

Regulation of host-seeking behavior is not unique to *A. aegypti*. It has also been observed in other blood-feeding arthropods including *A. gambiae* and *I. scapluaris* (Takken et al., 2001; Sonenshine 1991 and 1993). A shared mechanism of regulation by Head Peptide-I in *A. gambiae* would draw particular interest due to the higher health burden of malaria, but current evidence supports only weak behavioral similarity in feeding modulation between *A. aegypti* and *A. gambiae*.

Although evidence is lacking for a conserved Head Peptide-I mechanism in *A. gambiae*, observable inhibition following a blood meal preserves a glimmer of hope (Klowden and Briegel, 1994; Takken et al., 2001). Female anopheline mosquitoes display ~40 hours of host-seeking inhibition following a blood meal, in contrast to ~72h in *A. aegypti*, and subsequently exhibit attraction to specific odor cues for egg-laying (Rinker et al., 2013; Takken et al., 2001). Closer examination suggested that whereas *A. aegypti* require egg-laying to recover host-seeking behavior, *A. gambiae* may only require egg maturation (Takken et al., 2001). Although the details differ between the two vectors, a direct test of Head

Peptide-I was still warranted but unsuccessful. Injection of Head Peptide-I was not effective in altering host-seeking behavior in anopheline mosquitoes (Klowden, 1995). The final blow came when analysis of the *A. gambiae* genome showed that this vector lacks the Head Peptide-I gene, thus deterring any further studies.

Researchers have always assumed that *A. aegypti* and *A. gambiae* would be very similar, so that preventative strategies could also be similar. However, this may not be the case due to differences in selective pressure (Klowden, 1995). For instance, the difference in behavioral regulation has been attributed to *A. gambiae* being a nocturnal feeder, whereas *A. aegypti* is a day feeder (Klowden, 1995). When feeding during the day, human hosts are awake and alert, making them more adept at executing defensive responses to biting. Feeding at night has the benefit of a sleeping, defenseless human host who likely permits successful feeding more easily. Perhaps the selective pressure never existed for *A. gambiae* to evolve or maintain a Head Peptide-I mechanism of host-seeking regulation. Regardless, some inhibition does occur in the malaria vector and understanding Head Peptide-I action in *A. aegypti* may indicate new avenues of research for studying how *A. gambiae* regulate host-seeking behavior.

During the lengthy development of the Lyme disease vector *I. scapularis*, similarities to *A. aegypti* behavioral regulation have been observed (**Figure 1.3A and B**). Ticks mature through four developmental stages: egg, larva, nymph, and adult. All stages require a blood meal and each stage exhibits attraction to a different and progressively larger mammalian host. Molting between each life stage can take anywhere from 1-3 weeks where mechanisms for the inhibition of host-seeking are active. Though inhibition occurs for a longer time in the tick,

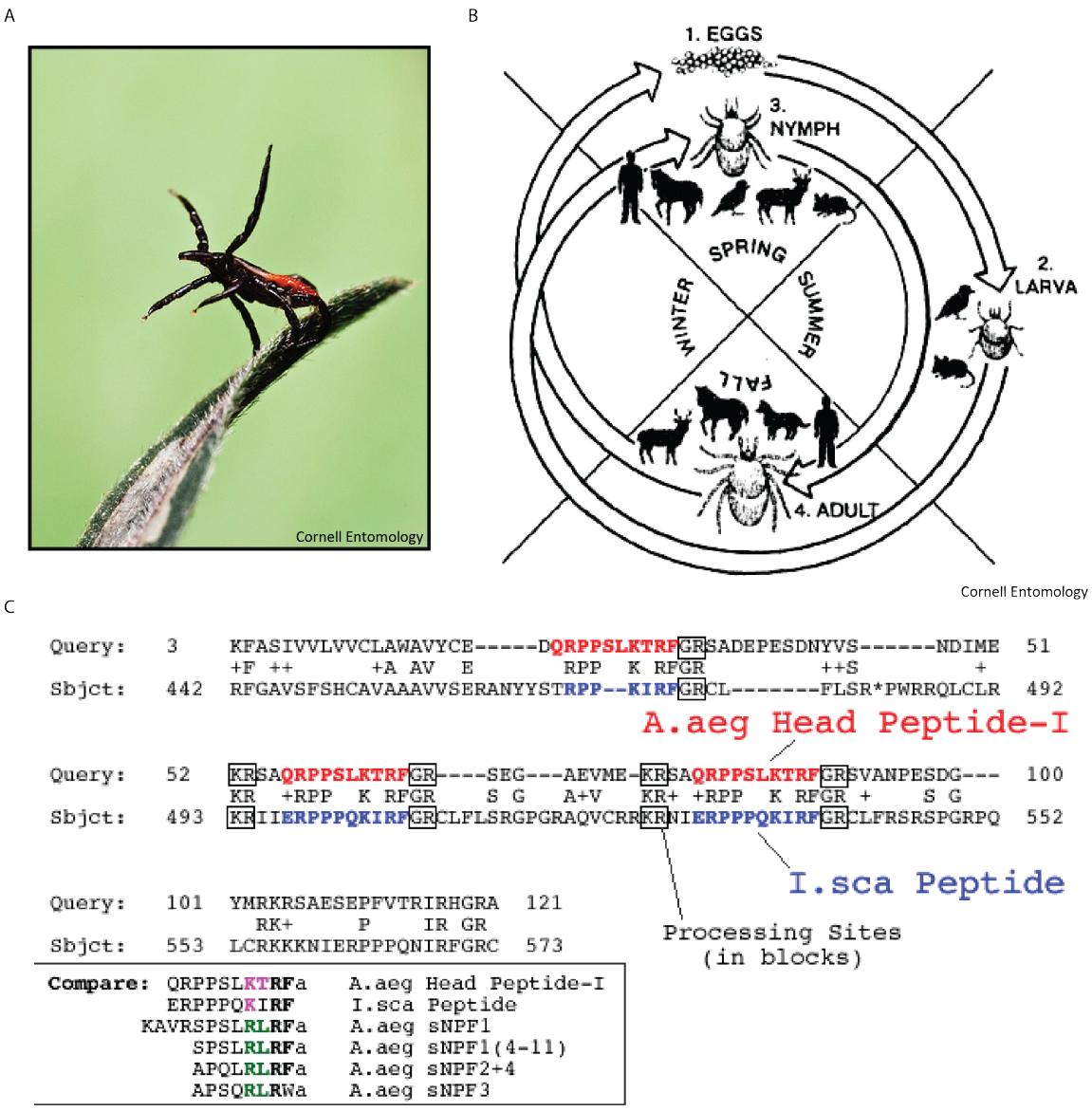


Figure 1.3 Identification of a Head Peptide-I Homologue in the tick, *Ixodes scapularis* (A) Image of an *I. scapularis* tick reaching for a host (Source: Cornell Entomology). **(B)** Illustration of 4-stage tick development with overlaid images of hosts (Source: Cornell Entomology). **(C)** Protein alignment of a putative *A. aegypti* (A. aeg, red) Head Peptide-I homologue found in trace reads of *I. scapularis* (I. sca, blue). Conserved prepropeptide processing sites are boxed. Below, comparison of propeptide sequences for *A. aegypti* HP-I, *I. scapularis* HP-I, and *A. aegypti* sNPFs noting the conservation of a Lysine (K) at position -4.

cyclical feeding indicates an opportunity for neuropeptide regulation. One notable difference is that unlike gravid female mosquitoes, blood-fed adult ticks do not recover host-seeking after laying eggs, but simply die. (Anderson and Harrington)

We identified a putative Head Peptide-I homologue in trace reads of the *I. scapularis* genome (**Figure 1.3C**). This is the first identification of a Head Peptide-I homologue in another organism. Although the mechanism for developmental inhibition in the tick is unknown, the evidence of a Head Peptide-I gene suggests a conserved role over 400-600 million years of arthropod evolution (Grimaldi and Engel, 2005). The existence of Head Peptide-I in *I. scapularis* leads us to propose that the neuropeptide may play an ancient conserved role in behavioral regulation.

1.6 Modulation of Olfactory-Dependent Behaviors by Neuropeptides

Modulation of behavior based on physiological need is essential for animal survival. Physiological needs are often communicated internally by the use of neuropeptides that modulate the activity of neurons and neural networks (Destexhe and Marder, 2004). In particular, neuromodulation of olfactory networks has been shown to produce profound alterations in several feeding related behaviors whose mechanisms may inform the action of Head Peptide-I. Neuropeptide signaling is typically redundant in that multiple neuropeptides often activate the same receptor (Kim et al., 2010; Wang et al., 2013; Valassi et al., 2008). To observe mutant phenotypes it is preferable to mutate a peptide receptor rather than the individual, and potentially redundant, neuropeptide gene.

1.6.1 NPF Modulates Foraging and Ingestion of Noxious Food

Neuropeptide-F was isolated in *D. melanogaster* by sequence conservation with Neuropeptide-Y (NPY), a pivotal regulator of food intake and other physiological processes in mammals (Brown et al., 1999; Gerald et al., 1996; Hu et al., 1996). The fly was proposed as a simpler model system to accelerate the study of NPY-Like peptides in behaviors such as olfactory-dependent food search (Brown et al., 1999; Larsson et al., 2004). Ablation of neuronal activity in both NPF- and NPF receptor (NPFR)-expressing cells had pronounced effects on food search behavior of *D. melanogaster* (Garczynski et al., 2002; Wu et al., 2003). Without NPF signaling, young larvae would wander away from food in a manner similar to older larva whose feeding is reduced in preparation for pupation (Wu et al., 2003). Complementary experiments showed that over-expression of NPF in older larva would prolong feeding (Wu et al., 2003). Follow-up studies found that over-expression of NPFR increased hunger, as observed by consumption of noxious food that is normally only performed by starved larva (Wu et al., 2005). The mechanism for NPF modulation of foraging and noxious food intake has yet to be fully elaborated, but recent studies have implicated a previously uncharacterized role for the neuropeptide in gating food odor excitation in the lateral horn of the central nervous system (Wang et al., 2013).

1.6.2 sNPF Modulates Odor Driven Food-Search in Adult Fruit Flies

Another member of the NPY-Like family of neuropeptides, Short-Neuropeptide-F (sNPF), was found by sequence similarity to NPF and has been implicated in odor-driven food search (Vanden Broeck, 2001). Initially, sNPF was

connected to food intake because overexpression of the neuropeptide or its receptor increased the percentage of fed *D. melanogaster* adults and larvae causing a noticeable increase in adult body-size (Lee et al., 2008; 2004). More recently, starvation-dependent food search of *D. melanogaster* was ablated by reduction of both sNPF neuropeptide and receptor independently (Root et al., 2011). Electrophysiological experiments observed that reduced sNPF expression weakens starvation-induced sensitivity of olfactory sensory neurons (OSNs) in response to the complex food odor, apple cider vinegar (Root et al., 2011). Interestingly, starvation increased OSN sensitivity by up-regulation of the sNPF receptor, not the neuropeptide. The mechanism for sNPF action is of particular interest to this thesis work because Head Peptide-I shows remarkable sequence similarity to sNPFs, which will be discussed in-depth later.

1.6.3 Behavioral Modulation in *C. elegans* by NPR-1 and NLP-1

The NPY-like receptor NPR-1 affects social behavior and food responses in the worm *Caenorhabditis elegans* (de Bono and Bargmann, 1998). Naturally occurring mutant variants of NPR-1 were observed to cause a solitary strain to take on social behavior, as indicated by increased clumping and bordering in food. Further work revealed that NPR-1 represses the activity of oxygen-sensing neurons thus enhancing food sensitivity (Chang et al., 2006). In another example, mutations of *C. elegans* neuropeptide NLP-1 increase certain behaviors by removing a feedback mechanism that initiates olfactory adaptation in response to starvation (Chalasani et al., 2010; Colbert and Bargmann, 1997). These discoveries highlight how neuropeptide activity in neuronal feedback and network integration can alter olfactory perception of food odors.

1.6.4 Tachykinins Modulate Fly Olfactory Preference

Outside of NPY-Like family neuropeptides, tachykinins have been linked to modulation of *D. melanogaster* olfactory perception. Tachykinins are structurally related to the mammalian neuropeptides substance P and neurokinin A and B. Expression of the neuropeptide was localized to olfactory neuropil (and the midgut) suggesting a role in olfactory perception (Winther et al., 2006). Reduction of tachykinin expression in adults and larva using RNA-interference caused behavioral indifference during olfactory choice tests to specific odors suggesting a role for the neuropeptide in modulating olfactory sensitivity (Winther et al., 2006).

1.7 How Does Head Peptide-I Regulate Host-Seeking Behavior?

Evidence suggests that Head Peptide-I is an important inhibitor of host-seeking behavior in *A. aegypti* and possibly *I. scapularis*. However, the receptor for this neuropeptide and how it regulates host-seeking behavior are unknown. In this dissertation, we developed *A. aegypti* into a genetically tractable model system to study Head Peptide-I regulation of mosquito host-seeking behavior.

1.7.1 What is the mechanism of Head Peptide-I Modulation?

Host-seeking behavior is an olfactory-dependent process and, therefore, Head Peptide-I may act by altering olfactory perception. Numerous examples, such as those described for NPF, sNPF, NPR-1, NLP-1, and Tachykinins, provide mechanisms for neuropeptides to regulate food search behaviors through modulation of olfactory perception. Furthermore, our hypothesis would agree with observations that mosquitoes injected with Head Peptide-I do not respond

to host odor cues, but can still blood-feed using vision, taste, and heat cues (Brown et al., 1994).

1.7.2 What are the cellular targets of Head Peptide-I?

To begin understanding the mechanism of Head Peptide-I inhibition, we decided that the cellular targets for the neuropeptide must be identified. Localization of these targets would suggest which sensory modalities, as well as neural circuits, are modulated by Head Peptide-I activity. Furthermore, we could then employ modern genetic techniques to synthetically manipulate their neuronal activity to further examine the impact on neural networks, behavior and physiology. To locate the cellular targets, we decided to identify the unknown Head Peptide-I receptor.

1.7.3 What is the Head Peptide-I receptor?

In chapter two of this dissertation, we use the recently published *A. aegypti* genome to bioinformatically predict candidate receptors and screen them for responses to Head Peptide-I in an HEK cell-based assay. In chapter three, we molecularly characterize a single candidate receptor named NPYLR1 and confirm that receptor agonists, Head Peptide-I and sNPF3, both functionally inhibit host-seeking behavior. Chapter four describes how we pioneer targeted mutagenesis of NPYLR1 using zinc-finger nucleases to isolate two types of null mutants. Finally, in chapter five we behaviorally characterize NPYLR1 mutants for general defects, changes in feeding, and behavioral modifications to the gonotrophic cycle.

1.8 Summary of Results

While we can say with certainty that NPYLR1 is a receptor for Head-Peptide-I, we found no behavioral effects for NPYLR1 mutants in locomotion, egg-laying, sugar feeding, blood-feeding, or host-seeking behavior throughout the gonotrophic cycle. This suggests that NPYLR1 is not required *in vivo* for Head Peptide-I action and that a redundant signaling mechanism for behavioral inhibition exists. Future work will determine the necessity of Head Peptide-I during host-seeking inhibition and attempt to identify additional Head Peptide-I and sNPF receptors causally related in host-seeking inhibition after a blood meal.

2 NPYLR1 Identified as a Candidate Head Peptide-I Receptor

2.1 Introduction and Summary of Results

To identify the neurons mediating Head Peptide-I behavioral modulation, we sought to identify the previously unknown Head Peptide-I receptor. Head Peptide-I sequence similarity to short-neuropeptide-F (sNPF), a class of insect neuropeptides implicated in insect feeding behavior, are known to signal through a highly conserved family of Neuropeptide-Y Like G-protein Coupled Receptors. We postulated that Head Peptide-I might signal through the same receptor class. We use bioinformatic analysis of the recently published *Aedes aegypti* genome to identify and clone nine putative NPY-like receptors (NPYLRs). These candidates were screened in a cell-based assay for sensitivity to select feeding-related neuropeptides. Four of the nine NPYLRs were sensitive to one or more neuropeptide, including NPYLR1, which responded to sNPFs and was the only receptor sensitive to Head Peptide-I. These experiments support the hypothesis that NPYLR1 is a candidate Head Peptide-I receptor and suggest that sNPF is a novel inhibitor of mosquito feeding behavior.

