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The Genetic and Neural Basis of Sexual Dimorphism in Mosquito Behavior

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Only female mosquitoes of the species *Aedes aegypti* have evolved to hunt humans in order to feed on their blood. In striking contrast, male mosquitoes have no drive to seek hosts or drink their blood. Through the act of blood feeding, only female mosquitoes can transmit pathogens that cause deadly diseases like dengue, chikungunya, and Zika. The genetic and neural circuit basis of this striking sex difference in behavior, one that is of critical importance to global public health, remain poorly characterized.

Across the insects, sex-specific alternative splicing is known to control sex-specific morphological and behavioral traits. We took advantage of this knowledge to conduct an unbiased comparative transcriptomic screen, and identified a number of genes that are alternatively spliced between the brains of males and females across blood-feeding mosquito species. Two of these genes were specifically expressed in the nervous system. The first gene, *fruitless*, encodes a set of male-specific transcription factors well-known to be required for male-specific mating behavior in *Drosophila* and other insects. We used CRISPR-Cas9 to generate *fruitless* mutant *Aedes aegypti* mosquitoes, and found that these mutants are unable to mate, consistent with the ancestral function of this gene. Surprisingly, *fruitless* mutant male mosquitoes also gained a strong and specific attraction to a live human subject that was specifically

elicited by human odor, and not heat or other human cues. *fruitless* mutant males did not gain the ability to blood-feed, suggesting that it is specifically required to inhibit attraction to humans in male mosquitoes, and that other genes specify host seeking in both male and female mosquitoes. These results indicate that *fruitless*, a conserved gene, has gained a new and unexpected function over the course of evolution, acting to repress host-seeking behavior in male mosquitoes.

The second gene identified from this screen, the previously undescribed *11211*, is sex-specifically spliced into a predicted short female and long male protein isoform, which we showed localize to the nucleus in both sexes. With the notable exception of non-mosquito flies, *11211* orthologs are sex-specifically spliced in other insects such as bees and beetles. However in contrast to the mosquito, all other insects encode longer female and shorter male 11211 protein isoforms, suggesting that sex-specific splicing of this gene has evolved a new role in the mosquito. In mosquitoes, but not in other insects, *11211* is enriched in neurons in a part of the brain known to control feeding behavior. We found that these neurons show sexual dimorphism in their inputs, as female 11211-expressing neurons receive input from sensory neurons that detect the taste of blood. When we silenced the activity of these *11211*-expressing neurons, females were able to feed on nectar and retain attraction to humans, but strikingly lost the ability to initiate blood feeding. These results suggest that a rapidly-evolving novel gene marks a population of neurons that are specifically required for blood-feeding behavior.

In memory of my grandparents Krishna Kant and Padma Basrur.

For my parents Sandeep and Roopa Basrur.

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CHAPTER 1. Introduction

Across animals, males and females of the same species show striking differences in behavior (Figure 1.1). Although an astonishing diversity of sexually dimorphic behaviors exists across species, most insights into the genetic and neural basis of sex-specific behaviors have come from a limited set of model organisms (Auer and Benton, 2016; Manoli et al., 2013; Matthews and Vosshall, 2020). Which genes control sexual dimorphism in specialist species that have evolved novel behaviors? Do conserved genes control sexual dimorphism in species-specific behaviors, or have novel genes evolved to control new behaviors?

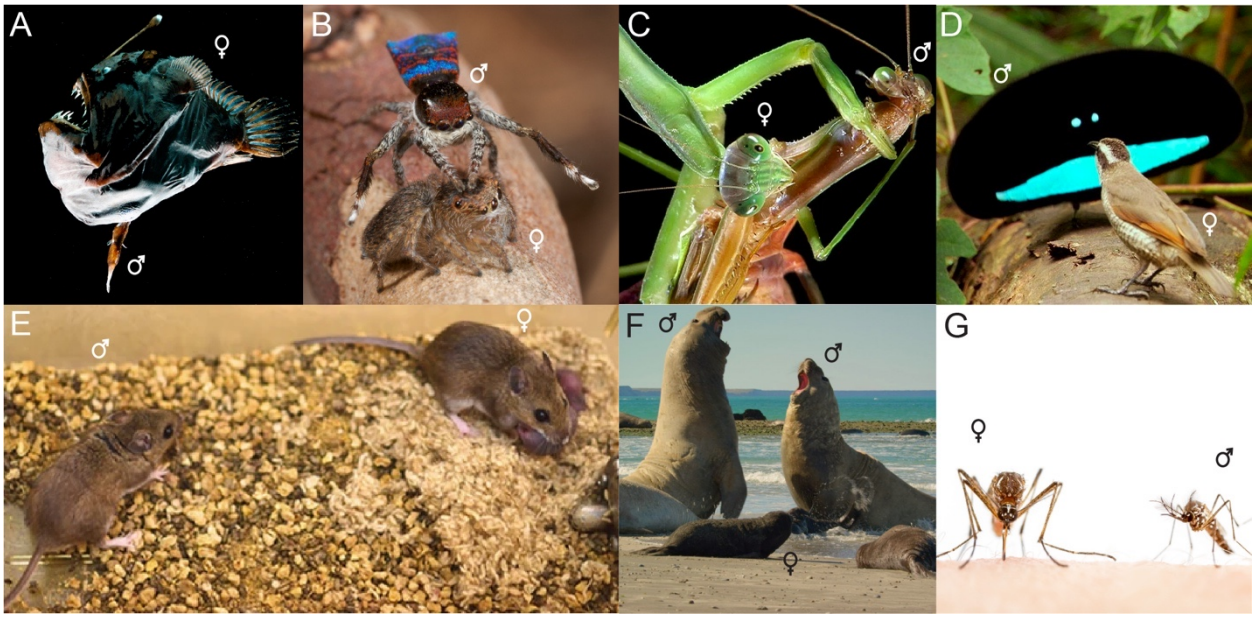

Figure 1.1 Sexual dimorphism in behavior across the animal kingdom

(A) Male *Melanocetus johnsonii* anglerfish latched on to the larger female. Photo credit: Edith A. Widder. (B) Male *Maratus mungaich* peacock spider courting female. Photo credit: Jurgen Otto. (C) Female *Tenodera sinensis* praying mantis cannibalizing male post-mating. Photo credit: Phil Hastings. (D) Male *Lophorina superba* superb bird of paradise courting female. Photo credit: Tim Laman/National Geographic (E) Male *Peromyscus maniculatus* deer mouse observing a female parenting. Photo from (Bendesky et al., 2017). (F) Male *Mirounga leonina* elephant seals fighting in front of females. Photo credit: Ruth Campbell. (G) Female and male mosquito *Aedes aegypti* on a human arm. Photo credit: Alex Wild.

Mosquitoes display striking sexually dimorphic mating and feeding behaviors. Only male mosquitoes initiate mating, and only females drink blood, which they require to develop their eggs (Bowen, 1991; DeGennaro et al., 2013; Dekker et al., 2005; Galun et al., 1963; Jové et al., 2020; Klowden, 1995; McMeniman et al., 2014; Zwiebel and Takken, 2004). Sexual dimorphism in blood feeding is one of the only instances of a completely sexually dimorphic feeding behavior since male mosquitoes never pierce skin or engorge on blood. While part of this dimorphism is enforced by sex-specific genitalia (Spielman, 1964) or feeding appendages (Jones and Pilitt, 1973), there is also a dramatic difference in the drive to hunt hosts between males and female mosquitoes (Bowen, 1991; Roth, 1948).

To blood-feed, females combine multiple behavioral modules (Bowen, 1991). Female *Aedes aegypti* mosquitoes take flight when exposed to carbon dioxide (Bowen, 1991; McMeniman *et al.*, 2014), and are attracted to human olfactory (DeGennaro *et al.*, 2013; Dekker *et al.*, 2005; Zwiebel and Takken, 2004), thermal, and visual cues (Liu and Vosshall, 2019; McMeniman *et al.*, 2014; van Breugel et al., 2015), and integrate at least two of these cues to orient toward and land on human skin. Engorging on blood is triggered by specific sensory cues tasted by the female (Galun *et al.*, 1963; Jové *et al.*, 2020).

It is not known which genes have evolved to control this unique sexually dimorphic and mosquito-specific feeding behavior. In the act of finding and biting humans, female mosquitoes act as vectors for pathogens that cause millions of cases of deadly diseases annually (Bartlow et al., 2019; Caragata et al., 2020). These pathogens include *Plasmodium*, the causative agent for malaria which is transmitted by *Anopheles* mosquitoes, and the arboviruses dengue, Zika, chikungunya, and yellow fever, which are transmitted by *Aedes aegypti*, the main mosquito species investigated in this work. Despite the devastating impact of these female-specific behaviors on global public health, there has been little to no investigation of the genetic and neural mechanisms that control sex-specific host-seeking and blood-feeding behaviors. Here, we hypothesize that comparing male and female gene expression will reveal novel regulators of female mosquito behavior, which in turn will suggest novel targets to intervene and control these behaviors.

1.1 Sexual dimorphism in behavior

There are many examples of sexual dimorphisms and sex differences in behavior across the animal kingdom (Kelley, 1988). Male Ceratiidae and Melanocetidae anglerfish seek out and latch onto the larger female fish, gaining nutrients from and providing sperm to the female (Pietsch, 1976) (Figure 1.1A). Female praying mantises are larger and more aggressive than males and routinely cannibalize their mates after copulation {Prokop, 2005 #184} (Figure 1.1C). While these are rare instances of

feeding-related sexual dimorphisms, most examples of sexually dimorphic behavior are related to courtship and sexual behaviors. Female peacock spiders are larger and more aggressive than males and routinely kill courting males if they are already mated, or unsatisfied with the courtship ritual (Girard et al., 2015) (Figure 1.1B). Males of most avian and mammalian species tend to court and initiate mating attempts with females, with some species having evolved elaborate courtship rituals. The male Paradisaeidae birds-of-paradise perform an elaborate courtship dance to seduce prospective female partners, contorting their bodies in forms resembling flowers, ballerinas, and smiling faces (Scholes, 2008) (Figure 1.1D). In addition to courtship behaviors, other social behaviors can show sex differences. Females of polygamous deer mouse species such as *Peromyscus maniculatus* tend to show more parenting behavior than males (Bendesky *et al.*, 2017) (Figure 1.1E). Many species show sex differences in aggressive behavior, such as the *Mirounga leonina* elephant seal where males fight for dominance and the ability to mate with females (McCann, 1981) (Figure 1.1F).

How are these sex-limited behaviors controlled by genes? Across animals, there are incredibly divergent mechanisms by which sex is specified (Blackmon et al., 2017). For example, in the mouse *Mus musculus* sex is determined by the action of the *SRY* gene on the Y-chromosome (Gubbay et al., 1990; Miyawaki et al., 2020; Morrish and Sinclair, 2002). In addition, many sex differences in mammalian behaviors are controlled by the action of steroid hormones such as estradiol and testosterone, which are released by the gonads and act through nuclear hormone receptors such as the estrogen receptor *Esr1* and the androgen receptor *Ar* (Arnold, 2009; Morris et al., 2004). In the fly

Drosophila melanogaster, the ratio of sex chromosomes to autosomes triggers female-specific alternative splicing of the gene *Sex-lethal*, which ultimately leads to the sex-specific splicing of the genes *fruitless*, and *doublesex* (Bopp et al., 2014). Sex-specific splicing is a conserved feature of sex determination across the insects, and will be explored in greater detail in Chapter 2. While the upstream mechanisms that determine sex are divergent across species, these mechanisms generally converge on genes that control sex-specific transcription. For example, *SRY, Esr1*, *fruitless,* and *doublesex* all encode transcription factors. Thus, by allowing for sex-specific gene expression, it is possible to encode distinct sex-specific behaviors within the brain (Williams and Carroll, 2009).

Many advances in understanding the genetics of sexually dimorphic behaviors have come from the study of *Drosophila melanogaster* fly courtship, where a male fly orients towards, taps, and follows a female fly, extending a wing to produce a courtship song before tasting, mounting, and copulating with her (Hall, 1994). Courtship comprises behavioral modules, which are simple discrete behaviors that must be combined to perform a complex behavior and are elicited by different sensory modalities. Courtship modules include orienting, which is driven by visual information (Ribeiro et al., 2018) and persistent following and singing, which are triggered by chemical cues on a female fly (Clowney et al., 2015) and guided by vision (Hindmarsh Sten et al., 2021; Ribeiro *et al.*, 2018). Many parts of the male-specific courtship ritual are specified by the action of the male-specific transcripts of the sex-specifically spliced *fruitless* gene, the functions of which will be elaborated in Chapter 3.

Mosquitoes display numerous sex-specific behaviors, including female-specific oviposition (Bentley and Day, 1989; Matthews et al., 2019) and male-specific mating initiation (Duvall et al., 2017; Hartberg, 1971). However, these behaviors are generally sex-specific across insects, including *Drosophila melanogaster*, a non-blood-feeding fly species. Blood feeding is female-specific, and mosquito-specific. Blood feeding is thought to have been gained once in the ancestor of mosquitoes (Peach and Gries, 2020), although intriguingly some mosquito species such as *Wyeomyia smithii* and the genus *Toxorhynchites* have independently lost the ability to blood-feed. Female mosquitoes drink blood to obtain protein and lipids to develop eggs and produce the next generation of mosquitoes. It is unclear how blood feeding is specified by genes and neural circuits.

In order to blood-feed, a female mosquito must first find and locate a host. This long-range behavior is termed host seeking. Mosquito species show a wide variation in their host-preferences. Some show limited preferences, feeding on any available vertebrate host. Others feed specifically on birds, or on amphibians, or fish, or even annelids (Reeves et al., 2018). The mosquito species of greatest public-health relevance have evolved a specific preference for feeding on human hosts. These include *the Anopheles gambiae* species complex, and the arboviral vector *Aedes aegypti*, which will be the primary focus of the remainder of this chapter.

1.2 Mosquito host-seeking behavior

Female mosquitoes are aroused and activated by carbon dioxide, which is exhaled in the breath of all vertebrates. After detecting carbon dioxide, mosquitoes begin to fly and persistently search for additional human host cues (Bowen, 1991; McMeniman *et al.*, 2014; Sorrells et al., 2021). Carbon dioxide is given off by most animals, and human-specific host seeking in *Aedes aegypti* is thus driven primarily by olfactory cues (DeGennaro *et al.*, 2013; McBride, 2016; McBride et al., 2014; Zhao et al., 2020b; Zwiebel and Takken, 2004). Humans possess a distinct olfactory signature, which is thought to be a result of the interaction between diet, human skin metabolites, and the skin microbiota (Verhulst et al., 2010). While the specific volatile cues given off by human skin are yet to be causally tested, it is thought that long-chain aldehydes are important (Zhao *et al.*, 2020b). Together, carbon dioxide and human skin odor drive robust female host-seeking behavior.

Mosquitoes detect carbon dioxide and human odor through their antennae and maxillary palps, which are pairs of sensory structures on their heads (Figure 1.2). The antennae and maxillary palps contain fine hair-like structures called sensilla. These olfactory sensilla contain fine pores through which odors can diffuse, and bind to receptors on the dendritic terminals of olfactory sensory neurons. Carbon dioxide is detected by a complex consisting of the Gustatory Receptors (GRs) Gr1, Gr2, and Gr3 (Kumar et al., 2020; McMeniman *et al.*, 2014), which are expressed in a single type of neuron in the maxillary palp. These cells are present in both male and female maxillary

palps, and carbon dioxide activates both male and female mosquitoes (Matthews et al., 2016; McMeniman *et al.*, 2014), although only female mosquitoes show persistent arousal after *Gr3* neuron activation (Sorrells *et al.*, 2021). Other odors are detected by the Odorant Receptors (ORs) and Ionotropic Receptors (IRs), which are large gene families encoding odorant-gated ion channels. Over 150 olfactory receptors are expressed in the antennae and maxillary palps (Matthews *et al.*, 2016; Younger et al., 2020), allowing for the detection of a large variety of human odors. Functional ORs consist of a complex between the ligand-specific OR and a co-receptor Orco (DeGennaro *et al.*, 2013; Larsson et al., 2004). Functional IRs are complexes of one or more ligand-specific IRs and one or more co-receptors such as Ir25a, Ir76b, and Ir8a (Benton et al., 2009). ORs and IRs detect distinct classes of chemicals, with ORs generally tuned to aldehydes, ketones, and esters, and IRs tuned to acids and amines (Benton *et al.*, 2009; Silbering et al., 2011). Together, these receptors and the cells in which they are expressed detect human volatiles, and enable robust host-seeking behavior.

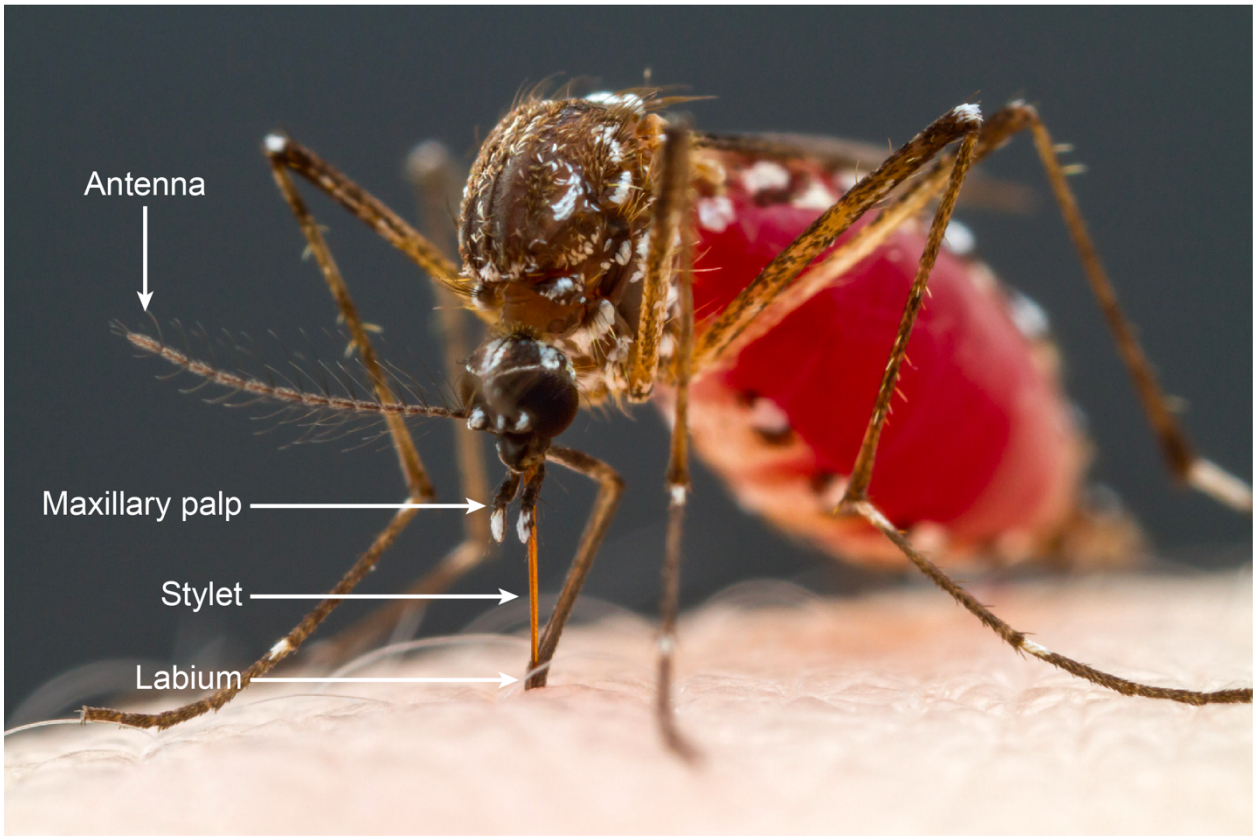

Figure 1.2 Peripheral sensory appendages of the female mosquito

Female *Aedes aegypti* mosquito feeding on human skin, with sensory tissues labeled. Photo credit: Alex Wild.

Mosquitoes display sexual dimorphism in the morphology of their antennae and maxillary palps. Males have long maxillary palps and fluffy antennae, with only 2 of the 13 segments containing olfactory sensilla. Females have short maxillary palps, and detect odors with all 13 antennal segments. In addition to this sexual dimorphism in antennal morphology, male and female mosquitoes show sex differences in host-seeking behavior. While still controversial, male mosquitoes are thought to show some attraction to humans over long distances, presumably in order to locate a conspecific female mate (Amos et al., 2021; Hartberg, 1971). However, in laboratory assays which measure host seeking over short distances, males show limited olfactory attraction to

humans in contrast to females (Basrur et al., 2020). This lack of short-range attraction could be due to the inability to detect humans at the sensory level, or could be due to suppression of host seeking in the central brain. We explore the role of host seeking in males in further detail in Chapter 3.

1.3 Mosquito blood-feeding behavior

After finding and locating a human host, a female mosquito must determine whether to pierce their skin and engorge on blood. Female mosquitoes retract their outer proboscis, known as the labium, and use their inner needle-like appendage known as the stylet to probe and pierce skin (Jové *et al.*, 2020; Klowden, 1995). Only female mosquitoes can retract their labium and pierce skin with their stylet, which is needle-shaped in females and blunt in males (Jones and Pilitt, 1973; Jové *et al.*, 2020; Wahid et al., 2007). Despite this morphological difference, even when blood is provided in a form where both males and females can drink, it is not palatable to males (Basrur *et al.*, 2020).

Landing on human skin is primarily driven by odor and heat (Bowen, 1991; Corfas and Vosshall, 2015; Liu and Vosshall, 2019; McMeniman *et al.*, 2014). Since all homeotherms such as birds and mammals maintain a body temperature above that of the environment, the integration of heat and odor is a sign of life, suggesting to the mosquito that she can safely pierce skin to taste blood. After detecting heat, a female mosquito begins to probe by moving her head in a distinctive motion, attempting to find

an appropriate site to retract her labium and pierce skin (Hol et al., 2020; Jones and Pilitt, 1973; McMeniman *et al.*, 2014; Sorrells *et al.*, 2021). This probing behavior is eliminated in *Ir25a* mutant mosquitoes that cannot detect heat and other human cues (Takeshi Morita, unpublished). In addition, males do not persist on human skin or heated objects (McMeniman *et al.*, 2014), suggesting that in addition to the morphological dimorphism, there are motivational and behavioral differences driving female-specific blood feeding.

Once a female mosquito has pierced skin, her stylet searches for a blood capillary from which she can pump blood into her midgut (Choumet et al., 2012). To initiate this pumping, a female must detect sensory cues in the blood. The female stylet contains chemosensory neurons which project to sensilla at the tip, and are poised to detect cues in the blood (Jové *et al.*, 2020). The most salient cues are adenosine triphosphate, sodium chloride, and sodium bicarbonate. These three cues together are sufficient to trigger robust pumping and engorgement on an artificial meal (Galun *et al.*, 1963; Jové *et al.*, 2020). Only female stylet neurons contain chemosensory pores, suggesting that only females can detect the taste of blood. However, the ability of the male stylet to detect chemosensory cues present in blood remains unexplored.

Female mosquito blood-feeding behavior is organized into discrete modules that can be performed independently, but which must all be combined in the appropriate sequence in order to successfully blood-feed. These modules are triggered by distinct, partly overlapping sets of sensory cues, such as odor that drives host seeking, heat and

odor that trigger probing, and the taste of blood that promotes engorgement. The modularity of these behaviors suggests that there must be discrete neural circuits within the mosquito brain that control these distinct, but overlapping behaviors. However, this hypothesis is yet to be formally tested. While the sensory structures that detect host cues in the periphery have been relatively well described, the circuits within the central mosquito brain that integrate these cues to trigger behavior are unknown. Olfactory sensory neurons send their axons to the antennal lobe, while gustatory neurons project to the subesophageal zone (Jové *et al.*, 2020; Younger *et al.*, 2020). Based on homology with *Drosophila*, it is thought that olfactory cues are further processed in the lateral horn and mushroom body, while gustatory cues are integrated locally within the subesophageal zone (McBride, 2016). However, to date, there have been no described neural circuits controlling either olfactory-driven host seeking or gustatory-driven blood feeding.

1.4 Concluding remarks

Here we show that host seeking and blood feeding are controlled by distinct genes and neural circuits within the mosquito brain. We characterized sex-biased gene expression and sex-specific alternative splicing across three blood-feeding mosquito species. We focused on sex-specific alternative splicing and identified two genes that are sex-specifically spliced in the brains of blood-feeding mosquitoes. The first, *fruitless*, encodes a male-specific transcription factor that is required for mating behavior across insects. The second, *11211*, is a novel gene that is sex-specifically spliced across

insects, but displays extreme divergence in the structure and sequence of the sex-specific proteins. In the mosquito, *11211* encodes a short female and long male protein isoform, both of which we show localize to the nucleus. Using CRISPR-Cas9, we generated mutant strains of *fruitless* and a driver line that enabled transgene expression within neurons that express *11211.* We found that *fruitless* mutant male mosquitoes did not blood-feed, but gained strong attraction to the smell of a human, unlike their male wild-type counterparts that normally never show strong attraction at close distances. This result suggests that *fruitless* has gained a new role in male mosquitoes where it inhibits host seeking, and that *fruitless*-expressing circuits in the mosquito brain regulate host-seeking behavior. We then focused on *11211*-expressing circuits, and found that in mosquitoes, but not other insects, there is an enrichment of 11211-expressing cells within the subesophageal zone, a region of the insect brain known to control feeding behavior. In *Aedes aegypti*, female *11211*+ neurons show dense projections that are poised to receive input from stylet neurons that detect the taste of blood. When we silence the activity of these neurons, host-seeking and nectar-feeding behaviors remain intact, but we significantly disrupt the ability of female mosquitoes to initiate blood feeding. Together, these results suggest that distinct neural circuits control host seeking and blood feeding in a modular fashion within the female mosquito brain, and that the action of distinct genes is required to specify the function of these host-seeking and blood-feeding controlling neurons.

CHAPTER 2. The evolution of sex-specific splicing in the mosquito brain

How are sex-specific behaviors encoded by the genome? Even closely-related species can show dramatically different sex-determination mechanisms, and these traits are some of the fastest evolving traits across the animal kingdom (Bachtrog et al., 2014). In this chapter, we focus on insects, and review the evidence supporting sex-specific splicing as a conserved mechanism controlling sex-specific traits across the insects. We then focus on the mosquitoes, and perform comparative transcriptomics to identify sex-specifically spliced transcripts that are unique to the mosquitoes. We also perform differential expression analysis to identify sex-biased transcripts that may also be of interest in controlling sex-specific behaviors. This chapter lays the foundation for identifying the sex-specifically spliced candidate genes *fruitless* and *11211*, which will then be functionally characterized in later chapters.

2.1 Sex-specific splicing is a conserved mechanism controlling sexual dimorphism across the insects

How is sex determined in the insects? The vast majority of insects reproduce sexually, are gonochoristic (have two distinct sexes that do not change over an animal's lifetime), and show genotypic sex determination (Blackmon *et al.*, 2017). This is in contrast to other modes of sex determination, such as asexual reproduction seen in many unicellular organisms (de Meeus et al., 2007), are hermaphroditic as in many molluscs (Jarne and Auld, 2006), or have environmental sex determination as in the

water flea *Daphnia magna* (Kato et al., 2011). All these sex determination systems are relatively common outside the insects (Bachtrog *et al.*, 2014; Blackmon *et al.*, 2017). Within the insects, there is a stunning diversity in the mechanism by which genotype determines sex (Figure 2.1). The most common sex-determination system is heterogamety, where one sex is heterozygous for the sex-determination locus, while the other is homozygous. Male heterogamety leads to the well-known X and Y chromosomes, where a male has an XY karyotype and a female has an XX karyotype, as is seen in *Drosophila melanogaster* (Cline, 1993). Female heterogamety is seen in the Lepidoptera, where females are ZW and males are ZZ (Traut et al., 2007). The mechanism by which genotype leads to sex determination can vary within heterogametic systems. For example, *Drosophila melanogaster* uses the ratio of X to autosomes to determine sex, where females have a ratio of 1.0 and males have a ratio of 0.5 (Bridges, 1916; Cline, 1993). The silkworm *Bombyx mori* has a dominant gene, *fem* on the female W chromosome, which encodes a piRNA that silences the *masc* gene encoded by the Z chromosome (Kiuchi et al., 2014). The medfly *Ceratitis capitata* has a dominant sex-determining gene *MoY* on the Y-chromosome (Meccariello et al., 2019). Moving beyond heterogamety, another commonly found sex determination system is haplodiploidy, where females are diploid and males are haploid, most common in the order *Hymenoptera* (Heimpel and de Boer, 2008). In the honeybee *Apis mellifera,* through complement sex determination (csd), animals carry a large number of *csd* alleles (Beye et al., 2003). Female *Apis mellifera* eggs are fertilized and heterozygous at the *csd* locus, while males are either hemizygous at *csd* by developing

from unfertilized haploid eggs, or are rare homozygotes at *csd*. Therefore, there is a huge diversity in sex-determination systems across the insects.

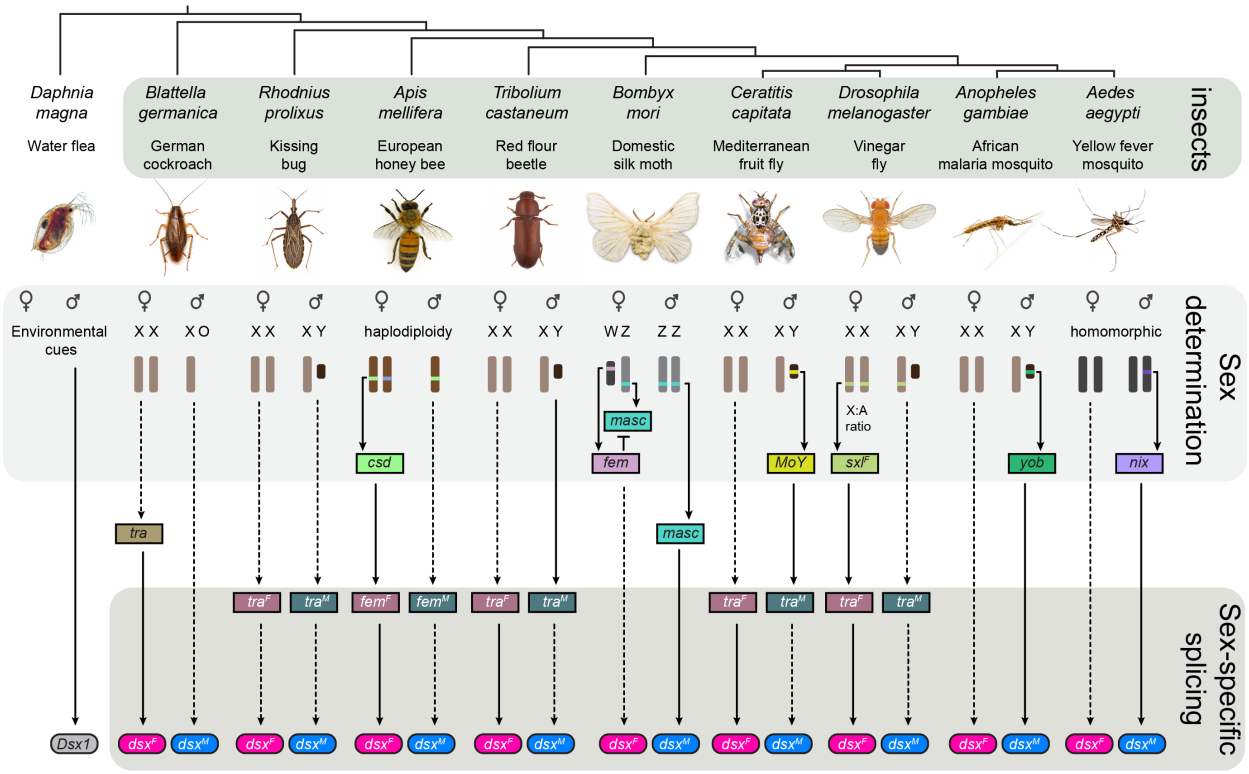

Figure 2.1 Sex-specific splicing is conserved across the insects

Phylogeny of insect species, with mechanism of sex-determination, sex-determining genes (if any), and mechanism by which they lead to sex-specific splicing of *doublesex*.

The greatest insight into the mechanism by which karyotype or genotype lead to sex determination comes from *Drosophila melanogaster*. As described above, the presence of two X chromosomes triggers female sex determination through the embryonic transcription of the gene *Sex-lethal* (*Sxl*) (Cline, 1993; Salz, 2011). The third exon of *Sxl* contains a premature stop codon, and sex-specific splicing is required for normal sexual development. Female *Sxl* transcripts lack the third exon and produce a functional Sxl^F protein, while male transcripts include the exon and produce truncated Sxl^M protein. Sxl^F

is able to regulate its own splicing in a positive feedback loop, thereby maintaining the exon-skipping event in females, while by default males include the exon (Bell et al., 1991; Bell et al., 1988; Salz, 2011). Sxl^F regulates the sex-specific splicing of other genes, the most notable of which is *transformer* (*tra*). Just like *Sxl*, *tra* contains an exon with a premature termination codon which is included by default. Sxl^F blocks usage of the male splice site, and forces splicing to a downstream splice site which does not include the stop codon. Therefore, females have a functional Tra^F protein, while male Tra^M is non-functional (Cline and Meyer, 1996; Sosnowski et al., 1989). Tra^F, acting together with Tra-2, regulates the sex-specific splicing of downstream genes, the most notable of which is *doublesex* (*dsx*) (Burtis and Baker, 1989; Hoshijima et al., 1991). Sex-specific splicing of *doublesex* leads to the production of either Dsx^F or Dsx^M proteins, both of which encode full-length transcription factors (Clough et al., 2014). Dsx proteins are members of the doublesex/mab-3 related (*dmrt*) family of transcription factors, which have conserved roles in sex determination in diverse species from mammals to insects (Kopp, 2012). Sex-specific Dsx proteins are expressed within specific tissues to control sex-specific differentiation in traits such as morphology and behavior. Expression of *doublesex* within subsets of cells in the gonad (Hempel and Oliver, 2007) and the nervous system (Rideout et al., 2010; Robinett et al., 2010) determines sex-specific gonadal differentiation and aspects of sex-specific behaviors, all through sex-specific transcription in a cell-type specific manner. Therefore, Sxl^F and Tra^F act to control female-specific RNA splicing of key effector genes such as *doublesex.*

What is the mechanism of sex determination in other insect species? As we described above, there are incredibly diverse sex-determination signals that initially determine sex. Remarkably, across the insects, these upstream signals converge on sex-specific splicing of *doublesex* (Hopkins and Kopp, 2021) (Figure 2.1). In many cases, the mechanisms linking the sex-determination signal to splicing are unknown, but sex-specific splicing of *doublesex* is observed, as in the cockroach *Blatella germanica*, kissing bug *Rhodnius prolixus*, and flour beetle *Tribolium castaneum* (Shukla and Palli, 2012a; Wexler et al., 2019). In *Rhodnius prolixus*, the honey bee *Apis mellifera*, *Tribolium castaneum*, and the medfly *Ceratitis capitata*, there are homologues of *tra* which are also sex-specifically spliced into functional Tra^F proteins, or Fem^F in the case of the honey bee (Hasselmann et al., 2008; Pane et al., 2002; Shukla and Palli, 2012b; Wexler *et al.*, 2019). In each of these species, the Tra^F homolog is thought to control the female-specific splicing of *doublesex*. Even in species that lack identifiable *tra* homologs, such as the silkmoth *Bombyx mori*, *doublesex* is sex-specifically spliced (Kiuchi *et al.*, 2014). Despite the divergence in sex-determination systems, sex-specific splicing of effector genes such as *doublesex* is incredibly conserved across insects that shared a common ancestor over 500 million years ago (Figure 2.1). This feature has led to an 'hourglass' model where sex-specific splicing is a conserved feature across insect sex-determination systems, while the upstream effectors and downstream targets are divergent across species (Blackmon *et al.*, 2017; Bopp *et al.*, 2014; Geuverink and Beukeboom, 2014). While *doublesex*, and to a lesser extent *tra,* are the best characterized and most conserved of the sex-specifically spliced genes, there are other examples such as *fruitless*, which we will describe in greater detail in Chapter 3.

What about the mosquito? In both *Anopheles gambiae* and *Aedes aegypti*, sex determination is male-heterogametic. *Anopheles gambiae* males are XY, and *Aedes aegypti* males carry a Y-chromosome like region called the M-locus on the homomorphic first chromosome. In both species, sex is determined by a male-dominant factor that is carried by the male-specific chromosome or locus. In *Anopheles gambiae*, this gene is named *Yob*, while in *Aedes aegypti*, it is named *Nix* (Hall et al., 2015; Krzywinska et al., 2016). These genes are unrelated and rapidly evolving, adding further evidence to the divergence of sex-determination mechanisms across insects. However, in both mosquito species, *doublesex* is sex-specifically spliced (Salvemini et al., 2011; Scali et al., 2005), and the male-determining genes are required for male-specific splicing of *doublesex* (Hall *et al.*, 2015; Krzywinska *et al.*, 2016), though the exact mechanisms remain undiscovered. Therefore, as in the other insects, sex-specific splicing is likely a key mechanism by which sexual dimorphism is controlled in the mosquitoes.

2.2 Sex-specific splicing in the mosquito brain

We have described how sex-specific splicing control sexual dimorphism in traits such as morphology and behavior across the insects. Most research has focused on a limited number of genes, namely *tra, doublesex,* and *fruitless,* which are all known to control sexual dimorphism in behavior across the insects. Are there undiscovered genes controlling sex-specific mosquito behaviors? Since sex-specific alternative splicing is a

conserved feature of insect sex-determination, we reasoned that any novel genes controlling sex-specific behaviors were likely to also be sex-specifically alternatively spliced. It would be possible to identify any novel sex-specifically spliced genes through the analysis of transcriptomic data.

We collected five different mosquito species, the blood-feeding *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*, and the non-blood-feeding *Wyeomyia smithii*, and *Toxorhynchites amboinensis*. We dissected brains from adult males and females of each species, extracted RNA, and prepared libraries for next-generation sequencing. Since only the blood-feeding mosquitoes have high-quality genomes, we focused our splicing analysis on these species. We also used data from *Drosophila melanogaster* as a non-blood-feeding outgroup comparison (Khodursky et al., 2020).

For each species, we aligned RNA-seq reads to the genome, and analyzed differential exon usage between the sexes (Anders et al., 2012). Using standard thresholds for fold change (fold-change>1.5) and significance ($p<0.1$), we identified a number of genes which showed sex-biased exon usage for each species, ranging from 50 in *Aedes aegypti* to 66 in *Drosophila melanogaster* (Figure 2.2A). We then asked whether the sex-specific splicing was conserved across species (Figure 2.2B). Only two genes were sex-specifically spliced across all four species. These genes were *fruitless* and *doublesex*, which as described above, are well-known to be sex-specifically spliced across the insects. Which sex-biased splicing events are species-specific? We identified multiple events specific to *Aedes aegypti*, including sex-specific splicing of the

neuropeptide *corazonin,* and the angiotensin-converting enzyme (*Ace*) gene. In *Drosophila melanogaster*, 2 of the 60 genes were the canonical sex-determining *Sxl* gene and the dosage compensation gene *msl-2* (Bell *et al.*, 1988; Merendino et al., 1999), neither of which are known to be sex-specifically spliced in non-Drosophilid species. The identification of these known genes thereby serves as a validation of this method, suggesting it is likely to predict important new sex-specifically spliced genes in the mosquito.

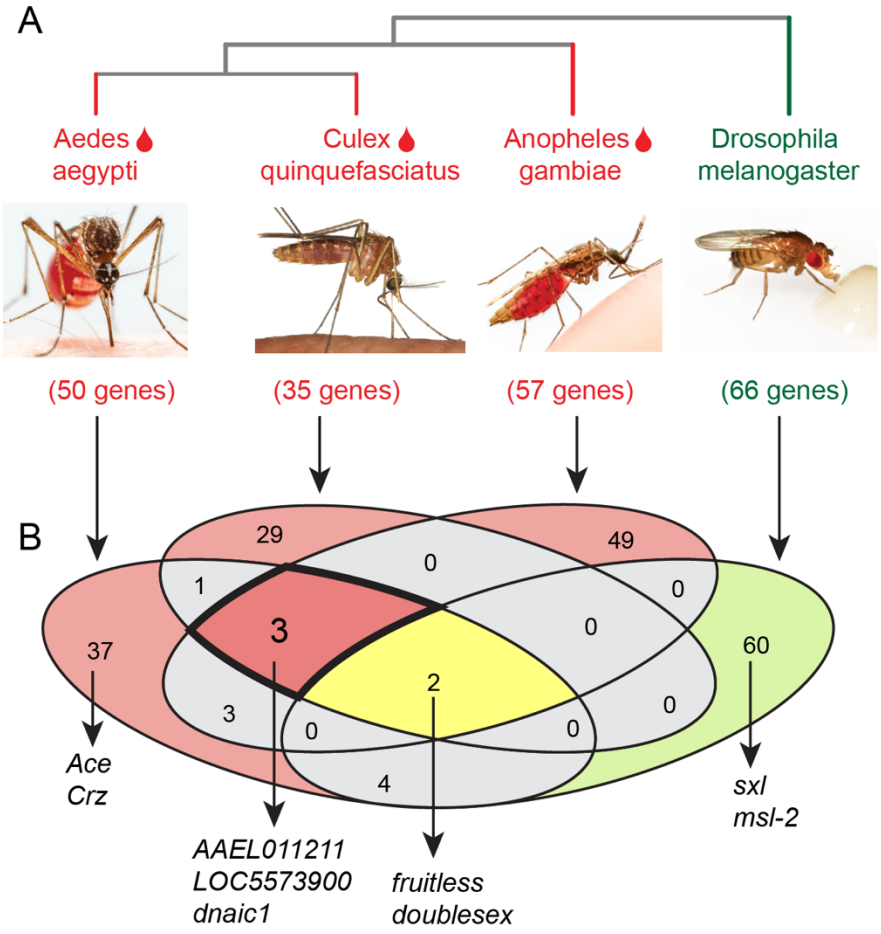

Figure 2.2 Sex-specific splicing of genes in the brain across mosquito species
(A) Phylogeny of species for which differential exon usage analysis from brain RNA-seq data was conducted, listing the number of sex-specifically spliced genes for each species. (B) Venn diagram showing the overlap of sex-specifically spliced genes between species, highlighting genes sex-specifically spliced only in *Aedes aegypti*, genes sex-specifically spliced in all mosquitoes only, genes sex-specifically spliced across all species, and genes sex-specifically spliced only in *Drosophila melanogaster*.

We then examined the genes which were sex-specifically spliced across multiple mosquito species. Only three genes were sex-specifically spliced across all mosquito species, but not *Drosophila melanogaster*. These genes were *AAEL011211, LOC5573900,* and *Dnaic1*. *AAEL011211* (*11211*) is a novel gene that is well conserved within the mosquitoes, but shows poor conservation outside the mosquitoes. Initially, a homolog could not be identified in *Drosophila melanogaster*, which is why this splicing was annotated as mosquito-specific. However, as will be described in further detail in Chapter 4, we eventually identified a *Drosophila melanogaster 11211* homolog, and showed that it was indeed not sex-specifically spliced. *LOC5573900* is annotated as the 'intracellular protein transport protein uso1', but is of unknown function although it is relatively well-conserved across the insects. The third gene *Dnaic1* encodes the dynein cytoplasmic 1 intermediate chain 2 protein, and is conserved across Metazoans.

What is the effect of sex-specific splicing on sex-specific transcripts and sex-specific proteins? There were three main categories of splicing events (Figure 2.3). The largest category was sex-specific coding exons, which accounted for 104 of the 188 genes. This category of events includes exons within the coding region of the gene, as well as alternative promoters. Here, the inclusion of a sex-specific exon would lead to an additional domain in the sex-specific protein. The next-largest category, accounting for

79 of the 188 genes, was genes with sex-specific splicing in either the 5’ or 3’ untranslated regions of the transcript. These splicing events had no predicted effect on the sex-specific protein. More rarely, there were sex-specific exons which included sex-specific termination codons, thereby truncating the encoded protein in one sex. This mode of splicing is known to be conserved and important across the insects, and we therefore focused on them. Within the sex-specific terminations, there are two categories of splicing events: destructive or constructive. Destructive splices include termination codons early in the coding region which severely truncate the protein in one sex, such that it is likely non-functional. An example of such a gene is *fruitless*. Constructive splices include termination codons later in the coding region, such that both the sex-specific proteins are functional, as is the case with *doublesex*. *11211* was the only other gene that had a putative constructive splice.

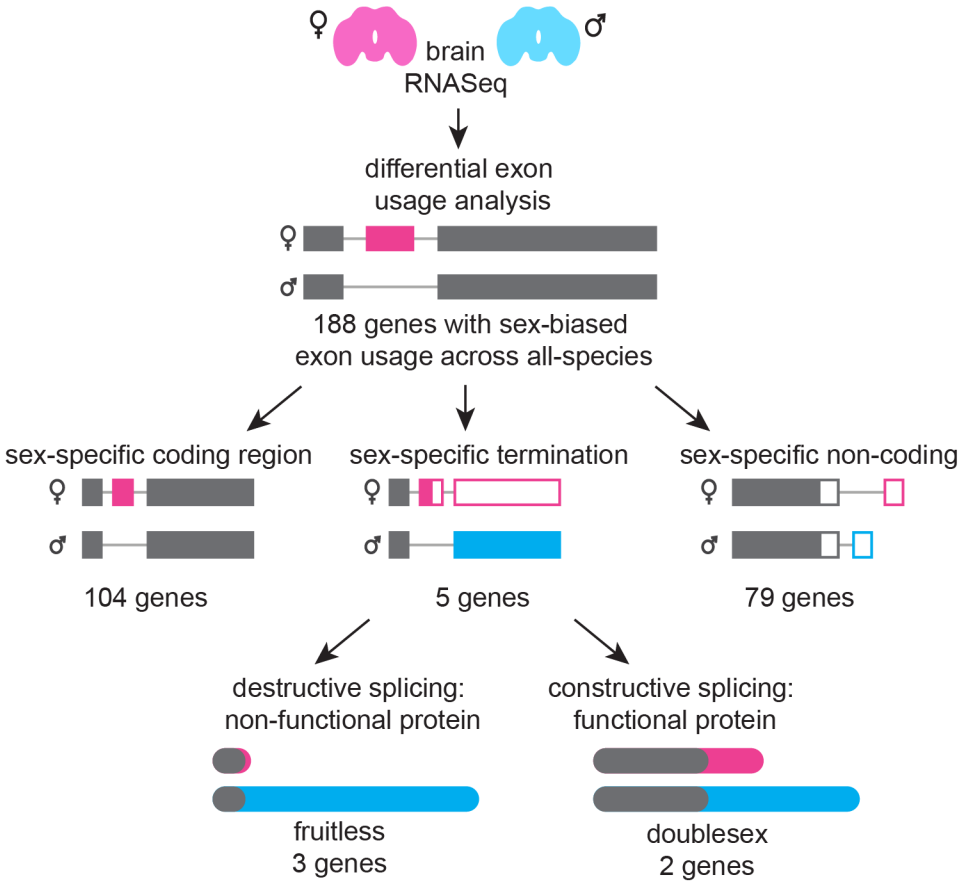

Figure 2.3 Categories of sex-specific splicing events

Flowchart showing the analysis pipeline, with representative schematics of sex-specific splicing events in each category, with the number of genes listed. RNA and protein diagrams are purely schematics and do not correspond to a given locus.

Finally, in which tissues are the sex-specifically spliced genes expressed? We examined the different categories of genes we described above in *Aedes aegypti*, and analyzed the expression of these genes across female and male tissues using published RNA-seq data (Matthews *et al.*, 2016). Intriguingly, genes with sex-biased coding exons are enriched in the antenna, expressed at moderate levels in the legs, and relatively lower expressed in the brain. (Figure 2.4). Genes with non-coding exons were relatively enriched in the brain, and uniformly expressed across the other tissues

with the notable exception of the female ovary. The only gene with destructive splicing, *fruitless*, was enriched in the brain, antenna, and ovary. Focusing on genes with constructive splicing, *11211* and *doublesex*, these genes were most strongly enriched within the brain. This enriched expression within the nervous system suggests a role of the putative constructively-spliced genes in controlling behavior.

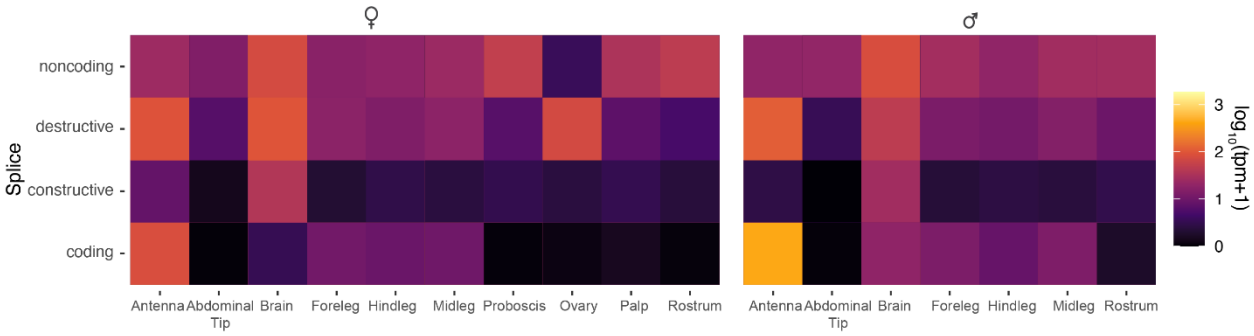

Figure 2.4 Expression of different categories of sex-specifically spliced genes across mosquito tissues

Heatmap showing the mean level of expression of different categories of sex-specifically spliced genes across mosquitoes. Scale in transcripts per million (TPM) (log scale).

In summary, we have identified a number of genes that are sex-specifically spliced between the male and female mosquito brain. These genes, particularly constructively spliced genes, and sex-specifically spliced genes that are conserved across the mosquitoes, are promising candidates for controlling sexual dimorphism in mosquito behavior.

2.3 Sex-biased gene expression in the mosquito brain

We have described how sex-specific splicing is conserved across the insects. In addition to splicing, another mechanism by which sexual dimorphism is controlled is through sex-biased gene expression. To completely capture any potential candidates controlling sex-specific mosquito behavior, we analyzed differential gene expression between male and female brains across the four species (Figure 2.5). Using standard cutoffs for the level of fold change (log2-fold-change>1.0) and significance (p-adj<0.1), we identified a number of sex-biased genes across species, ranging from 91 in *Drosophila melanogaster* to 214 in *Culex quinquefasciatus*. Surprisingly, no genes were sex-specifically biased across all four species. 16 genes showed sex-biased expression across the mosquitoes. Some of these genes appeared in the sex-specific splicing analysis, such as *uso1*/*LOC5573900* and *dnaic1*, suggesting that these predicted splicing events are either in addition to, or a spurious result of sex-biased expression. Many of the other sex-biased genes showed functions related to the cytoskeleton, such as actin, myosin, and dynein chains. These genes may be the result of sex-biased gene expression within the brain, or the result of contamination by muscle tissue attached to the brain, as is possible given that female mosquitoes possess enlarged head muscles to pump blood (Pappas, 1988).

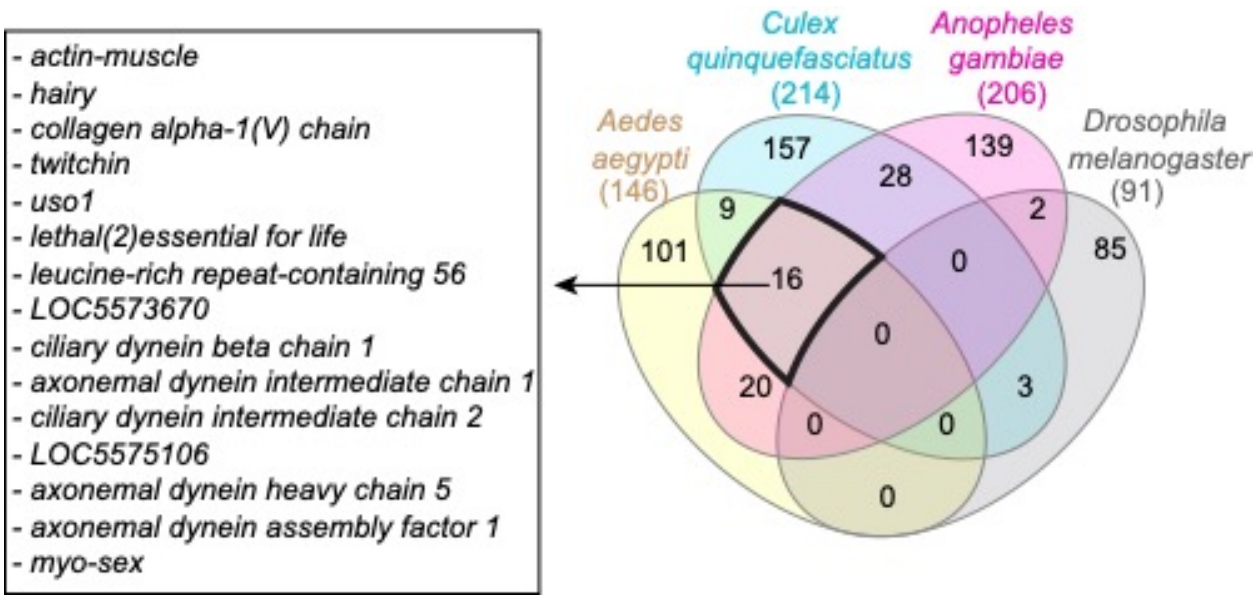

Figure 2.5 Sex-biased gene expression across mosquitoes

Venn diagram showing the intersection of sex-biased genes across three mosquito species and *Drosophila melanogaster*, listing the 16 genes that are sex-biased only in the mosquitoes.

Several of the sex-biased genes showed interesting patterns of expression and regulation. One of these genes was *hairy*, a well-known transcriptional repressor. The *hairy* mutant was originally described about a century ago, and it has since been well-described to bind to DNA and act as a repressor (Barolo and Levine, 1997; Mohr, 1922). *hairy* was between 1-3 fold upregulated in the brains of females of all three mosquito species, but not *Drosophila melanogaster* (Figure 2.6). This suggested a mosquito-specific role for the gene. We then explored *hairy* expression within *Aedes aegypti* using tissue-specific and state-specific RNA-seq data (Matthews *et al.*, 2016). *Hairy* was between upregulated in females across all tissues (Figure 2.7A). It showed notably high levels of expression in sensory organs, suggesting a role in controlling the morphology or function of female-specific sensory appendages. This potential role

would be consistent with the function of *hairy* in *Drosophila melanogaster*, where it acts to repress sensory-organ function (Ohsako et al., 1994). Mosquitoes show greater sexual dimorphism in the morphology of their sensory structures than *Drosophila melanogaster*. It is possible that *hairy* function has been co-opted in the mosquito to repress male sensory organ formation in the female.

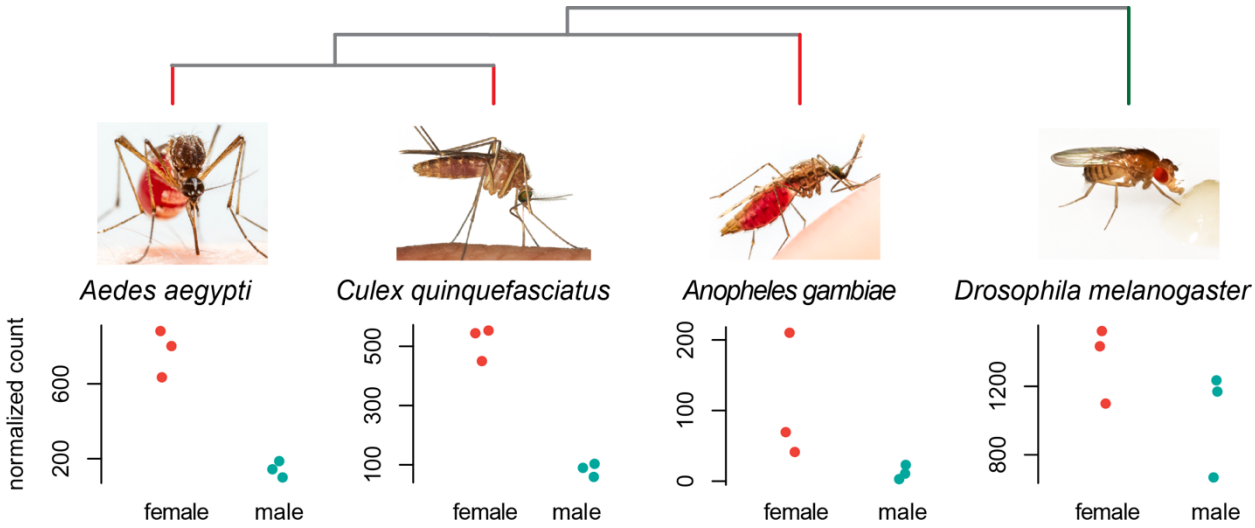

Figure 2.6 Expression of *hairy* in male and female brains of the indicated species

Normalized counts of expression levels of *hairy* in male and female brains.

In addition to the sex-biased gene expression, *hairy* was also temporally regulated within the brain. After a blood-meal, expression of *hairy* dropped by 2-3 fold by 2 days after a blood-meal, before recovering back to baseline levels by 4 days after a meal (Figure 2.7B). Blood feeding suppresses mosquito attraction to humans for around 3 days, and attraction recovers 4 days after a blood-meal when mosquitoes have laid their eggs (Duvall et al., 2019; Klowden, 1994). The expression levels of *hairy* after a blood-meal correlated with levels of human attraction, as does the sex-biased

expression. While we do not investigate *hairy* function further in this thesis, it is an intriguing candidate gene for controlling sex-differences in mosquito behavior.

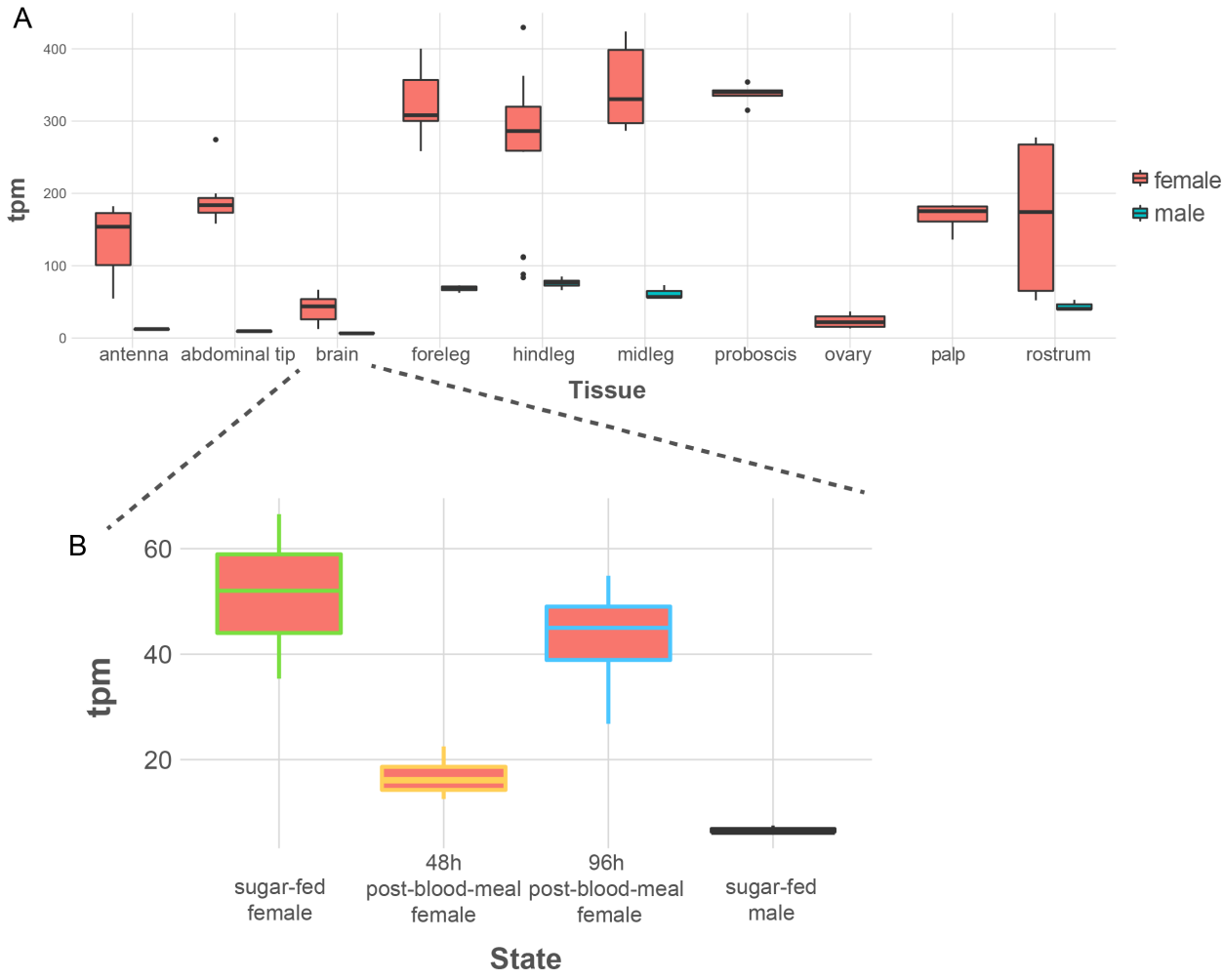

Figure 2.7 Expression of *hairy* across *Aedes aegypti* tissues and after a blood-meal.