2.2 Head Peptide-I similarity to sNPFs suggests activation of NPYLRs

We noticed that Head Peptide-I (pERPhPSLKTRFa, pE = pyroglutamic acid, hP = hydroxyproline, a = c-terminal amidation) shares striking similarity to insect Short-Neuropeptide-Fs (sNPFs) that signal through a highly-conserved family of seven-transmembrane Neuropeptide-Y Like G-protein Coupled Receptors (NPYLRs) (**Table 2.1**; (Garczynski et al., 2006; 2007; Mertens et al., 2002)). Interestingly, sNPFs are implicated in olfactory modulation of *D. melanogaster* food-search behavior, suggesting that Head Peptide-I may

Table 2.1 Head Peptide-I Similarities to NPY-Like Receptor Agonists

Name	Species	Peptide Sequence	Receptor (NPYLR)	Assay	Reference	Synthesized
Head Peptide-I	*A. aegypti*	pERPhPSLKTRFa	- -	- -	Brown 1994	RU/Bachem
Head Peptide-III	*A. aegypti*	pERPPSLKTRFa	- -	- -	Brown 1994	RU
sNPF-1	*A. aegypti*	KAVRSPSLRLRFa	- -	- -	Predel 2010	Bachem
sNPF-1 (4-11)	*A. aegypti*	SPSLRLRFa	- -	- -	Predel 2010	Bachem
sNPF-2+4	*A. aegypti*	APQLRLRFa	- -	- -	Predel 2010	Bachem
sNPF-3	*A. aegypti*	APSQRLRWa	- -	- -	Predel 2010	Bachem
sNPF-1	*D. melanogaster*	AQRSPSLRLRFa	sNPFR76f	Gα-16 - Ca2+	Mertens 2002	- -
sNPF-2	*D. melanogaster*	SPSLRLRFa	sNPFR76f	Gα-16 - Ca2+	Mertens 2002	- -
sNPF-3	*D. melanogaster*	PQRLRWa	sNPFR76f	Gα-16 - Ca2+	Mertens 2002	- -
sNPF-4	*D. melanogaster*	PMRLRWa	sNPFR76f	Gα-16 - Ca2+	Mertens 2002	- -
sNPF-1	*A. gambiae*	AVRSPSLRLRFa	sNPFR	cAMP	Garczynski 2007	- -
sNPF-2	*A. gambiae*	AIRAPQLRLRFa	sNPFR	cAMP	Garczynski 2007	- -
sNPF-3	*A. gambiae*	APSQRLRWa	- -	- -	- -	- -
sNPF-4	*A. gambiae*	TIRAPQLRLRFa	sNPFR	cAMP	Garczynski 2007	- -
sNPF-5	*A. gambiae*	APTQRLRWa	- -	- -	- -	- -
Neuropeptide-F	*D. melanogaster*	SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFa	NPFR1	cAMP	Garczynski 2002	- -
Neuropeptide-F**	*A. aegypti*	SFTDARPQDDPTSVAEAIRLLQELETKHAQHARPRFa	- -	- -	- -	Bachem
Neuropeptide-F	*A. gambiae*	TLVAARPQDSDAASVAAAIRYLQELETKHAQHARPRFa	- -	- -	- -	- -
Allatostatin	*D. melanogaster*	SRPYSFGLa	DalstR1	Xenopus Currents	Birgul 1999	- -
Bombesin	*R. pipiens*	GNLWATGHFMa	- -	- -	- -	- -
Dromyosuppressin	*D. melanogaster*	TDVDHVFLRFa	NM_13945	Gα-16 - Ca2+	Egerod 2003	- -
FMRF-1	*D. melanogaster*	DPKQDFMRFa	FMRFaR	Gα-16 - Ca2+	Cazzamali 2002	- -
Leukokinin	*D. melanogaster*	NSVVLGKKQRFHSYG	DLKR	- -	Terhzaz 1999	- -
Neuropeptide-Y	*H. sapiens*	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYa	NepYR*	Xenopus Currents	Li 1992	Bachem
Peptide-YY	*H. sapiens*	IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRYa	NepYR*	Xenopus Currents	Li 1992	Bachem
Sex Peptide	*D. melanogaster*	WEWPWNRKPTKFPIPSPNPRDKWCRLNLGPAWGGRC	SPR	Chimeric G - Ca2+	Yapici 2008	- -
SIFamide	*R. prolixus*	TYKKPPFNGSIFa	- -	- -	Ons 2009	- -
Sulfakinin	*D. melanogaster*	GGDDQFDDYGHMRFa	DSK-R1	Ca2+	Kubiak 2002	- -
Tachykinin	*D. melanogaster*	APTSSFIGMRa	TAKR86C/99D	IP3 - Ca2+	Johnson 2003	- -
Vasopressin	*L. migratoria*	CLITNCPRGa	- -	- -	- -	- -

Legend	
- -	not available
pE	Pyro-Glutamic Acid
hP	Hydroxyproline
a	Amidation
*	*D. melanogaster* Receptor
**	Predicted Sequence

perform a similar function in *A. aegypti* (Lee et al., 2004; 2008). However, in the fly, sNPF activity increases olfactory sensitivity to food odors, whereas Head Peptide-I is hypothesized to reduce mosquito attraction to host odor (Root et al., 2011). It is possible that Head Peptide-I could reduce attraction through increasing olfactory sensitivity as is seen by the repulsive qualities of prolonged stimulation with CO_2 (Turner et al., 2011). If further parallels are found between sNPF and Head Peptide-I, the potential opposing effects on attraction is a noteworthy observation for future experiments. With both sequence and behavioral similarity to sNPFs, we postulated that Head Peptide-I likely signals through the same NPYLR class in mosquitoes.

2.3 Identification and Cloning of Nine Mosquito NPYLRs

NPYLRs are members of the Class A: Rhodopsin-Like Peptide G-Protein Coupled Receptor (GPCR) family of proteins of which 30 are predicted in *D. melanogaster*, 25 in *A. gambiae*, and 33 in *A. aegypti* (Nene et al., 2007). The most-studied family of NPYLRs exists in *D. melanogaster*, where six have been predicted and three are functionally characterized (Brown et al., 1999; Li et al., 1992; Mertens et al., 2002). Using the 2007 *A. aegypti* genome publication and BLAST, Genewise, and HMMER bioinformatics tools, we identified nine putative NPYLRs from *A. aegypti* mosquitoes as candidate Head Peptide-I receptors (**Figure 2.1**). Strong sequence conservation and Rapid Amplification of cDNA Ends (RACE) techniques permitted cloning of full-length transcripts for all nine predicted NPYLRs from purified mRNA isolates of female mosquito heads (NPYLR1, 2, 3, 4, 5, 6, 8) and bodies (NPYLR7A, 7B). Sequence similarity reveals

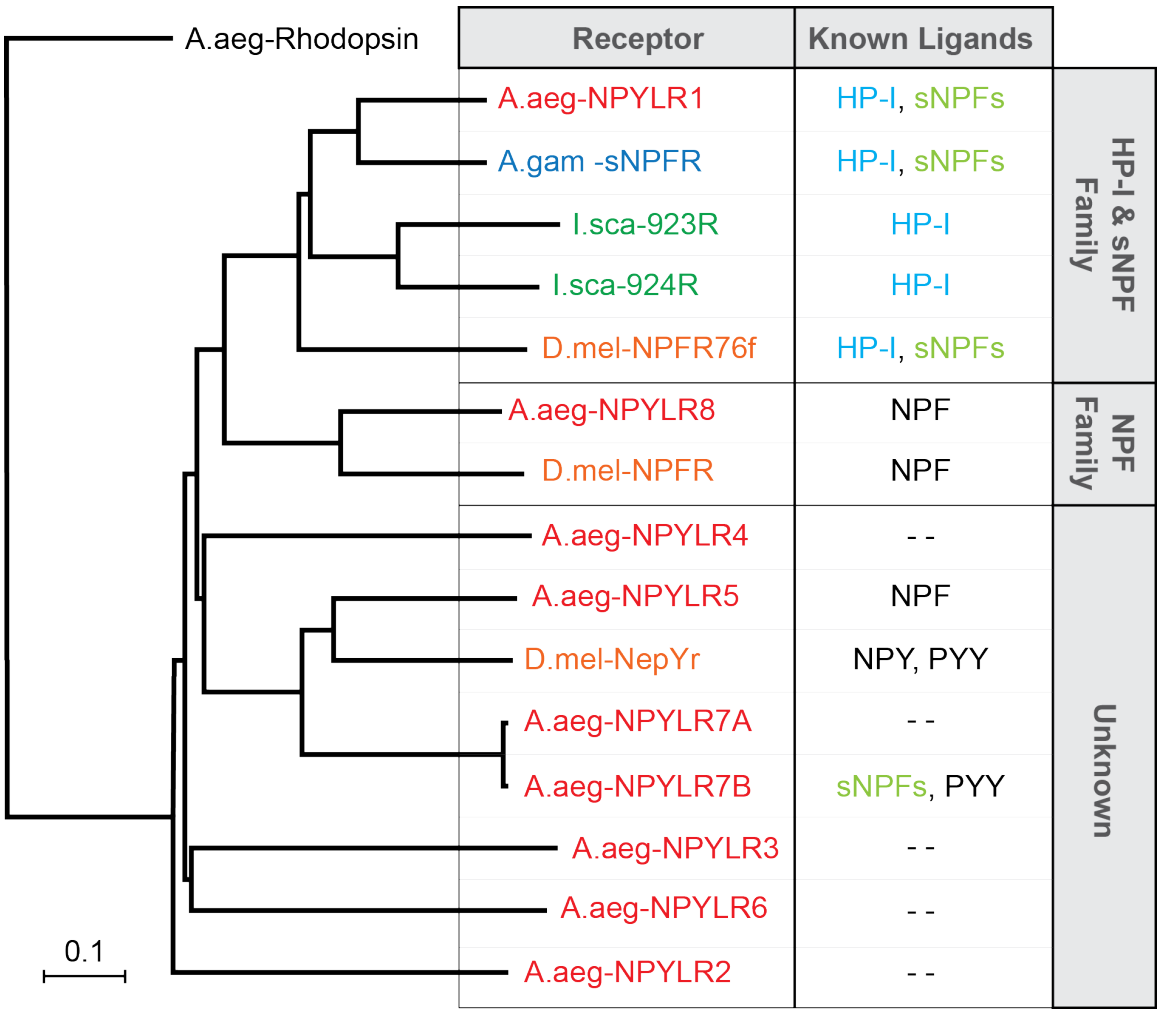


Figure 2.1 Phylogeny of Mosquito NPYLRs and their Similarity to Other Species

Nine *A. aegypti* NPY-Like Receptors (NPYLR1-8, red) are compared using ClustalW protein-alignments to receptors from *A. gambiae* (blue), *I. scapularis* (green), and *D. melanogaster* (orange). Receptor phylogeny and identified ligands from cell-based assays (second column) described in this dissertation suggest two conserved families (right in grey): one that responds to Head Peptide-I (HP-I) and Short-Neuropeptide-Fs (sNPF) and another that responds to Neuropeptide-F (NPF).

that the candidates include orthologues of the three functionally characterized *D. melanogaster* receptors: Short-Neuropeptide-F Receptor (NPYLR1 to sNPFR76f: 42% identity), Neuropeptide-F Receptor (NPYLR8 to NPFR: 48% identity), and Neuropeptide-Y Receptor (NPYLR5 to NepYr: 53% identity), all of which are linked to the regulation of feeding behaviors (Gehlert, 2004; Lee et al., 2004; 2008; Wu et al., 2005). Other NPYLRs share similarity to uncharacterized *D. melanogaster* NPY receptors except for NPYLR4, which shares 56% identity to putative Allatotropin receptors in the moth, *Bombyx Mori*.

2.4 Cell-based calcium-imaging screen of *A. aegypti* NPYLRs

Heterologous expression of GPCRs in an HEK cell-based assay is an established technique for discovering ligand-receptor interactions (**Table 2.1**). GPCRs have been intensively studied and couple to G-protein subunits for signal transduction. There are a number of different G-alpha subunits that then couple to distinct signal transduction cascades: $G_{\alpha s}$ stimulates cAMP production, $G_{\alpha i/o}$ inhibits cAMP production, $G_{\alpha q/11}$ activates Phospholipase-C-β and calcium release, and $G_{\alpha 12/13}$ causes Rho activation (Wettschureck and Offermanns, 2005). To circumvent NPYLR requirements for different G-protein subunits in our cell-based assay, we expressed the promiscuous murine $G_{\alpha\text{-}15}$ protein, which can interact with a wide range of GPCRs (Offermanns and Simon, 1995). In addition, $G_{\alpha\text{-}15}$ preferentially activates Phospholipase-C-β, leading to calcium release from intracellular stores that can be monitored with the calcium-sensitive dye Fura-2 following bath application of synthesized feeding-related neuropeptides (**Table 2.1**). We tested Head Peptide-I and III, the behaviorally inactive control peptide

Head Peptide-I [Cys10], four sNPF variants produced from the single sNPF gene, the predicted *A. aegypti* NPF, and the human NPY and PYY (Brown et al., 1994; Li et al., 1992; Predel et al., 2010; Stanek et al., 2002). The human neuropeptides were originally used to define the NPY-like receptor class in *D. melanogaster* and are useful for confirming receptor function in the cell-based assay (Li et al., 1992).

Our receptor screen found that only NPYLR1 responds to Head Peptide-I, with a half maximal effective concentration (EC50) value of 823 nM (**Figure 2.2**). In our assay, receptor agonists with EC50 values around or below 1 μM are considered to be medium to strong activators and suggest physiological relevance. Further testing revealed that NPYLR1 is also sensitive to sNPFs (EC50s range from 35-75 nM) - an anticipated result due to strong homology to *D. melanogaster* sNPF receptor sNPFR76f - and the low abundance neuropeptide Head Peptide-III (pERPPSLKTRFa, EC50 = 954 nM). It is not uncommon for peptide receptors to be sensitive to multiple neuropeptides, especially in this case where the neuropeptides have striking similarity (Garczynski et al., 2006; Kim et al., 2010). The dual sensitivity of NPYLR1 to both Head Peptide-I and sNPFs suggests that sNPFs may be novel inhibitory neuropeptides for mosquito host-seeking behavior.

Other responses of note include NPYLR8 sensitivity to the predicted *A. aegypti* NPF (EC50 = 989 nm), providing further support that it is an NPF receptor orthologue. The putative NPY receptor orthologue, NPYLR5, is activated weakly by NPF (EC50 = 7 μM), but not by human NPY. Lastly, NPYLR7B exhibits moderate sensitivity to sNPF1 (EC50 = 634 nM) and Human PYY (EC50 = 791 nM), and weak sensitivity to sNPF3 (EC50 = 6 μM). Because NPYLR5, 7B and 8 were active in the cell-based assay but displayed no response

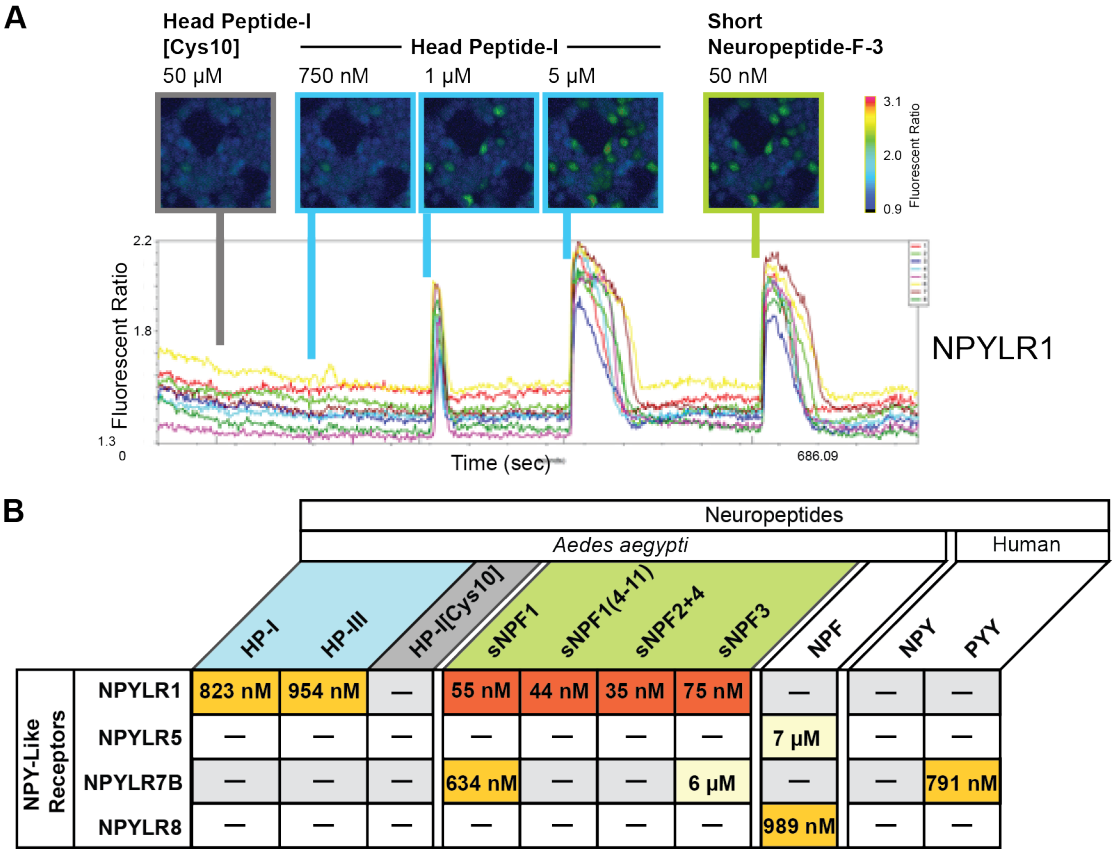


Figure 2.2 NPYLR1 Responds to Head Peptide-I and sNPFs

(A) Top: Representative images of NPYLR1 transiently transfected HEK293 cells illustrating the calcium flux after application of peptides. Bottom: Individual cellular traces of fluorescent ratios representing Ca^{+2} flux for the same HEK293 cells following application of the indicated peptides. **(B)** Summary of results for the cell-based calcium-imaging assay of *A. aegypti* NPYLRs against *A. aegypti* and Human peptides. (Red = EC50<100nM, Orange = 1μM>EC50>100nM, Yellow = EC50>1μM. HP = Head Peptide, sNPF = Short Neuropeptide-F, NPF = Neuropeptide-F, NPY = Neuropeptide-Y, PYY = Peptide-YY). NPYLR2, 3, 4, 6, 7A were tested but did not respond to any peptides.

to Head Peptide-I they are unlikely candidates for a Head Peptide-I receptor. However, these three receptors likely play a conserved role in mosquito feeding behavior as indicated by their similarity to *D. melanogaster* receptors.

NPYLR2, 3, 4, 6, and 7A did not display any activity in the cell-based assay. The receptors may simply not be sensitive to any of the tested peptides, not expressed or trafficked to the cell surface to function properly, or unable to interact with the promiscuous $G_{\alpha-15}$. Encouragingly, four of nine candidate receptors were sensitive to at least one of the tested feeding-related neuropeptides and all known agonists of NPY-Like receptors in *D. melanogaster*. Most remarkably, NPYLR1 was the only receptor sensitive to Head Peptide-I, warranting additional study as a strong candidate Head Peptide-I receptor.