(A) Levels of expression of *hairy* in TPM for different sugar-fed *Aedes aegypti* tissues. (B) Levels of expression of *hairy* in TPM in the *Aedes aegypti* brain after different blood-feeding states.

We previously mentioned that we carried out RNA-seq on the brains of males and females of the non-blood-feeding mosquito species *Wyeomyia smithii* and

Toxorhynchites amboinensis. Both these species have independently lost the ability to blood feed, and the rationale behind sequencing them was to compare sex-biased gene expression and splicing to identify genes that might control blood feeding. However, these species lack genome assemblies, making any thorough analysis of splicing or differential expression complicated. Once these genome assemblies are completed, these analyses will be promising routes to identify additional genes involved in sex-specific behaviors.

Instead of genome-based comparisons, we carried out a preliminary analysis by assembling *de novo* brain transcriptomes for males and females of each species (Grabherr et al., 2011). We then quantified transcript abundances (Patro et al., 2017) and carried out differential transcript expression analysis between the sexes for each species (Love et al., 2014). We then identified homologous transcripts between species (Altschul et al., 1997), and examined which transcripts showed sex-specific expression across mosquitoes (Figure 2.8A). Each step of this analysis pipeline, and particularly the transcript quantification and homolog identification, has greater levels of imprecision than genome-based differential expression, increasing the likelihood of spurious results. Therefore, this should be viewed as a preliminary analysis that can be repeated after the release of high-quality genomes for all species. Nevertheless, one interesting pattern that emerged was that all the transcripts that were sex-biased across all mosquito species were integrases or reverse-transcriptases of putative retroviral origin. The *Aedes aegypti* transcripts mapped to the genome, suggesting they are true genes, and not viral or bacterial contamination. Most of these transcripts showed male-enriched expression, with occasional tissue-specific expression as is seen for *LOC110674818* (Figure 2.8B).

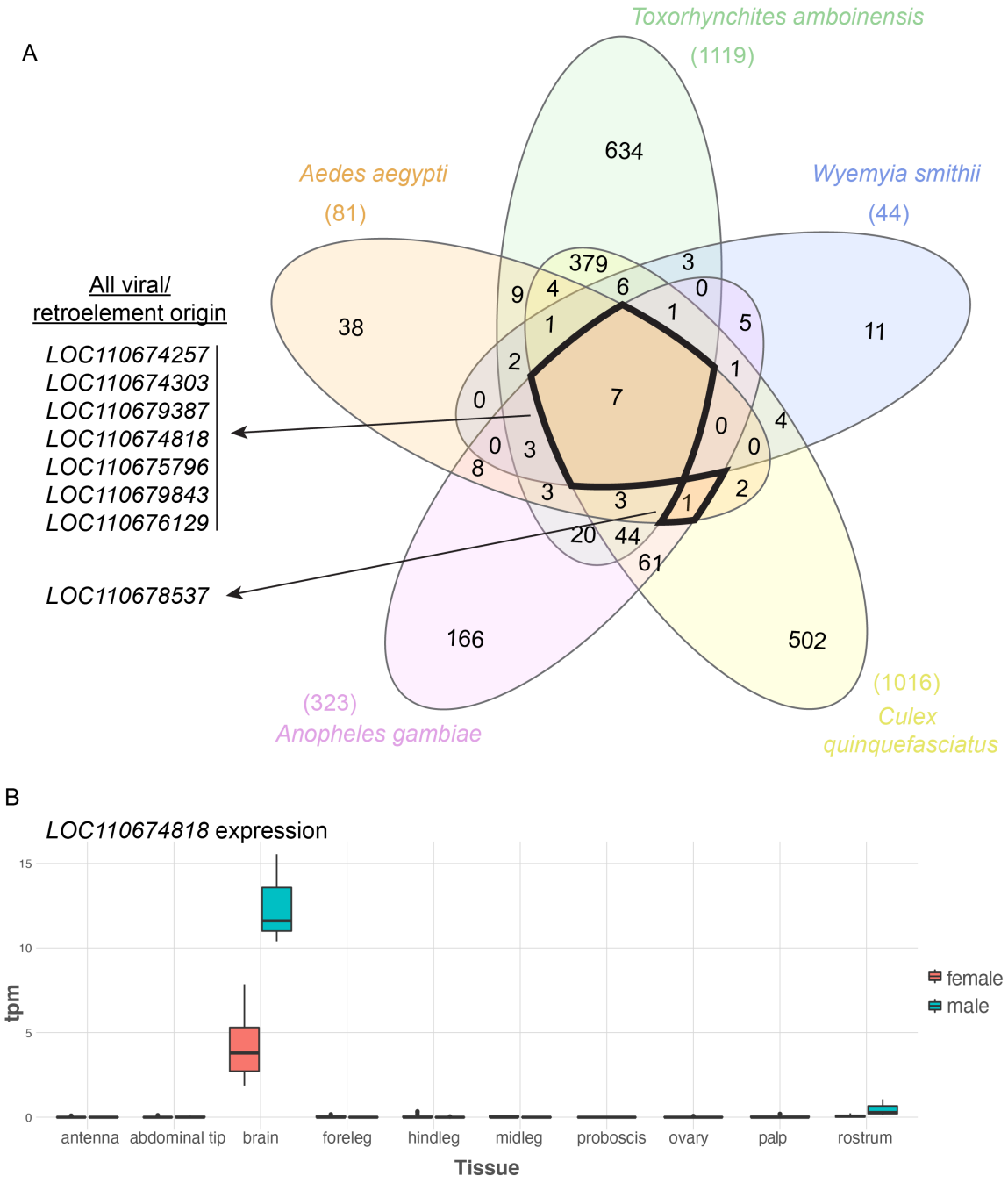

Figure 2.8 Sex-biased transcript expression in brains of blood-feeding and non-blood-feeding mosquitoes

(A) Venn diagram showing the overlap in sex-biased transcript expression, as determined by *de novo* transcriptome assembly, across blood-feeding and non-blood-feeding mosquito species.
(B) Expression levels (TPM) of *LOC110674818*, a putative retroviral integrase, across *Aedes aegypti* tissues.

In summary, in addition to sex-specific splicing, there are interesting patterns of sex-biased gene expression in the mosquitoes, with potential candidate genes such as *hairy* and retroviral genes like *LOC110674818*.

CHAPTER 3. *fruitless* mutant male mosquitoes gain attraction to human odor

Sex-specific alternative splicing plays a critical role in controlling sexual dimorphism in both the morphology and the behavior of insects across the phylogeny (Bopp *et al.*, 2014). Since the discovery that *fruitless* mutant male *Drosophila melanogaster* flies showed specific defects in their courtship and mating behavior, *fruitless* has become one of the best characterized genes in the field of behavioral genetics (Hall, 1978; Ito et al., 1996; Ryner et al., 1996). Study of the neurons that express *fruitless* has yielded rich insights into the neuronal mechanisms that drive courtship behavior (Ruta et al., 2010; Seeholzer et al., 2018; Stockinger et al., 2005). More recently, *fruitless* has been shown to be required for male mating behavior across *Drosophila*, as well as in several other insects such as moths and cockroaches (Clynen et al., 2011; Seeholzer *et al.*, 2018; Xu et al., 2020). Together these studies and observations have led to the broadly accepted model that *fruitless* is the master-regulator of male mating behaviors across the insects (Salvemini et al., 2010). Here, we investigated the role of *fruitless* in the male *Aedes aegypti* mosquito, and made the surprising discovery that in addition to its conserved role controlling mating, *fruitless* has gained a role in repressing host-seeking behavior in the male mosquito. Our results demonstrate that sexual dimorphism in a single module of a mosquito-specific behavior is controlled by a conserved gene that we speculate has gained a new function in the course of evolution.

3.1 *fruitless* plays a role in mating behavior across insects

This section will provide a brief overview of the *fruitless* gene in *Drosophila melanogaster. fruitless* was first identified as a mutant male-sterile *Drosophila melanogaster* line generated through X-ray mutagenesis (Gill, 1963). This sterility was specifically due to a defect in the courtship behavior of mutant male flies. Courtship behavior is an intricate, stereotyped behavioral ritual where a male fly samples the pheromones on a female fly, orients toward and follows her while singing a courtship song using an extended wing, before eventually mating (Hall, 1994). In contrast to wild-type males that mostly court female flies, *fruitless* mutant males regularly courted both males and females, and were unable to progress beyond courtship to successfully mounting and mating with a female (Hall, 1978). Many male-sterile fly lines have been described, with most of them linked to defects in spermatogenesis or morphology (Castrillon et al., 1993). However, *fruitless* is one of the rare examples of a male-sterile mutant that shows specific defects in behavior (Hall, 1994). In addition, *fruitless* is required for sex-specific aggressive behaviors (Vrontou et al., 2006).

fruitless was cloned independently by two groups (Ito *et al.*, 1996; Ryner *et al.*, 1996), both of which showed that *fruitless* was alternatively spliced in both sex-specific and non-sex-specific modes. These transcripts encoded protein products with a predicted BTB domain and a zinc-finger domain, suggesting that *fruitless* is a transcription factor. *fruitless* expression is driven by multiple promoters, and transcripts driven by the most upstream P1 promoter are specifically expressed in the nervous

system, while transcripts from the P2-4 promoters are broadly expressed (Lee et al., 2000). Moreover, only P1 transcripts show sex-specific alternative splicing, where the female transcript contains a premature stop codon which truncates the protein before the DNA-binding domain, suggesting that the female protein product is non-functional, although this prediction has not been functionally validated (Usui-Aoki et al., 2000). Sex-specific splicing of the *fruitless* gene controls several aspects of courtship behavior. Male flies mutant for *fruitless* promiscuously court other males and cannot successfully mate with females (Ito *et al.*, 1996; Ryner *et al.*, 1996). Forcing male *fruitless* splicing in females triggers orientation and singing behaviors normally only performed by males (Demir and Dickson, 2005).

fruitless is transcribed into a number of different non-sex-specific transcripts, which collectively encode for four distinct zinc-finger binding domains, named fruitless A-D. These proteins are expressed in distinct subsets of cells both within and outside the nervous system (Dalton et al., 2013; Neville et al., 2014; von Philipsborn et al., 2014). Within the nervous system, the mechanism by which these male-specific proteins act to control courtship behavior has only recently begun to be unraveled. Briefly, fruitless proteins interact with chromatin remodeling enzymes to enact cell-type specific, sex-specific, and isoform-specific transcription, likely acting to repress transcription of genes in the male (Brovkina et al., 2021; Ito et al., 2012; Neville *et al.*, 2014). These transcriptional changes lead to the presence or absence of specific cells in the male nervous system, such as the P1 neurons (Kimura et al., 2008), or cells that are present

in both sexes but differ in their wiring and response properties, such as the mAL or p-SPg neurons (Ito *et al.*, 2012; Kimura et al., 2005; Kohl et al., 2013).

fruitless is expressed in an interconnected set of neurons, all the way from peripheral olfactory and gustatory sensory neurons which detect the scent and taste of a female fly, to neurons that project to motor regions of the ventral nerve cord to elicit courtship song (Ruta *et al.*, 2010; von Philipsborn et al., 2011). Sensory cues given off by a female are integrated in the P1 neurons (not to be confused with the P1 *fruitless* transcripts), which control the decision to initiate courtship (Clowney *et al.*, 2015; Kimura *et al.*, 2008; Kohatsu et al., 2011; Pan et al., 2012; von Philipsborn *et al.*, 2011). Optogenetic activation of the P1 neurons leads to increased arousal and sustained courtship of stimuli such as a moving magnet (Clowney *et al.*, 2015; Kohatsu *et al.*, 2011; Pan *et al.*, 2012). The function of the P1 neurons is conserved in multiple *Drosophila* species, but the balance of the excitation and inhibition onto these neurons is retuned to allow for species-specific courtship (Seeholzer *et al.*, 2018).

In every species in which its role has been examined by either CRISPR/Cas9 based mutagenesis or RNAi-mediated knockdown, including various *Drosophila* species (Ding et al., 2019; Seeholzer *et al.*, 2018), cockroaches (Clynen *et al.*, 2011), and silkworms (Xu *et al.*, 2020), *fruitless* has been shown to be required for male-specific courtship and mating behavior. This deficit is specific to behavior, and not to morphological development or infertility. Moreover, sex-specific *fruitless* splicing is conserved across wasps (Bertossa et al., 2009) and mosquitoes (Gailey et al., 2006; Salvemini et al.,

2013). The conserved splicing and behavioral role of *fruitless* across insects has led to the proposal that it may act as a master regulator of sexually dimorphic mating behaviors across insects (Salvemini *et al.*, 2010).

3.2 *fruitless* is sex-specifically spliced in the mosquito nervous system

We used an arm-next-to-cage assay (Figure 3.1A-B) to monitor attraction of male and female *Aedes aegypti* mosquitoes to a live human arm. Consistent with their sexually dimorphic blood-feeding behavior, only females were strongly attracted to the arm (Figure 3.1C). What is the genetic basis for this extreme sexual dimorphism?

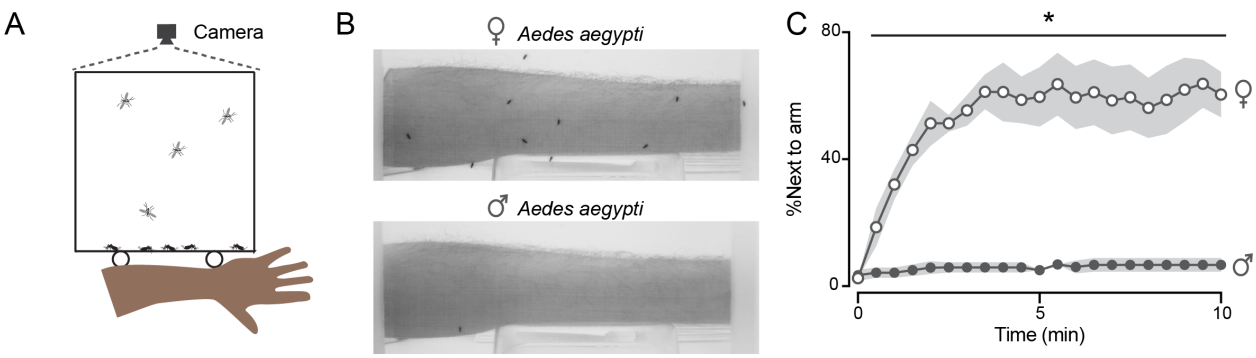

Figure 3.1 Female-specific host seeking in *Aedes aegypti*

(A-B) Arm-next-to-cage assay schematic (A) and image (B) with male (bottom) and female (top) *Aedes aegypti* mosquitoes.
(C) Percent mosquitoes next to arm measured every 30 sec. Data are mean±s.e.m., n = 6 trials, n = 20 mosquitoes/trial; *p < 0.05, Mann-Whitney test for each time point.

We reasoned that *fruitless*, which is alternatively spliced in a sex-specific manner and promotes male courtship and copulation in *Drosophila melanogaster* flies (Ito *et al.*, 1996; Ryner *et al.*, 1996), may play similar roles in controlling sexually dimorphic

behaviors in *Aedes aegypti*. *fruitless* is a complex gene with multiple promoters and multiple alternatively spliced exons. Downstream promoters drive broadly expressed non-sex-specific *fruitless* transcripts and proteins (Lee *et al.*, 2000). A previous study showed (Salvemini *et al.*, 2013) and we confirmed that transcripts from the upstream neuron-specific (P1) promoter in the *Aedes aegypti fruitless* gene are sex-specifically spliced (Figure 3.2A-B). Both male and female P1 transcripts include a short male 'm' exon, and female transcripts additionally include a longer female 'f' exon with an early stop codon, predicted to yield a truncated Fruitless protein in the female. However, it is unlikely that any sex-specific Fruitless protein or peptides are stably expressed in adult females. In *Drosophila*, sex-specific female fruitless peptides are not detected (Lee *et al.*, 2000), and *tra* is thought to inhibit translation by binding to female *fruitless* P1 transcripts (Usui-Aoki *et al.*, 2000). P1 transcripts of both sexes splice to the first common 'c1' exon, but only male transcripts are predicted to encode full-length Fruitless protein with BTB and zinc-finger domains (Figure 3.2B).

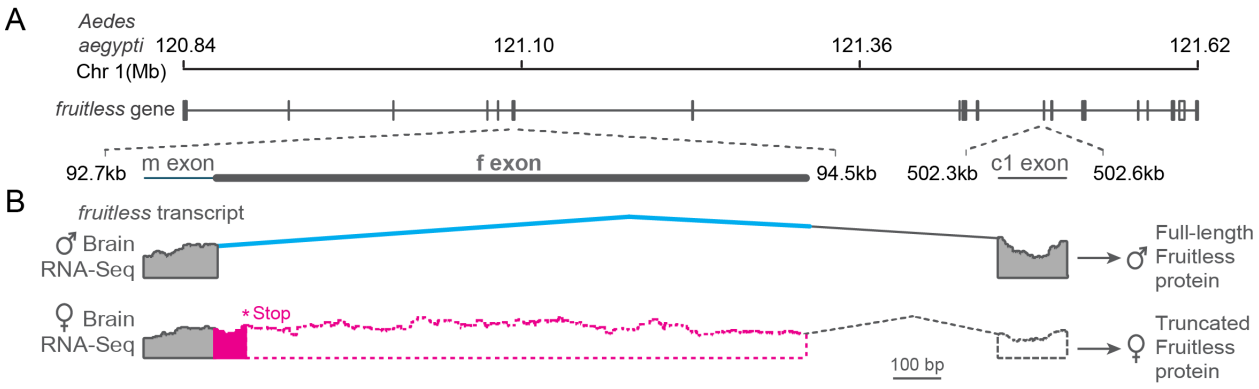

Figure 3.2 Sex-specific splicing of the *fruitless* gene

(A) Schematic of *Aedes aegypti fruitless* genomic locus
(B) Sex-specific splicing region with RNA-seq read evidence. Coding and non-coding exons are represented by filled and open dashed bars, respectively.

Is the f exon the only sex-specifically spliced exon of *fruitless*, and in which tissues is this exon sex-specifically spliced? By analyzing previously published tissue-specific RNA-seq data (Matthews *et al.*, 2016), we verified that of all the *fruitless* exons, only the f exon was sex-specific in *Aedes aegypti* brains (Figure 3.3A). Moreover, while the c1 exon was broadly expressed through downstream promoters, P1 transcripts were specifically expressed in the brain and the antenna, the major olfactory organ of the mosquito (Figure 3.3B), consistent with *fruitless* expression in *Drosophila* (Stockinger *et al.*, 2005).

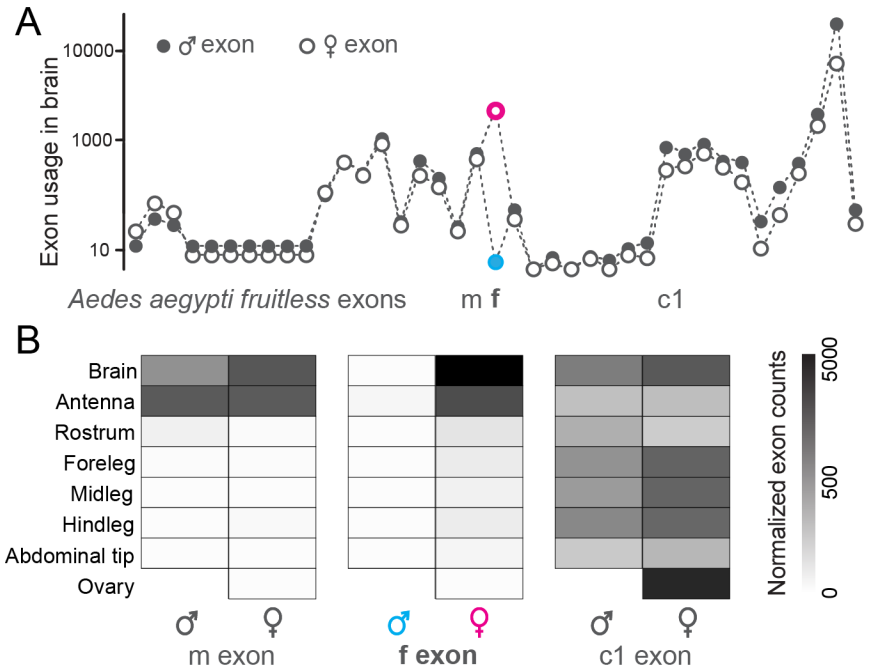

Figure 3.3 Sex-specific splicing of *fruitless* is enriched in the nervous system

(A, B) *Aedes aegypti fruitless* exon usage based on male and female RNA-seq data (normalized counts) from the indicated tissue plotted for each exon (A) and m, f, and c1 exons (B) n = 3-4 independent RNA-seq replicates. Data from (Matthews *et al.*, 2016).

To ask if *fruitless* splicing was conserved across mosquitoes, we sequenced RNA from male and female brains of five different species and assembled *de novo* transcriptomes for each sex. Three of these species are important arboviral disease vectors because their females blood feed on humans, whereas the two other species only feed on plants (Bradshaw et al., 2018; Zhou et al., 2014) (Figure 3.4). We identified orthologues of *fruitless* in each species and found that all had conserved ‘m’ and ‘c1’ exons and distinct ‘f’ exons. *fruitless* was sex-specifically spliced in each of these species with a female-specific ‘f’ exon and early stop codon, predicted to produce a full-length fruitless protein only in males.

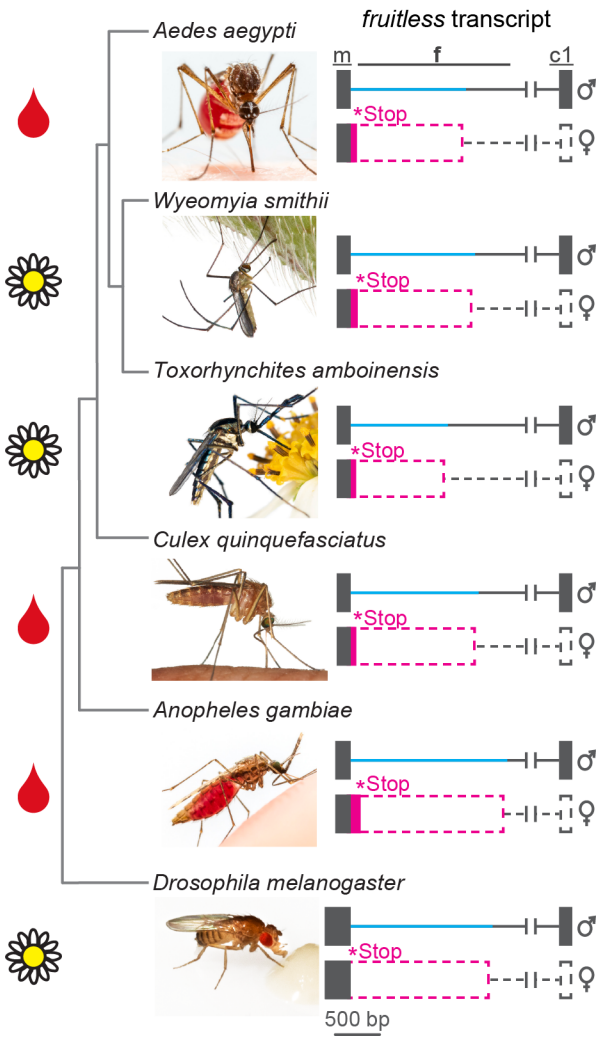

Figure 3.4 Sex-specific splicing of *fruitless* is conserved across the mosquitoes

Phylogeny of mosquito species with outgroup *Drosophila melanogaster*, with conserved *fruitless* exon structure inferred from *de novo* transcriptome assembly. Coding and non-coding exons are represented by filled and open dashed bars, respectively. *Toxorhynchites rutilus* and *Culex salinarius* images were used to represent *Toxorhynchites amboinensis* or *Culex quinquefasciatus*, respectively. Cartoons indicate blood-feeding (blood drop) and non-blood-feeding (flower) species.

3.3 Generation of *fruitless* mutant alleles

We used CRISPR-Cas9 genome editing (Kistler et al., 2015) to disrupt P1 neural-specific *fruitless* transcripts in *Aedes aegypti* to investigate a possible role of *fruitless* in

sexually dimorphic mosquito behaviors. We generated two alleles, *fruitless*$^{\Delta M}$, which introduces a frameshift that is predicted to produce a truncated protein in males, and *fruitless*$^{\Delta M\text{-}tdTomato}$, in which the *fruitless* gene is disrupted by a knocked-in CsChrimson:tdTomato fusion protein (Figure 3.5). In both alleles, the protein is truncated before the downstream BTB and zinc-finger domains. The *fruitless*$^{\Delta M\text{-}tdTomato}$ line allowed us to visualize cells that express the fluorescent tdTomato reporter under the control of the endogenous *fruitless* regulatory elements. To control for independent background mutations, we used the heteroallelic *fruitless*$^{\Delta M}$/*fruitless*$^{\Delta M\text{-}tdTomato}$ mutant strain in all subsequent behavior assays (Figure 3.5). In this heteroallelic mutant, *fruitless* P1 transcripts are disrupted in both males and females. Since full-length fruitless protein is male-specific, we expected that only *fruitless*$^{\Delta M}$/*fruitless*$^{\Delta M\text{-}tdTomato}$ male mosquitoes would display altered behavioral phenotypes.

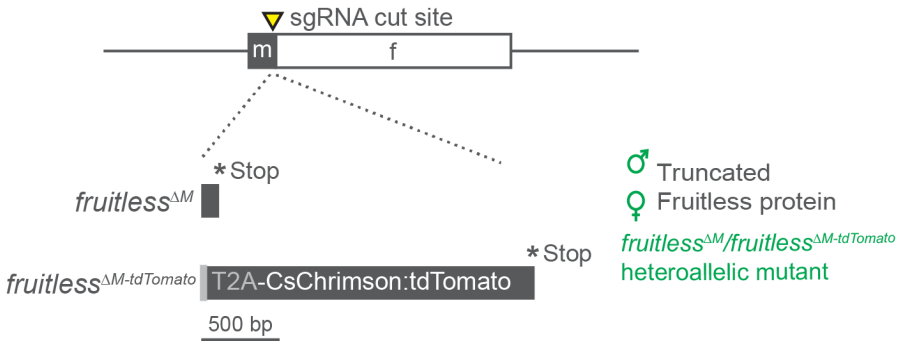

Figure 3.5 Generation of a knock-out and a knock-in/knock-out *fruitless* allele

Schematic of generation of *fruitlessΔM* and *fruitlessΔM-tdTomato* mutants.

CsChrimson is a red light-activated cation channel and we originally generated this animal with the intention of optogenetically manipulating behavior. However,

CsChrimson:tdTomato intrinsic fluorescence was not visible under a confocal microscope, even at high laser intensities, and required immunofluorescent amplification in all our images. When animals were fed with retinal, the necessary co-factor which was absent in all other experiments, and we attempted to substitute human odor and CO_2 with optogenetic activation of *fruitless*+ neurons in a blood-feeding assay, we did not observe increased feeding (preliminary data not shown). Although we were unable to see evidence of CsChrimson activity in these optogenetic experiments, potential background levels of CsChrimson-driven activation of *fruitless*-expressing neurons is an important concern to rule out when considering the behavioral data in this paper. We note that animals were not fed trans-retinal, the necessary cofactor for CsChrimson activity. *fruitless*$^{\Delta M\text{-}CsChrimson\text{-}tdTomato/+}$ males and females were able to mate normally, and *fruitless*$^{\Delta M\text{-}CsChrimson\text{-}tdTomato/+}$ females show normal blood-feeding and egg-laying behavior (Figure 3.15A-C). We have not examined *fruitless*$^{\Delta M/\Delta M}$ animals due to the difficulty of obtaining these animals without molecular genotyping of each individual. However, given the weak expression of CsChrimson and the robust behavior of heterozygote animals, we consider it unlikely that this allele is significantly affecting mosquito behavior.

We attempted to generate *fruitless* P1-specific driver lines by knocking in a cassette containing the ribosomal-skipping peptide T2A followed by the transcriptional activator QF2 (Riabinina et al., 2015), with 3xP3-dsRed as an insertion marker as previously described (Matthews *et al.*, 2019). In this knock-in/knock-out strain we aimed to disrupt the *fruitless* gene as well as generate a driver line that would allow us to label

and manipulate *fruitless*-expressing neurons. We recovered 7 independent 3xP3-dsRed positive G1 families. However, all females with one copy of the correct integration did not blood-feed after many attempts using multiple different human hosts. Males with one copy of this insertion did not mate with wild-type females. Since blood meals are required for *Aedes aegypti* egg development, this line could not be maintained. We next tried to knock-in the weaker QF2w transcriptional activator, and recovered 6 independent families, all of which showed the same blood feeding and mating defects (Table 3.1). We speculate that toxicity of QF2 or QF2w may affect the function or viability of *fruitless*-expressing neurons, leading to the behavioral defects we observed. The cause of Q-system toxicity, even attenuated from Q to QF2 to QF2w is unknown (Riabinina *et al.*, 2015). We speculate that this toxicity is unrelated to the *fruitless* locus, because *fruitless*$^{\Delta M/+}$ animals had no phenotype as heterozygotes, unlike *fruitless*$^{\Delta QF2/+}$ and *fruitless*$^{\Delta QF2w/+}$ animals.

Table 3.1 List of successful and attempted genetic manipulations of the *fruitless* locus

Construct	Purpose	#hatched /injected (% eggs hatched)	#G1 families verified hits	Hit rate (%)	Notes
SUCCESSFUL *FRUITLESS M* CONSTRUCTS					

fruitless∆M-T2A-CsChrimson-tdTomato	Disrupt FruitlessM and express a fusion protein to label and optogenetically activate fruitless neurons in *fruitlessM* mutant males	820/2000 (41%)	4	0.97	Viable line. CsChrimson-tdTomato was not detectable in the brain without antibody amplification from antibody staining, suggesting that expression is too low for optogenetic stimulation
fruitless∆M	Disrupt FruitlessM	376/2000 (20%)	1	0.53	Viable line. Resulted from imprecise insertion of the *fruitless∆M-T2A-QF2* construct at the fruitless locus
ATTEMPTED *FRUITLESS M* CONSTRUCTS					
fruitless∆M-T2A-QF2	Disrupt FruitlessM and express the bipartite Q system in fruitless neurons (regular QF2)	376/2000 (20%)	7	4.43	All males did not mate, all females did not blood-feed
fruitless∆M-T2A-QF2w	Disrupt FruitlessM and express the bipartite Q system in fruitless neurons (attenuated QF2)	462/2000 (22%)	6	2.61	All males did not mate, all females did not blood-feed. In G0s crossed to QUAS-CD8-GFP, saw labeling in G1 brains that resembled *fruitless∆M-tdTomato* expression
fruitless∆M-T2A-GCaMP6s-T2A-TdTomato-T2A-3XFLAG-DsRed-NLS	Disrupt FruitlessM and express cytoplasmic and nuclear markers and GCaMP for calcium imaging	349/2000 (16%)	4	1.15	Viable line but weak marker
ATTEMPTED *FRUITLESS F* CONSTRUCTS					

fruitlessΔF	Force expression of FruitlessM in females (unmarked allele)	205/1000 (20%)	G1s pooled for quick screening	?	G1 males pooled and crossed to +/+ females for PCR screening. G2 females had blood-feeding defects and did not lay eggs
fruitlessΔF-dsRed-3XP3	Force expression of FruitlessM in females (marked allele)	740/2000 (37%)	6	1.6	G1/G2 Females blood-fed normally. We suspect that the 3xP3 insertion interferes with splicing

3.4 Sexually dimorphic expression of *fruitless* in the mosquito brain and antenna

In *Drosophila melanogaster*, P1 *fruitless* transcripts are expressed in several thousand cells comprising about ~2% of the neurons in the adult brain (Stockinger *et al.*, 2005). To examine the distribution of cells expressing *fruitless* in male and female *Aedes aegypti* mosquitoes, we carried out whole mount brain staining to reveal the tdTomato marker expressed from the *fruitless* locus. *fruitless>tdTomato* is expressed in a large number of cells in both male and female brains (Figure 3.6A-F), as well as in the ventral nerve cord (Figure 3.7A-B). *fruitless>tdTomato*-expressing cells innervate multiple regions of the mosquito brain, including the suboesophageal zone, the lateral protocerebral complex, and the lateral horn. These areas have been implicated in feeding (Jové *et al.*, 2020), mating (Seeholzer *et al.*, 2018), and innate olfactory behaviors (Datta et al., 2008) respectively, and also receive projections from *fruitless*-expressing neurons in *Drosophila* (Seeholzer *et al.*, 2018; Stockinger *et al.*, 2005). The

projections of *fruitless>tdTomato* neurons are dramatically sexually dimorphic, with denser innervation in the female suboesophageal zone and the male lateral protocerebral complex (Figure 3.6A-F). We did not detect any gross anatomical differences between heterozygous and heteroallelic *fruitless* mutant male brains or the pattern of *fruitless>tdTomato* expression (Figure 3.6B,C,E,F). We cannot exclude the possibility that there are subtle differences that can only be observed with sparse reporter expression in subsets of cells.

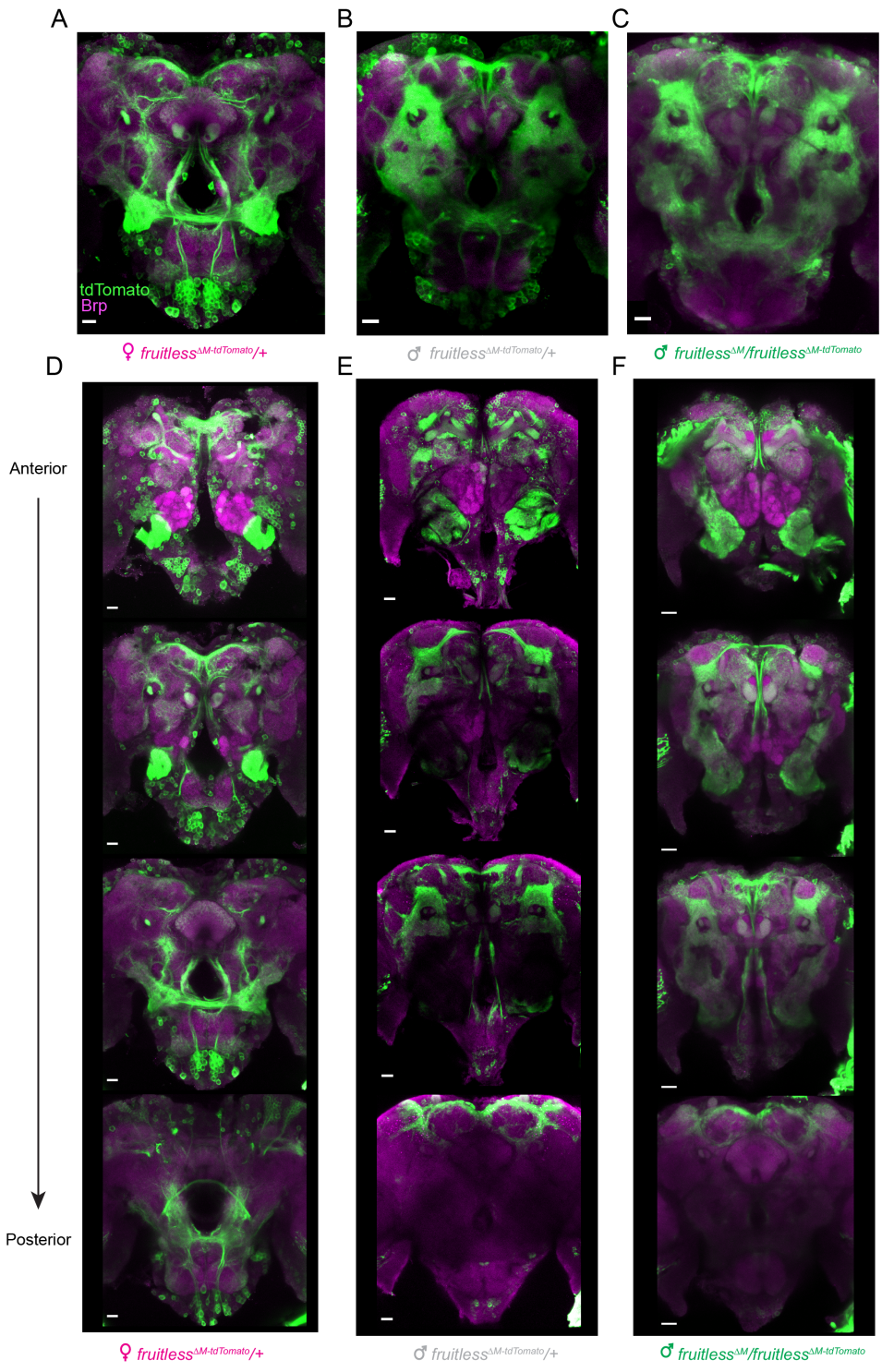

Figure 3.6 Expression of fruitless in the mosquito brain

(A-F) Confocal images of brains of the indicated genotypes showing *fruitless>tdTomato* (green) and Brp (magenta) expression. Scale bars, 20 μm.
(D-F) Top-to bottom images are optical sections of the same brain, arranged from anterior to posterior.

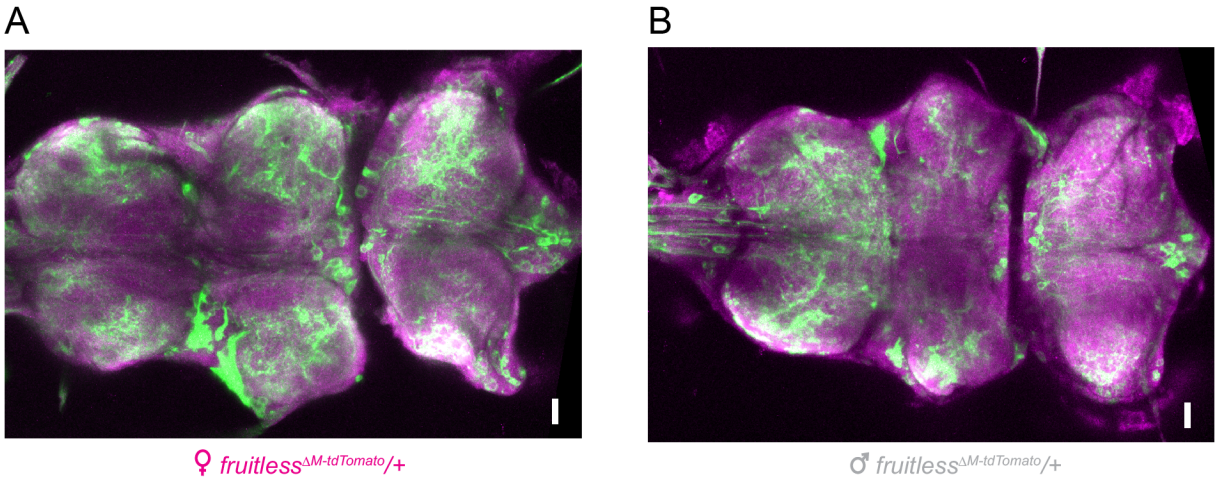

Figure 3.7 Expression of fruitless in the female mosquito ventral nerve cord

(A-B) Confocal images of ventral nerve cords of the indicated genotypes showing *fruitless>tdTomato* (green) and Brp (magenta) expression. All scale bars, 20 μm.

We also examined *fruitless* expression in the periphery. Odors are sensed by olfactory sensory neurons in the mosquito antenna, and each type of neuron projects to a single glomerulus in the antennal lobe of the mosquito brain (Figure 3.8A). We found that, as is the case in *Drosophila* (Stockinger *et al.*, 2005). *fruitless>tdTomato* is expressed in olfactory sensory neurons in the antenna of both male and female mosquitoes, and that some of these neurons co-express the olfactory receptor co-receptor Orco (Figure 3.8B-E). *fruitless>tdTomato* labels a subset of glomeruli in the antennal lobe, with females having about twice as many positive glomeruli compared to males of either genotype (Figure 3.9A-L). There was no difference in the number of *fruitless>tdTomato*-labeled glomeruli between wild-type and *fruitless* mutant males (Figure 3F), suggesting that *fruitless* does not control sexual dimorphism in the number of glomeruli labeled by *fruitless>tdTomato*.

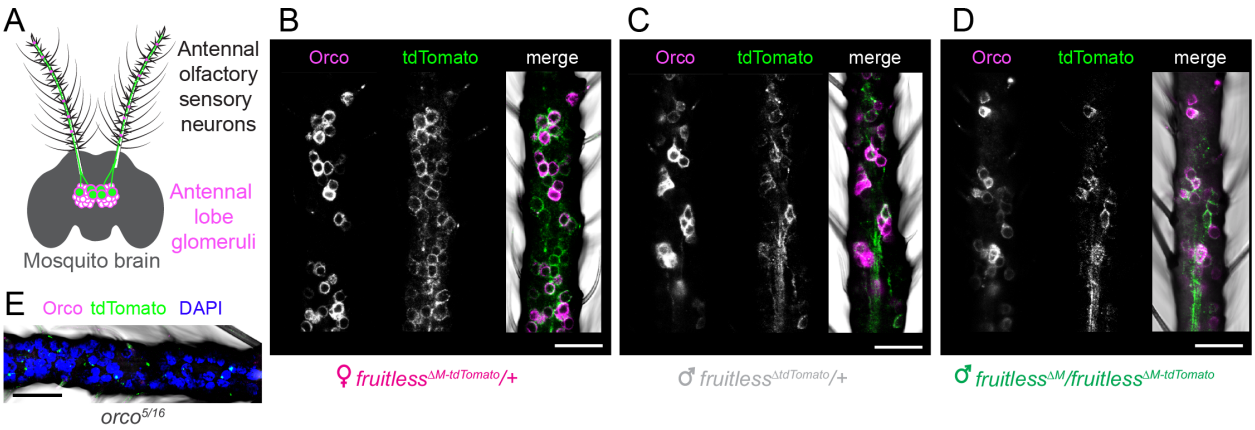

Figure 3.8 Expression of *fruitless* in the mosquito antenna

(A) Schematic of antennal olfactory sensory neurons and their projections to the antennal lobe of the mosquito brain.
(B-D) Confocal images of antennae of the indicated genotypes with *fruitless>tdTomato* (green) and Orco (magenta) expression.
(E) Confocal image of orco mutant antenna, as negative control for Orco and tdTomato antibodies, with DAPI (blue). All scale bars are 20 μm.

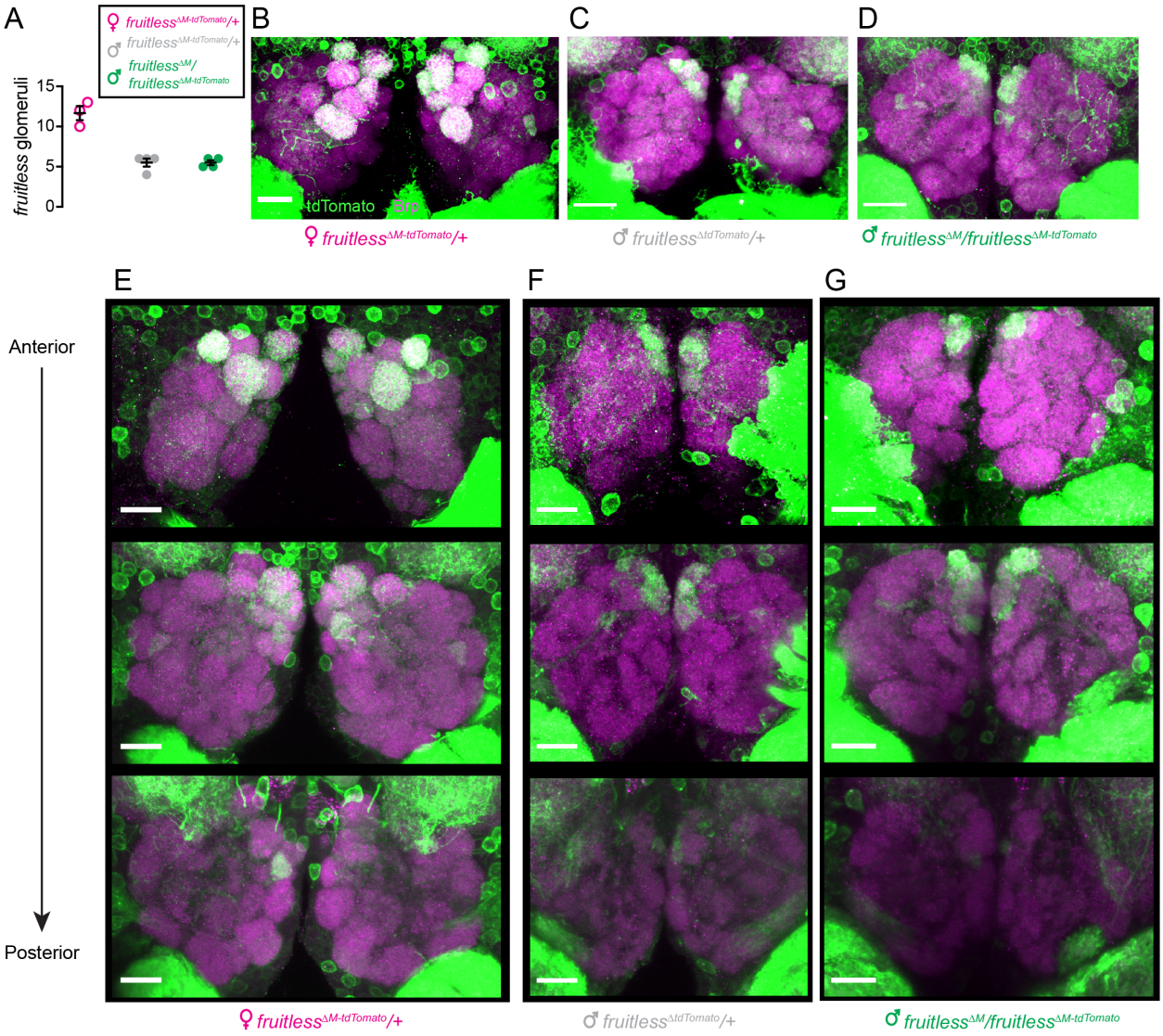

Figure 3.9 Expression of fruitless in the mosquito olfactory system.

(A) Number of antennal lobe glomeruli labeled by *fruitless>tdTomato* in the indicated genotypes.
(B-D) Confocal images of antennal lobes of the indicated genotypes with fruitless>tdTomato (green) and Brp (magenta) expression.
(E-G) Confocal images of antennal lobes of the indicated genotypes showing *fruitless>tdTomato* (green) and Brp (magenta) expression. Top-to bottom images are optical sections of the same lobes, arranged from anterior to posterior. All scale bars, 20 μm.

3.5 *fruitless* mutant males gain attraction to live human hosts

Given the broad neural expression and sexual dimorphism in *fruitless* circuits, we asked if *fruitless* mutant males showed any defects in sexually dimorphic feeding and mating behaviors. Since only female mosquitoes have the anatomical capacity to pierce skin and artificial membranes (Jové *et al.*, 2020; Klowden, 1995), we developed a feeding assay in which both females and males are able to feed from warmed liquids through a net without having to pierce a membrane to access the meal (Figure 3.10A). Both wild-type males and females reliably fed on sucrose and did not feed on water. Only wild-type females fed on blood. Even when warm blood was offered and available to males for ready feeding, they still did not find it appetizing (Figure 3.10B). *fruitless* mutant males fed similarly to their wild-type male counterparts on all meals, suggesting that this behavioral preference is not under the control of *fruitless* in males (Figure 3.10B).

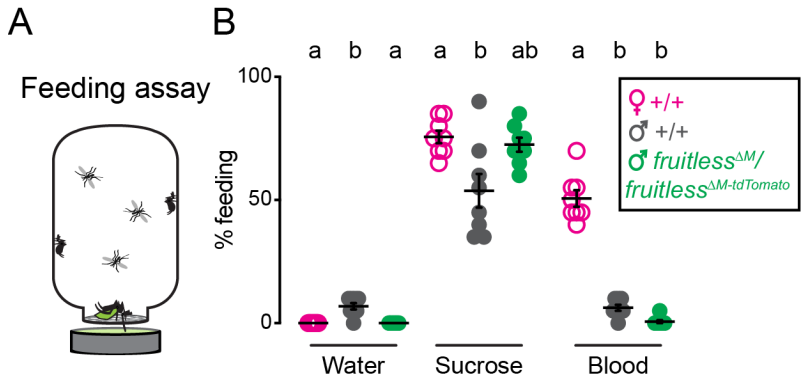

Figure 3.10 Male *fruitless* mutant mosquitoes do not feed on blood

(A) Feeding assay schematic.
(B) Feeding on indicated meal (n = 8 trials/meal; n = 20 mosquitoes/trial). Data are mean±s.e.m. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$). Comparisons are made between genotypes for each meal.

Because *fruitless* plays a key role in male courtship and mating in *Drosophila*, we asked if it is similarly required in *Aedes aegypti*. Since mosquitoes show extremely rapid in-flight mating behavior that is completed in less than 30 seconds, it is difficult to directly observe or quantify (Hartberg, 1971). We used previously developed insemination assays (Degner and Harrington, 2016; Duvall *et al.*, 2017) to quantify the ability of males to successfully mate (Figure 3.11A). We found that *fruitless* mutant males appeared to contact females but were unable to successfully inseminate wild-type females (Figure 3.11B). This mating failure is consistent with the established role of *fruitless* in *Drosophila* male sexual behavior (Demir and Dickson, 2005; Ryner *et al.*, 1996).

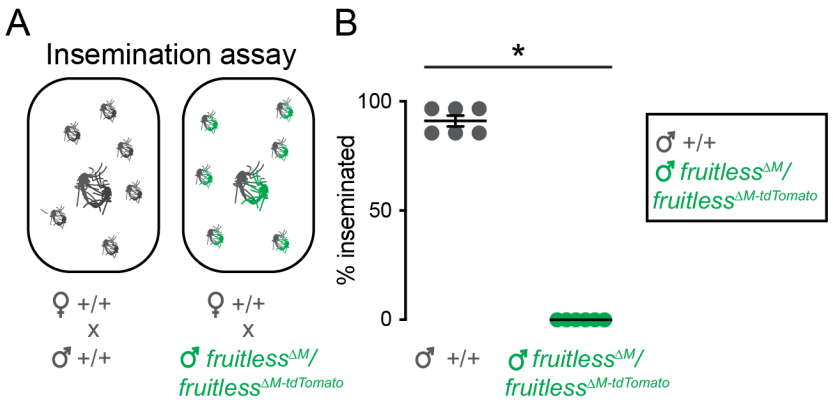

Figure 3.11 Male *fruitless* mutant mosquitoes are unable to inseminate females

(A) Insemination assay schematic.
(B) Insemination of wild-type females by males of the indicated genotype (n = 6 trials/male genotype, n = 20 females/trial; *p = 0.0022, Mann-Whitney test. Data are mean±s.e.m.

We then turned to innate olfactory behaviors that govern the search for nectar, which is used as a source for metabolic energy by both males and females, and blood, which is required only by females for egg production. Consistent with the use of these meals, nectar-seeking behavior is not sexually dimorphic, but human host-seeking behavior is sexually dimorphic.

To measure these behaviors, we adapted the Uniport olfactometer (Liesch et al., 2013), which is only able to test one stimulus at a time, and developed the Quattroport, an olfactometer that tests attraction to four separate stimuli in parallel (Figure 3.12A). The Quattroport measures both the activation, the participation of the animals in the assay, and attraction, short range attraction to the stimulus (Figure 3.12B). In control experiments we examined activation responses of wild-type male and females offered a blank, CO_2, a human arm, or the floral odor of honey. While males and females showed equivalent activation with a blank and honey, females were more strongly activated to the host-related cues of CO_2 and the human arm (Figure 3.12C). To model nectar-seeking behavior, we used honey as a floral odor and glycerol as a control odor as previously described (Figure 3.13A) (DeGennaro *et al.*, 2013). There was no difference in nectar-seeking as defined by attraction in the Quattroport between wild-type females, males, and *fruitless* mutant males (Figure 3.13B).

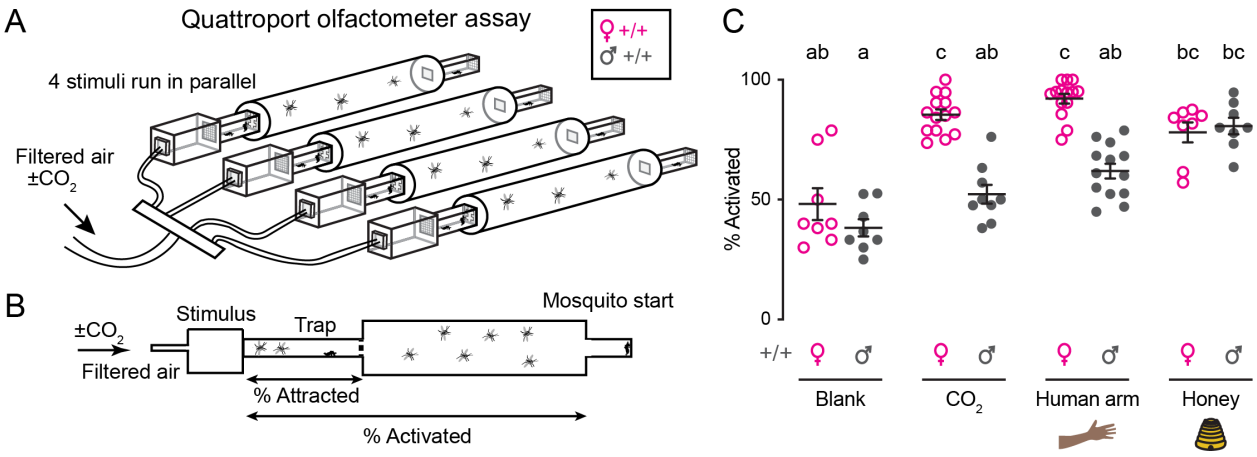

Figure 3.12 The Quattroport assays host-seeking behavior

(A) Schematic of Quattroport assay, highlighting ability to run multiple stimuli and genotypes simultaneously.
(B) Side-view schematic of Quattroport, highlighting close-range (attraction) and long-range (activation) metrics
(C) Percent activated animals, n = 8-14 trials/group, n = 17-28 mosquitoes/trial. Data are mean±s.e.m. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$).

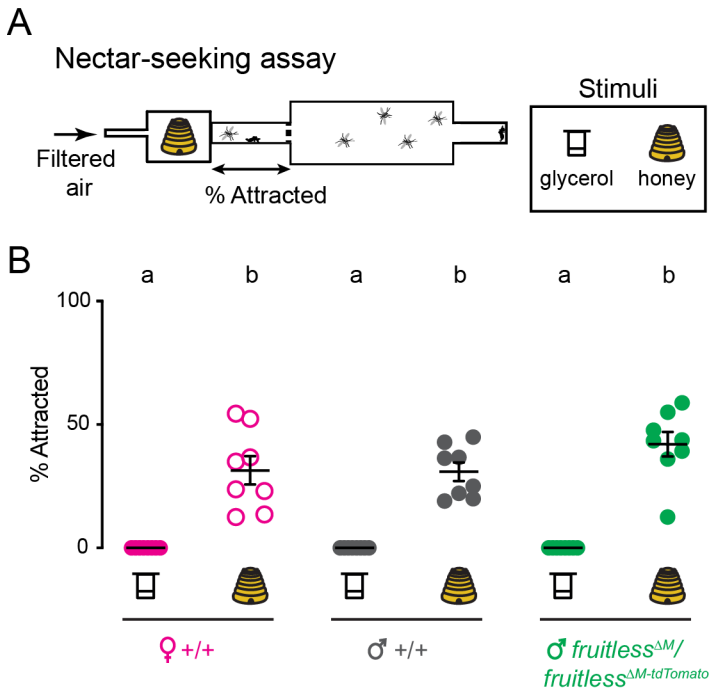

Figure 3.13 Male *fruitless* mutant mosquitoes show normal nectar-seeking behavior

(A) Quattroport assay schematic for nectar seeking.
(B) Percent of attracted animals (n = 8-14 trials per group, n = 17-28 mosquitoes/trial). Data are mean±s.e.m. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$). Comparisons are made between all genotypes and stimuli.

We next used the Quattroport with a live human host as a stimulus (Figure 3.14A). As expected, wild-type females robustly and reliably entered traps in response to a live human forearm. In contrast, zero wild-type males entered the trap, consistent with our observations in the arm-next-to-cage assay (Figure 3.1A-C). If *fruitless* function in *Aedes aegypti* were limited to mating and aggression as it is in *Drosophila*, we would expect *fruitless* mutant males to show no interest in a live human host. Unexpectedly, *fruitless* mutant males were as attracted to a live human host as wild-type females (Figure 3.14B). This indicates that fruitless males have gained the ability to host-seek, displaying the signature sexually dimorphic behavior of the female mosquito.

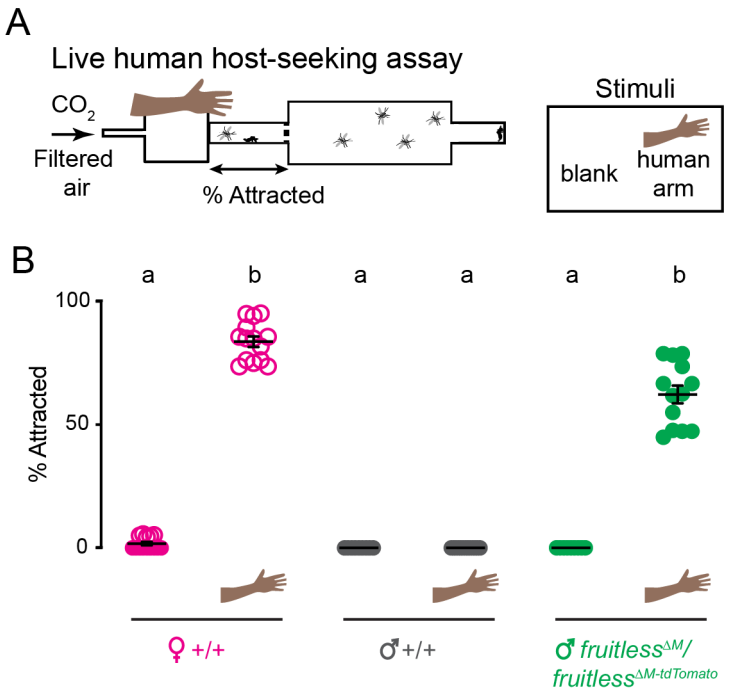

Figure 3.14 Male *fruitless* mutant mosquitoes gain attraction to a live human host

(A) Quattroport assay schematic for live human host seeking. 1% CO2 is added to the airstream.
(B) Percent of attracted animals (n = 8-14 trials per group, n = 17-28 mosquitoes/trial). Data are mean±s.e.m. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$). Comparisons are made between all genotypes and stimuli.

3.6 The role of *fruitless* in female mosquitoes

How do *fruitless* mutant females behave? Normal host seeking in *fruitless*$^{\Delta M}$ mutant females is expected since full-length fruitless protein from the P1 transcript is translated only in males. We tested the behavior of these females, and found normal blood-feeding, egg-laying, and mating behaviors (Figure 3.15A-C), confirming our prediction that sex-specific splicing of *fruitless* leads to protein function specifically in the male.