3 Molecular Characterization of NPYLR1 and Specific Homologues

3.1 Introduction and Summary of Results

Building from NPYLR1 responses obtained in the cell-based assay, we carried out peptide injections into unfed *A. aegypti* females to confirm the activity of both Head Peptide-I and sNPF3 as inhibitors of host-seeking behavior. With behavioral validation of NPYLR1 ligands, we looked for conserved responses of NPYLR1 homologues in other blood-feeding disease vectors such as the malaria vector, *A. gambiae*, and the tick, *I. scapularis*. All NPYLR1 homologs were sensitive to Head Peptide-I, indicating that the receptor may be part of a conserved mechanism for behavioral regulation in several species. In preparation for targeted mutagenesis of the receptor, we fully characterize the NPYLR1 genetic locus, clone and functionally test NPYLR1 cDNA variants, and determine that NPYLR1 expression is regulated by blood-feeding. These experiments support a conserved role for NPYLR1 in the modulation of arthropod behavior in response to Head Peptide-I and sNPFs.

3.2 sNPF3 is a Novel Inhibitor of *A. aegypti* Host-Seeking Behavior

To confirm the reported activity of Head Peptide-I, we injected the neuropeptide into non-fed female mosquitoes and monitored their attraction to human odor cues in the uniport olfactometer (**Figure 3.1**) (Brown et al., 1994). Based on NPYLR1 pharmacology from our cell-based assay we also injected sNPF3 (APSQRLRWa), as a representative of the sNPF family, to see if it could inhibit behavior as well. Our results confirm that both Head Peptide-I and sNPF3 significantly inhibit host-seeking behavior when injected at a dosage of 10 mM (**Figure 3.1B**). In contrast, injection of the C-terminal modified control

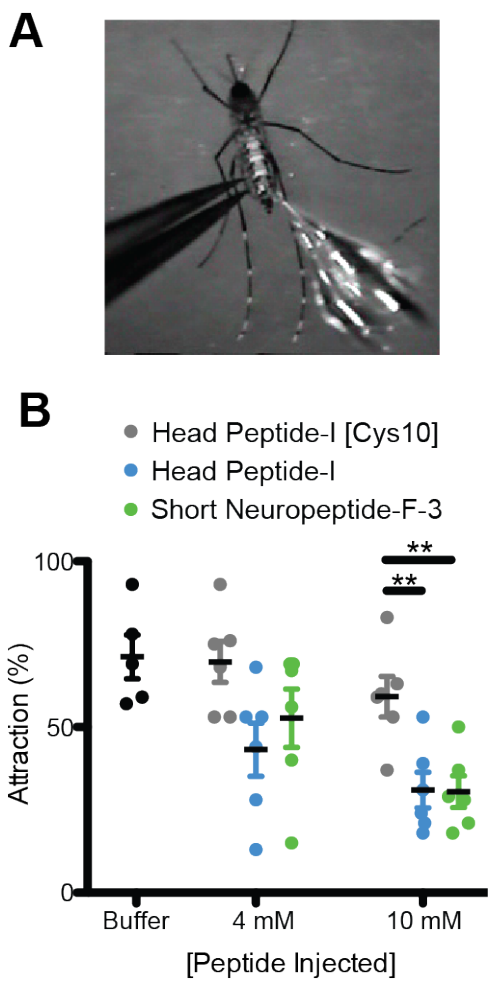


Figure 3.1 Injection of Head Peptide-I or sNPF3 Inhibits Host-Seeking Behavior. (A) Intra-thoracic injection of a female *A. aegypti* mosquito (photo courtesy of Dr. Marc Klowden, University of Idaho). **(B)** Percent attraction of non-blood-fed female mosquitoes to human hand odor cues after injection of buffer (black) or 4 and 10 mM of the indicated neuropeptide. (n=6; ~20 mosquitoes per trial, ANOVA with Bonferroni Correction for Multiple Comparison; ** = $p < 0.01$).

Head Peptide-I [Cys10] (pERPhPSLKTR**C**a) did not cause inhibition confirming previously reports (Brown et al., 1994). One could predict that since NPYLR1 exhibits greater sensitivity to sNPFs in the cell-based assay, there should also be a stronger inhibition of host-seeking behavior when injected. However, several factors could influence the *in vivo* activity of these peptides such as protein stability in the hemolymph or unidentified protein chaperones. Nevertheless, our results support sNPFs are a novel class of behavioral inhibitors in *A. aegypti* that may act redundantly with Head Peptide-I through NPYLR1.

3.3 NPYLR1 Homologues in the Tick and Malaria Mosquito

Using similar bioinformatics approaches employed for NPYLRs in *A. aegypti*, we identified and cloned candidate NPYLR1 homologues in *A. gambiae* and *I. scapularis* (**Figure 2.1**) (Garczynski et al., 2007). Interestingly, our analysis confirmed a gene duplication of the *I. scapularis* NPYLR1 homologue referred to here as I.sca-923R and I.sca-924R (37% and 33% identity respectively to NPYLR, 48% identity to each-other). In the cell-based assay, the sensitivity of the *A. gambiae* receptor homologue was weak (EC50 = 3.7 μM) which may be indicative of the loss of Head Peptide-I inhibition in this species (**Figure 3.2**). Previous publications report that the NPYLR1 homologue in *A. gambiae* is sensitive to sNPFs (3-653 nM) and therefore were not duplicated here (Garczynski et al., 2007). Both *I. scapularis* homologues are also responsive to Head Peptide-I (**Figure 3.2**, I.sca-923R EC50 = 427 nM and I.sca-924R EC50 = 10 μM), though I.sca-923R is more sensitive, even compared to *A. aegypti* NPYLR1. As the most studied homologue of NPYLR1, we confirmed that the *D. melanogaster* sNPFR76f is also sensitive to Head Peptide-I (EC50 = 1 μM), even though no

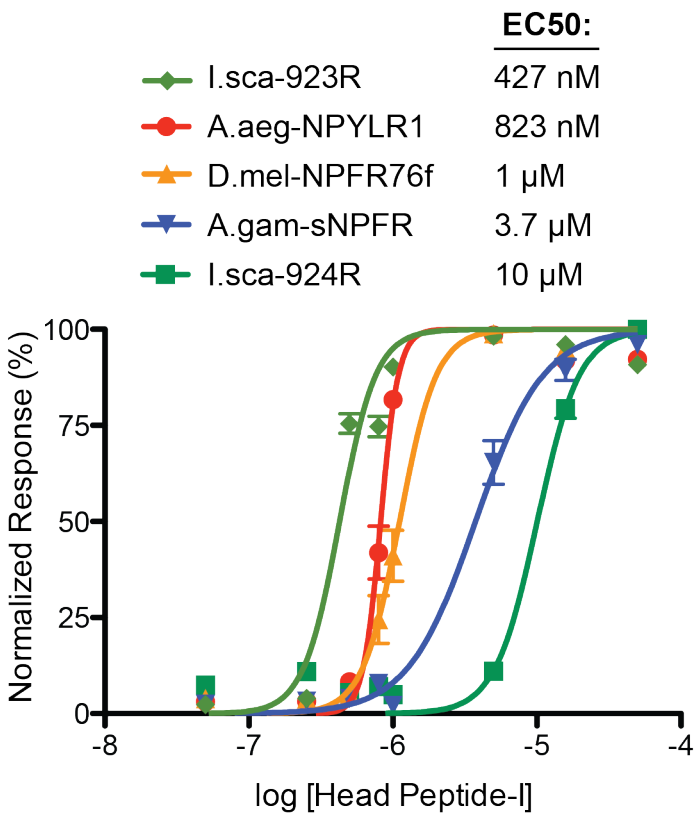


Figure 3.2 NPYLR1 Homologues are sensitive to Head Peptide-I

Head Peptide-I dose-response curves for NPYLR1 and receptor homologues in *I. scapularis* (I.sca-923R and -924R), *A. gambiae* (A.gam-sNPFR), and *D. melanogaster* (D.mel-NPFR76f) as determined in the cell-based assay.

Head Peptide-I gene has been found in the *D. melanogaster* genome. These experiments support that NPYLR1 homologues possess conserved sensitivity to Head Peptide-I in other important disease vectors.

3.4 Characterization of the NPYLR1 Genetic Locus

The genome of *A. aegypti* is nearly ten times larger than *D. melanogaster* because of an expansion of repeat-rich transposable elements (Nene et al., 2007). As a result, genomic sequencing and assembly proved quite difficult and led to an overabundance of predicted gene duplications (Lobo et al., 2007). Our analysis of the *A. aegypti* genome as well as cloning experiments suggest that a duplication of the NPYLR1 locus might exist. To create a NPYLR1 null mutant mosquito to confirm a role in host-seeking behavior modulation, we first needed to determine how many genetic loci to target.

Using southern blot techniques, we confirmed that there were two distinct NPYLR1 loci in the *A. aegypti* Orlando strain (**Figure 3.3A and B**, referred to as NPYLR1-1 and NPYLR1-2). However, we were unable to clarify whether the two loci represented a gene duplication event or simply two alleles of one NPYLR1 genetic locus. To resolve this discrepancy, we identified a small 10 bp insertion directly upstream of the NPYLR1 coding region that is unique to the NPYLR1-1 allele. We used the microsatellite marker to prove predictable Mendelian segregation of the two loci, confirming that they were in fact two alleles of one gene (**Figure 3.4**).

To determine the transcripts produced in our polymorphic strain of wild-type *A. aegypti* Orlando mosquitoes, we carried out extensive cloning of NPYLR1 cDNAs and identified four slightly different variant transcripts

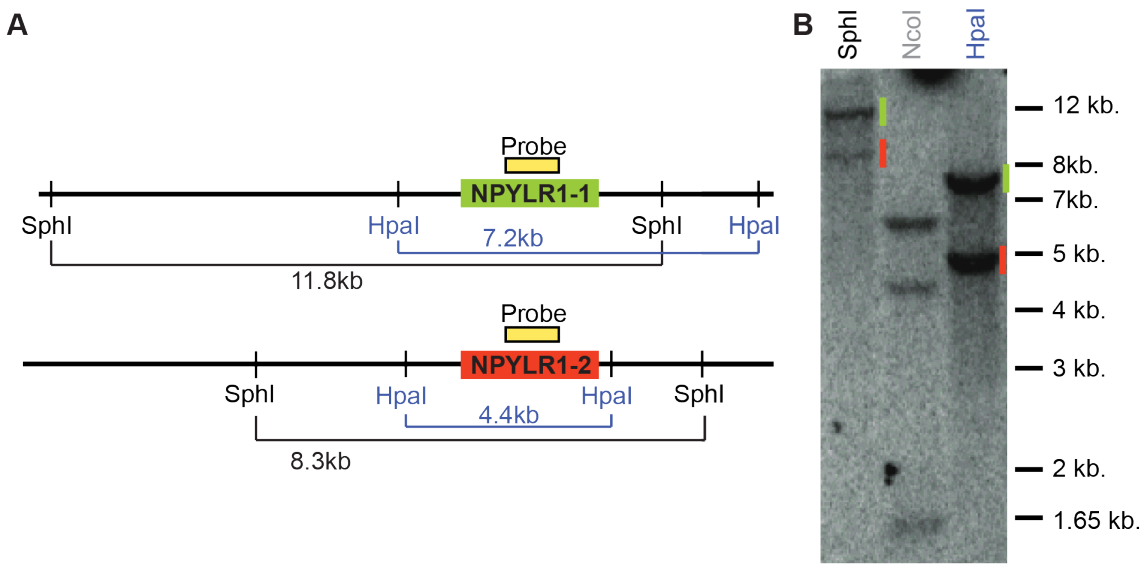


Figure 3.3 Southern Blot Confirmation of two NPYLR1 Alleles

(A) Restriction map of the NPYLR1-1 and NPYLR1-2 loci with the probe (yellow) used for Southern hybridization. **(B)** SphI and HpaI restriction enzymes were used to digest *A. aegypti* Orlando genomic DNA into predictable fragments of NPYLR1-1 (green) and NPYLR1-2 (red) that were identified by southern blot. Bands produced with the NcoI digest could not be interpreted given the current genome annotation.

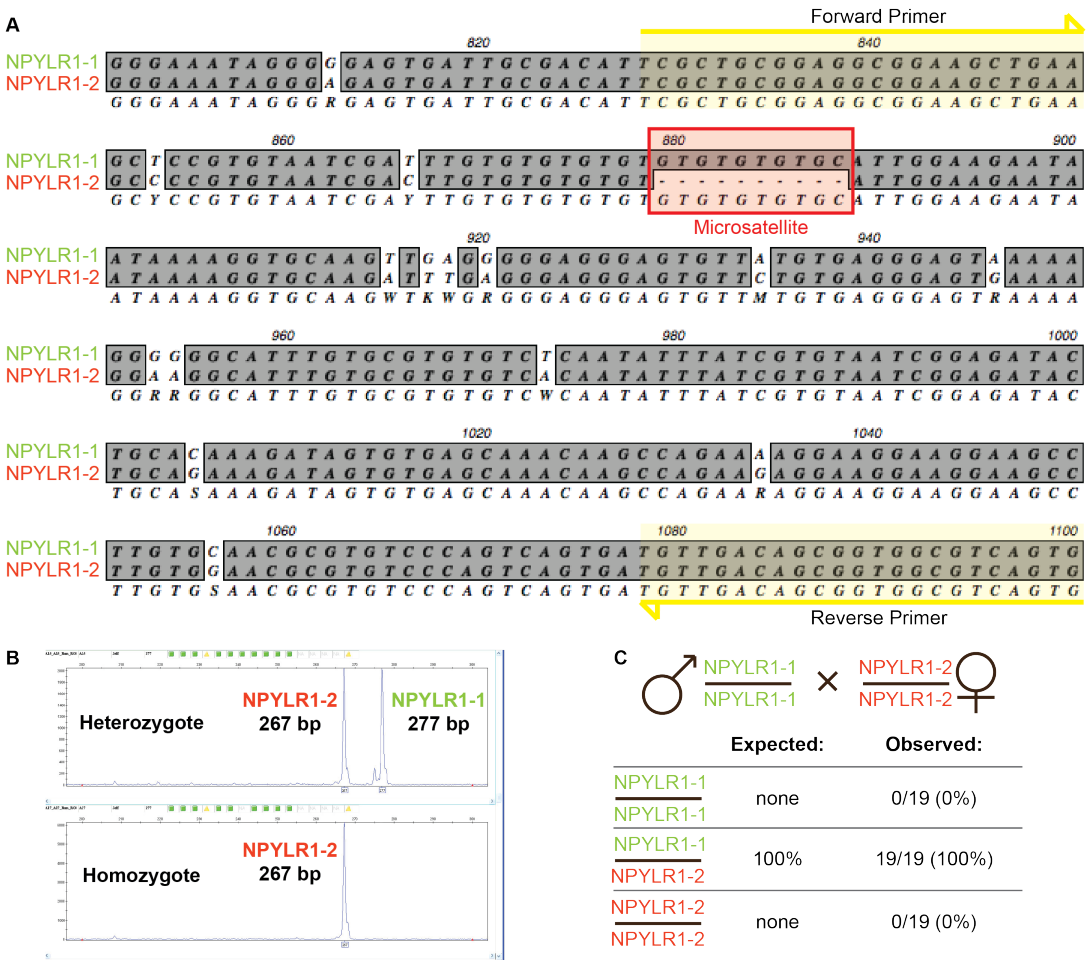


Figure 3.4 Mendelian Inheritance of a Microsatellite Confirms One NPYLR1 Gene

(A) DNA sequence alignment of NPYLR1-1 and NPYLR1-2 with the 10 bp microsatellite indicated in orange that is PCR amplified using the highlighted primers (yellow). **(B)** PCR products are separated using small capillary size-based gel electrophoresis to reveal the genotype of individual mosquitoes for NPYLR1-1 (277 bp) and NPYLR1-2 (267 bp). **(C)** Observed results from a genetic cross between an NPYLR1-1 homozygous male and NPYLR1-2 homozygous female confirms predicted Mendelian segregation of NPYLR1-1 and NPYLR1-2 as two alleles of one gene.

(**Figure 3.5A**). Using the cell-based assay, we confirmed that all cDNA variants had equal sensitivity to both Head Peptide-I and sNPF3 (**Figure 3.5**).

3.5 NPYLR1 Expression is Regulated by Blood Feeding

sNPF modulation of olfactory perception in *D. melanogaster* is controlled by increased expression of the receptor, not the neuropeptide, during starvation (Root et al., 2011). Due to the similar function of Head Peptide-I and sNPF in our experiments, we were interested to know if a similar change in NPYLR1 expression occurs after female mosquitoes blood-feed. Reverse transcriptase-polymerase chain reactions (RT-PCR) from various tissues suggest that NPYLR1 might indeed be up-regulated in heads and bodies of female mosquitoes following a blood meal (**Figure 3.6**). Quantitative PCR (qPCR) of female whole body tissue confirmed that NPYLR1 expression dramatically increases to roughly fifteen times baseline levels at 48 hours post-blood meal then drops at 72 hours, coinciding well with the transition from egg maturation to egg-laying site location (**Figure 3.7**). Interestingly, levels are only moderately increased at 24 hours post-blood meal when females are known to exhibit behavioral inhibition from abdominal distension and not Head Peptide-I (Klowden and Lea, 1979b). These changes, which were not evident in non-fed controls, also occur in blood-fed virgins who are behaviorally inhibited for numerous days after a blood meal despite an inability to fertilize their eggs or seek out egg-laying sites (**Figure 3.7 and data not shown**). Intriguingly, NPYLR1 displays a temporally appropriate expression pattern for increased sensitivity to neuropeptides during host-seeking inhibition drawing parallels to sNPF mechanisms of olfactory modulation.

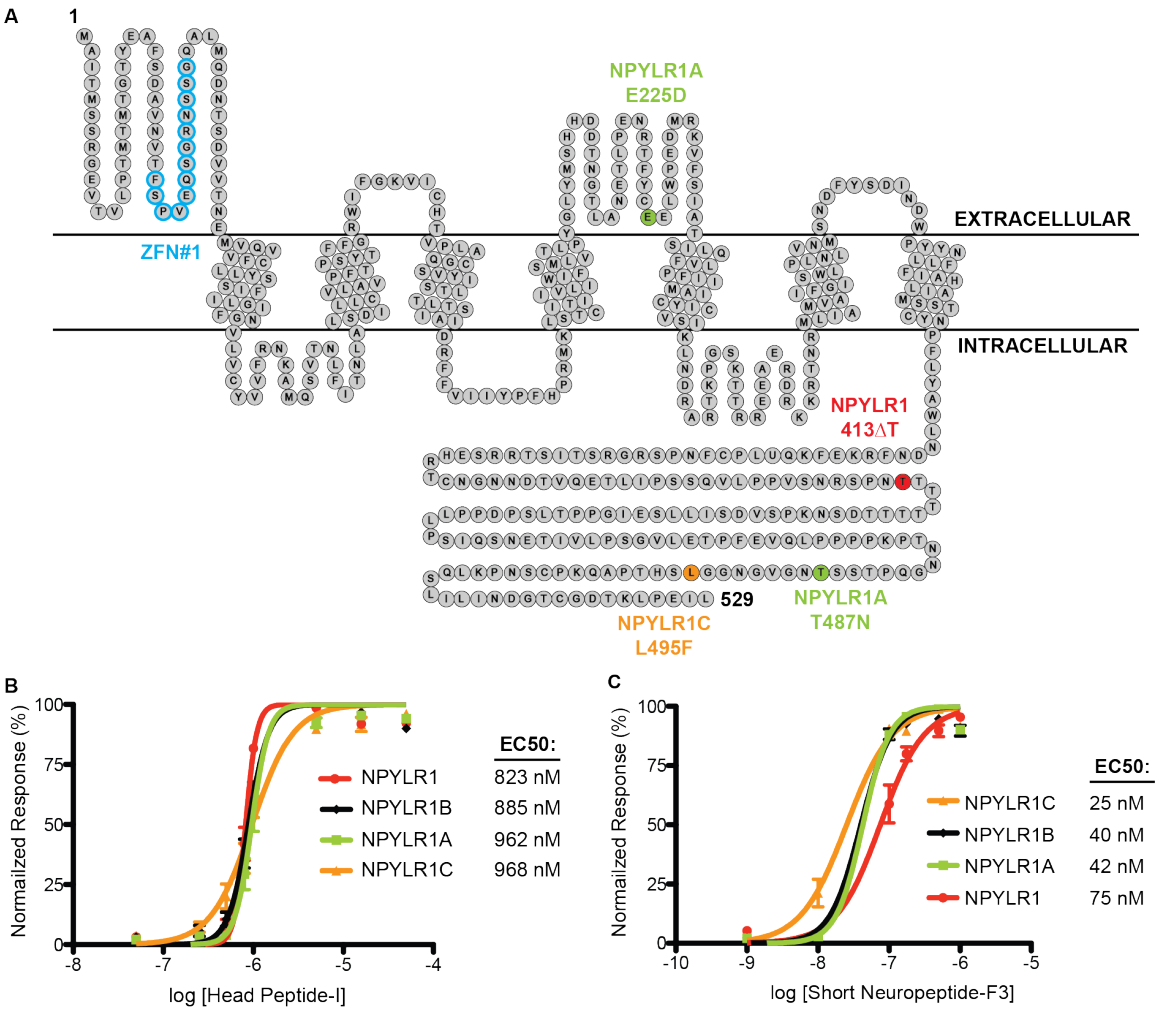


Figure 3.5 Variant NPYLR1 cDNAs are Sensitive to Head Peptide-I and sNPF3
(A) Snake plot of predicted NPYLR1B membrane topology indicating the polymorphisms contained in other receptor alleles (NPYLR1 413ΔT, NPYLR1A E225D and T487N, and NPYLR1C L495F). Also highlighted in blue is the protein region targeted by the NPYLR1 ZFN. **(B and C)** Dose-response curves indicate that the four variant cDNAs of NPYLR1 are all similarly sensitive to Head Peptide-I **(B)** and short Neuropeptide-F3 **(C)** in the cell-based assay. NPYLR1 (red) was the cDNA used in the original Head Peptide-I receptor screen.

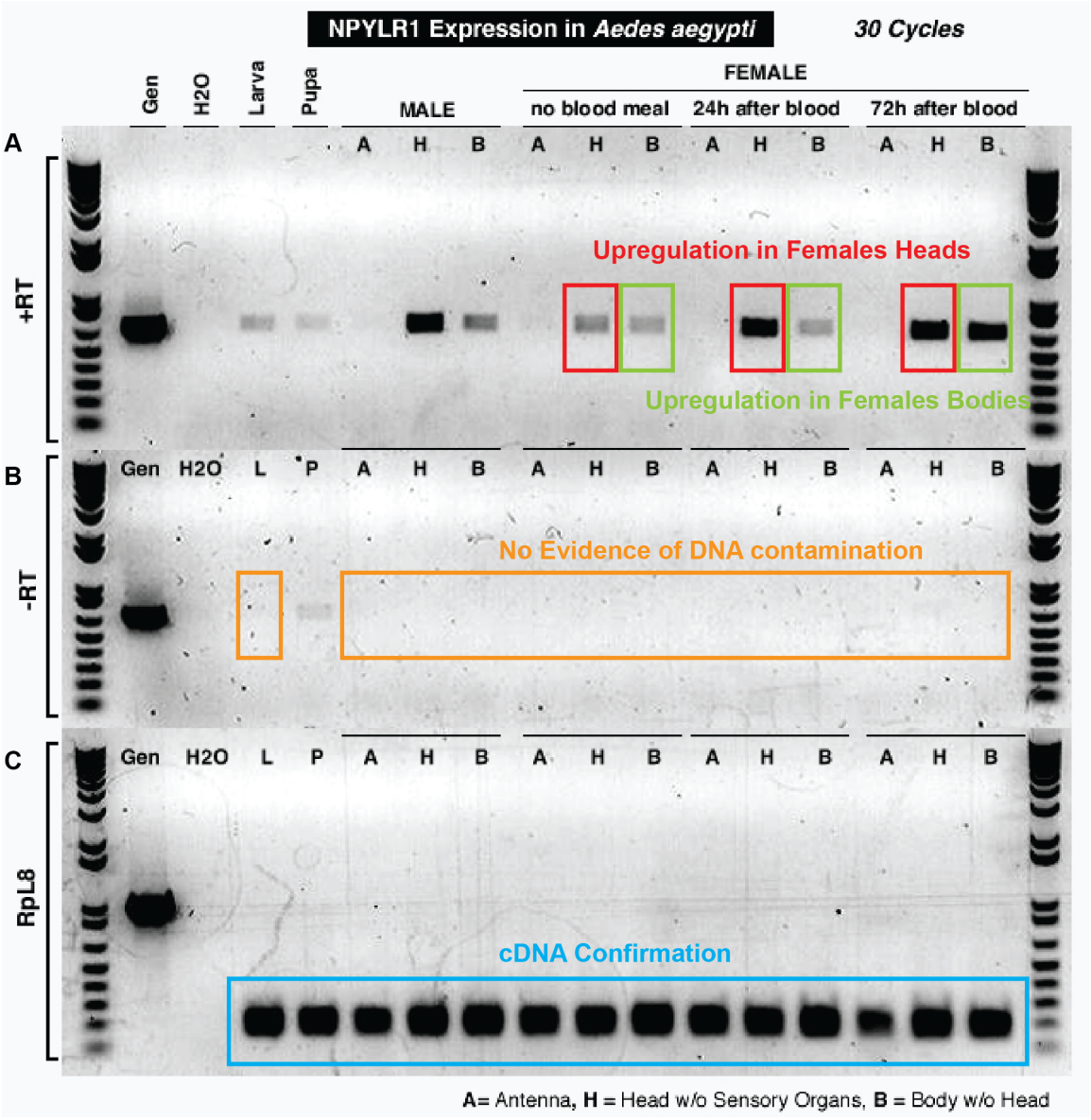


Figure 3.6 NPYLR1 Expression and Suggested Regulation by Blood-feeding (A, B, and C) RT-PCR of NPYLR1 transcripts in various life-stages including larvae, pupae, and dissected antenna (A), head without antenna or maxillary palp sensory organs (H), and body without head (B) derived from sugar-fed males or females dissected 24 or 72 hours after a blood meal. **(A)** NPYLR1 expression. **(B)** Control cDNA synthesis reactions without Reverse Transcriptase. **(C)** Positive control reactions with RpL8, a ubiquitous ribosomal sub-unit (~1 kb genomic, 200 bp cDNA).

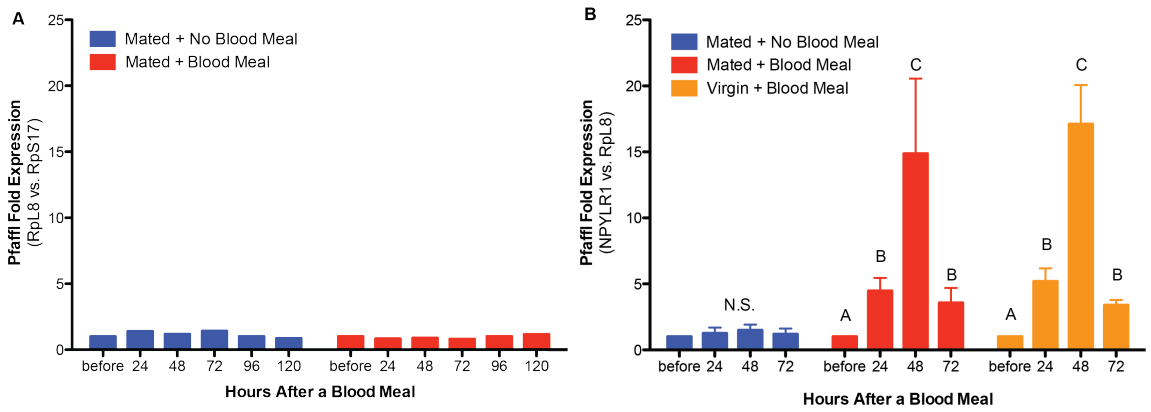


Figure 3.7 NPYLR1 Expression in Females Increases After a Blood Meal

(A) Control reactions for qPCR comparing the Pfaffl Fold Expression of two house-keeping genes RpL8 and RpS17 (ribosomal subunits) for non-bloodfed (blue) and bloodfed (red) female whole bodies. No relative change in expression was observed for the control genes at any point from before to 24, 48, 72, 96 and 120 hours after a blood meal. **(B)** Pfaffl Fold Expression of NPYLR1 compared to the reference RpL8 gene for female whole body tissue from groups of Mated + No Blood Meal (blue), Mated + Blood Meal (red), and Virgin + Blood Meal (orange) at times before and 24, 48, and 72 hours after a blood meal. Levels of NPYLR1 transcript significantly increase for both Mated and Virgin mosquitoes following a blood meal with a peak at 48 hours (n=3, ANOVA-Tukey's Multiple Comparison of ΔCt values, not Pfaffl Expression which is displayed here).

4 Pioneering Targeted Mutagenesis of NPYLR1

4.1 Introduction and Summary of Results

We have shown that NPYLR1 responds to Head Peptide-I in cell-based assays and injection of ligands for the receptor inhibit attraction to human hosts. However, these results do not exclude the possibility that the neuropeptides also signal through another receptor not represented in our screen. To test the role of NPYLR1 in mosquito behavior, we pioneered targeted mutagenesis strategies to create NPYLR1 null mutants. Zinc-finger nuclease technology was employed to isolate two types of genetic lesions at the NPYLR1 locus. Our prediction is that by removing NPYLR1, mosquitoes will no longer display inhibition of host-seeking behavior after a blood meal.

4.2 Zinc-Finger Nucleases and NPYLR1 Mutagenesis Strategies

To be a viable model system for genetic research, an organism must have a published and assembled genome, be amendable to transgenesis, and accessible to techniques for generating mutants. *A. aegypti* already has a published genome (nearing complete assembly) and successful techniques for transgenesis (Jasinskiene et al., 1998; Nene et al., 2007; Nimmo et al., 2006). As a goal of this thesis, we worked to establish targeted mutagenesis techniques for generating mutants for the first time in *A. aegypti* mosquitoes.

Zinc-finger nuclease (ZFN) technology is a targeted mutagenesis strategy successfully applied to several model organisms including *D. melanogaster* and the zebrafish, *Danio rerio* (Carroll et al., 2010; Ekker, 2008). Zinc-finger nucleases are proteins that contain a DNA binding domain fused to an obligate heterodimer *fok-1* DNA nuclease domain (**Figure 4.1A**) (Durai et al., 2005).

Working in pairs, ZFNs recognize unique DNA sequences within a genome spanning ~40bp. Upon binding, the two nucleases dimerize to create a functional nuclease that makes double-stranded breaks in the chromosome between the DNA binding sites. Damaged DNA triggers host repair mechanisms such as error-prone Non-Homologous End-Joining (NHEJ) that creates small insertions and deletions, or Homologous Recombination (HR) which repairs using an exogenous DNA template with homology to the damaged region (Durai et al., 2005). Null mutants can then be isolated by screening NHEJ events for frame-shift mutations or by visual selection of HR events that incorporate a visible marker.

NPYLR1 ZFNs were designed in collaboration with Sigma-Aldrich to target a 40bp sequence located 100 bp downstream of the NPYLR1 start codon (**Figure 4.1A**). Frame-shift mutations at this location that generate premature stop codons would prevent translation of ~90% of NPYLR1 coding sequence. Over 3,000 mosquito eggs were injected with mRNA for each custom NPYLR1 ZFN together with a DNA vector containing ~2.7kb of sequence homology to the target location and a dominant marker expressing a fluorescent protein under a ubiquitously expressed promoter. Following translation by host machinery to produce ZFN protein, nuclease activity occurs in all accessible cells including the reproductively competent primordial germ cells. If there is neither a critical developmental role for NPYLR1 nor excessive damage to the egg during injection, we expect that eggs will develop into adults where they are mated to propagate mutant germ cells to future offspring. A portion of the offspring will be heterozygous for NHEJ or HR mutagenesis events at the NPYLR1 locus, which can be isolated by appropriate screening protocols.

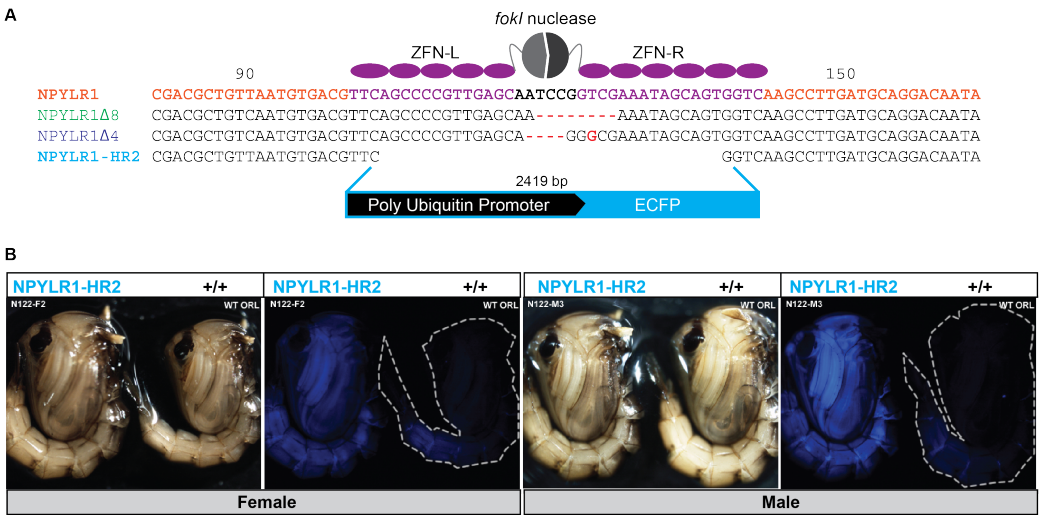


Figure 4.1 Targeted Mutagenesis of NPYLR1 with Zinc-Finger Nucleases

(A) Illustration of the NPYLR1 ZFN target sequence and alignment to mutations generated in NHEJ events NPYLR1Δ8 and NPYLR1Δ4 as well as HR events that integrated a Poly-Ubiquitin Promoter>ECFP marker into NPYLR1-HR2. **(B)** Representative images of female and male NPYLR1-HR2 mutants showing the broad expression of the ECFP fluorescent marker that clearly distinguishes mutants from wild-type (+/+).

4.3 Isolation of NPYLR1 Mutants: Homologous Recombination

Three independent homologous recombination lines named NPYLR-HR1, -HR2 and -HR3, were isolated by insertion of a marker into the NPYLR1 locus containing a broadly expressed *A. aegypti* Poly-Ubiquitin promoter driving fluorescent ECFP (**Figure 4.1B**) (Anderson et al., 2010). Successful targeted mutagenesis by HR occurred at an approximate rate of 0.09% (51 fluorescent/~55,000 total F1 larva screened). Recombination of the HR vector introduced novel restriction sites into the NPYLR1 locus allowing for the identification of targeted versus non-targeted events by predictable changes in DNA length (**Figure 4.2A**). To confirm single insertion events, Southern blots were carried out with an ECFP probe that identified the predicted single DNA fragment for an event at the NPYLR1 locus (**Figure 4.2A**). HR mutants were homozygosed by an observable increase in fluorescence due to the presence of two ECFP copies. For further confirmation, PCR genotyping over the integration site produced a 2.4 kb shift in amplicon size that could be easily differentiated from the smaller wild-type allele (**Figure 4.2B**). To confirm disruption of NPYLR1 protein production, amplification of transcripts in the mutant revealed three major splice products, all of which contain a premature stop codons ~100 bp into the sequence as expected by integration of the HR vector at the target locus (data not shown).