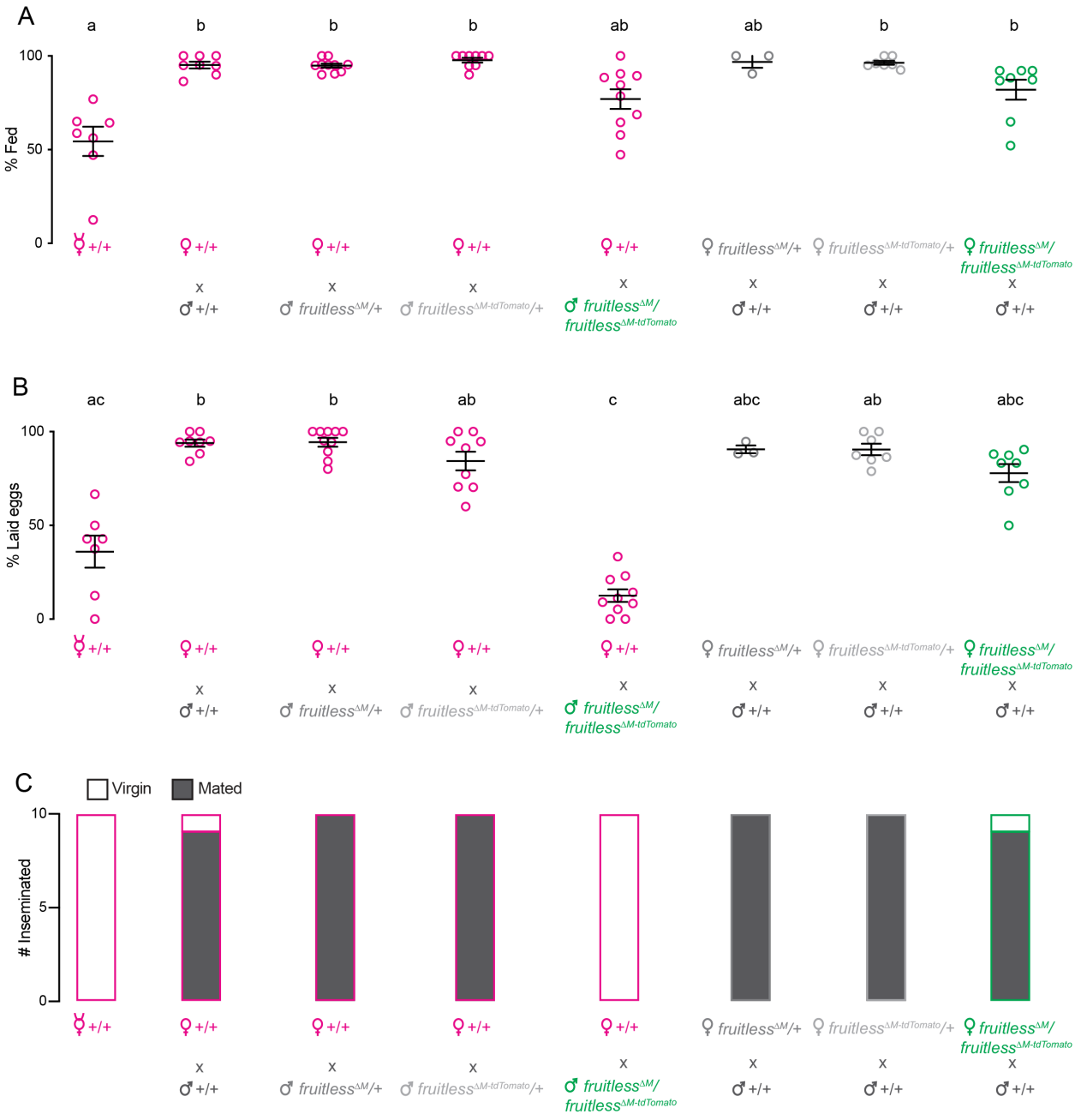

Figure 3.15 No significant blood-feeding or mating defects in *fruitless*$^{\Delta M}$ females

(A) Percent of females crossed to indicated male genotype blood feeding on a live human arm.
(B) Percent of females crossed to indicated male genotype laying eggs. Data in A, B, are mean±s.e.m., n = 3-10 trials/group, n = 12-27 mosquitoes/trial. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$). Comparisons were made across all genotypes.
(C) Number of inseminated females by males of indicated genotype. n = 10 females.

We then asked if expression of fruitless protein was sufficient to inhibit host-seeking behavior in females. To do this we attempted to force females to express male fruitless protein by deleting the female exon of *fruitless* P1 transcripts and forcing male *fruitless* splicing in female brains (Figure 3.16A-C), as has been done for *Drosophila melanogaster* previously (Demir and Dickson, 2005). We recovered multiple G1 animals with the correct integration, as verified by PCR and sequencing. Male *fruitless* splicing in *fruitless*$^{\Delta F}$ females was verified with reverse-transcription PCR (data not shown). G2 *fruitless*$^{\Delta F}$ females did not fully blood-feed or lay eggs even though they were successfully inseminated by wild-type males (Figure 3.16D-I). It was therefore impossible to maintain these lines. We do not know if the blood-feeding defect was due to a failure to respond to the host or some other behavioral or anatomical defect. Since *fruitless* is tightly linked to the male-determining locus, it was not an option to maintain this targeted allele in males. Integrations on the male chromosome would yield ~1/500 females with the recombinant allele, and integrations on the female chromosome yield inviable females and rare recombinant males. In either scenario, the *fruitless*$^{\Delta F}$ insertion is unmarked and would need to be followed by PCR genotyping.

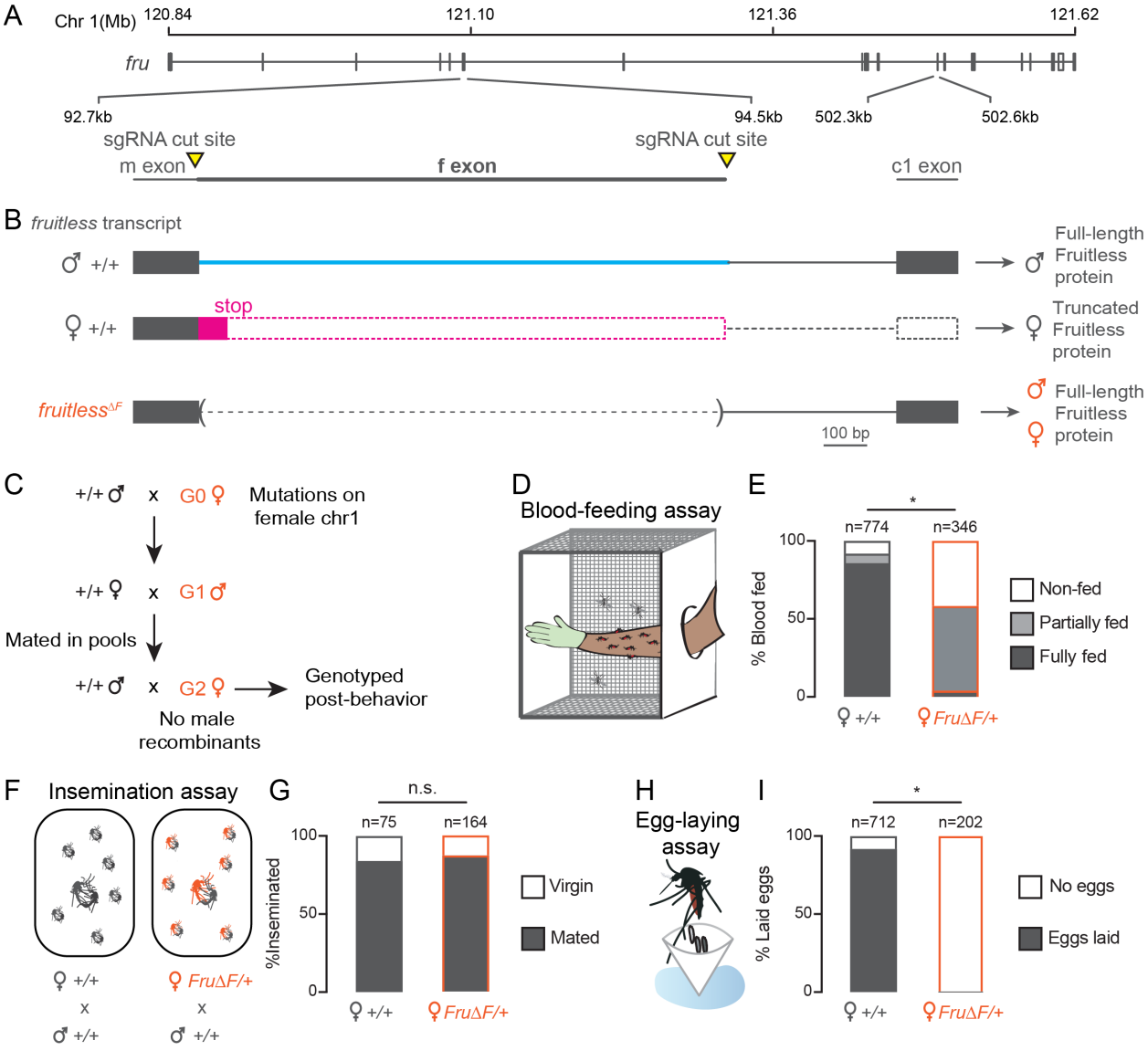

Figure 3.16 Female *fruitless*$^{\Delta F}$ mutant mosquitoes have blood-feeding and egg-laying defects

(A) Schematic of *Aedes aegypti fruitless* genomic locus.
(B) Sex-specific fruitless transcripts and generation of *fruitless*$^{\Delta F}$ mutant, with effect of splicing on Fruitless protein.
(C) Crossing scheme to generate female mutants and potential male recombinants.
(D) Blood-feeding assay schematic.
(E) Feeding on live human arm; $p<0.0001$, Chi-square test.
(F) Insemination assay schematic.
(G) Insemination of females of indicated genotype by wild-type males; $p = 0.5464$, Fisher's exact test.
(H) Egg-laying assay schematic.
(I) Egg laying by females of indicated genotype; $p<0.0001$, Fisher's exact test.

We also attempted to generate a line where we both deleted the female *fruitless* exon and knocked-in an intronic 3xP3 fluorescent marker, which would allow us to maintain this line in males and use the marker to select rare recombinants for behavioral analysis. However, females with this integration did not have any behavioral phenotypes, suggesting that the intronic 3xP3 marker interfered with regular *fruitless* splicing in both males and females. These difficulties precluded any further investigation of the phenotype of expressing full-length fruitless protein in females.

3.7 Olfactory and not heat cues attract *fruitless* mutant males to hosts

A live human arm gives off multiple sensory cues that are known to attract female mosquitoes, the most salient of which are body odor and heat. *fruitless* mutant males might be attracted by heat alone or only the human odor, or to the simultaneous presentation of both cues. To disentangle the contribution of these complex sensory cues to the phenotype we observed, we tested the response of *fruitless* mutant males to each cue in isolation. We first used a heat-seeking assay (Corfas and Vosshall, 2015; McMeniman *et al.*, 2014) to present heat to mosquitoes in the absence of human odor (Figure 3.17A). Neither *fruitless* mutant nor wild-type males were attracted to the heat cue at any temperature (Figure 3.17B). In contrast, wild-type females showed typical heat-seeking behavior that peaked near human skin temperature (Figure 3.17B).

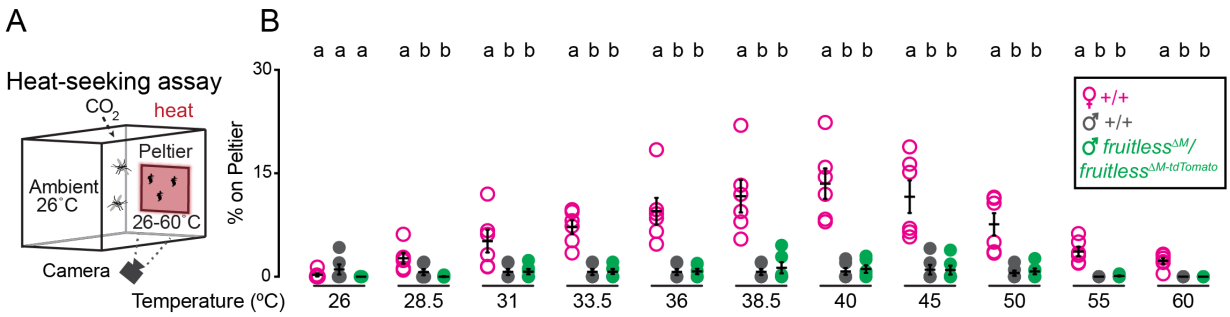

Figure 3.17 Male *fruitless* mutant mosquitoes are not attracted to heat

(A) Heat-seeking assay schematic. A 20 second pulse of 10% CO2 is added to the assay.
(B) Percent of animals on Peltier. Data are mean±s.e.m., n = 6 trials/temperature, n = 50 mosquitoes/trial. Data labeled with different letters are significantly different from each other, within each temperature. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$). Comparisons are made between genotypes at each temperature.

To ask if *fruitless* mutant males are attracted to human host odor alone, we collected human scent on nylon stockings and presented this stimulus in the Quattroport (Figure 3.18A) to both male and female mosquitoes. Whereas wild-type males and heterozygous *fruitless* mutant males showed no response to human odor, wild-type females and *fruitless*$^{\Delta M}$/*fruitless*$^{\Delta M\text{-}tdTomato}$ females were strongly attracted to human odor (Figure 3.18B). Remarkably, heteroallelic *fruitless* mutant males were strongly attracted to human scent, at levels comparable to wild-type females (Figure 3.18B). These results demonstrate that *fruitless* mutant males have gained a specific attraction to human odor, which drives them to host-seek.

There are field reports of *Aedes aegypti* males being collected near human hosts (Hartberg, 1971), which the experimenters interpreted as male *Aedes aegypti* attraction

to humans. We note, however, that these field experiments did not control for the presence of females, suggesting that the collected males may have been attracted to the female mosquitoes that attempt to bite humans. In our well-controlled laboratory assays, we were unable to find any evidence of strong attraction to humans in mosquito males at close-range (Figure 3.1C, Figure 3.14B) or at long distances (Figure 3.12C). In these same assays, wild-type male mosquitoes showed strong attraction to floral cues (Figure 3.13B). We cannot exclude that male mosquitoes in the field show some long-range attraction to a human host, as has been suggested by recent work (Amos *et al.*, 2021). Given that males do not blood-feed and are thought to host-seek only in order to find mates, it is possible that any attraction in males would be weaker than in wild-type females, or the attraction we demonstrate in *fruitless* mutant males here.

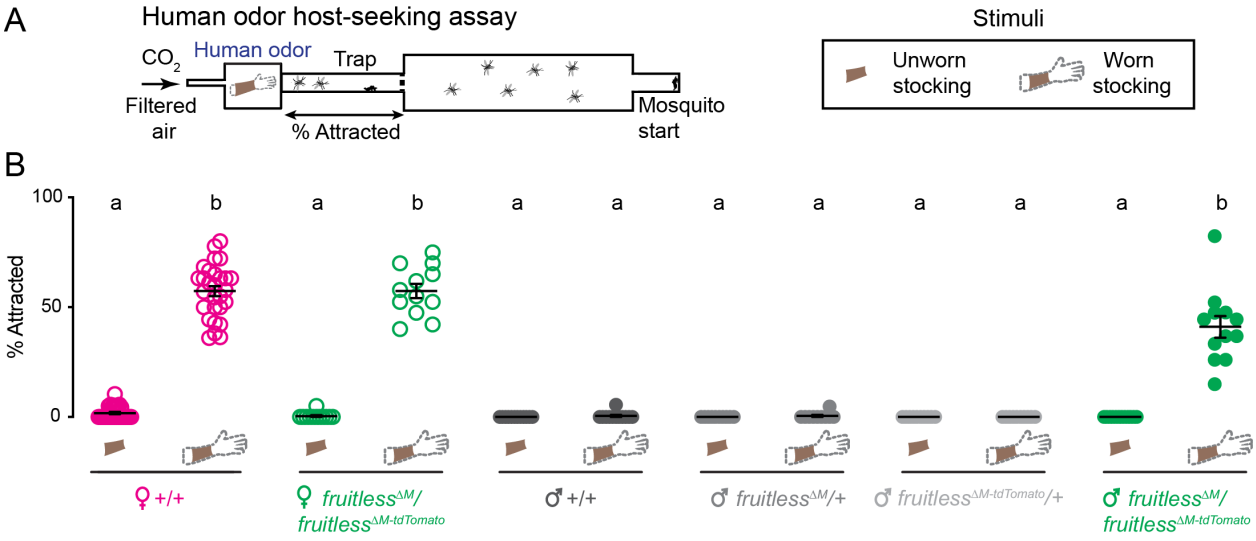

Figure 3.18 Olfactory cues selectively drive male *fruitless* mutant attraction to humans

(A) Schematic of human odor host-seeking assay (left) and stimuli (right).
(B) Percent of attracted animals. Data are mean±s.e.m., n = 8-14 trials/group, n = 17-28 mosquitoes/trial. Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$. Comparisons are made across all genotypes and stimuli.

Only female *Aedes aegypti* mosquitoes host-seek, and we have shown that mutating *fruitless* reveals an attraction to human odor in the male mosquito (Figure 3.19A). Previously, *fruitless* was shown to be required for male mating behavior in both *Drosophila melanogaster* (Demir and Dickson, 2005) and *Bombyx mori* silkmoths (Xu *et al.*, 2020). Our work demonstrates that in *Aedes aegypti* mosquitoes, *fruitless* has acquired a novel role in inhibiting female host-seeking behavior in the male (Figure 3.19B).

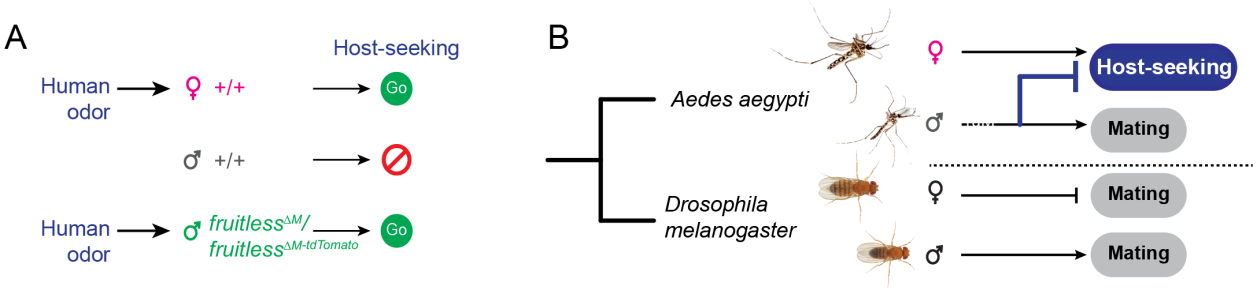

Figure 3.19 Model of *fruitless* function in the mosquito

Summary of results (A) and model of gain of fruitless function in *Aedes aegypti* (B). Photo credit: *Aedes aegypti* (Alex Wild); *Drosophila melanogaster* (Nicolas Gompel).

CHAPTER 4. *11211* is a novel sex-specifically spliced gene

In Chapter 2, we described the analysis of sex-specific alternative splicing across blood-feeding mosquitoes, and mentioned several sex-specifically spliced genes, including *11211*. The original name of this gene was *AAEL011211*, and it will be referred to as *11211* hereon. Many sex-specific spliced genes have been described in *Drosophila melanogaster*, the most prominent of which include *doublesex* and *fruitless*, which was described in the previous chapter. *11211* was unique as it was sex-specifically spliced in mosquitoes, but not in *Drosophila melanogaster.* This species-specific splicing hinted that this gene may play a role in mosquito-specific behavior, warranting closer investigation of this gene. In this chapter, we describe the evolution of *11211* and its expression across insects, characterize the subcellular localization of sex-specific proteins in the mosquito, and begin to describe the role of the gene in mosquito behavior.

4.1 Sex-specific splicing of *11211* is conserved across the insects

We found that *11211* was sex-specifically spliced in the brains of all three blood-feeding mosquito species, but not in non-blood-feeding *Drosophila melanogaster*, suggesting that this gene may have a role in mosquito-specific behavior such as blood feeding. Initially, *11211* did not have an identifiable homolog in *Drosophila*, due to the well-described weaknesses of BLAST homology searches (Weisman et al., 2020). This led to the initial idea that *11211* was a mosquito-specific gene. However, using more

sensitive methods of homology detection such as jackhmmer and PSI-BLAST (Altschul *et al.*, 1997; Johnson et al., 2011), we discovered that *11211* has orthologues in species across the insect phylogeny, including *Diptera* (flies), *Coleoptera* (beetles), *Hymenoptera* (ants and bees), and *Hemiptera* (bugs). How do we know that these putative *11211* orthologs are truly homologous, and not spuriously annotated genes without any relationship to mosquito *11211*? There are three lines of evidence: sex-specific splicing, sequence homology, and synteny.

11211 was identified on the basis of mosquito-specific sex-specific splicing. The *Drosophila melanogaster* homolog is not sex-specifically spliced, and neither is the homolog in other flies such as *Ceratitis capitata* and *Stomoxys calcitrans*. However, in every other insect species for which we have RNA-seq data, *11211* is sex-specifically spliced into a male and female specific transcript (Figure 4.1A-B). In all these species, female *11211* transcripts contain a female-specific exon, such that the female transcript is always longer (Figure 4.1B). In the mosquitoes, this female-specific exon contains a premature stop codon that truncates the female-specific protein. Both male and female mosquito proteins share the first 300-400 amino acids, with a short female-specific C-terminal end, while males encode a longer 900-1000 amino acid protein with a long male-specific C-terminus. In all other insects, including ants, bees, beetles, and bedbugs, the female-specific exon of *11211* encodes a longer female-specific portion, leading to a longer female and shorter male protein where the first portion of the protein is common between the sexes (Figure 4.1C). What are the functional domains of these proteins? There are no annotated protein domains in any species, and no predicted

secretion signal sequences, or transmembrane domains. *In-silico* prediction suggested that most 11211 proteins contain nuclear localization sequences as predicted by three different algorithms (Brameier et al., 2007; Kosugi et al., 2009; Nguyen Ba et al., 2009) (Figure 4.1C). However, given the low fidelity of these prediction algorithms, this prediction will need to be experimentally validated in each species. In addition, *11211* has duplicated in the *Hymenopterans*, with a paralog located adjacent to the original gene. This paralog is sex-specifically spliced, but shows divergence in the effect of sex-specific splicing on the sex-specific protein. In *Harpegnathos saltator,* the paralog is sex-specifically spliced in the 3' untranslated region (UTR), leading to no sex-differences in the protein. In *Apis mellifera*, the paralog is sex-specifically spliced to lead to a short female and long male protein, opposite to the *11211* homolog, and similar to *11211* in the mosquitoes. Therefore, sex-specific splicing of *11211* is conserved across the insects, but the effect of this splicing on the sex-specific protein is dramatically different across divergent species.

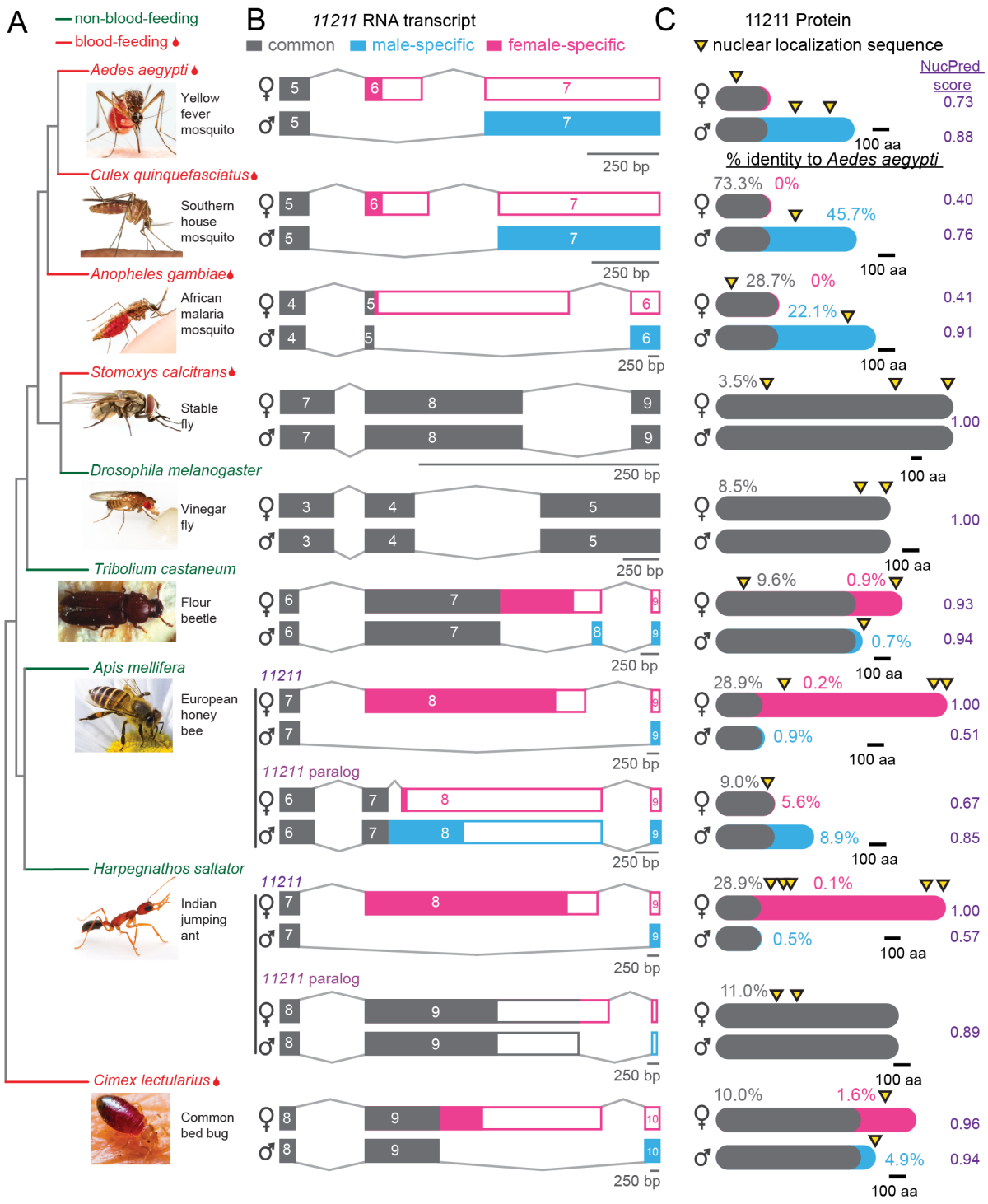

Figure 4.1 Sex-specific splicing of *11211* across the insects

(A) Selected insect species. Phylogenetic relationships are true, but distances are not to scale.
(B) Structure of *11211* RNA sex-specific splicing in each species as inferred from sex-specific RNA-seq data.
(C) Predicted sex-specific 11211 proteins with annotated nuclear localization sequences as predicted by cNLSmapper, NLStradamus, and NucPred scores.

Sequence homology is based on the alignment of protein sequences and detection of conserved sequences across species. As mentioned above, this method can fail to detect homologous genes that show elevated rates of sequence divergence. *11211* orthologues show variable levels of conservation, with Hymenopteran *11211* proteins showing relatively higher levels of sequence identity to *Aedes aegypti 11211*, while *Drosophila melanogaster* shows lower levels of homology. There are orthologs in basal insect species such as the cockroach *Blattella germanica,* and the dragonfly *Ladona fulva*, but since these species lack high-quality genomes and annotations, they were excluded from further analysis. One notable insect order with high-quality genomes but without an identifiable *11211* ortholog was the *Lepidoptera* (butterflies and moths), suggesting that this gene has been lost in this order. Together, we have identified orthologs of *11211* in most major insect families. However, in all the putative *11211* orthologs described, there is a domain of sequence with relatively high protein identity (Figure 4.2). This putative domain is located within the first 100-400 amino acids of each 11211 protein, and shows relatively higher levels of conservation across species relative to the rest of the protein. The rest of the protein shows weak levels of conservation across species outside the mosquito. This line of evidence suggests that there is a relatively well conserved domain of *11211*, which is identifiable across insect species.

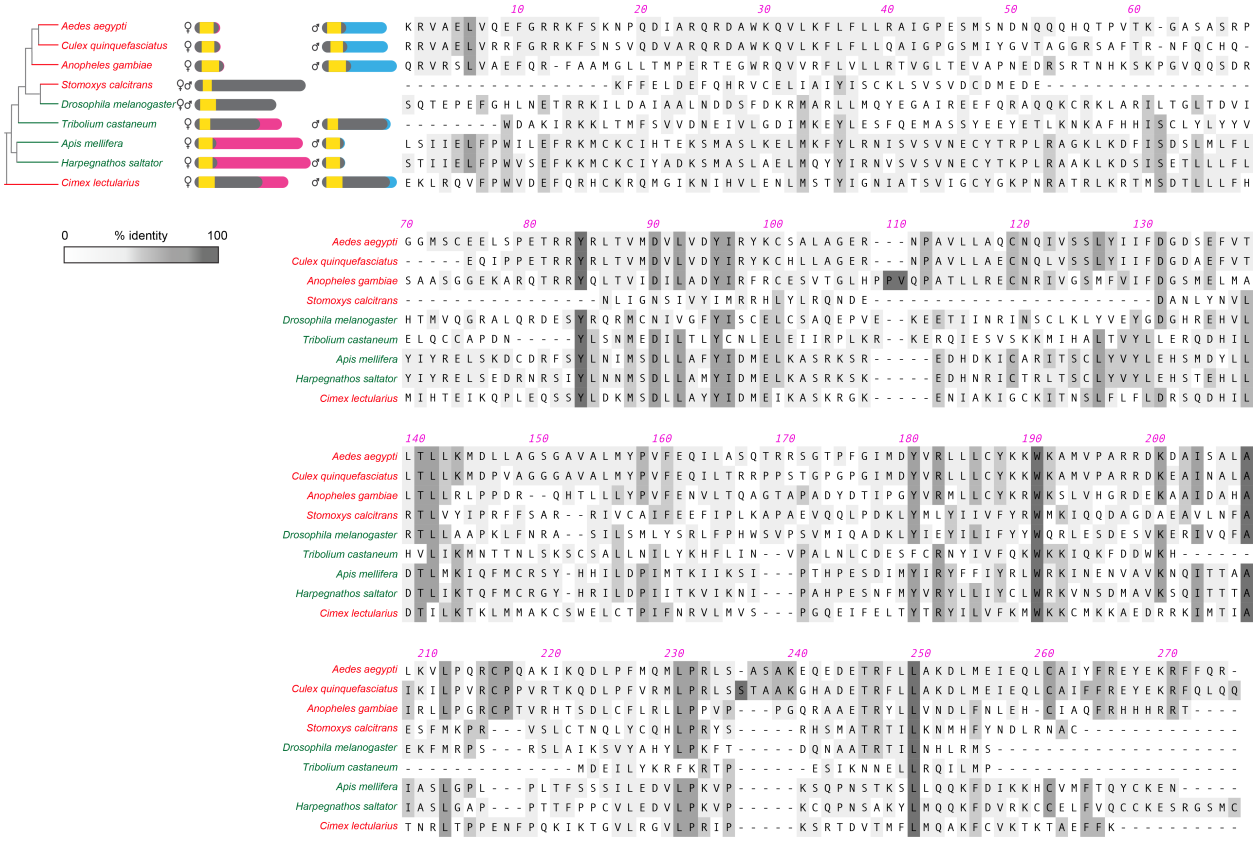

Figure 4.2 Conserved domains in the common region of *11211*

Schematic of the sex-specific 11211 proteins, with a conserved protein domain highlighted in yellow. Multiple sequence alignment of the conserved domain in each species colored by percent identity.