4.4 Isolation of NPYLR1 Mutants: NHEJ Deletion Mutants

Two independent NHEJ deletion lines were isolated with 4 and 8 bp deletions predicted to cause disruptive frame-shift mutations (**Figure 4.1A**,

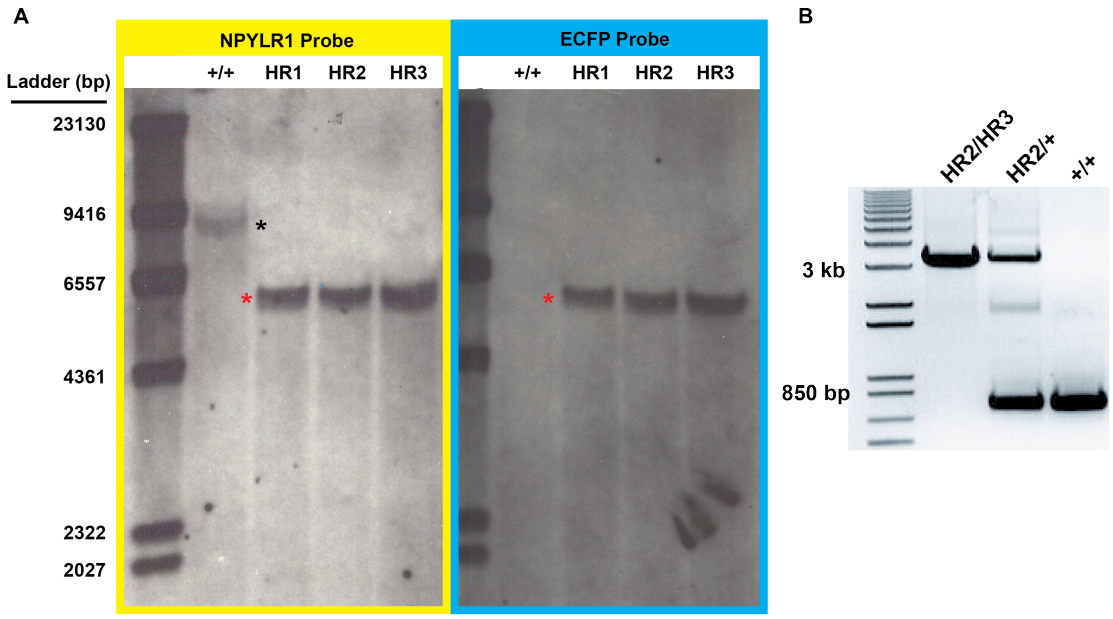


Figure 4.2 Molecular Validation of NPYLR1 Homologous Recombination Alleles

(A) Left: Southern blot using a NPYLR1 probe indicates a predicted shift in genomic DNA digested from wild-type (black star, +/+, 9 kb) to a successful targeted insertion (red star, 6 kb). Right: Southern blot using an ECFP probe confirms a single insertion event of predicted size to the NPYLR1 locus (red star, 6 kb). **(B)** Example of PCR products used for genotyping HR alleles to establish homozygous lines. Absence of the wild-type allele (~800 bp) and the presence of the insertion allele (~3.5 kb) confirm homozygosity of hetero-allelic lines.

named NPYLR1Δ4 and NPYLR1Δ8). The rate of NHEJ mutagenesis was 28%, significantly higher than the HR rate. However, working with the deletion mutants is much more laborious because they lack any visible marker for genotyping. We genotyped these deletion strains using small capillary size-based gel electrophoresis of amplicons near the deletion site obtained from individual mosquitoes to identify those carrying mutations (**Figure 4.3**). As expected, NPYLR1Δ4 and NPYLRΔ8 produced transcripts with frame-shift mutations that yielded 22 and 9 premature stop codons respectively.

4.5 Predicted NPYLR1 Mutant Protein Structures

Both HR and NHEJ mutants create significant disruption of NPYLR1 protein sequence producing no more than 47/529 wild-type amino acids and therefore eliminating 91% of the protein (**Figure 4.4**, compare with wild-type NPYLR1 in **Figure 3.5A**). Unfortunately, two attempts to produce custom antibodies to corroborate the absence of NPYLR1 protein in mosquito tissue failed in control experiments to exhibit binding specificity (data not shown). Regardless, we are confident that both HR and NHEJ genetic lesions remove all NPYLR1 protein function in each of the mutant lines.

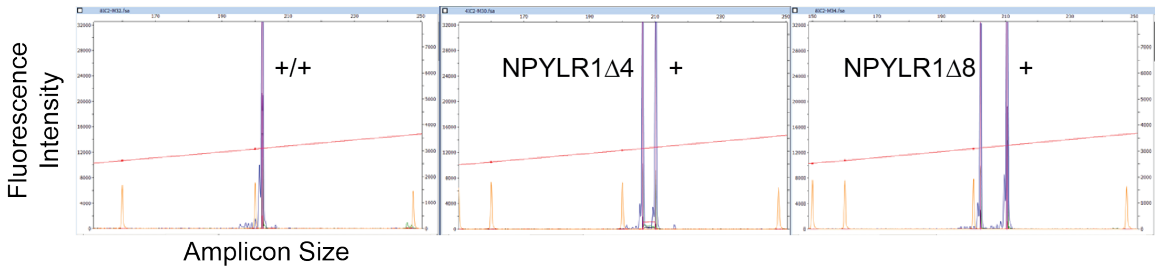


Figure 4.3 NPYLR1 NHEJ Mutant Genotyping

Screen shots of plots illustrating fluorescent PCR products separated by small capillary size-based gel electrophoresis, comparing fluorescent intensity (y-axis) and amplicon size (x-axis) that clearly identify peaks distinguishing mutant NHEJ alleles NPYLR1Δ4 and NPYLR1Δ8 from wild-type (+/+).

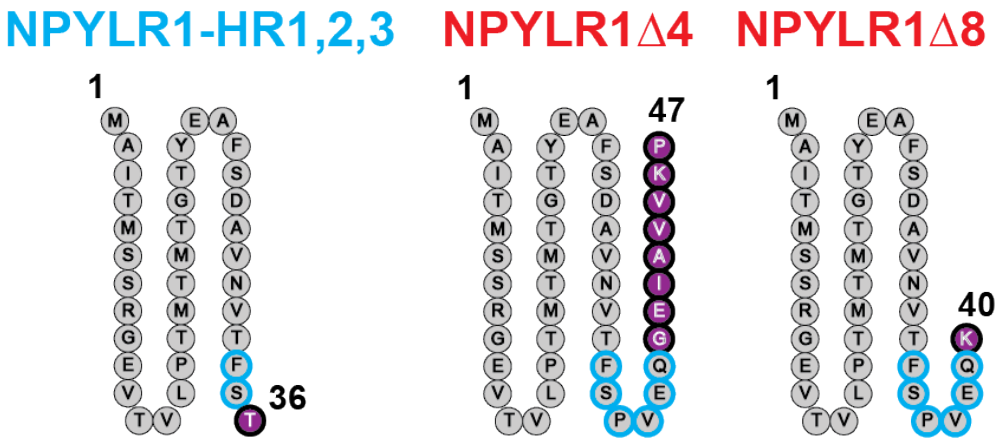


Figure 4.4 Truncated Protein Products Predicted for NPYLR1 Mutants

Snake plots illustrating predicted protein truncations for NPYLR1 mutant alleles that produce 36/529 (4%) amino acids in HR mutants, while 47/529 (8%) and 40/529 (7.5%) are produced for NPYLR1Δ4 and NPYLR1Δ8 respectively. Blue circles indicate the protein region targeted by the NPYLR1 ZFN and the purple circles indicate missense mutations from inserted sequence or frame-shifts (compare to Figure 3.5A).

5 Behavioral Analysis of NPYLR1 Mutants

5.1 Introduction and Summary of Results

Based on evidence from our cell-based assay and behavioral validation of NPYLR1 ligands, we have compelling evidence for NPYLR1 playing an important role in behavior modulation. Therefore, we predicted that NPYLR1 mutant mosquitoes would be insensitive to the inhibitory neuropeptides Head Peptide-I and sNPF, thus preventing the inhibition of host-seeking behavior after a blood meal. NPYLR1 null mutant mosquitoes were tested for general defects in locomotion and egg laying, changes to feeding related behaviors such as sugar and blood consumption, as well as aspects of the gonotrophic cycle: host-seeking behavior, inhibition of host-seeking behavior, and recovery of host-seeking behavior after egg-laying. Despite the evidence arguing for an important NPYLR1 role, no changes in mutant behaviors were observed, suggesting redundant mechanisms for behavioral control.

5.2 Genotypes Used for Behavioral Experiments

Behavioral tests can be extremely sensitive to changes in background mutations so we took several precautionary steps before analyzing NPYLR1 mutants. First, multiple mutant lines for both HR and NHEJ mutagenesis were isolated for parallel testing to corroborate any behavioral phenotypes. Second, HR lines were outcrossed for five generations to the wild-type *A. aegypti* Orlando strain to recombine away potential background genetic differences. NHEJ mutants were outcrossed for two generations due to the laboriousness of genotyping. Third, independent mutant lines were combined in hetero-allelic combinations to heterozygous any remaining background events. For example,

HR lines NPYLR1-HR2 and NPYLR1-HR3 were combined into an NPYLR1-HR2/NPYLR1-HR3 (HR2/HR3) and NPYLR1Δ4 and NPYLR1Δ8 were combined into NPYLR1Δ8/Δ4 (Δ8/Δ4) for behavioral analysis. Lastly, heterozygous control lines were created for each mutant allele, by crossing to the wild-type *A. aegypti* Orlando strain, to reveal any dominant effects.

5.3 Investigation of Possible General Defects of NPYLR1 Mutants

As a likely receptor for neuromodulation, NPYLR1 may be involved in a broader physiological role than we anticipated, such as motor coordination or egg development (Nässel et al., 2008). Therefore, we first tested NPYLR1 mutants for general defects of locomotion and fecundity. Small changes in both general behaviors were observed in various mutant lines, but none were consistently different from all controls lines leading us to conclude that NPYLR1 mutants do not possess general defects.

5.3.1 No Apparent Locomotion Defects of NPYLR1 Mutants

To test general locomotion, individual female mosquitoes were placed in glass vials, large enough for limited flight, which were electronically monitored for movement for three standard day-night cycles. Δ8/Δ4 were indistinguishable from control lines whereas HR2/HR3 displayed a 50% decrease in average daily activity counts in comparison to wild-type controls only (**Figure 5.1A**, HR2/HR3: 105.4±20.58 and +/+: 249.1±45.14, $p < 0.05$). HR2/HR3 and wild-type lines were not significantly different from either HR2/+ or HR3/+ controls (189.3±33.64 and 190.7±34.36 respectively). Since there was no defect observed for Δ8/Δ4 mutants,

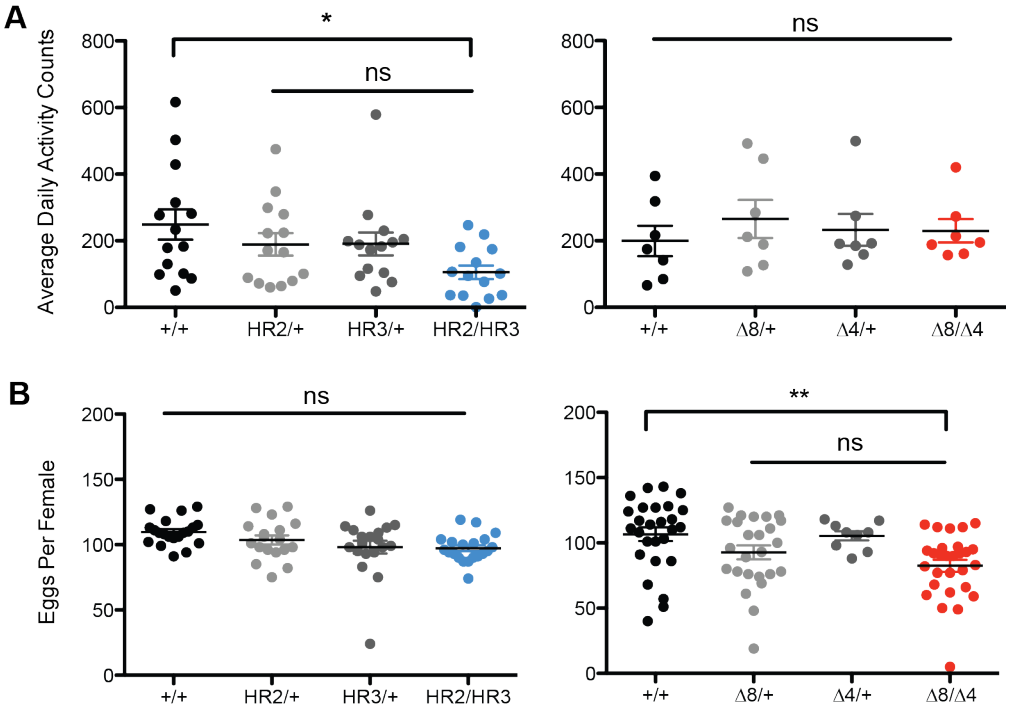


Figure 5.1 Normal Locomotor Behavior and Egg-Laying in NPYLR1 Mutants

(A) Average daily activity counts for individual female HR mutants (left, n = 14) and NHEJ mutants (right, n = 7). Only HR2/HR3 is significantly different from wild-type controls, but not from HR2/+ or HR3/+ controls. **(B)** Eggs laid per individual female HR mutants (left, n = 18-20) and NHEJ mutants (right, n = 9-28). Δ8/Δ4 is significantly different from wild-type controls, but not from Δ8/+ or Δ4/+ controls. In A-B, data are plotted as mean ± SEM. 1-Way ANOVA with Bonferroni correction for multiple comparisons. * = $p<0.05$; ** = $p<0.01$, ns = not significant.

and HR2/HR3 is not consistently different from all controls, we believe that NPYLR1 mutants have normal locomotor behavior.

5.3.2 No Apparent Egg Laying Defects of NPYLR1 Mutants

Eggs were manually counted from individual females blood-fed to completion on a human arm. No differences in eggs laid were observed for HR2/HR3 (**Figure 5.1B**). A slight decrease was observed for Δ8/Δ4 compared to wild-type (82.54±4.59 and 106.7±5.26 respectively, $p < 0.01$), but not in Δ8/+ or Δ4/+ controls (92.76±5.36, and 105.4±3.48). We conclude that egg-laying behavior is normal in NPYLR1 mutants.

5.4 Studying Mosquito Feeding Behaviors

Our hypothesized role for Head Peptide-I as a modulator of host-seeking behavior might be a consequence of the neuropeptide communicating a "fed" internal state. Studies in *D. melanogaster* support the existence of feeding states in insects. For example, sNPF has been shown to regulate olfactory sensitivity in response to starvation, indicating that fruit flies have fed and starved internal states (Root et al., 2011). Interestingly, *D. melanogaster* do not feed on blood but rather eat yeast growing on rotting fruit; and in lab environments, a combination of sugar and protein (in the form of yeast paste), whose hunger drives are differentially regulated (Ribeiro and Dickson, 2010). In contrast, wild *A. aegypti* mosquitoes feed on floral nectar and humans; and in the lab are maintained on a sugar solution and obtain protein from blood meals. It is not known whether *A. aegypti* mosquitoes, like *D. melanogaster*, separately regulate the drive for sugar and protein. As a consequence of NPYLR1 sensitivity to ligands implicated in

blood and non-blood-feeding behaviors, we decided to quantify consumption of both sugar and blood to learn if either was modulated by the receptor.

5.4.1 Normal Sugar Consumption in NPYLR1 Mutants

To measure sugar consumption of NPYLR1 mutants we designed two feeding assays called the CAFE and BUFFET. The CAFE (Capillary Feeder Assay; Adapted from (Ja et al., 2007)) houses a group of five female mosquitoes in a vial with access for 2 hours to a glass capillary filled with 10% sucrose (**Figure 5.2A**). During the experiment, females are capable of locating and feeding from the open-end of the glass capillary whose change in volume can be measured by the change in the meniscus. Preliminary experiments in the CAFE revealed that non-fasted mosquitoes were not motivated to feed on sugar under experimental conditions. We therefore tested groups of wild-type mosquitoes after 0, 12, 24, 48, and 72 hours of fasting to optimize feeding conditions. Results indicate that on average less than 1.5 μl of total sucrose was withdrawn after fasting for 0, 12, and 24 hours (**Figure 5.2B**). At 48h, a significant increase to 2.48±0.50 μl of sucrose was consumed per group. Continuing the trend at 72h, increased consumption to 3.70±0.30 μl was observed, though numerous mosquitoes also died from desiccation. We therefore decided that 48h was an appropriate fasting time to motivate mosquitoes to sugar feed without imparting fatal stress. To test whether NPYLR1 mutants modulate feeding state, we studied mosquitoes in the CAFE after 0 and 48 hours of fasting and found that they modulate their consumption of sugar at similar levels to controls (**Figure 5.2C**).

Due to limitations with the throughput and accuracy of the CAFE assay, we designed the BUFFET to remove competition for the single capillary food

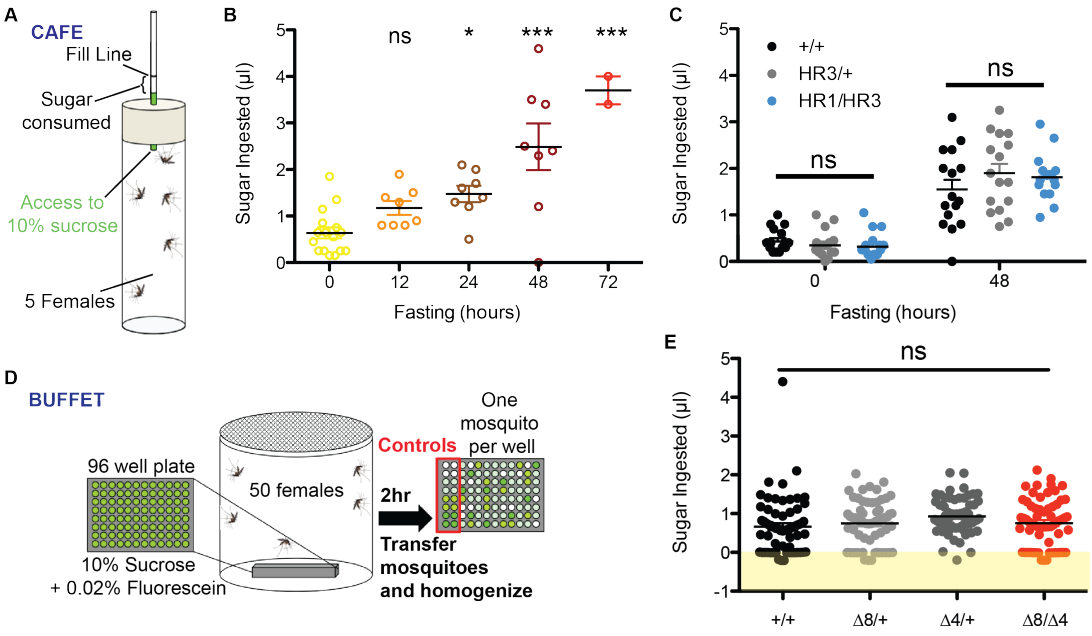


Figure 5.2 Sugar Consumption Is Not Modulated in NPYLR1 Mutants

(A) Diagram of the CAFE assay. **(B)** Female mosquitoes were fasted for 0, 12, 24, 48, and 72 hours, and tested in the CAFE assay for feeding modulation (n = 8-16, except 72h, n = 2; 5 mosquitoes per trial. Mean ± SEM. 1-way ANOVA compared to 0h with Dunnett's Multiple Comparison Test. * = $p<0.05$, *** = $p<0.001$, ns = not significant). **(C)** After 48h of fasting, HR mutants are able to modulate food consumption in the CAFE at similar levels to controls (n = 16; 5 mosquitoes per trial. Mean ± SEM, 2-way ANOVA with Bonferroni correction for multiple comparisons). **(D)** Diagram of the BUFFET assay. **(E)** After 48h of fasting, individual NHEJ mutants on average consume similar levels of sugar to controls. The yellow region indicates individuals that did not feed and whose numbers are also not significantly different from controls (n = 64; 50 mosquitoes per trial. Mean ± SEM. 1-way ANOVA with Bonferroni correction for multiple comparisons).

source, implement a larger more free-flying test enclosure, reduce potentially traumatic handling prior to testing, and greatly improve the precision of measurements by examining individual mosquitoes. After 48h of fasting, a group of fifty female mosquitoes was allowed access to an open-faced 96-well micro-titer plate containing 10% sucrose + 0.02% Fluorescein Dye in each well (**Figure 5.2D**). After 2 hours of feeding, mosquitoes were individually homogenized and the amount of fluorescein dye released from their gut was quantified in a fluorescent plate reader. Results from the BUFFET assay indicated that individual Δ8/Δ4 mutants, fasted for 48h, ate on average 0.75±0.08 μl of sugar solution which is not significantly different from wild-type controls (0.66±0.09 μl), Δ8/+ (0.74±0.07 μl) or Δ4/+ (0.92±0.06 μl) (**Figure 5.2E**). Experiments were not performed on HR mutants because no phenotype was observed in the NHEJ deletion mutants. These data suggest that NPYLR1 does not play a significant role in regulating sugar ingestion during fasting in *A. aegypti*.

5.4.2 Normal Blood Consumption of NPYLR1 Mutants

To measure blood consumption, we designed an artificial feeding assay called the MEMBRANE FEEDER. Similar to the BUFFET, sheep's blood was mixed with 0.02% Fluorescein Dye and loaded into a heated glass device with one accessible surface covered with Parafilm that female mosquitoes could puncture to withdraw blood (**Figure 5.3A**). To encourage feeding, the apparatus was placed within an enclosed container that provided 5% CO_2 to synergize with the heated blood and trigger blood-feeding. After a 15-minute assay, approximately 40% of both control and NPYLR1 mutant mosquitoes had successfully consumed blood (**Figure 5.3B**). Individual mosquitoes were

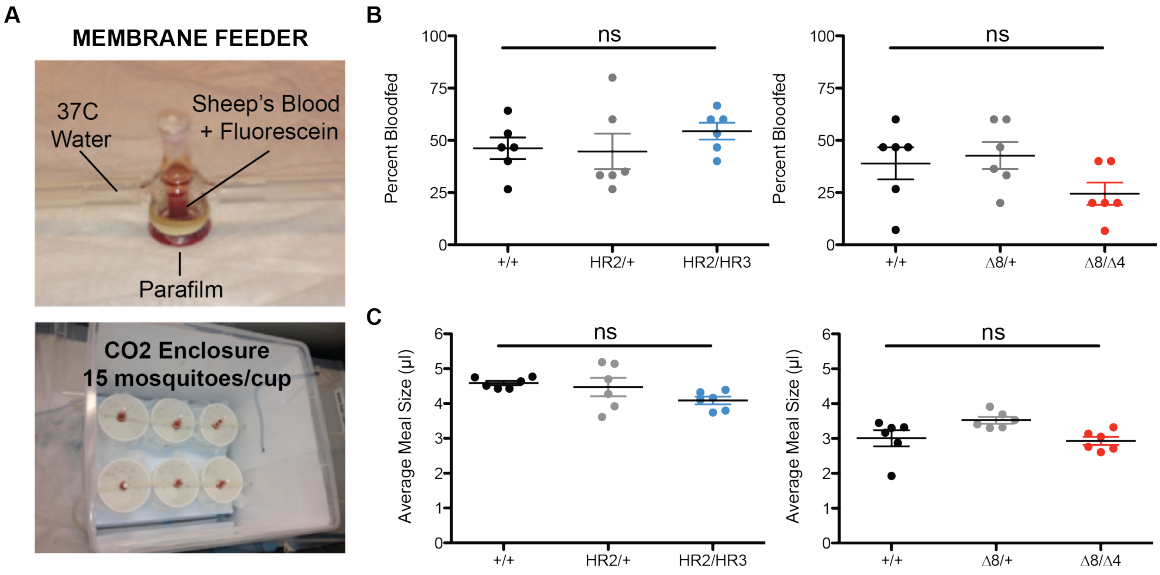


Figure 5.3 Blood Consumption is Not Modulated by NPYLR1

(A) Diagram of the artificial membrane feeder (top) containing Sheep's Blood + 0.02% Fluorescein Dye within a jacket compartment of 37°C water and feeding access through 2x layer of Parafilm. Bottom: Image of six artificial membrane feeders placed on top of cups containing 15 female mosquitoes within the enclosure perfused with 5% CO_2. **(B)** Percent of mosquitoes that blood-fed in the MEMBRANE FEEDER for HR mutants (left) and NHEJ mutants (right). **(C)** Average blood-meal size of NHEJ (left) and HR (right) mutants, for only those confirmed to have blood-fed within each cup of 15 female mosquitoes as measured on a plate reader. (n = 6, 1-12 mosquitoes confirmed as fed per trial. Mean ± SEM. 1-way ANOVA with Bonferroni correction for multiple comparisons)

homogenized to release the fluorescein dye and measured using a fluorescent plate reader to determine the volume of blood consumed. Excluding those that did not feed, we found no significant difference in blood volume consumed for HR2/HR3 (4.08±0.10 μl) and wild-type controls (4.59±0.06 μl) or HR2/+ (4.47±0.26 μl) (**Figure 5.3C**, left). In agreement, there was no difference observed between Δ8/Δ4 (2.93±0.11 μl) compared to wild-type controls (3.00±0.23 μl) or Δ8/+ (3.52±0.09 μl) (**Figure 5.3C**, right). In general, the average volumes were slightly higher in the HR experiment likely due to different aliquots of Sheep's blood and do not indicate any behavioral difference between NPYLR1 mutants. Overall, these results do not support a role for NPYLR1 in modulating blood consumption.

5.5 The Gonotrophic Cycle

The gonotrophic cycle is a complex set of behaviors where unfed female mosquitoes find a host for a blood meal, inhibit host-seeking behavior while eggs develop, locate and deposit eggs, and recover attraction to hosts for a subsequent blood meal. Head Peptide-I is known to inhibit host-seeking behavior and therefore we predicted that an NPYLR1 mutant would be insensitive to the behavioral inhibition of the neuropeptide. However, since inhibition is part of a cyclical process, removing Head Peptide-I signaling may interfere with progression through several aspects of the gonotrophic cycle. We studied each aspect carefully but determined that NPYLR1 does not play an observable role in modulating any gonotrophic behaviors.

5.5.1 Normal Host-Seeking Behavior in NPYLR1 Mutants

Host-seeking behavior was tested in the uniport olfactometer by measuring the percent of female mosquitoes that flew approximately one meter upwind towards human host odor (**Figure 1.1A**). Prior to a blood meal, host-seeking behavior of HR2/HR3 (85.96%±5.57) was not significantly different than wild-type mosquitoes (95.41%±1.17), HR2/+ (92.55%±2.90), or HR3/+ (87.67%±2.76) (**Figure 5.4A**, "before"). Similarly, Δ8/Δ4 (74.56%±6.64) was comparable to wild-type mosquitoes (82.04%±6.34), Δ8/+ (87.04%±3.08), and Δ4/+ (86.20%±4.76) (**Figure 5.4B**, "before"). All NPYLR1 mutants and control lines displayed robust host-seeking behavior indicating no baseline defect in the ability to find a host.

5.5.2 Normal Inhibition of Host-Seeking Behavior in NPYLR1 Mutants

Based on previous results, we predicted that if NPYLR1 were the Head Peptide-I receptor, then a NPYLR1 null mutant would show reduced inhibition of host-seeking behavior after abdominal distension is relieved at 24h. Following a blood meal, inhibition is observed in all tested lines at 24h as expected (**Figure 5.4A and B**, 24h). As the most direct test for NPYLR1 inhibition, at 48 and 72 hours after a blood meal HR2/HR3 and Δ8/Δ4 mutants still display strong inhibition of host-seeking behavior (**Figure 5.4**, 48 and 72h). This result indicates that NPYLR1 is not required for the inhibition of host-seeking behavior and that behavioral modulation must be controlled by a different or a redundant mechanism.

Closer scrutiny of the behavioral timeline following a blood meal led us to question whether Head Peptide-I and NPYLR1 inhibition may be limited to the

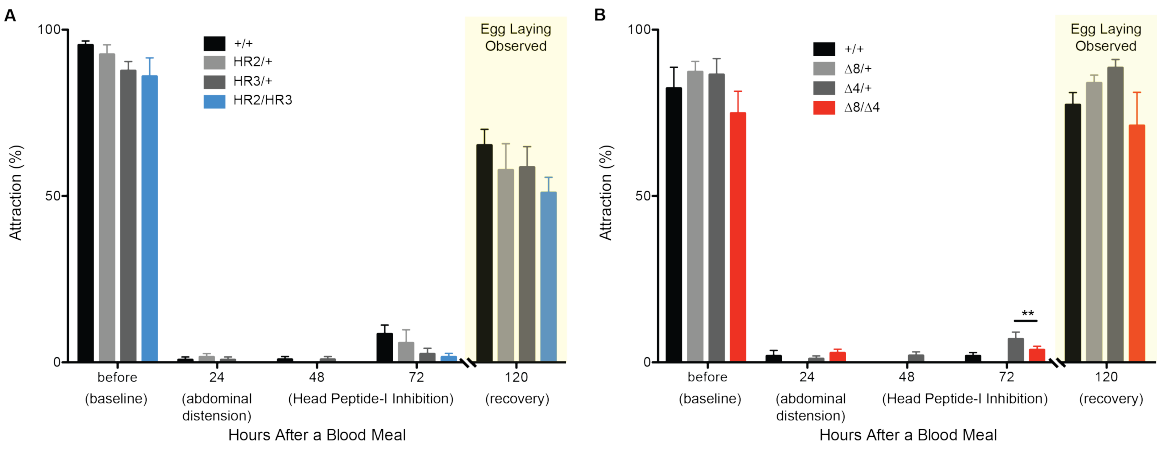


Figure 5.4 Normal Host-Seeking Behavior of NPYLR1 Mutants during the Gonotrophic Cycle

Host-seeking behavior of HR **(A)** and NHEJ **(B)** NPYLR1 mutants before and at 24, 48, 72, and 120 hours after a blood meal as observed in the uniport olfactometer. "Before" is a measure of baseline host-seeking behavior of mature non-blood-fed female mosquitoes. 24h after a blood meal has been shown to be inhibition by abdominal distension. Head Peptide-I inhibition is predicted for 48 and 72h. Egg-laying was observed after 72h and completed by 120h. Recovery of host-seeking behavior was tested at 120h (indicated by yellow shading). (n = 6-12; ~20 mosquitoes per trial. Mean ± SEM. 1-way ANOVA with Bonferroni correction for multiple comparisons within each time point).

time between abdominal distension and early stages of egg development, rather than throughout egg development. To test this idea, we decided to increase the temporal resolution following relief of abdominal distension at 24h. Again, Δ8/Δ4 displayed robust inhibition of host-seeking behavior at 32 and 40 hours after a blood meal (**Figure 5.5**). These results further supported that NPYLR1 is not required for the inhibition of host-seeking behavior.

5.5.3 Normal Recovery of Host-Seeking Behavior in NPYLR1 Mutants

Egg-laying was observed in all mosquito lines between 72-120 hours after a blood meal. Like wild-type mosquitoes, we found that both HR2/HR3 and Δ8/Δ4 recovered host-seeking behavior when tested at 120h following the completion of egg-laying (**Figure 5.4A and B**, 120h). Therefore, NPYLR1 also does not affect the recovery of host-seeking behavior following egg deposition.

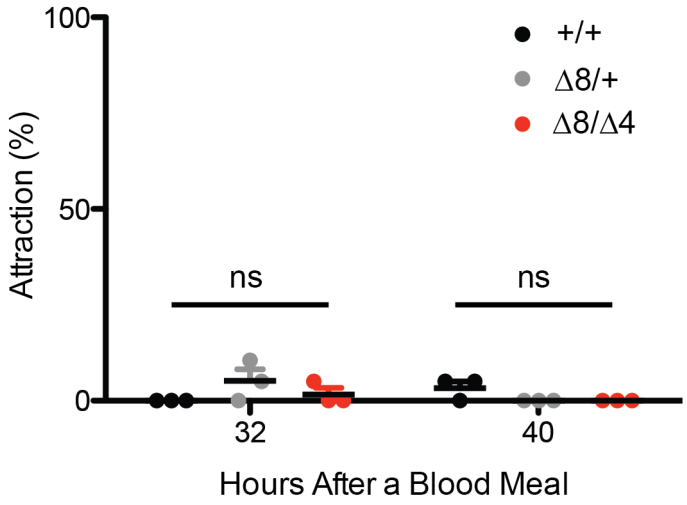


Figure 5.5 NPYLR1 Mutants Show Normal Host-Seeking Inhibition at 32 and 40h After a Blood Meal

NHEJ mutants were tested in the uniport olfactometer for attraction to human host odor at 32 and 40h after a blood meal. No significant differences were observed (n = 3, with ~20 mosquitoes per trial. Mean ± SEM. 1-way ANOVA with Bonferroni correction for multiple comparisons within each time point).

6 Discussion

The work presented in this dissertation explains the development of *A. aegypti* as a modern genetic model system to pursue novel strategies for reducing the spread of vector-borne diseases. We used modern techniques to advance the study of the important neuropeptide regulator, Head Peptide-I, by identifying the candidate receptor NPYLR1, pioneering targeted mutagenesis with zinc-finger nucleases, and testing the loss of NPYLR1 function on several important mosquito behaviors. In addition, we functionally identified several ligand-receptor interactions within the NPY-Like Receptor family that likely play pivotal roles in the regulation of mosquito feeding behaviors. The techniques and results presented here will be valuable for future work addressing important aspects of *A. aegypti* biology and developing innovative disease prevention strategies.

6.1 The Role of NPY-Like Receptors in *A. aegypti*

In pursuit of a Head Peptide-I candidate receptor, we used bioinformatics and molecular cloning to characterize the *A. aegypti* NPY-Like Receptor family. Difficulties with accurate gene prediction due to a large amount of repetitive DNA elements in the genome were clarified to confirm accurate gene models of at least nine NPYLRs. The possibility still exists for additional NPYLRs to be discovered which are currently inaccessible to bioinformatics techniques because of poor sequencing and assembly. Efforts are currently underway within the Vosshall Lab to use modern RNA sequencing technology to profile the entire set of expressed genes in *A. aegypti* mosquitoes for several tissues and during several points during their life cycle.

Of the nine confirmed NPYLRs, we linked four receptors with peptide ligands in a heterologous HEK cell-based assay. Most interestingly, NPYLR1 had medium to strong sensitivity to both sNPFs and Head Peptide-I. No other receptor in our screen was sensitive to Head Peptide-I. These results vaulted NPYLR1 into the top candidate position as the Head Peptide-I receptor.

NPYLR8 was predicted as a NPF receptor orthologue and later confirmed to possess moderate sensitivity to a predicted *A. aegypti* NPF. Studies from *D. melanogaster* suggest that as a NPF orthologue, NPYLR8 may perform critical developmental functions in larval food-search behavior and food quality assessments (Wu et al., 2003; 2005). Molecular analysis of the receptor could help to understand larval olfactory preferences as well as motivational circuits for foraging.

The function of NPYLR5 in mosquito biology has yet to be determined. NPYLR5 displays weak sensitivity to NPF and therefore may be involved in NPYLR8/NPFR associated feeding behaviors. To test this idea, localization of NPYLR5 during larval development would be an appropriate first step to uncovering parallels to NPFR expression in CNS and midgut (Garczynski et al., 2002). However, weak NPF activation suggests that other ligands may exist that can now be identified using the functional NPYLR5 clone in future cell-based assays. Sequence similarity initially suggested that NPYLR5 might be the orthologue to *D. melanogaster* NepYR, which is related to mammalian Neuropeptide-YY receptor (PYY) and involved in gastrointestinal regulation (Gehlert, 2004). No evidence was found in our cell-based assay for NPYLR5 sensitivity to PYY.

Lastly, NPYLR7B is the most mysterious receptor confirmed for activity in our cell-based assay. Homologues to NPYLR7B have no known function and broad sensitivity to sNPF1, sNPF3, and PYY confound any clear relationship to other NPY-Like Family members. However, every NPY-Like receptor has been implicated in some aspect of feeding behavior, so the chances are likely that studies of NPYLR7B would lead to a similar role.