Synteny is the conservation of the order of neighboring genes. Insect genomes are rapidly evolving, so it is possible to identify blocks of synteny in every single species. In all mosquito species, the same five genes were found on either side of *11211*. One of these genes, encoding the predicted melatonin receptor, was found next to the putative *11211* ortholog in both the Indian jumping ant *Harpegnathos saltator* and the European honey bee *Apis mellifera (*Figure 4.3). The melatonin receptor has been lost in multiple insect orders, including *Lepidoptera* and most *Diptera* including *Drosophila.* It is thus

surprising that *11211* and the melatonin receptor show synteny in the mosquito and the ant/bee, insects that diverged over 300 million years ago, adding one line of evidence that these genes are orthologous.

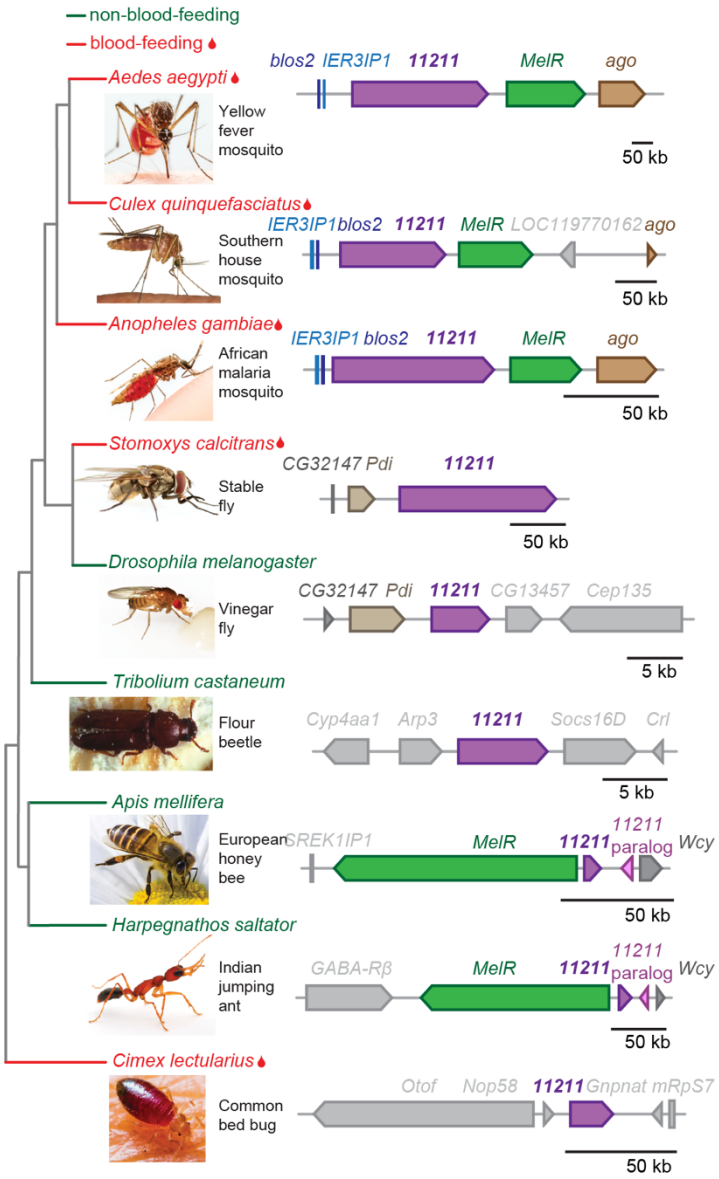

Figure 4.3 Synteny of *11211* in insects

Selected insect species (phylogenetic relationships are true, but distances are not to scale) with organization of *11211* locus (in purple) in each species.

In summary, *11211* is a new example of a sex-specifically spliced gene that encodes distinct proteins in the sexes. These proteins have generally diverged in sequence, but contain a conserved domain that has identifiable sequence homology across insects. *11211* shows some level of synteny within the mosquitoes, and between mosquitoes and ants/bees. While non-mosquito flies have lost sex-specific splicing of *11211*, all other insects with an identifiable homolog and high-quality RNA-seq data show sex-specific splicing of this gene. However, the mode of this splicing is divergent between mosquitoes and other insects, with mosquitoes encoding a short female protein, while in other insects females encode a longer protein than males. This gene therefore displays an interesting pattern of evolution across the insects, but particularly within the mosquitoes.

4.2 *11211* is expressed in the subesophageal zone of the mosquito brain

We next asked where in the brain *11211* was expressed in a number of species. We turned to *in situ* hybridization with probes targeting a pan-neuronal gene *Syt1* and the sexually isomorphic region of the gene, thus recognizing the *11211* transcript in both males and females. We carried out these experiments in male and female brains of *Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles gambiae, Drosophila melanogaster, Tribolium castaneum*, and *Harpegnathos saltator*. As expected, *Syt1* was broadly expressed in neurons throughout the brain. We found that in all the non-mosquito species, *11211* was broadly expressed in neurons throughout the brain in both males and females (Figure 4.4). In contrast to *Syt1*, which were expressed in a ‘donut’ like

pattern in the cytoplasm of a cell, *11211* showed punctate expression in the nucleus and the cytoplasm. This suggests that while *11211* is broadly expressed in these basal insect species, it has relatively low levels of expression within each cell.

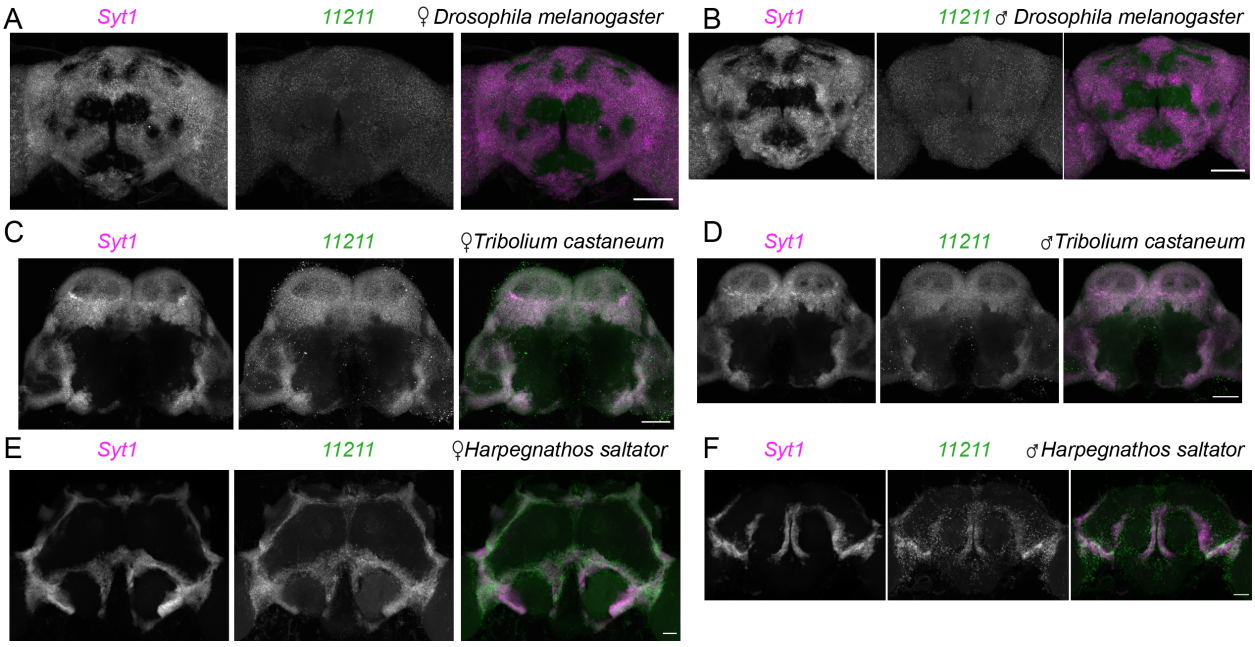

Figure 4.4 Expression of *11211* and *Syt1* in the brains of non-mosquito insects

(A-F) Confocal image maximum projections of RNA *in situ* hybridization with probes against *Syt1* (magenta) and the sexually isomorphic region of *11211* (green) in *Drosophila melanogaster* female brain (A), *Drosophila melanogaster* male brain (B), *Tribolium castaneum* female brain (C), *Tribolium castaneum* male brain (D), *Harpegnathos saltator* female brain (E), and *Harpegnathos saltator* male brain (F). All scale bars are 100 μm.

We then examined the expression of *11211* in the brains of male and female mosquitoes. In *Anopheles gambiae, 11211* was relatively broadly expressed throughout the brains of both males and females. However, in *Culex quinquefasciatus* and *Aedes aegypti*, *11211* is expressed at higher levels within a subset of cells (Figure 4.5).

Notably, there was no obvious sexual dimorphism in either the level of expression or the number of cells expressing *11211* in either species.

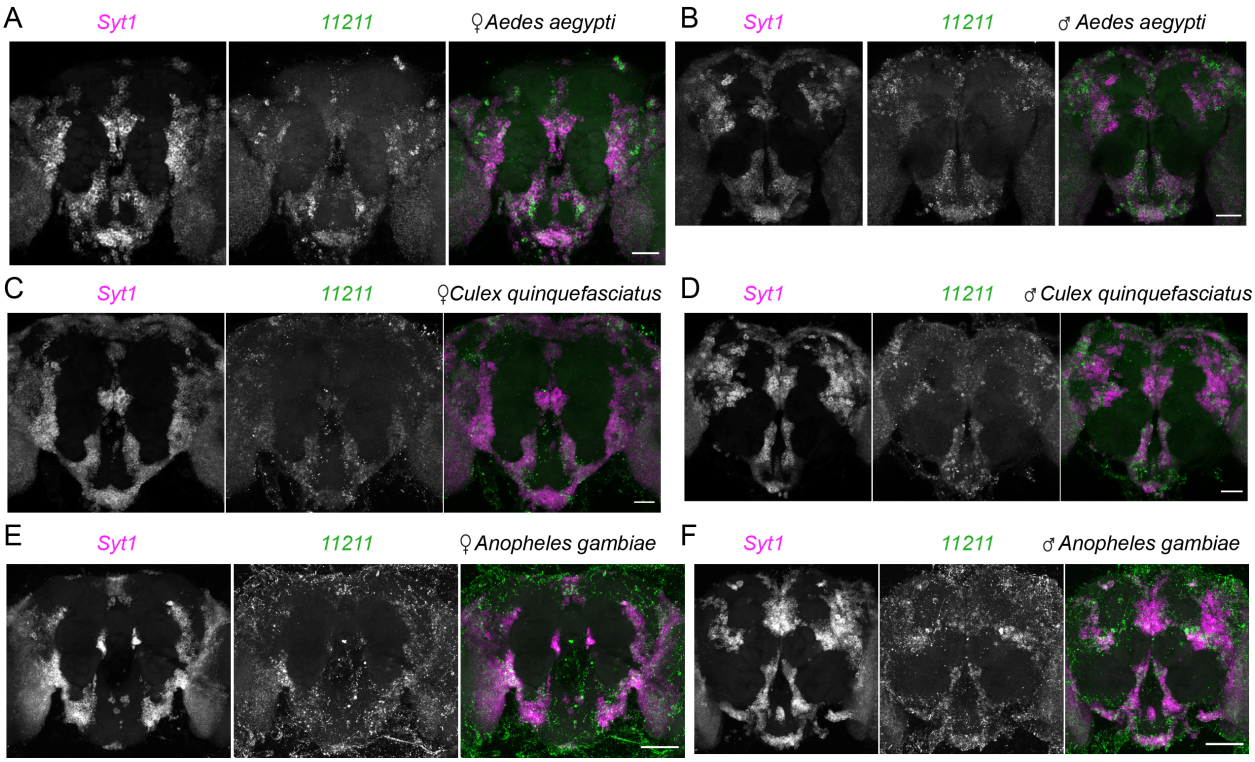

Figure 4.5 Expression of *11211* and *Syt1* in the brains of mosquitoes

(A-F) Confocal image maximum projections of RNA *in situ* hybridization with probes against *Syt1* (magenta) and the sexually isomorphic region of *11211* (green) in *Aedes aegypti* female brain (A), *Aedes aegypti* male brain (B), *Culex quinquefasciatus* female brain (C), *Culex quinquefasciatus* male brain (D), *Anopheles gambiae* female brain (E), and *Anopheles gambiae* male brain (F). All scale bars are 100 μm.

In *Aedes aegypti*, these cells are dispersed through the brain, with notable concentrations in the superior protocerebrum and in the subesophageal zone (Figure 4.6) in both male and female brains. The superior protocerebrum is a region of the brain

that receives input from various sensory inputs, including olfactory, visual, and thermosensory neurons, and integrates these inputs to guide multiple behaviors. The subesophageal zone is a region that receives input from gustatory sensory neurons in the stylet, labium, and legs, and is thought to guide feeding and oviposition behaviors. The expression of *11211* in the subesophageal zone was hence notable, and suggested a potential role of this gene in guiding feeding behavior.

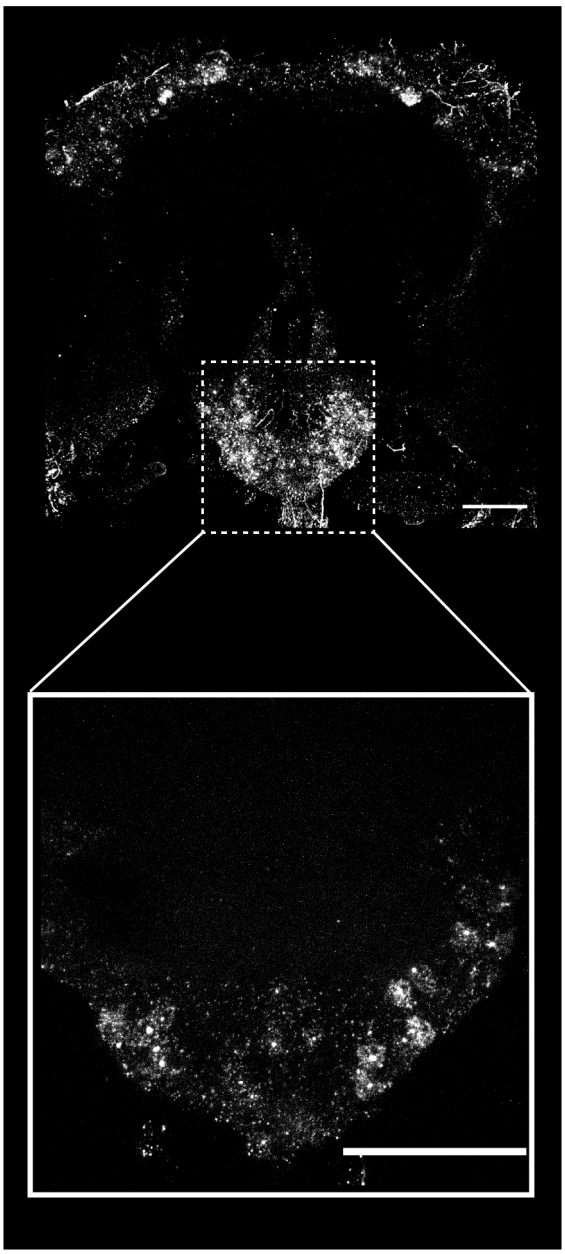

Figure 4.6 Expression of *11211* in the subesophageal zone

Confocal image maximum projection of RNA *in situ* hybridization with probes against *11211* in a female *Aedes aegypti* brain, zooming in on a projection of 10 slices in the subesophageal zone on the bottom panel. Scale bars are 50 μm.

In *Aedes aegypti,* we asked whether *11211* is expressed in neurons or glia, and if neurons, what the identity of the neurotransmitters these cells express. Using probes against *11211,* the pan-neuronal marker *nsyb*, and the glial marker *repo*, we found that

11211 was co-expressed with *nsyb* and never with *repo* (Figure 4.7). We then probed against the genes *vacht*, *vglut*, and *gad1*, which are markers of cholinergic, glutamatergic, and GABAergic neurons respectively. We found that *11211* was co-expressed with all three neuronal subtypes (Figure 4.8).

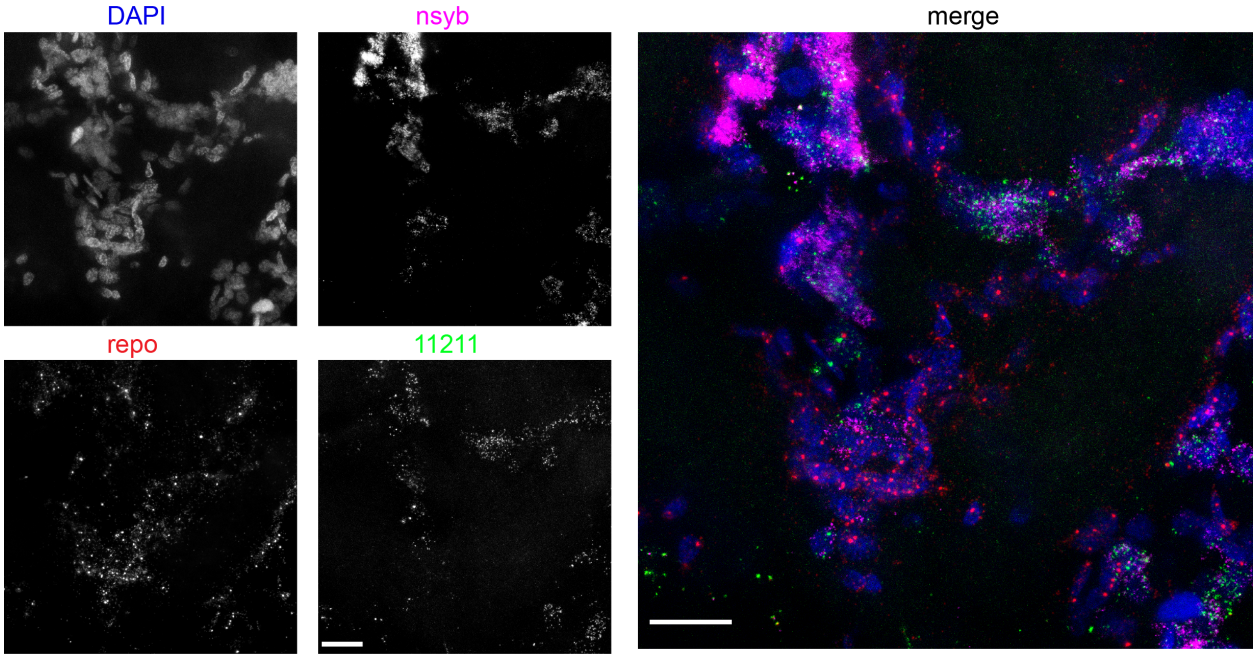

Figure 4.7 Expression of *11211* within neurons

Confocal image maximum projection of RNA *in situ* hybridization with probes against *nsyb* (magenta), *repo* (red), *11211* (green) and DAPI counterstain for nuclei (blue) in female *Aedes aegypti* brains, zooming in on the superior protocerebrum. Scale bars are 20 μm.

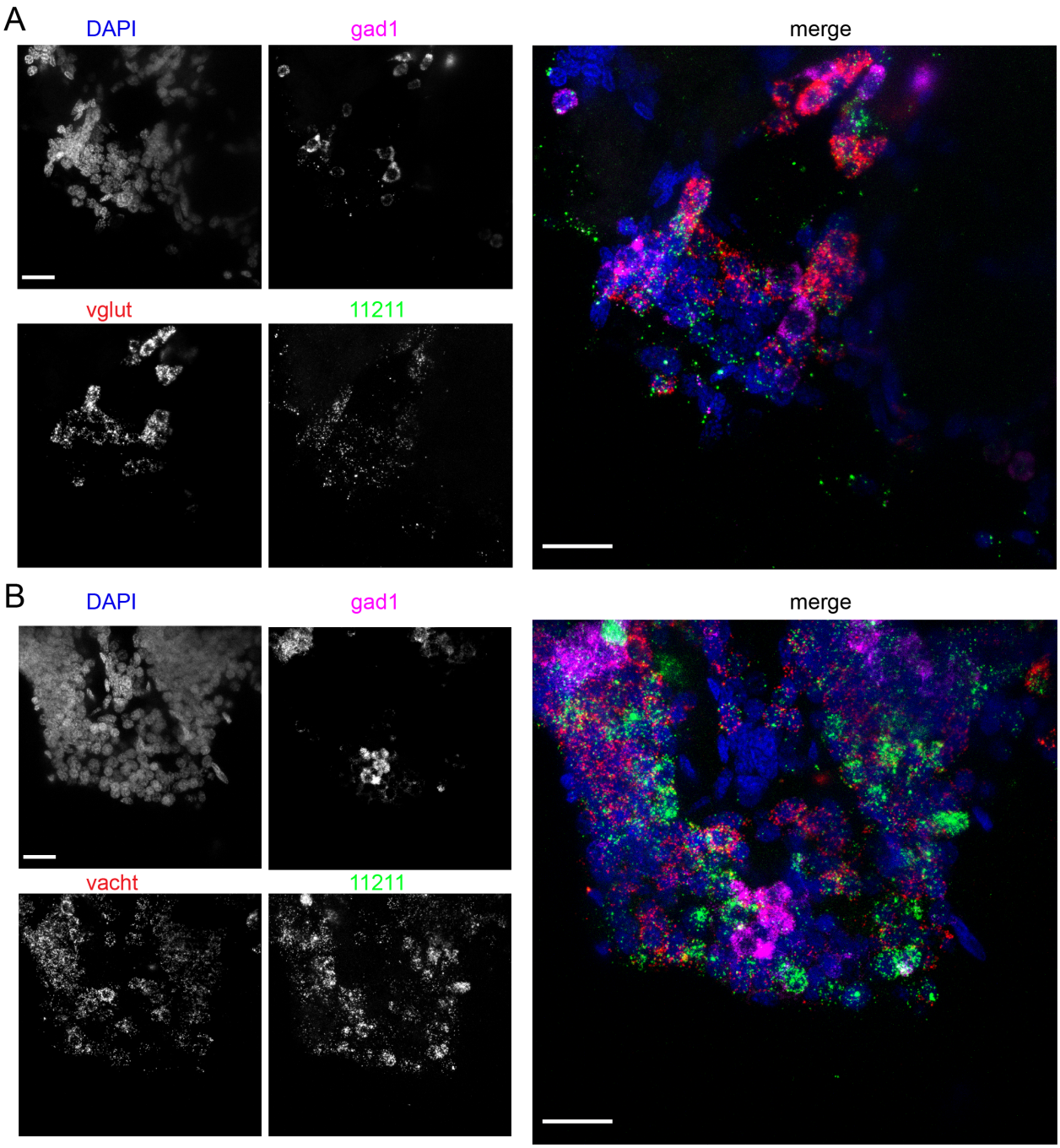

Figure 4.8 Expression of *11211* within neuronal subtypes

(A) Confocal image maximum projection of RNA *in situ* hybridization with probes against *gad1* (magenta), *vglut* (red), *11211* (green) and DAPI counterstain for nuclei (blue) in female *Aedes aegypti* brains, zooming in on the subesophageal zone.
(B) Confocal image maximum projection of RNA *in situ* hybridization with probes against *gad1* (magenta), *vacht* (red), *11211* (green) and DAPI counterstain for nuclei (blue) in female *Aedes aegypti* brains, zooming in on the subesophageal zone. All scale bars are 20 μm.

In summary, our RNA *in situ* hybridization data suggest that in addition to the evolution of the protein and mode of splicing, the expression pattern of *11211* has diverged over the course of evolution. In the basal insects and *Drosophila melanogaster*, this gene is broadly expressed at lower levels in most neurons. However in the mosquitoes, and particularly in the Culicine mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*, *11211* shows enriched expression within a subset of neurons. Some of these neuronal cell bodies are within the subesophageal zone, which is a region of the brain thought to control feeding.

4.3 *11211* expression in *Aedes aegypti* across tissues and time

We now focus on the expression of *11211* in *Aedes aegypti*. We showed that it is expressed in the brain, where it is sex-specifically spliced. A number of questions remain. Is it expressed during development? Is *11211* expressed in other tissues? Is it sex-specifically spliced in other tissues where it is expressed? Is there any change in brain expression or splicing during distinct internal states, such as before or after a blood-meal? Here, we leverage previously collected RNA-sequencing datasets to profile the expression of *11211* across tissues and states.

We first asked when *11211* was expressed in development, using previously published RNA-seq data from pooled whole bodies of all larval and pupal stages of *Aedes aegypti* (Matthews et al., 2018). In general, *11211* was expressed at very low levels in both male and female larvae, with a median expression of under 0.5 transcripts

per million (TPM) at each larval stage. However, *11211* expression rose in pupae, to 1.5 TPM in females and 0.5 TPM in males (Figure 4.9). During the pupal phase, adult neural circuits begin to develop and refine. The rise in expression of *11211* in this stage could suggest a potential role in controlling pupal or adult behavior. Since we lack tissue specific RNA-seq data or RNA *in situ* hybridization of the pupal brain, it is unclear whether this expression is specific to the brain or broad. There is also a slight sexual dimorphism in expression levels, as female pupae express higher levels of *11211* than male pupae. However, as this is bulk data, additional transcripts from male-specific cells may artificially dilute the expression of *11211,* so this potential sexual dimorphism in pupal expression will need to be verified with additional methods such as RNA *in situ* hybridization or single-cell RNA-seq.

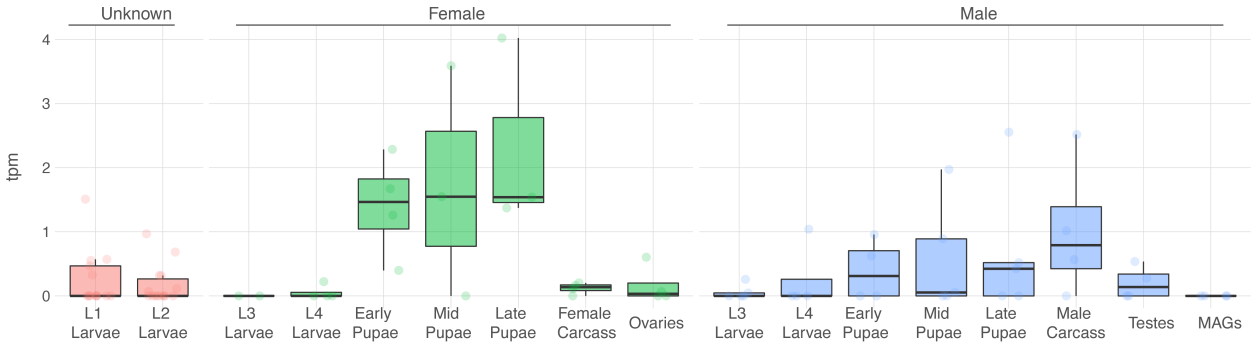

Figure 4.9 Expression of *11211* during development

Expression levels of *11211* (in TPM) from RNA-seq data of whole larvae and pupae during development. Data from (Matthews *et al.*, 2018). L1 and L2 larvae were not sexed prior to sequencing.

We then asked in which tissues *11211* was expressed in the adult. Analyzing tissue-specific deep RNA sequencing (Matthews *et al.*, 2016), we found that *11211* was expressed at high levels in the brain and antenna of both males and females (Figure 4.10). It was expressed at very low levels (median < 2 TPM) in all other tissues. In the brain, it was expressed at comparable levels in females and males, suggesting that there is not much sexual dimorphism in expression levels, consistent with results from RNA *in situ* hybridization. In the antenna, there was higher expression of *11211* in females than in males. However, since male antennae only dedicate 2 of their 13 antennal segments to olfaction, this dimorphism in expression would be expected if *11211* was expressed in olfactory sensory neurons, and would not reflect a difference in expression level per cell (Matthews *et al.*, 2016). *11211* is sex-specifically spliced in the antenna as it is in the brain (Figure 4.11). We looked at two additional RNA-seq datasets (Nadav Shai, Leah Houri-Zeevi, unpublished), including the midgut (Figure 4.12) and ventral nerve cord (Figure 4.13). 11211 was not expressed at high levels in the midgut, but was expressed at moderate levels in the ventral nerve cord. In summary, *11211* is expressed in the brain, ventral nerve cord, and antenna, consistent with the idea that it is enriched within the nervous system.

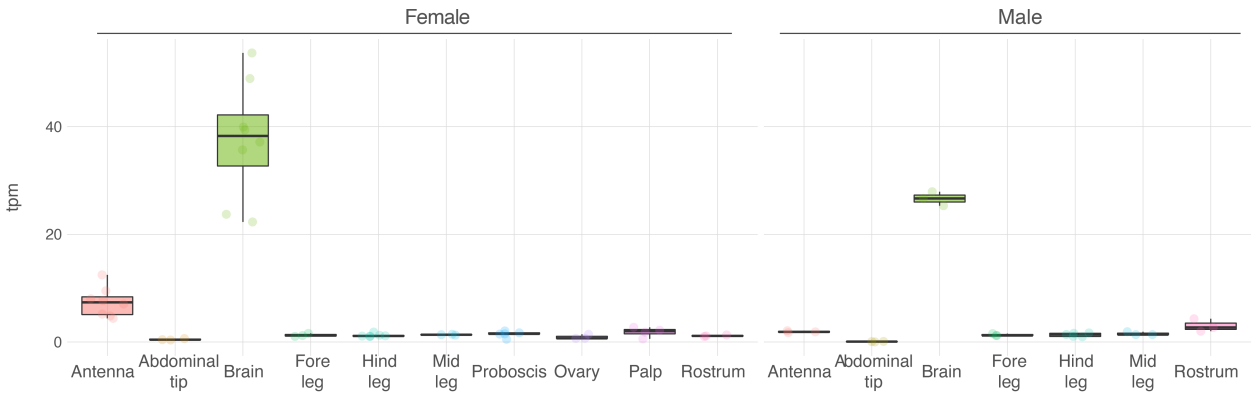

Figure 4.10 Expression of *11211* in adult tissues

Expression levels of 11211 (in TPM) from RNA-seq data of sugar-fed mated adult tissues. Data from (Matthews *et al.*, 2016).