6.2 Targeted Mutagenesis Establishes *A. aegypti* as a Model System

Targeted mutagenesis is a vital technique needed to advance *A. aegypti* as a modern model disease-vector system to compliment an already published genome and transgenesis (Jasinskiene et al., 1998; Nene et al., 2007; Nimmo et al., 2006). Beyond zinc-finger nucleases, modern genomic editing techniques, such as TALENs and CRISPR-Cas, are developing quickly to accelerate the time by which targeted mutagenesis can be accomplished (Gaj et al., 2013). It is not unreasonable to assume that within the next 5-10 years the adoption of these new genomic editing techniques could produce mutant lines for all NPYLRs identified in this dissertation.

As the most understood genomic editing system available, zinc-finger nucleases were a promising technique for us to apply to *A. aegypti*. The dual opportunity for generating Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) mutants described here are valuable successes that can be implemented in future genomic editing techniques. In addition, screening methods have been optimized so that isolation of mutants can occur within a week or two from injection and be ready for behavioral testing within a few months. Likely, the techniques developed for *A. aegypti* could also be easily

amended for converting *A. gambiae* to a genetic model system, although other obstacles, such as producing robust host-seeking behavior in laboratory environments, will still need to be overcome.

As additional mutants are generated, reliable behavioral assays are needed for testing host-seeking and feeding related behaviors. The CAFE, BUFFET, MEMBRANE FEEDER, and uniport olfactometer are fundamental and reliable experiments that will be valuable for describing future mosquito behavior relevant for preventing disease transmission.

6.3 NPYLR1 is Not Required for Behavioral Inhibition

Previous work and our cell-based assay suggest a critical role for NPYLR1 in Head Peptide-I-mediated inhibition. However, NPYLR1 null mutants showed no mutant phenotype in sugar or blood consumption or any aspect of the gonotrophic cycle. Our interpretation is that there is a redundant signaling mechanism that functions even after removal of NPYLR1 activity.

Early experiments validated multiple agonists, sNPFs and Head Peptide-I, for NPYLR1. Our initial concern was that this result, in combination with previous difficulties describing neuropeptide mutant phenotypes, strongly argued that a Head Peptide-I null mutant would not be effective due to sNPF redundancy ((Kim et al., 2010; Wang et al., 2013, Valassi et al., 2008). Targeting NPYLR1 appeared as the safer option to eliminate activity of both neuropeptides and had the added benefit of known mutant feeding behaviors established for the *D. melanogaster* homologue, sNPFR76f.

The most pessimistic response to the lack of a mutant phenotype would be to argue that NPYLR1 is not a physiologically relevant Head Peptide-I receptor.

However, we firmly believe that the injection experiments argue against this position. In the cell-based assay, NPYLR1 was not only sensitive to Head Peptide-I, but also to sNPFs that were not previously connected to behavioral inhibition. Our discovery that sNPF is a novel inhibitor, as shown when injected into non-bloodfed females, corroborates that NPYLR1 sensitivity mimics the profile of validated behavioral inhibitors. The only conceivable argument against a role for NPYLR1 is that its expression limits access to neuropeptides in the hemolymph and therefore is not involved in inhibitory signaling. Unfortunately, this is a difficult hypothesis to test because we currently lack tissue localization data for this receptor and would still be challenged to prove that hemolymph neuropeptides could not be accessed. This then presents several difficult situations where redundancy may be occurring that would confound further analysis of *A. aegypti* behavioral inhibition.

The first possibility is that the neuropeptides signal through an additional receptor that is not currently known. It is conceivable that the NPY-Like Receptor family members not yet linked to a ligand (NPYLR2, 3, 4, 6, and 7A) may also be sensitive to Head Peptide-I and sNPFs. Additional control experiments would need to be performed to confirm proper expression and function of these receptors in a cell-based assay. Furthermore, there are numerous receptors outside of the NPYLRs with no known ligands that could also be sensitive to Head Peptide-I and sNPF. Pursuit of this possibility is more difficult, though I would suggest considering members of the more inclusive Class A: Rhodopsin-Like GPCRs, of which NPYLR1 is a member. Ultimately, any molecular characterization of a subsequent candidate receptor will likely have to be

genetically recombined with the NPYLR1 mutant to remove all redundant signaling pathways.

The second possibility is that although Head Peptide-I and sNPFs are sufficient to inhibit behavior, additional neuropeptides are also competent inhibitors of behavior. In this scenario, an additional unknown neuropeptide and cognate receptor would need to be identified to further study inhibition of host-seeking behavior. Evidence already exists that an ovarian factor is released into the hemolymph between 6-10 hours to trigger release of a longer lasting inhibition presumably including Head Peptide-I (Klowden, 1981). Both this unknown ovarian peptide and additional candidate inhibitors could be identified by proteomic analysis of hemolymph at various stages of the gonotrophic cycle. It may also be possible to reduce the number of candidates by injection of biochemically separated hemolymph fractions. This project is currently being pursued by other members of the Vosshall laboratory.

6.4 NPYLR1 as the sNPF Receptor in *A. aegypti*

Beyond a role for NPYLR1 in the inhibition of host-seeking, possibilities exist for behavioral changes related to sNPF signaling that were not fully pursued in the work described here. Evidence from *D. melanogaster* implicates sNPF signaling as a regulator of olfactory sensitivity during starvation (Root et al., 2011). This finding was the basis for our expansion of NPYLR1 mutant characterization with the CAFE and BUFFET sugar feeding assays. However, removal of sNPF signaling in *D. melanogaster* only exhibited weak behavioral phenotypes in food search behavior to a complex odor, Apple Cider Vinegar (Root et al., 2011). No equivalent complex odor has been established in *A. aegypti*

mosquitoes as a proxy for attraction to non-host sources of sugar such as flower nectar. The closest experiment established within the Vosshall lab was the attraction of male and female *A. aegypti* to un-processed honey odor. However, these experiments were dependent on long periods of fasting and still were difficult to reproduce reliably. We therefore decided not to pursue these experiments because at best, they would recapitulate an already known behavior. Manipulation of sNPF signaling also was described to have effects on *D. melanogaster* body size, though no superficial change was observed in NPYLR1 mutants.

More interesting would be to pursue characterization of potential changes in olfactory sensitivity in NPYLR1 mutants to various host and plant odors. The best technique to perform this experiment would be to adapt calcium imaging of the olfactory bulb using the molecular calcium indicator GCaMP (Root et al., 2011). This technique provides a global view of responses within the entire olfactory system and would not only be useful for NPYLR1 characterization but also for analyzing responses to host odors during different stages of the gonotrophic cycle. This would be a wonderful technique to test whether modulation of olfactory perception is a potential mechanism by which host-seeking inhibition occurs. This project is also being pursued by a team of Vosshall lab members.

Localization experiments for sNPF in *D. melanogaster* also argue for a wide-spread role for the neuropeptide in several neural circuits. Particularly, extensive expression has been described in intrinsic interneurons of the mushroom body, the central nervous system, OSNs, and circadian pacemaker neurons (Nässel et al., 2008). Other behaviors controlled by these neural circuits that may be

influenced by an NPYLR1 null mutant are circadian rhythm, memory, and coordinated muscle contraction. These reports were the basis for our concern that deletion of NPYLR1 function may prove fatal during development or lead to generic defects in locomotion and egg-laying. However, NPYLR1 mutants were clearly viable and did not possess gross defects. But inclusion of NPYLR1 mutants in behavioral experiments outside of host-seeking and food behaviors would be advisable.

Overall, though NPYLR1 was a strong candidate for Head Peptide-I inhibition of host-seeking behavior, it is almost more astounding that such a highly conserved receptor of sNPF signaling could have little noticeable effect on behavior. This is an important lesson about the adaptability of living organisms to adjust to genetic changes whose loss could be extremely damaging to survival.

6.5 Genetic Manipulation of Head Peptide-I

Targeted mutagenesis of NPYLR1 was a strategy we pursued to avoid neuropeptide redundancy, though the lack of a mutant phenotype redirected our efforts to creating a Head Peptide-I mutant. Our first consideration was to test for a redundant signal through behavioral characterization of NPYLR1 mutants injected with Head Peptide-I or sNPF3. However, in our hands, behavioral analysis following peptide injections was difficult and only produced modest levels of inhibition. For comparison, previous publications reported complete inhibition of host-seeking behavior at comparable doses to our experiments that yielded only ~30% inhibition (**Figure 3.1A**) (Brown et al., 1994). Differences in the injections could be attributed to technique or possibly the stability of the custom synthesized peptides. Regardless, we decided that testing the redundancy

hypothesis through injections would be a difficult experiment that would only confirm what we already knew about the inadequacy of NPYLR1 mutants. We preferred to pursue a more impactful and independently useful project, namely, creating a Head Peptide-I null mutant.

Our data already suggests that a Head Peptide-I null mutant would not be sufficient to reduce inhibition of host-seeking behavior due to sNPF redundancy. But we propose that combining a Head Peptide-I null mutant with the NPYLR1 mutant would remove all Head Peptide-I activity and sNPF activity through NPYLR1. Combined, a double mutant may disrupt inhibitory signals enough to show a behavioral phenotype. Furthermore, it would validate Head Peptide-I as a bona fide inhibitor critical for *A. aegypti* host–seeking inhibition - an achievement that is currently weakened due to our results that a validated Head Peptide-I receptor is not required for inhibition. My waning hours as a graduate fellow have been devoted to this project in collaboration with Dr. Laura Duvall, a post-doc in the Vosshall lab who will carry the Head Peptide-I torch when I ride off into the proverbial sunset.

An additional project idea concerning genetic manipulation of Head Peptide-I is to create a transgenic line that over-expresses the neuropeptide. We initially considered this project at the beginning of my graduate career but decided to go for the higher impact NPYLR1 null mutant. The idea is that over-expression could lead to continual release of Head Peptide-I which would maintain an inhibitory state. Propagation of such a mutant line could occur due to observations that females inhibited by Head Peptide-I injections still feed when placed directly on human arms (Brown et al., 1994). Our concern was that Head Peptide-I undergoes post-translational modifications, which only occurs in

limited endocrine cells, that are likely critical to function and stability (Park et al., 2008).

Over-expression may not produce the desired increase in mature Head Peptide-I. Furthermore, release of Head Peptide-I, as measured in a radioimmunoassay, is temporally controlled. So we could not assume that overexpression would lead to Head Peptide-I release. Contrary to our trepidations, overexpression of neuropeptides has been successful in other cases (Lee et al., 2004; 2008). If over-expression does work, then the hypothesis that Head Peptide-I modulates olfactory perception of relevant host-odors would be more easily testable because the mutants would permanently exhibit the desired olfactory state. To be more cautious with this approach, over-expression could be triggered by an inducible promoter, which has already been validated in *A. aegypti* (Carpenetti et al., 2012; Wise de Valdez et al., 2011).

6.6 Implications For Other Research Areas

Results presented here may have implications in *A. gambiae* research as well as recent findings that describe Head Peptide-I transfer in seminal fluid from *A. aegypti* males to females.

We propose that the unknown mechanism *of A. gambiae* inhibition for approximately 40 hours after a blood meal may involve sNPF signaling. Sensitivity of NPYLR1 to Head Peptide-I and sNPF motivated us to confirm sNPF as a novel inhibitor of host-seeking behavior in *A. aegypti*. Previous injection experiments in anopheline mosquitoes only found Head Peptide-I to be ineffective (Klowden, 1995), but never tested sNPF. There is chance that injection of sNPF into *A. gambiae* may prove effective for inhibiting host-seeking behavior

in the malaria vector. A positive result would open a new avenue for dissecting the internal regulation of host-seeking behavior in *A. gambiae*. These experiments could also provide additional support for NPYLR1 and its homologues in the regulation of host-seeking behavior.

Previously, researchers found that male accessory gland (MAG) substances significantly reduce host-seeking behavior when injected into gravid unmated *A. aegypti* females (Fernandez and Klowden, 1995). Typically, if female *A. aegypti* do not fertilize their developed eggs, they will slowly recover host-seeking behavior to locate males that hover in host proximity (Klowden, 1999). The hemolymph of mated versus unmated gravid females was analyzed by radioimmunoassay to determine if Head Peptide-I levels may indicate a role for the neuropeptide, but no significant difference was found (Fernandez and Klowden, 1995). Surprisingly, the molecules responsible have not been pursued until only recently, where researchers found that Head Peptide-I is produced in male accessory glands and transferred during mating (Naccarati et al., 2012). This led the same group to test whether Head Peptide-I activated the conserved Sex Peptide-Receptor (SPR), which has been implicated in altering female behavior after mating in *D. melanogaster* (Yapici et al., 2008). Unfortunately, no activity was found therefore stalling progress understanding a mechanism for mating inhibition due to Head Peptide-I transfer.

We propose that NPYLR1 could be involved in MAG-associated inhibition of host-seeking behavior. We did not pursue any experiments to test this hypothesis because it was outside of our project aims. To do so would require either injection of MAG isolates or mating gravid unmated NPYLR1 mutant females to

male mosquitoes and testing for inhibition of host-seeking behavior. Presumably, if NPYLR1 were involved then no inhibition would occur with either approach.

6.7 Conclusion

The importance of developing novel strategies to combat vector-borne diseases cannot be overstated. Head Peptide-I has been implicated as a natural inhibitor of host-seeking behavior in *A. aegypti* and may also act to control *I. scapularis* behavior. This dissertation has provided valuable advancements to *A. aegypti* as a model system to continue pursuing the mechanism of Head Peptide-I action in hopes that it will provide innovative opportunities to interfere with host location. Even though NPYLR1 is not required to inhibit host-seeking behavior, elimination of the receptor as the sole candidate for Head Peptide-I activity is a noteworthy and informative result to guide future work in mosquito biology.

7 Materials and Methods

7.1 Mosquito Maintenance

A. aegypti Orlando mosquitoes were maintained at 25-28ºC with 70-80% relative humidity under a 14h light: 10h dark cycle (lights on 8am). Eggs were hatched in de-oxygenated, deionized water containing powdered Tetramin tropical fish food (Tetra, Melle, Germany). Larva were cultured in deionized water and fed Tetramin tablets as needed. Adults were given unlimited access to 10% sucrose solution. Adult females were fed on mice for stock maintenance and on human arm for isolation of mutants, egg-laying and host-seeking behavior experiments. All blood-feeding procedures with mice and humans were approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB), respectively. All human subjects gave their informed consent to participate in these experiments.

7.2 Bioinformatics and Protein Alignment

In the *A. aegypti* genome publication, 33 predicted Class A: Rhodopsin-like GPCRs in the "Peptide" and "Orphan" categories were analyzed for similarity to *D. melanogaster* and *A. gambiae* NPY-Like Receptors. From this group, eight NPYLRs were supported but further analysis revealed that two were fragments of another predicted GPCR and therefore there were actually six candidate receptors. BLAST, Genewise, and HMMER bioinformatics tools were used to identify two additional candidates from published raw genomic sequence reads, returning the total candidate list to eight. NPYLR7A and 7B were identified through cloning.

Vectorbase - *A. aegypti* genome

https://www.vectorbase.org/

BLAST – Basic Local Alignment Search Tool

http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download

Genewise - Wise2 compares a protein sequence to a genomic DNA sequence, allowing for introns and frame-shifting errors.

http://www.ebi.ac.uk/Tools/psa/genewise/help/

HMMER – biosequence analysis using profile hidden Markov Models.

http://hmmer.janelia.org/

All protein alignments and phylogentic analyses were performed using default ClustalW and Neighbor Joining Tree Building methods in MacVector (www.macvector.com). Snake plots of predicted receptor topology were created using toppred:Mobyle@pasteur (http://mobyle.pasteur.fr/).

7.3 Molecular Biology

RNA isolation was accomplished using RNeasy Mini Kits (Qiagen, Valencia, CA, USA) from various tissues as noted. DNA isolation from whole mosquitoes was completed using Qiagen's DNAeasy Blood & Tissue Kit, homogenized with 2mm glass beads (Sigma Aldrich Cat#Z273627-1EA, St. Louis, MO, USA) using a Qiagen TissueLyzer II at 1800 rpm for 1 min. Unless otherwise noted, Synthesis of cDNA was performed using SuperScript® III Reverse Transcriptase (Invitrogen, Grand Island, NY, USA) and PCR reactions were performed using EMD Millipore KOD polymerase (Billerica, MA, USA). All DNA sequencing reactions were performed by Genewiz (South Plainfield, NJ, USA).

7.4 Receptor Cloning

Homology to known NPYLRs from other species permitted cloning of full-length cDNA from purified *A. aegypti* female head and body RNA for NPYLR2, 5, 6, and 8. Full length sequences for NPYLR1, 1A, 1B, 1C, 3, 4, 7A, and 7B were verified using Clontech's Rapid Amplification of cDNA Ends (RACE) and Advantage 2 PCR polymerase (Clontech/Takara Bio, Mountain View, CA, USA). *A. gambiae* sNPFR was amplified based on published sequences by Garczynski et al. (2007). *D. melanogaster* sNPFR76f clone GH23382 was obtained from the Drosophila Genomics Resource Center (DGRC, https://dgrc.cgb.indiana.edu/). *I. scapularis* receptors were identified using bioinformatics and cloned by homology from tissue provided by Rick Ostfeld (Cary Institute – Millbrook, NY). All primers used for cloning can be found in section 7.5.

All receptors were cloned into Invitrogen's TOPO-TA Cloning system for propagation in TOP10 or DH5alpha cells. For the cell-based assay, all receptors were cloned into the XhoI-NotI sites of the mammalian expression vector pME18S, except for A.gam sNPFR and D.mel sNPFR76f, which were cloned into EcoRI-NotI.

Genbank accession numbers: NPYLR1 KC439528, NPYLR1A KC439529, NPYLR1B KC439530, NPYLR1C KC439531, NPYLR2 KC439532, NPYLR3 KC439533, NPYLR4 KC439534, NPYLR5 KC439535, NPYLR6 KC439536, NPYLR7A KC439537, NPYLR7B KC439538, NPYLR8 KC439539, IX923R KC439540, IX924R KC439541.

7.5 Primers

All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA)

#	Experiment	Species	Gene Target	Primer FOR (top) / REV (bottom)
1	Receptor Cloning	A.aeg	NPYLR1	ATGGCCATAACGATGTCATCACG
				TTACAGTATCTCCGGCAGCTTGG
2	Receptor Cloning	A.aeg	NPYLR2	ATGCTGGCAAGTACCGCTAAGAC
				TTACAAACGTGTAATGTCTTCTTGGAAGC
3	Receptor Cloning	A.aeg	NPYLR3	ATGAAGTCCAAGGAGACCGCGTCGGATGC
				CTCGCCCGTAATCTTTGGCACCGC
4	Receptor Cloning	A.aeg	NPYLR4	CGTTGTCAGCTTCGACGATGAGTGT
				CGCCAGGAAACGTGCAGCTTCG
5	Receptor Cloning	A.aeg	NPYLR5	ATGAGCGGCGCGCCATTCACGGTC
				TCACCGTAGCAGGGACGTTTCCGT
6	Receptor Cloning	A.aeg	NPYLR6	CACGCCACAATGGATTACCC
				CATCACTTGAACAGGATCCGC
7	Receptor Cloning	A.aeg	NPYLR7A	GCGATGAACTTCACTGCCGAGTT
				CTACAACCCCTTCCGGCACCACT
8	Receptor Cloning	A.aeg	NPYLR7B	GCGATGAACTTCACTGCCGAGTT
				CTACAACCCCTTCCGGCACCACT
9	Receptor Cloning	A.aeg	NPYLR8	ATGGACGTGGTCCTGTCCAGGCTG
				TCACGGCATGAGCTCGGTAAGC
10	Receptor Cloning	A.gam	sNPFR	TTATAGAATAGCGGGCACTTTCGAGTC
				GACGCCTCGGAATGCTGACG
11	Receptor Cloning	I.sca	IX-923R	AACCCAAGCTTGTTCAATCC
				ATGCTGACATCTGGGGGTAG
12	Receptor Cloning	I.sca	IX-924R	TTCTTGCAGATGTCGGATCA
				TTTCTCCATGTTGCAGTGCT
13	Receptor Cloning	D.mel	sNPFR76f	ATGGCCAACTTAAGCTGGCTGAG
				CCTATCTCAGTTGATTCGCCTC
14	Receptor Cloning	A.aeg	NPYLR1A	ATGGCCATAACGATGTCATCACG
				TTACAGTATCTCCGGCAGCTTGG
15	Receptor Cloning	A.aeg	NPYLR1B	ATGGCCATAACGATGTCATCACG
				TTACAGTATCTCCGGCAGCTTGG
16	Receptor Cloning	A.aeg	NPYLR1C	ATGGCCATAACGATGTCATCACG
				TTACAGTATCTCCGGCAGCTTGG
17	Southern Blot	A.aeg	NPYLR1	CCACCCTCGAATGAAACTATCAAC
				TAGTACTTGGGAGGATGGGATGAG
18	Southern Blot	- -	ECFP	ATGGTGAGCAAGGGCGAGGAGCTGTTC
				CTTGTACAGCTCGTCCATGCCGAGAGTG
19	Mendelian Inheritance	A.aeg	NPYLR1 5'UTR	CGCTGCGGAGGCGGAAGCTGAAGC
				CGCCTCACTGACGCCACCGCTGTC
20	qPCR	A.aeg	NPYLR1	GCTATCTGCTACATCTGTGTGTCAA

				GTCCGAGTAGAAGTCGTTGCTCAT
21	qPCR	A.aeg	RpL8	TCACTGCCCACACCAAGAAGCG
				CGGCAATGAACAACTGCTTGCG
22	qPCR	A.aeg	RpS17	CACTCCCAGGTCCGTGGTAT
				GGACACTTCCGGCACGTAGT
23	HR Construct	A.aeg	Left Arm	TGCTGGCGTTACGGCAAACTGATTC
				GAACGTCACATTAACAGCGTCGCTG
24	HR Construct	A.aeg	Right Arm	GGTCAAGCCTTGATGCAGGACAATAC
				AGTATCTCCGGCAGCTTGGTGTCG
25	NHEJ Genotyping	A.aeg	NPYLR1	CGGAACTTACGAAGCATTCAGCGAC
				GAACACTACGTAGCATACCAACACG
26	HR Genotyping	A.aeg	NPYLR1	TAATCGTGTGGACTAGAAGAGGG
				AGCTCTTCGCAGTAGAATGTACG

7.6 HEK293 Cell-Based Calcium-Imaging Assay

HEK293 cells were cultured using standard protocols in a Thermo Scientific FORMA Series II – Water Jacketed CO2 incubator (Waltham, MA, USA). Invitrogen's Lipofectamine 2000 was used for transfection of 1 μg pME18S>Candidate Receptor and 1μg pME18S> $G_{\alpha-15}$. Transfected cells were loaded with the calcium sensitive dye Fura-2 (Invitrogen) according to product instructions. Cells were imaged on a Nikon Eclipse TE-2000-U (Melville, NY, USA) using fluorescent excitement from a Lambda DG-4 (Sutter Instruments Co., Novato, CA, USA). Bath application of ~300 μl solutions containing neuropeptides diluted in PBS was accomplished using a diaphragm pump (Gilson Minipuls3, Middleton, WI, USA) with live recording and analysis in Metafluor software (Molecular Devices, Sunnyvale, CA, USA). Normalization was accomplished by setting the highest response to 100%.

7.7 Host-Seeking Behavior in the Uniport Olfactometer

The uniport olfactometer was custom built by Vadim Sherman in the Rockefeller Instrument Shop. Approximately 15-25 female mosquitoes aged 5-14 days were

placed within loaders (small plastic cylinder with mesh covering both openings and a sliding door on one end obtained from the World Health Organization (WHO), Vector Control Research Unit, Penang, Malaysia) and attached to one end of a 1 m. long plastic tube (19 cm diameter) that led to another "goal" trap (14 cm long, 5 cm diameter), followed by a sealed chamber containing a volunteer's arm or forearm (see image in **Figure 1A**). Humidified room air was carbon-filtered (Donaldson Ultrac-A, Bloomington, MN), combined with 10% CO2 using flowmeters (Cole Parmer, Cat#023-92-ST, Vernon Hills, IL) and passed over the volunteer's arm into the olfactometer at 3.8 L/min. Mosquito loaders were attached to the olfactometer and given 5 minutes to acclimatize to air-flow prior to a 5 minute host-seeking test. Mosquitoes are described as host-seeking if they flew upwind through the 1 meter tube and into the "goal" trap within the allotted time.

7.8 Custom Neuropeptides Synthesis

Head Peptide-I (pERPhPSLKTRFa), Head Peptide-III (pERPPSLKTRFa), and Head Peptide-III (pERPhPSLKTRC) were synthesized by the Rockefeller University Proteomics Resource Center. sNPF-1 (KAVRSPSLRLRFa), sNPF-1(4-11) (SPSLRLRFa), sNPF-2+4 (APQLRLRFa), and sNPF-3 (APSQRLRWa), sNPF-2 (SIRAPQLRLRFa), sNPF-4 (TIRAPQLRLRFa), NPF (SFTCARPQDDPTSVAEAIRLLQELETKHAQHARPRFa), human NPY (YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYa), and human PYY (IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRYa) were synthesized by Bachem Bioscience Inc. (King of Prussia, PA, USA).

7.9 Neuropeptide Injections

Solutions of Head Peptide-I, sNPF3 and Head Peptide-I [Cys10] were made at a concentration of 4 and 10 mM in saline solution (0.1M NaCl, 4mM KCl, 2mM CaCl2) for injection. ~30 female mosquitoes aged 5-14 days were anesthetized on ice for 3 min, moved individually onto a chill-plate (BioQuip Cat#1429, Rancho Dominguez, CA, USA), and injected with 200 nl of desired solution using a Drummond Nanoject II (Drummond Cat# 3-000-204, Broomall, PA, USA) attached to 3.5" needles (Drummond, Cat#3-000-203-G/X) shaped on a needle puller (Sutter Instruments Co., Model P-97). The injection occurred into the hemolymph by piercing under the second abdominal tergite from the ovipositor. Injected mosquitoes were placed in plastic loaders and allowed one hour to recover from injection before being tested for host-seeking behavior in the uniport olfactometer.

7.10 Locomotion Assay

Locomotor activity was monitored using LAM25 Locomotor Activity Monitors (Trikinetics Inc., Waltham, MA, USA). 5 to 14 day-old sugar-fed females mosquitoes were individually placed in glass tubes (25 mm diameter, 125 mm long). A 10% sucrose-soaked cotton plug sealed one end of the glass tube to serve as a food source for the mosquito during the experiment. The vials were inserted into the monitor and housed within a Digitherm incubator (Tritech Research Inc., Los Angeles, CA, USA) set to 25°C and 70-80% relative humidity under a 14 hr light: 10 hr dark cycle (lights on at 8am). Infrared beam breaks triggered by the mosquito's movement were recorded continuously and tabulated into 1 min

bins. Bins with 60 or more beam breaks per minute (>1/sec) and trials with 2000 or more beam breaks per day were excluded from the analysis.

7.11 Egg-laying

5-14 day old female mosquitoes were blood-fed on a human arm or leg for 15 minutes. 72-96 hours after feeding, individual mosquitoes were placed in plastic fly vials (25 mm diameter, 95 mm long) containing 5 ml water and a Whatman filter paper (55 mm diameter, GE Healthcare, Buckinghamshire, UK) folded into a cone. The filter paper would become moist from the water and act as a substrate for females to lay eggs. At 144h post-blood meal, egg papers were removed and eggs were counted by eye using a Nikon SMZ1500 microscope.

7.12 CAFE Assay

Female mosquitoes aged 5-14 days were starved from sugar for the indicated amount of time. Afterwards, five mosquitoes were transferred into plastic fly vials (25 mm diameter, 95 mm long) containing a cotton plug pierced through by a 5 μl calibrated pipet (VWR International, Cat#53432-706) filled with a known volume of 10% sucrose. After 2 hours, the pipets were removed and the change in sucrose volume was measured by ruler. A control vial was set up to measure the loss of volume due to evaporation which was applied to all test vial. Approximately 50 female mosquitoes were starved for 48 hours in a mosquito bucket cage. 96-well plates (Biorad, Cat#HSP-9661) filled with 200 μl 10% sucrose + 0.02% fluorescein dye (Sigma Aldrich, Cat#16377) were placed inside the cage and mosquitoes were allowed to feed for 2 hours. Afterwards, mosquitoes were frozen at -20C and prepared for fluorescent measurement (see section 8.15).

7.13 MEMBRANE FEEDER Assay

Glass feeders (20 mm Glass Jacketed Membrane Feeder, Chemglass Life Sciences, Vineland, NJ, USA) were filled with 400 μl Defibrinated Sheep's Blood (Hemostat Laboratories, Dixon, CA, USA) + 0.02% fluorescein dye. The larger opening of the feeder was covered with two layers of Parafilm stretched as much as possible to make puncturing easier. The other end was also sealed with Parafilm to prevent spillage. Fifteen mosquitoes aged 5-14 days were placed into 6 separate small cups (16 oz SOLO paper cups, www.webstaurantstore.com) sealed on top by white mesh and loaded into a sealable plastic container. One blood-filled feeder was placed on top of each cup and connected by hosing to a 37°C water bath. The container was cleaned with 70% ethanol to remove residuals, sealed, and left for 5 minutes so that mosquitoes could acclimatize. To begin the experiment, water pumps began circulation of 37°C water through the membrane feeder hosing and 5% CO_2 was pumped into the container to initiate feeding. After 15 minutes, the small cups were frozen at -20°C and prepared for fluorescent measurement (see section 8.15).

7.14 Fluorescence Measurements

Frozen mosquitoes were loaded individually into wells of a 96-well plate (Biorad, Cat#HSP-9661) containing 100 μl PBS plus one 2 mm glass bead (Sigma Aldrich, Cat#Z273627-1EA) and covered with PCR Sealers (BioRad, Cat#MSB1001, Hercules, CA, USA). Control wells containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 μl of 0.02% fluorescein dye were combined with unfed control female mosquitoes to create a reference dilution curve. Plates were homogenized using a Qiagen TissueLyzer II at 1800 rpm for 1 min. Homogenized plates were

taken to the Rockefeller University High Through-Put Screening Resource Center, where 15 μl of homogenized solution from each well was transferred to a 384 well plate (Greiner Bio One, Cat#784201, Monroe, NC, USA) alongside 15 μl of a 1:10 dilution in PBS dispensed using a Thermo Multi-Drop Combi and Perkin Elmer JANUS Mini (Waltham, MA, USA). Samples were vortexed briefly and fluorescent intensity for each well was measured using a Biotek Synergy NEO plate reader (Winooski, VT, USA). Using the reference dilution curve, fluorescent measurements were converted back to volume (μl) of solution ingested.

7.15 Gonotrophic Cycle

Large groups of ~300 female mosquitoes/genotype were fed simultaneously on human volunteer arms and legs (typically at 1pm) then separated under cold anesthesia to isolate individuals who successfully fed. Approximately 15-25 fed mosquitoes were transferred to loaders 4 hours before testing at 0 (before blood), 24, 48, 72, 96, and 120 hours post-blood-meal in the uniport olfactometer. Access to egg laying substrate was provided after 72h and egg laying typically finished by ~110 hours. Mosquitoes were only tested for one time point and then discarded.

7.16 Southern Blot

Southern blots were performed using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland) with Protran BA83 Nitrocellulose membranes (Whatman) from 10 μg mixed mosquito genomic DNA digested individually with XhoI, SphI, NcoI, and HpaI (Figure 3.3, New England BioLabs,

Ipswich, MA, USA) or XhoI and XmaI (Figure 4.2). NPYLR1 and ECFP probes were synthesized by PCR amplification (see section 7.5 for primers) following Roche DIG-labeling protocols.

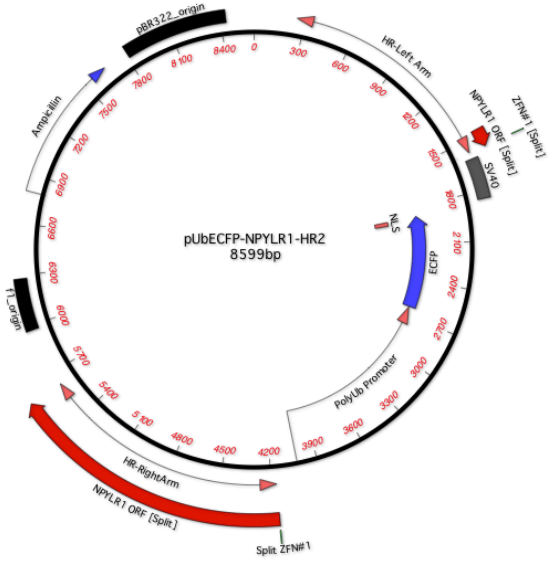
7.17 Qualitative PCR (qPCR)

RNA was purified from whole bodies of female mosquitoes before and 24, 48, and 72 hours after blood-feeding. Purified RNA was converted to cDNA and aliquoted at 250 ng/μl RNA equivalents for each qPCR reaction. Reactions were prepared as instructed by BioRad iQ SYBR Green SuperMix (Cat#170-8882) in BioRad iQ 96-well Plates (Cat#223-9441) covered with BioRad Microseal "B" Adhesive Seals (Cat#MSB-1001) to be run on a iQ5 iCylcer (BioRad). Primers were designed to meet the following criteria: 90-110% efficiency and an R^2 above 0.98 in control reactions and are listed in section 7.5. Each reaction was performed in triplicate with a total n=3.

7.18 Targeted Mutagenesis with Zinc-Finger Nucleases

Zinc-finger nucleases were synthesized in collaboration with Sigma-Aldrich Life Science. Genetic Services Inc. (Cambridge, MA, USA) injected purified NPYLR1 ZFN mRNA into ~3000 *A. aegypti* Orlando embryos (batches of 1000, 800, and 1200) at a concentration of 200 ng/μl plus a Homologous Recombination vector at 850 ng/μl using embryo preparation methods described previously (Jasinskiene et al., 2007)The homologous recombination vector contained 1319 and 1451 bp of homology to the left and right flanking sequence of the intended cut site (see primers in section 7.5). The marker contained 1391 bp of the *A.*

aegypti Poly-Ubiquitin promoter driving expression of ECFP, and in total was 2419 bp (Anderson et al., 2010).



36% of injected embryos hatched and were sexed as male and female prior to eclosion. 94% of the hatched individuals developed into adults and each injection batch were in-crossed after reaching sexual maturity (~2 days). Each of the three in-crossed groups were considered to contain independent ZFN events. Isolation occurred by aliquoting 3-day old F1 larva into 96-well plates (VWR International, Cat#29444-018) and screening for ECFP fluorescence on a Nikon SMZ1500 + Intensilight C-HGFI. 27, 6, and 18 larva were positive for fluorescence in each respective batch of an estimated 55,000 total screened.

NHEJ events were isolated a few months later from the same batch of HR injected mosquitoes. At that time, only eight F1 larva hatched (due to prolonged storage) and were outcrossed to wild-type *A. aegypti* Orlando. Genomic DNA was prepped for each of the eight individuals and NPYLR1 PCR amplicons were sequenced to confirm ZFN activity in two individuals. Individual PCR clones for

the two positive individuals were Sanger sequenced by Genewiz to confirm 4 and 8 bp deletions in each line.

7.19 Genotyping and Mendelian Inheritance Test

Genotyping of individual NHEJ mutants occurred in collaboration with Genewiz using a capillary gel electrophoresis DNA analyzer (ABI3730xl, Applied Biosystems, Carlsbad, CA, USA) of PCR amplicons from 6-FAM fluorescently labeled primers spanning over the NPYLR1 ZFN cut site. Data was analyzed using Peak Scanner software (Applied Biosystems). HR genotyping of individuals occurred by gel electrophoresis of PCR amplicons spanning over the inserted DNA. Mendelian inheritance experiments were also accomplished in collaboration with Genewiz using the capillary gel electrophoresis DNA analyzer. Refer to primer list in Section 7.5.

7.20 Statistics

Statistics were performed as indicated in each figure legend using Graph Pad PRISM (La Jolla, CA, USA) except for statistical analysis of qPCR results, which also used BioRad iQ5 software.

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