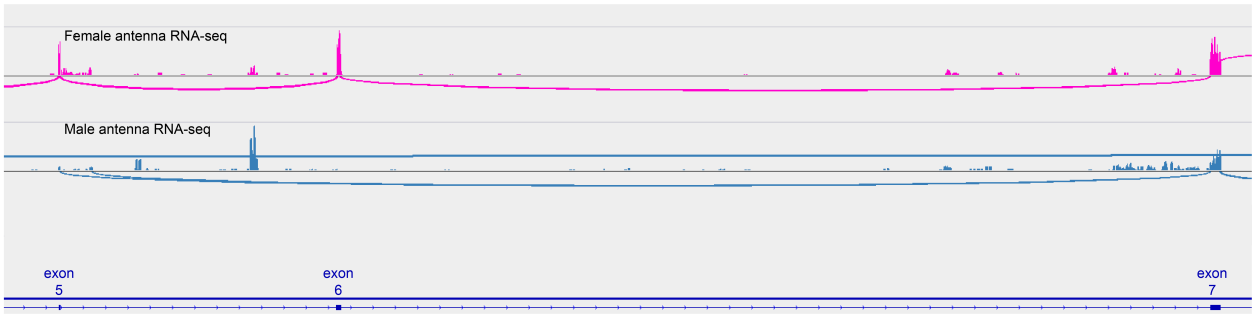

Figure 4.11 Sex-specific splicing of *11211* in the antenna

Sashimi plot showing *11211* locus with RNA-seq tracks from male and female antennae. Data from (Matthews *et al.*, 2016).

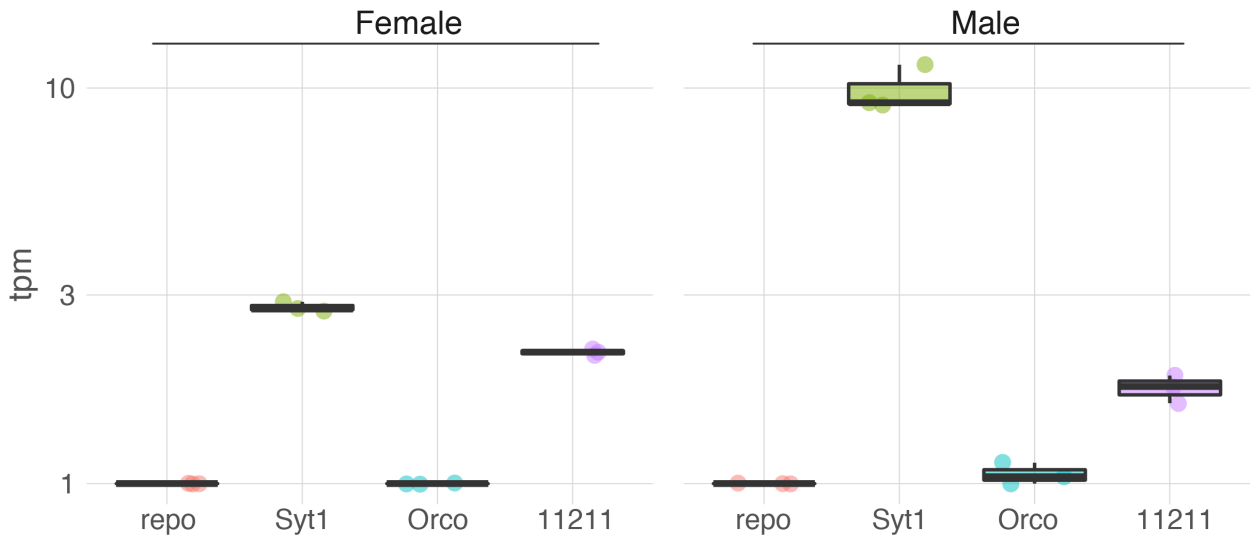

Figure 4.12 Expression of *11211* in the sugar-fed adult midgut

Expression levels of *repo*, *syt1*, *orco*, and *11211* (in TPM) from RNA-seq data of sugar-fed mated adult midguts. Data from Nadav Shai (unpublished).

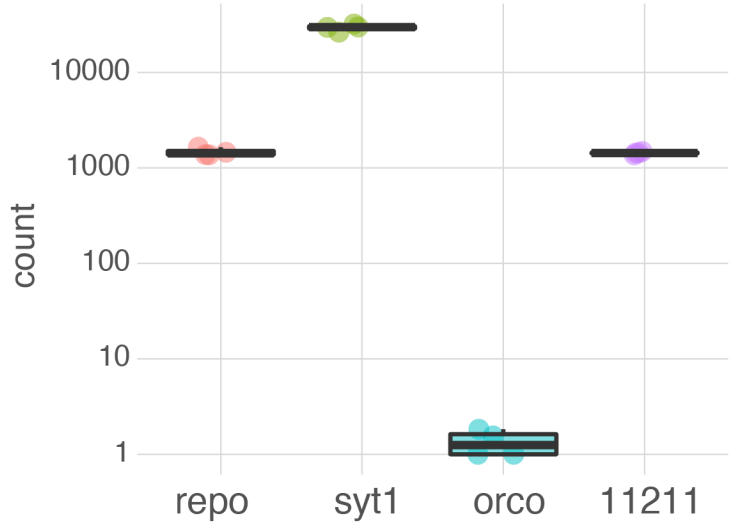

Figure 4.13 Expression of *11211* in the female ventral nerve cord

Expression levels of *repo*, *syt1*, *orco*, and *11211* (in normalized counts) from RNA-seq data of sugar-fed virgin adult female ventral nerve cords. Data from Leah Houri-Zeevi (unpublished).

Does *11211* expression change in the brain over different internal states? After a blood-meal, female mosquitoes suppress their host-seeking and blood-feeding behaviors. We found that *11211* expression did not significantly change in female brains 48 or 96 hours after being blood-fed (Figure 4.14). However, in the antenna, *11211* expression was ~3-fold reduced 48 hours after a blood meal, and recovered to baseline levels by 96 hours post blood meal (Figure 4.15). A female mosquito also shows differences in behavior before and after mating with a male. There are no significant changes in *11211* expression in the brain before and after mating (Figure 4.16). We then looked at splicing, and saw that sex-specific splicing of *11211* was consistent in the brain and antenna in all blood-feeding states (Figure 4.17). Therefore, while there may be blood-meal dependent regulation of *11211* in the antenna, *11211* splicing and expression remain relatively stable in the brain over different internal states.

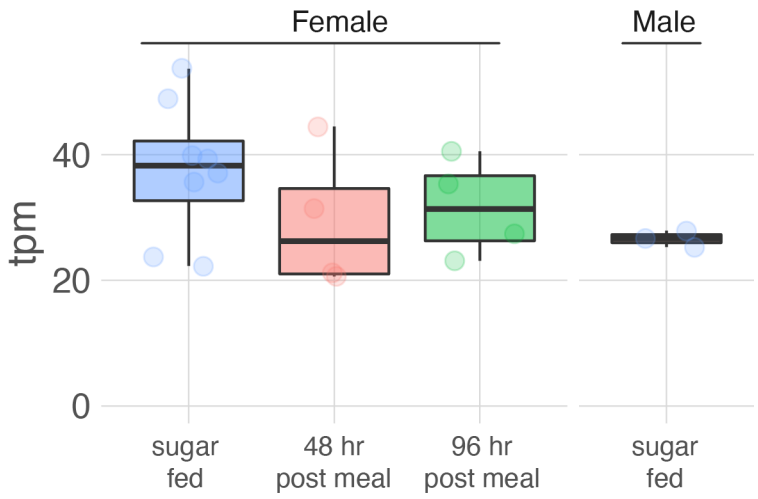

Figure 4.14 Expression of *11211* in the brain after a blood-meal

Expression levels of *11211* (in TPM) from RNA-seq data of mated adult brains from sugar-fed males and females or blood-fed females at 48 or 96 hours post blood-meal. Data from (Matthews *et al.*, 2016).

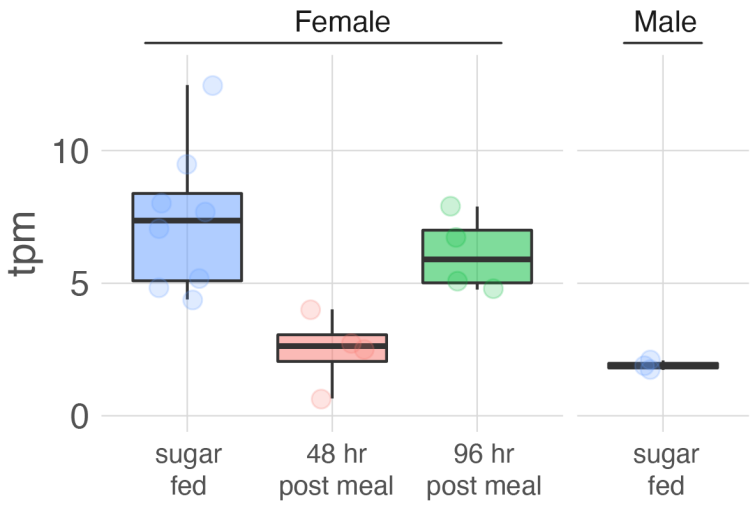

Figure 4.15 Expression of *11211* in the antenna after a blood-meal

Expression levels of *11211* (in TPM) from RNA-seq data of mated adult antennae from sugar-fed males and females or blood-fed females at 48 or 96 hours post blood-meal. Data from (Matthews *et al.*, 2016).

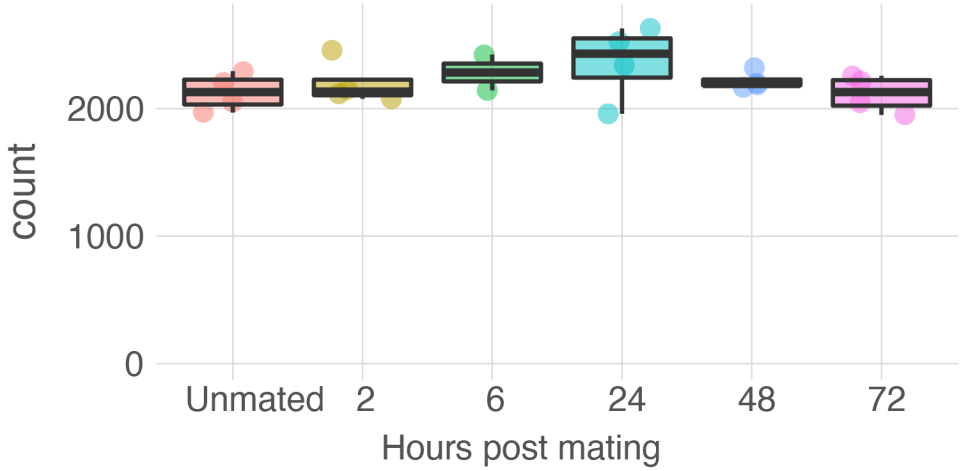

Figure 4.16 Expression of *11211* in the female brain after mating

Expression levels of *11211* (in normalized counts) from RNA-seq data of sugar-fed adult female brains at selected time-points after mating. Data from Leah Houri-Zeevi (unpublished).

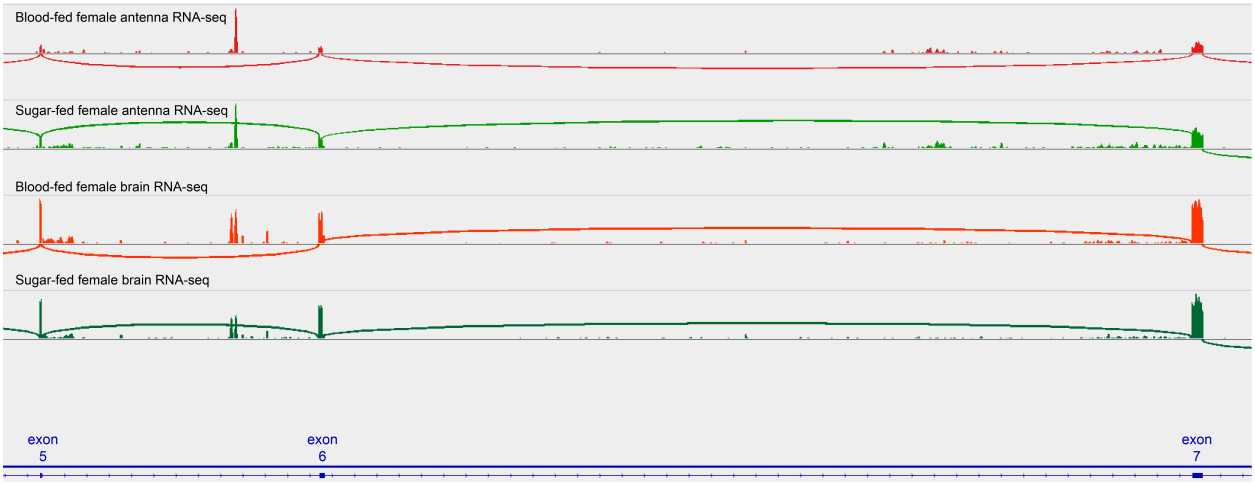

Figure 4.17 No change in sex-specific *11211* splicing after a blood-meal

Sashimi plot showing *11211* locus with RNA-seq tracks from 48 hours post blood-meal and sugar-fed female antennae and brains. Data from (Matthews *et al.*, 2016).

4.4 *11211* encodes nuclear proteins in both sexes

We have extensively described the sex-specific splicing of *11211*. What is the functional consequence of sex-specific splicing on sex-specific protein expression? Splicing could be “destructive”, where the female-specific premature stop codon leads to the lack of a female protein due to nonsense-mediated decay. Alternatively, splicing could be “constructive”, leading to a functional protein in both sexes. What is the function of the protein, if it is present in either sex? A hint to this question can be found by looking at where in the cell the protein localizes too. Attempts to generate specific antibodies against the common region of 11211 were unsuccessful. Therefore, to address these linked questions, we used CRISPR-Cas9 to knock-in an epitope tag into the endogenous *11211* locus in a sex-specific manner.

We used CRISPR-Cas9 to knock-in an epitope tag into the end of the female coding region in the 6th exon of *11211* (Figure 4.18). We knocked in 2 copies of the hemagglutinin tag (2x-HA), which is a nine amino-acid long epitope derived from the human influenza virus, that is specifically recognized by anti-HA antibodies. In the *11211*$^{F\text{-}HA}$ strain, sex-specific splicing should ensure that the HA tag is only detected in females, and not in males of the same genotype. We also knocked-in a 2x-HA tag into the end of the male coding region in the 9th exon (Figure 4.19). In the *11211*$^{M\text{-}HA}$ strain, only males should express the HA tag, and not females of the same genotype.

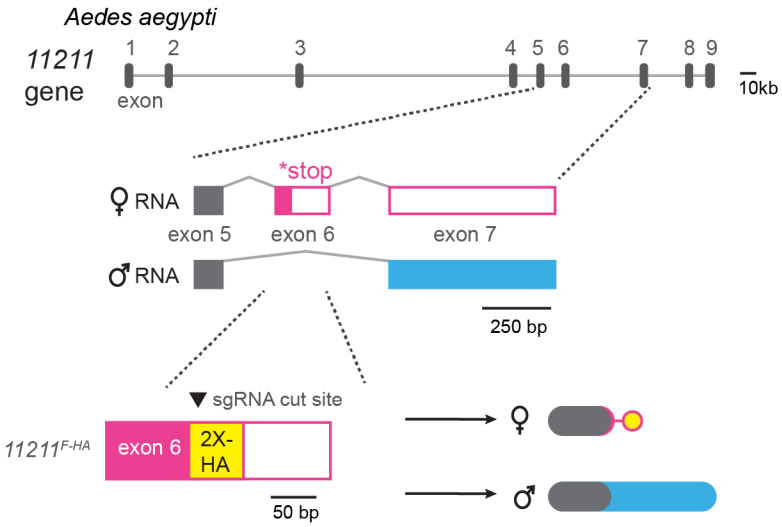

Figure 4.18 Generation of a *11211*$^{F\text{-}HA}$ strain

Schematic of the *11211* locus, showing sex-specific RNA splicing, and location of the CRISPR/Cas9-mediated 2x-HA epitope tag insertion with predicted sex-specific proteins. The 2X-HA epitope is indicated by the yellow circle.

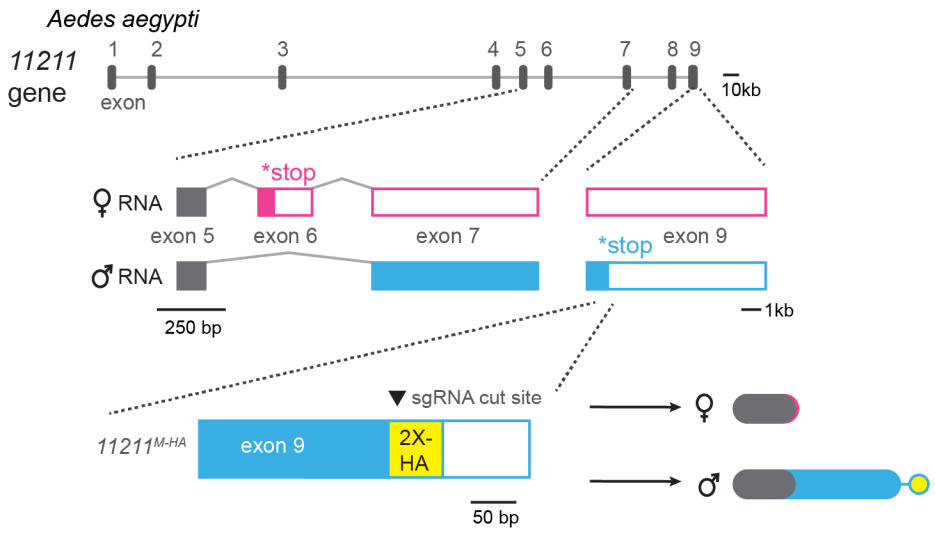

Figure 4.19 Generation of a *11211^{M-HA}* strain

Schematic of the *11211* locus, showing sex-specific RNA splicing, and location of the CRISPR/Cas9-mediated 2x-HA epitope tag insertion with predicted sex-specific proteins. The 2X-HA epitope is indicated by the yellow circle.

We immunostained with an HA antibody in the brains of males and females of both the *11211^{F-HA}* and *11211^{M-HA}* genotypes. We focused on the subesophageal zone (Figure 4.20A), since we knew from RNA *in situ* hybridization that *11211* was densely expressed in this region of the brain. We found that HA-tagged 11211 protein was detected only in females of the *11211^{F-HA}* strain, and not in males of the same genotype (Figure 4.20B-C). Likewise, we only found HA-tagged 11211 protein in males of the *11211^{M-HA}* strain, and not females of the same genotype (Figure 4.20D-E). This result confirms that sex-specific splicing leads to distinct proteins in the two mosquito sexes, and is constructive in the female.

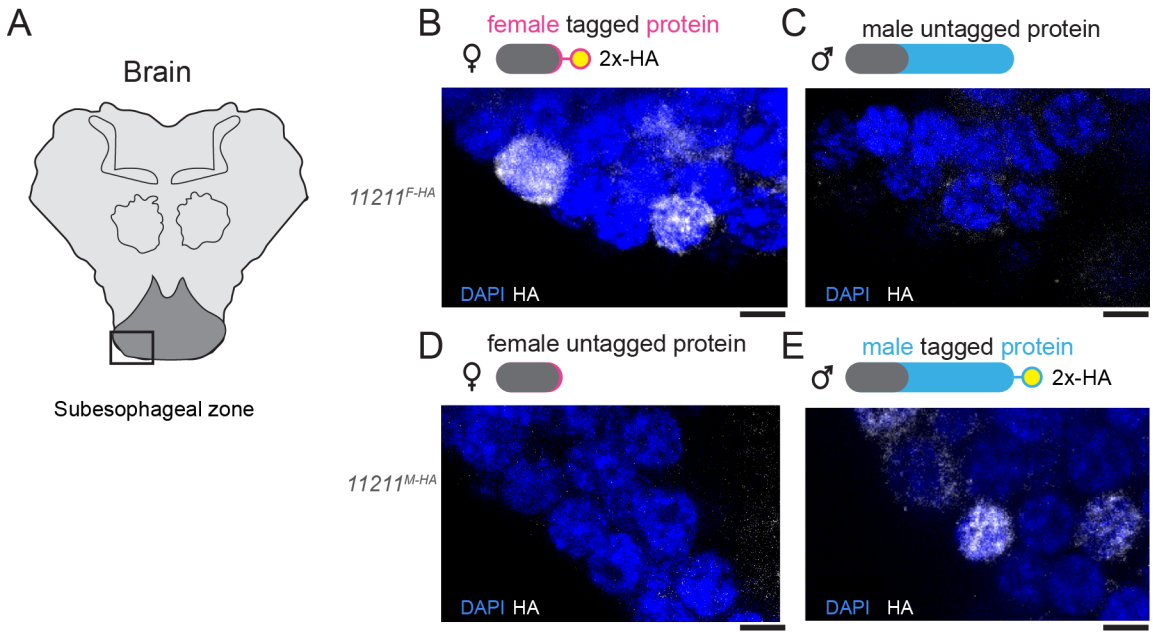

Figure 4.20 *11211* splicing leads to sex-specific proteins that localize to the nucleus

(A) Schematic of the mosquito brain with the subesophageal zone highlighted.
(B)-(E) Immunostaining of the subseophageal zone of brains of the indicated genotypes with anti-HA antibody (white) and DAPI (blue). Scale bars represent 5 μm.

We previously described how both male and female 11211 proteins contain a nuclear localization sequence. Using our endogenously tagged strains, we were able to ask where in the cell these proteins are localized. Consistent with the predictions, 11211 proteins localized to the nucleus in both sexes (Figure 4.20B,E). The proteins were localized in puncta throughout the nucleus. In summary, *11211* sex-specific splicing is constructive, with transcripts encoding nuclear proteins in both sexes.

4.5 Sex-specific splicing of *11211* may be required for blood-feeding behavior

Given the expression of *11211* in the subesophageal zone of the brain, we wondered whether it had a role in controlling mosquito blood-feeding behavior. To test this, we generated a knock-in/knock-out *11211* mutant line using CRISPR-Cas9 (Kistler *et al.*, 2015). We targeted the third exon of *11211*, and used homologous recombination to knock-in a cassette encoding the ribosome-skipping peptide T2A followed by the transcriptional activator QF2 (Matthews *et al.*, 2019; Potter et al., 2010; Riabinina et al., 2016), an SV40 transcriptional terminator (Figure 4.21). This 11211^{QF2} construct disrupts *11211* in both males and females, while also allowing QF2 to be expressed in the cells in which *11211* is normally expressed. In order to rescue the function of *11211* we also generated transgenic lines that express either the full-length male and female 11211 proteins downstream of the QUAS binding site. QF2 binds to the QUAS sequences to drive expression of the desired transgene in the cells in which QF2 is expressed. We verified that QF2 is faithfully expressed within cells that express endogenous *11211* mRNA using RNA *in situ* hybridization (Figure 4.22)

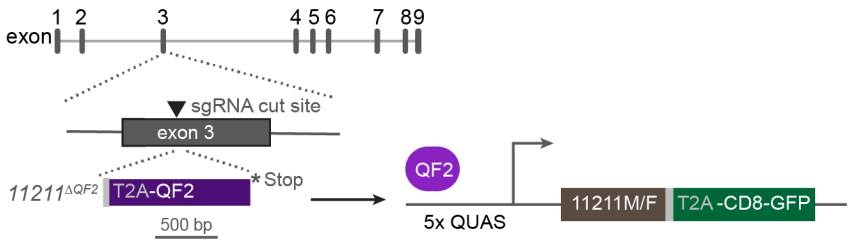

Figure 4.21 Generation of a $11211^{\Delta QF2}$ strain

Schematic of the *11211* locus, showing CRISPR/Cas9-mediated homology-directed repair and insertion of the plasmid driving QF2, which can drive expression of transgenic effectors within the cells that express *11211.*

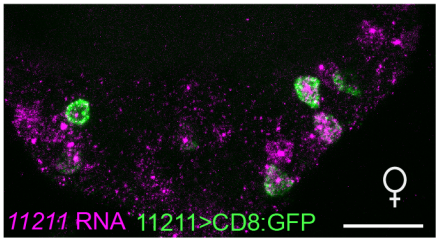

Figure 4.22 Expression of *11211* mRNA and reporter driven GFP

Confocal image of RNA *in situ* hybridization with probes against *11211* (magenta) and CD8-GFP (green) in *11211>CD8-GFP* female brains, zooming in on the subesoophageal zone. Scale bar is 20 μm.

We then tested the role of *11211* in blood-feeding behavior by allowing heterozygous and homozygous 11211^{QF2} mutant females to feed on a glytube, which is parafilm-covered meal that is maintained at a warm temperature by heated glycerol (Costa-da-Silva et al., 2013). We found that *11211* mutant females were significantly impaired in their ability to initiate blood feeding (Figure 4.23). However, when we attempted to rescue this phenotype by overexpressing the female isoform of *11211* under the control of *11211*-driven QF2 in the homozygous mutant background, we found that females still had severe deficits in blood feeding (Figure 4.23). The failure to rescue suggested one of three possibilities: first that the rescue was incomplete as the QF2 line did not express in all of the cells that normally express *11211*, or second, that both overexpression and underexpression of *11211* expression lead to a failure to blood-feed, or third,that the mutant phenotype was not caused by the loss of *11211* but by overexpression of QF2 as was seen with the original Q-lines (Riabinina *et al.*, 2015). We generated another mutant allele 11211^{ie1}, and found that heteroallelic $11211^{ie1/QF2}$ females did not show any deficits in blood-feeding behavior (Figure 4.24). These results suggest that overexpression of QF2 in *11211* expressing neurons interferes with blood-

feeding behavior, although conclusive evidence would require disruption of QF2 in the *11211*^{QF2} animals. While the mechanism by which QF2 leads to toxicity is unclear (Riabinina *et al.*, 2015), it is likely interfering with transcription either broadly or selectively, thereby disrupting the function of the cells in which it is expressed.

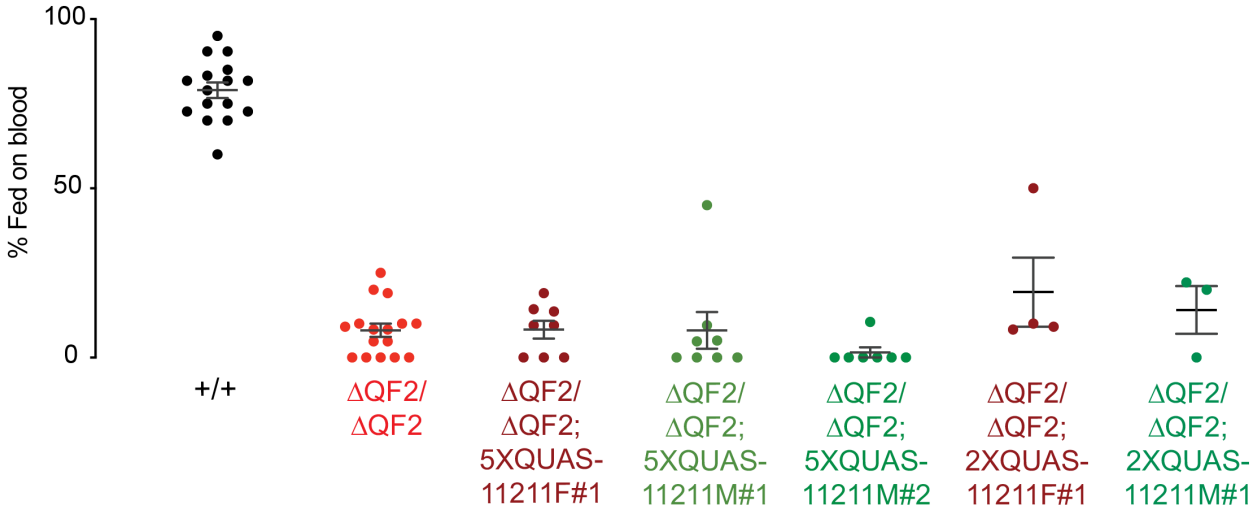

Figure 4.23 *11211*$^{\Delta QF2}$ females are unable to feed, but this phenotype cannot be rescued by overexpression of 11211F

Percent of females blood feeding on a glytube. Each point represents the % of 20 female mosquitoes that engorged on blood. n=3-18.

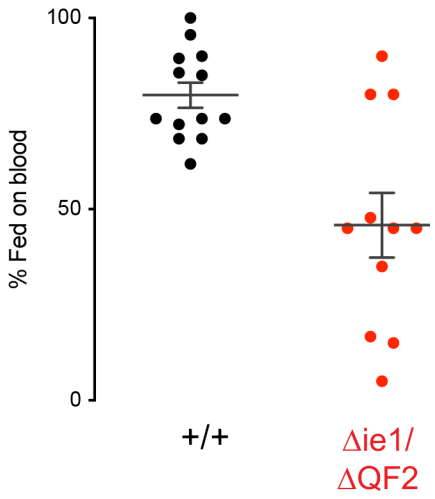

Figure 4.24 *11211*$^{\Delta ie1/\Delta QF2}$ females do not show significant defects in blood feeding

Percent of females blood feeding on a glytube. Each point represents the % of 20 female mosquitoes that engorged on blood. n=11-13.

Using the heteroallelic mutant females, we then tested whether overexpression of the male isoform of 11211 would disrupt blood-feeding behavior. We found that 'masculinized' *11211* females indeed showed deficits in blood feeding (Figure 4.25). However, this phenotype relied on overexpression of the male isoform of 11211 at potentially non-physiologically relevant conditions. In order to bypass this issue, we began to generate a mutant allele where we deleted the female-specific exon, *11211*$^{\Delta e6}$. In this mutant, females should express the male RNA transcript and protein isoform, due to the lack of the sixth exon. Characterization of these mutants is ongoing, and will reveal whether sex-specific splicing of this gene plays a role in blood-feeding behavior.

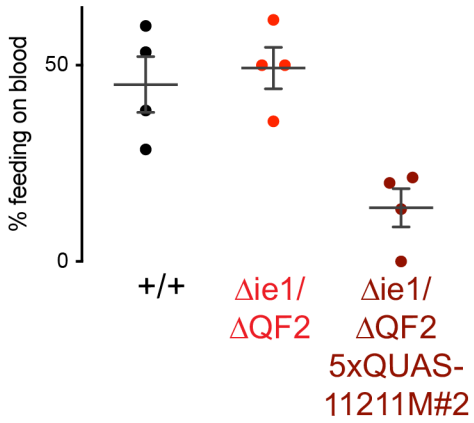

Figure 4.25 Overexpression of the male isoform of *11211* suppresses blood-feeding behavior.

Percent of females blood feeding on a glytube. Each point represents the % of 20 female mosquitoes that engorged on blood. n=4.

In summary, sex-specific splicing of *11211* may be required for blood-feeding behavior.

CHAPTER 5. *11211*-expressing neurons are required for blood-feeding behavior

We previously showed that *11211*^{QF2} homozygous females were impaired in the ability to blood-feed, and that this potentially was not due to the loss of *11211*. This result suggested that, rather than disrupting *11211*, impairing the function of *11211* expressing neurons may be required for blood feeding. Since we knew that heterozygous *11211*$^{QF2/+}$ females were unimpaired in their ability to feed on blood, all experiments in this chapter were carried out in heterozygous females that carry one copy of QF2 and one copy of a transgene. This experimental setup prevents any potential toxicity from homozygous animals that carry two copies of QF2. In this chapter, we characterize the expression-pattern of *11211*+ neurons, and test their role in behavior by chronically silencing their activity.

5.1 *11211*-expressing neurons innervate the subesophageal zone of the brain

We crossed the *11211*^{QF2} driver line to a reporter that expressed either a membrane bound fluorescent protein CsChrimson-tdTomato, or cytosolic dTomato. We used CsChrimson-tdTomato (hereon *11211>tdTomato*) as a marker to examine expression in peripheral sensory organs (Figure 5.1A). Consistent with the prediction from RNA-seq data, the *11211*^{QF2} driver line drove expression in the antenna, with putative olfactory sensory neurons labeled in both male and female antennae (Figure 5.1B-C). There was no expression in the labium or the maxillary palps, which are thought to detect sugar to drive nectar feeding, and carbon dioxide to drive host seeking respectively (Figure 5.1D-

E,H-I). When we looked at the stylet, we found that both male and female stylets had two *11211*+ neurons (Figure 5.1F-G).

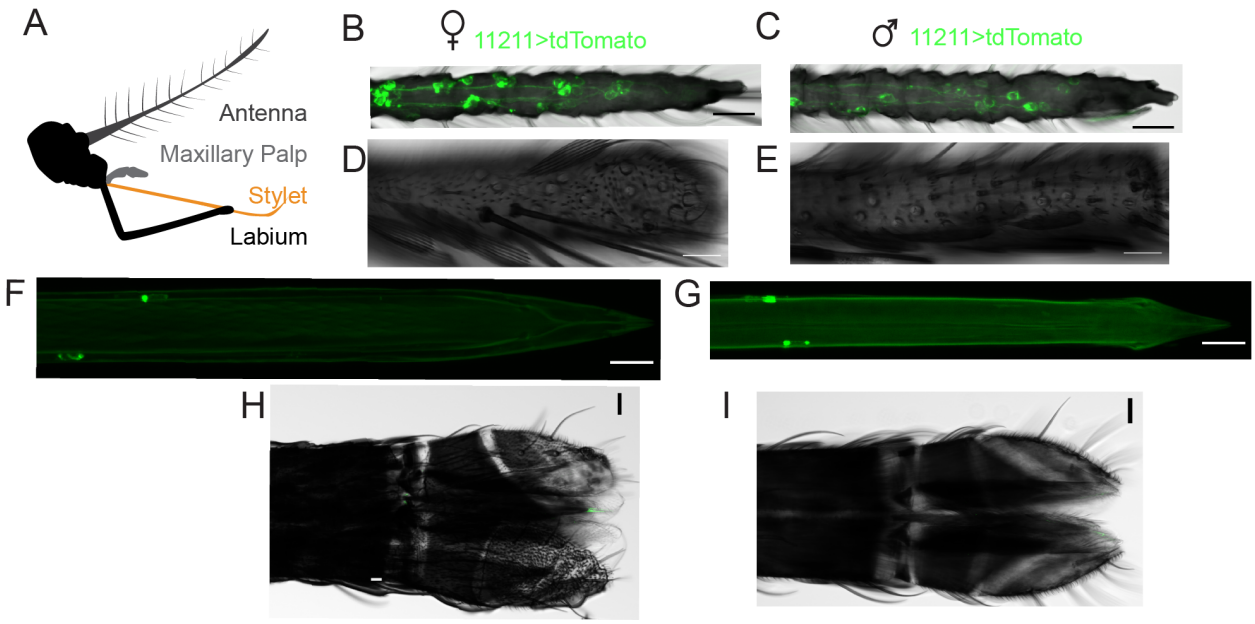

Figure 5.1 Expression of the *11211* reporter in peripheral sensory tissues

(A) Schematic of the mosquito head with sensory organs.
(B-I) Confocal images of antennae (B-C), maxillary palps (D-E), stylets (F-G), and labiums (H-I) showing *11211>tdTomato* (green) expression. All scale bars are 20 μm.

We then examined expression of the *11211*^{QF2} driver line using dTomato-T2A-GCaMP6s (hereon *11211>dTomato*) as a reporter within the central nervous system. Consistent with our expectation, we found expression in a number of cells in the superior brain and in the subesophageal zone in both male and female brains (Figure 5.2A-B). In addition, there were projections either to or from the ventral nerve cord in both males and females (Figure 5.3A-B). The subesophageal zone projections appeared to show some level of sexual dimorphism, with denser projections in the

female brain. However, this dimorphism may be due to the difference in size of the brains, as the female brain is larger.

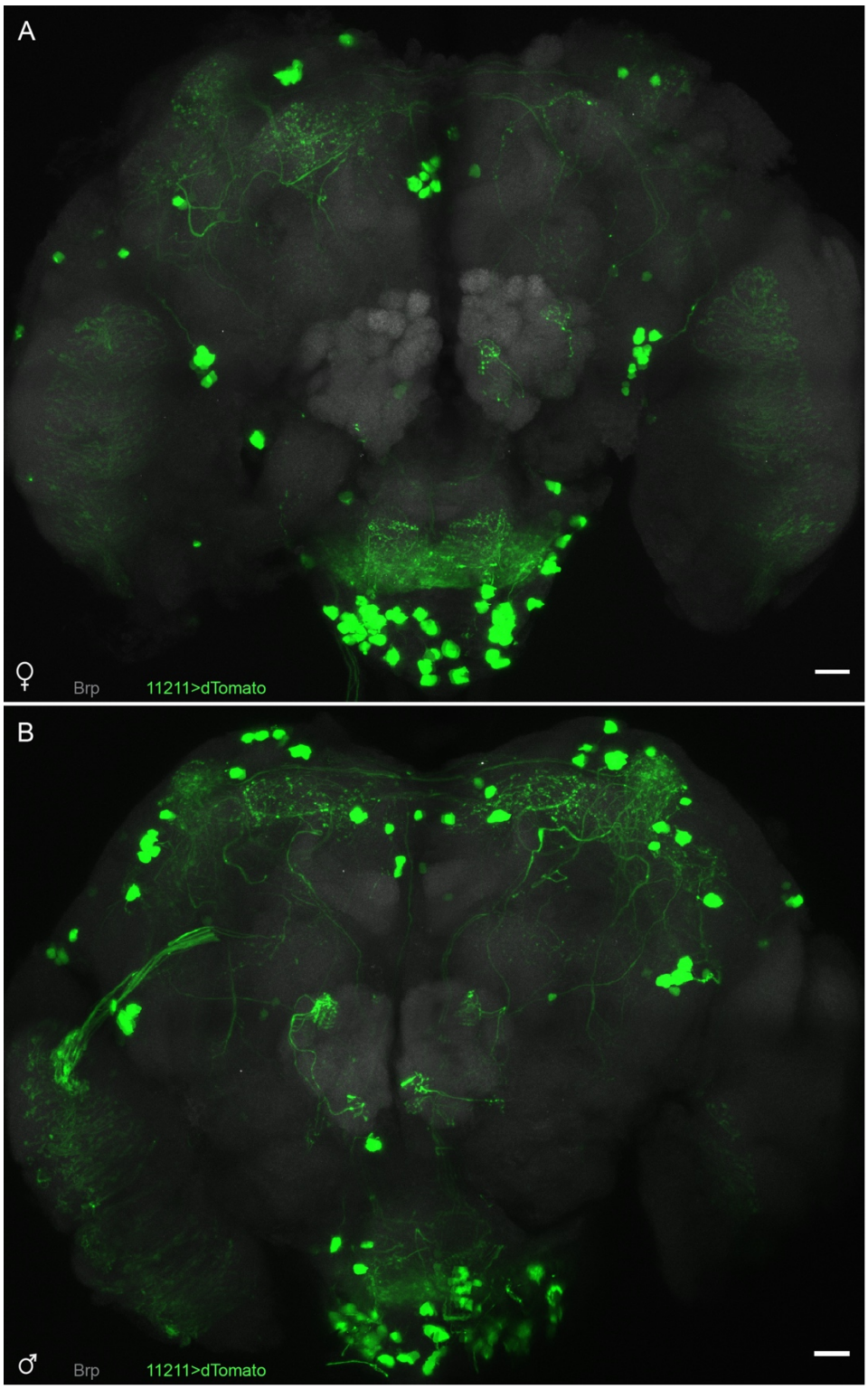

Figure 5.2 Expression of the *11211* reporter in the central brain

(A-B) Confocal images of the female (A) and male (B) adult brains showing *11211>dTomato* (green) expression with counterstained Brp (grey). All scale bars are 20 µm.

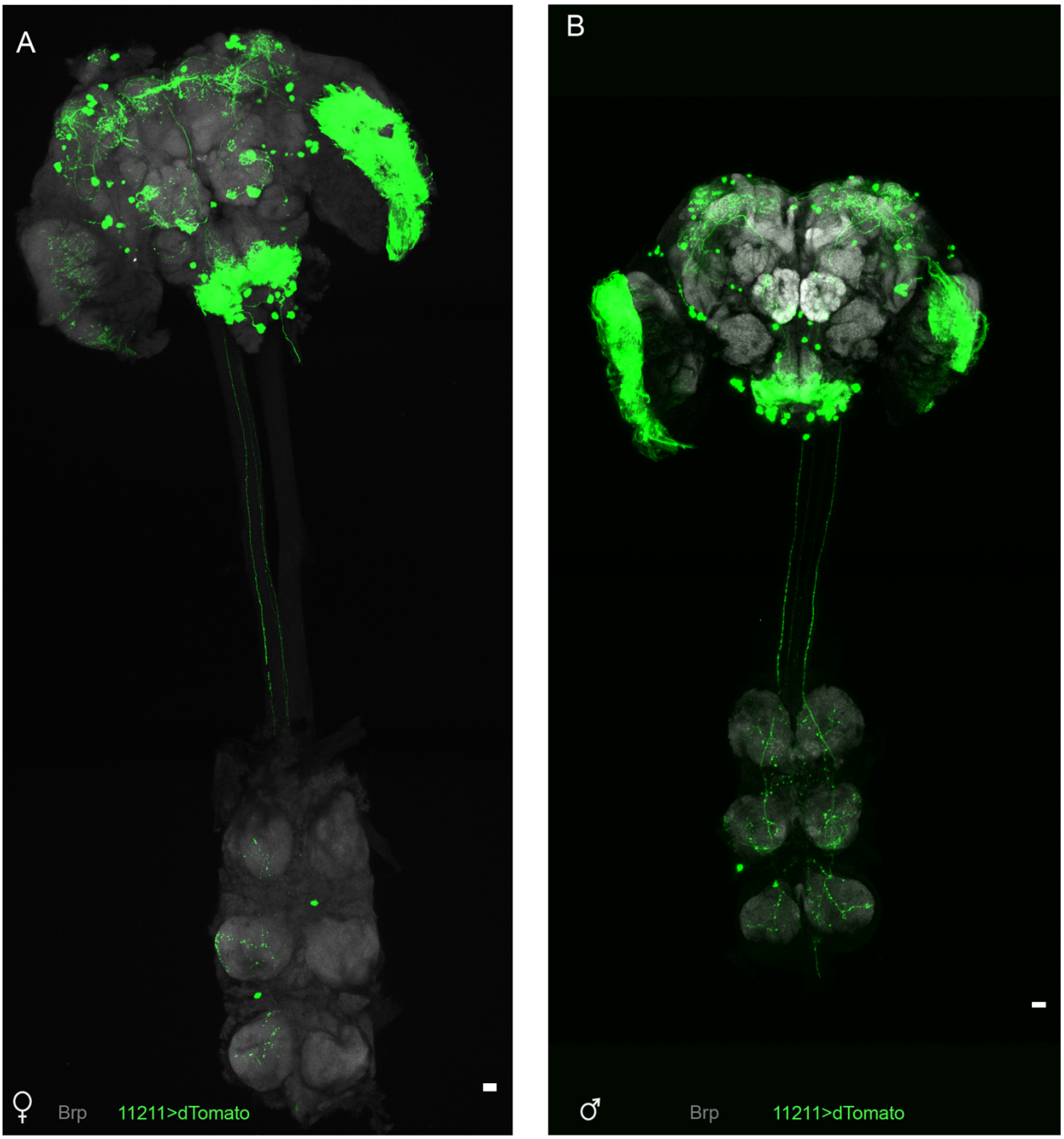

Figure 5.3 Expression of the *11211* reporter in the central brain and ventral nerve cord

(A-B) Confocal images of the female (A) and male (B) adult brains and ventral nerve cords showing *11211>dTomato* (green) expression with counterstained Brp (grey). All scale bars are 20 μm.

Given that the subesophageal zone receives input from gustatory sensory neurons, we were curious as to whether *11211+* neurons overlapped with these projections, there was any preference for input from the stylet or the labium. We carried out dye-fill experiments, where we cut the stylet and labium so the neurons could take up one of two dyes, allowing for tracing of the projections of these neurons in the central brain (Figure 5.4A-B). We found that female stylet and labium projections were distinct, with the stylet projecting to anterior regions of the subesophageal zone, while the labium projected more posteriorly (Figure 5.4C). We used membrane-bound CD8-GFP (henceforth *11211>CD8-GFP*) as a marker of *11211* expression. When we examined the overlap of *11211>CD8-GFP* neurons with the stylet and labium projections, we found greater overlap with the stylet relative to the labium. This anatomical evidence suggests that *11211* neurons may receive input from neurons that detect the taste of blood. However, this anatomical prediction needs to be supported by functional data, such as *in vivo* imaging of *11211+* neurons during blood feeding.

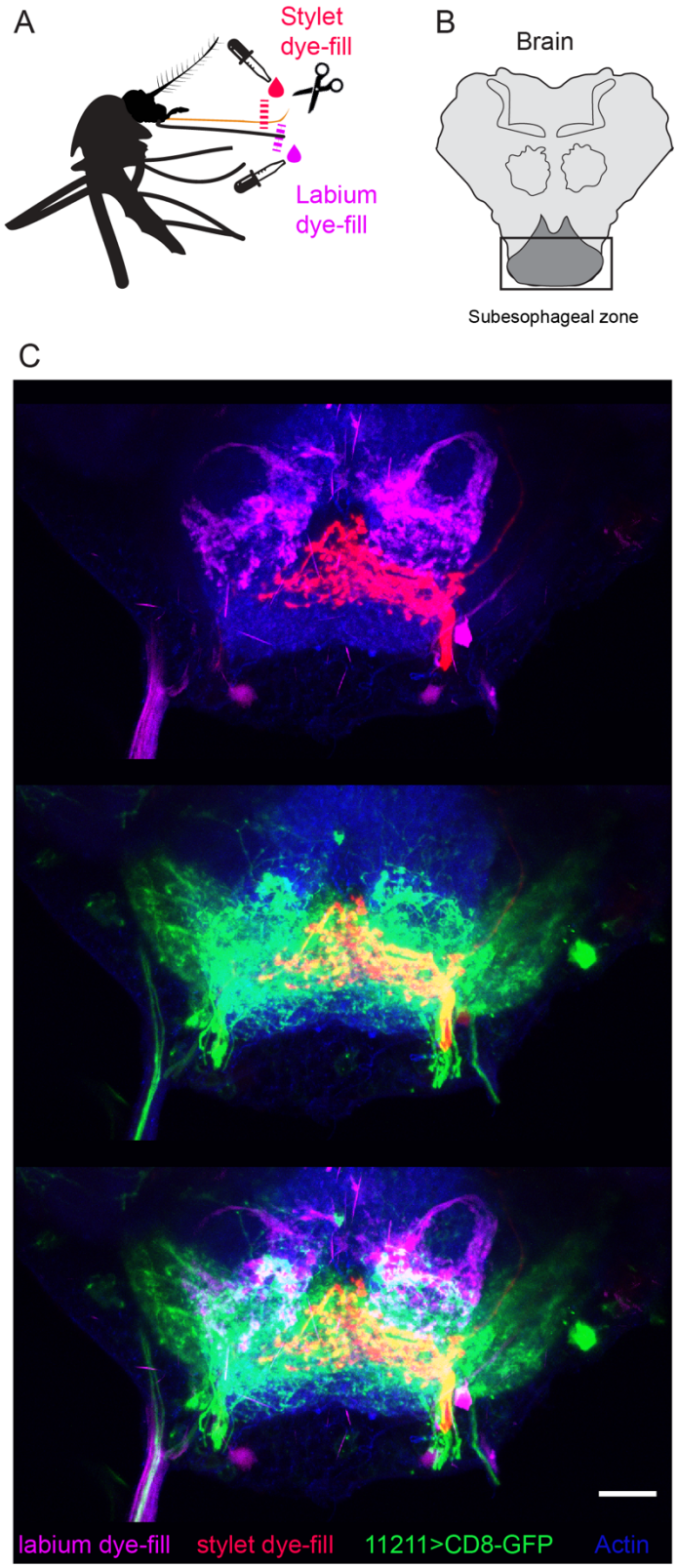

Figure 5.4 Overlap of *11211* reporter neurons with stylet projections

(A-B) Cartoon of dye-fill experiment (A) and mosquito brain (B)
(C) Confocal images of female adult brain subesophageal zone showing *11211>CD8-GFP* (green) expression with dye-filled projections of labium neurons (magenta) and stylet neurons (red) counterstained with phalloidin to mark actin (blue). Scale bar is 20 µm.

5.2 *11211*-expressing neurons are specifically required for blood feeding

We used previously developed reagents to silence the activity of *11211*+ neurons (Trevor Sorrells, unpublished). These silencing lines are the inward rectifying potassium channel Kir2.1 which hyperpolarizes neurons and Tetanus toxin (TetX) which blocks the cleavage of synaptobrevin and prevents synaptic vesicle release. We expect that either the control $11211^{QF2/+}$, QUAS-TetX/+ or QUAS-Kir2.1/+ should exhibit no phenotype. However, expression of either of these two reagents under the control of one copy of 11211-driven QF2 should chronically silence the activity of these neurons.

We found that silencing of *11211* neurons with either Kir2.1 or TetX led to moderate levels of larval lethality (~20% for 11211>TetX#5, ~50% for 11211>Kir2.1#8, ~70% for 11211>TetX#6), suggesting that these neurons are required for larval survival. This is despite low levels of *11211* expression in larval RNA-seq data. Given this caveat, silencing of *11211* neurons with conditional methods such as optogenetic silencing reagents would likely allow for more specific testing of the role of these neurons in adult blood-feeding behavior. Since validated optogenetic silencing tools in the mosquito are lacking, we proceeded with characterizing the chronically silenced lines. Despite the larval lethality we observed, in all cases we were able to recover sufficient adults to carry out behavioral experiments.

We first tested whether *11211*-silenced females were generally healthy by characterizing their host-seeking and sugar-feeding behaviors. Silenced females showed host-seeking behavior at similar levels to control unsilenced females, and did not display significant deficits in their attraction to humans (Figure 5.5). Similarly, silenced females fed on sugar at similar rates to control females (Figure 5.6). These data imply that silencing of *11211*+ neurons does not cause gross deficits in female behavior, as these females are able to host-seek and sugar-feed normally.

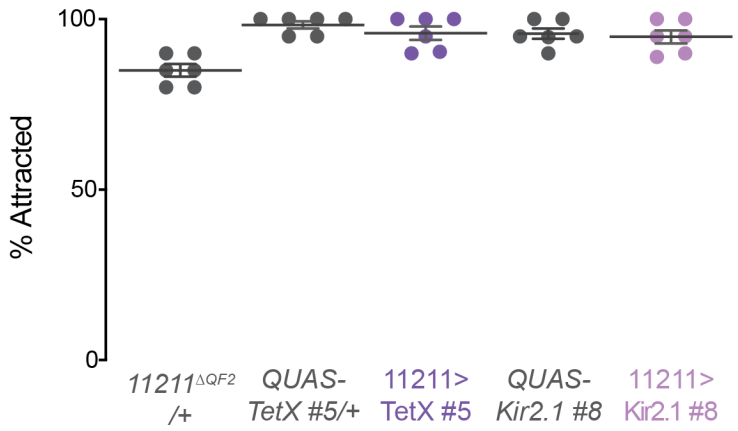

Figure 5.5 *11211*-expressing neurons are not required for female mosquito attraction to humans

Percent of attracted females of the indicated genotypes to a live human arm in the quattroport assay (n = 6 trials per group, n = 18-21 mosquitoes/trial). Data are mean±s.e.m.

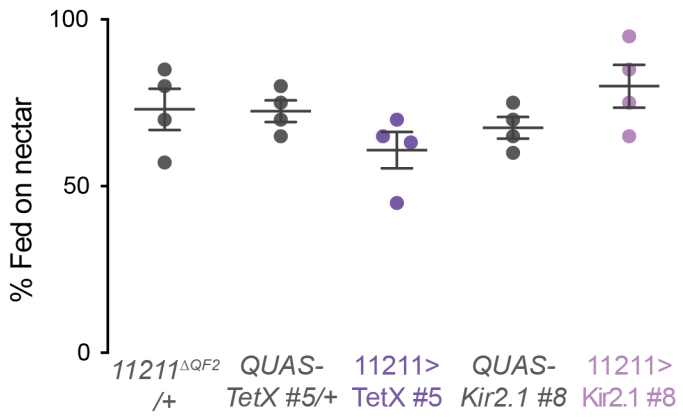

Figure 5.6 *11211*-expressing neurons are not required for female mosquito nectar feeding

Percent of females of the indicated genotypes feeding on sugar in a nectar-feeding assay (n = 5 trials per group, n = 20 mosquitoes/trial). Data are mean±s.e.m.

Finally, we characterized the ability of these silenced females to blood feed, allowing them to feed on either a live human arm or a blood meal delivered through a glytube. Silenced females showed a severe deficit in their ability to initiate blood feeding on either meal source, while control females were unaffected (Figure 5.7). A number of silenced females did engorge on blood, and these females appeared to take meals of comparable size to control females, although meal size was not quantified in these experiments. Therefore, *11211*-expressing neurons are specifically required for the initiation of blood feeding, and not strictly required during the process of engorgement.

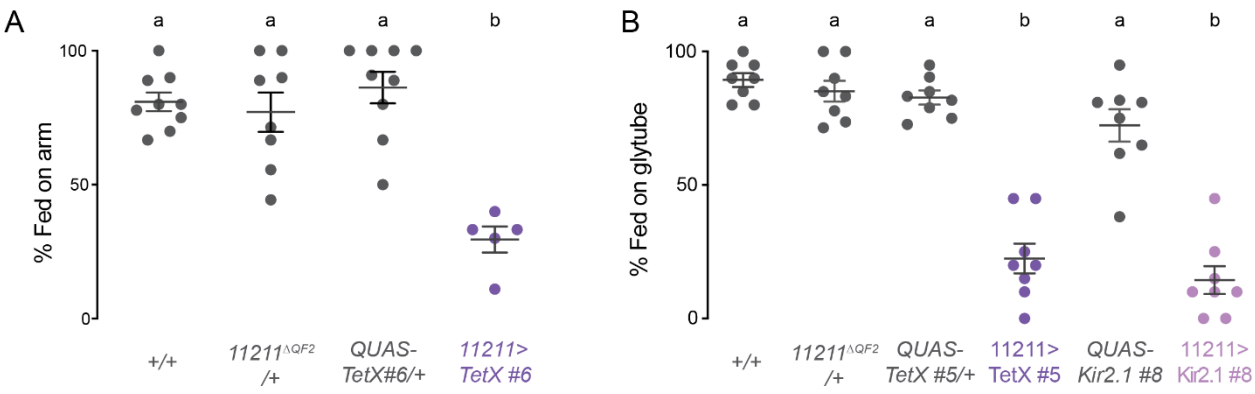

Figure 5.7 *11211*-expressing neurons are required for female blood-feeding behavior.

(A) Feeding of female mosquitoes of the indicated genotypes on a human arm (n = 5-9 trials; n = 20 mosquitoes/trial). Data are mean±s.e.m.

(B) Feeding of female mosquitoes of the indicated genotypes on blood delivered through a glytube (n = 8 trials; n = 20 mosquitoes/trial). Data are mean±s.e.m.

Data labeled with different letters are significantly different from each other (Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.05$).

CHAPTER 6. Discussion

The work presented in this thesis has revealed the regulators of female-specific host-seeking and blood-feeding behaviors in the mosquito *Aedes aegypti*. We demonstrate that a number of genes are sex-specifically spliced in mosquito brains, and that two of these genes regulate mosquito behavior. *Fruitless* is required to repress host-seeking behavior in male mosquitoes. Normal activity of *11211*-expressing neurons are required for the initiation of female blood-feeding behavior. Here we conclude with a final summary of our work, and a discussion of future open questions for the field.

6.1 Analysis of sex-specific splicing in the mosquito brain reveals novel regulators of mosquito behaviors

Sex-specific splicing is a conserved feature of sex determination across the insects. However, in our analysis, relatively fewer sex-specific splicing events are predicted to have dramatic effects on the sex-specific proteins. Even more strikingly, despite the conservation of sex-specific splicing, very few genes show conserved sex-specific splicing across species In our analysis, we identified only 3 genes that are sex-specifically spliced across multiple species and lead to sex-specific proteins. These genes are *fruitless, doublesex,* and *11211*. *fruitless* and *doublesex* were well-known, and *11211* is a novel sex-specifically spliced gene.

We note that our analysis relied on reference genome annotations, and that any unannotated exons or splice donor or acceptor sites may not be captured in the analysis (Anders *et al.*, 2012). Additionally, we focused on sex-biased exon usage, since this was the most common type of sex-specific splicing previously described in other insects (Salz, 2011). There may be other types of conserved sex-specific splicing events which are not detected in our analysis, such as intron retention (Kim et al., 2008). Despite the shortcomings of this method, it was able to reliably identify interesting candidate genes that had a role in sex-specific mosquito behavior. Such an approach may be of interest in investigating the genetic basis of other sexually dimorphic insect behaviors, such as Hymenopteran social behavior.

How might sex-specific splicing first have emerged in the insects? A well-described mechanism to resolve sexually antagonistic gene function is gene duplication and sex-specific expression of the paralogs (Connallon and Clark, 2011; VanKuren and Long, 2018). Sex-specific splicing is an alternative mechanism to resolve any intralocus sex conflicts. It is unclear precisely when sex-specific splicing originated within the insects, but it is conserved at least to cockroaches (Wexler *et al.*, 2019). Deeper genomic and transcriptomic coverage of basal insect lineages would help resolve the question of when sex-specific splicing emerged, and might provide hypotheses as to why it arose.

Why is sex-specific splicing so conserved, despite the rapid evolution of sex-determination signals? This is an open question across species, given that sex-determination mechanisms evolve rapidly in general, but still display some conserved

genetic players (Bachtrog *et al.*, 2014; Capel, 2017). One example is the Dmrt family of transcription factors, which control sex-determination and gonadal development in species as diverse as insects and fish (Kopp, 2012). Multiple factors can cause the evolution of sex-determination signals, such as genomic conflict or sexually antagonistic selection. If sex-biased selfish genetic elements can distort sex-ratios, then new sex-determination systems that restore ratios will be favored (Bachtrog *et al.*, 2014; Kozielska et al., 2010; McLaughlin and Malik, 2017). Similarly, sexually antagonistic mutations that are linked to sex-determination genes can trigger the turnover of sex chromosomes (Bachtrog *et al.*, 2014; van Doorn and Kirkpatrick, 2007). In contrast to sex-determination genes that transform sex across the whole organism, genes like *fruitless* and *doublesex* show tissue-specific dedicated functions, and may therefore not be under the same kinds of selective pressures as sex-determination genes. The conservation of the sex-specific splicing of these genes may be due to constraints on the types of genes that can act as 'switch' genes (Bachtrog *et al.*, 2014; Graves and Peichel, 2010). However, in the case of *11211*, there are clear losses of sex-specific splicing. It is possible that sex-specific splicing may show gains and losses across the insects.

6.2 *fruitless* represses host-seeking behavior in the male mosquito

We showed that *fruitless* is required to repress host-seeking behavior in the male mosquito. Our results suggest that the neural circuits that promote female attraction to human scent are latent in males and suppressed by expression of *fruitless* either during

development, or during adulthood. This is in contrast to a model where the ability to host-seek develops exclusively in females. Since males are able to host-seek in the absence of *fruitless,* other components of the sex-determination pathway do not intrinsically regulate the development and function of brain circuits controlling host-seeking behavior, even though this behavior is normally sex-specific. The concept that a latent sex-specific behavior can be revealed by knocking out a single gene was elegantly demonstrated in the mouse (*Mus musculus*). Only male mice court and initiate sexual contact with females and yet knocking out the *Trpc2* gene causes female mice to display these male-specific behaviors (Kimchi et al., 2007).

fruitless has gained a role in suppressing female-specific host-seeking behavior in the male mosquito, which is in addition to its ancestral role promoting mating. Interestingly, *fruitless* also acts to suppress female-specific aggressive behaviors in male *Drosophila* in addition to its role in promoting male-specific courtship and aggression (Vrontou *et al.*, 2006), suggesting a common theme where this gene can repress specific aspects of female-specific behavior. We cannot exclude the possibility that *fruitless* had a broader ancestral role in repressing male-specific host seeking or feeding but consider this extremely unlikely given the rarity of sexually dimorphic feeding behaviors relative to sexually dimorphic mating behaviors.

Where in the nervous system is *fruitless* required to suppress host seeking in male mosquitoes? *fruitless* might function in the antenna to modulate the detection of human odor in male mosquitoes, perhaps by tuning the functional or anatomical properties of

olfactory sensory neurons. Such a role has been recently demonstrated in the aging-dependent sensitization of the male *Drosophila* antennal response to pheromones (Sethi et al., 2019; Zhang et al., 2020; Zhao et al., 2020a). Alternatively, both wild-type males and females might detect human odor, and *fruitless* could function in the central brain to reroute these signals to drive different motor outputs, as has been demonstrated with the sexually dimorphic response to *Drosophila* pheromones (Datta *et al.*, 2008; Kohl *et al.*, 2013; Ruta *et al.*, 2010).

We would require significant advances in technology and mosquito genetics to answer these questions, including a *fruitless* driver line to image neural responses to human odor. Despite significant effort, we were unable to generate a viable *fruitless* driver line both because of tight genetic linkage of *fruitless* to the sex-determining M locus (Hall *et al.*, 2015) and because gene-targeted females failed to blood-feed and were therefore sterile. To explore central brain *fruitless*+ circuits, we would need to be able to subset expression to label and drive reporters or rescue *fruitless* expression in sparse populations of neurons, a technology that is still out of reach. Advances in mosquito genetic tools, such as the successful implementation of orthogonal transcriptional activator reagents, combined with sparse labeling approaches will be required to gain mechanistic insight into *fruitless* function within mosquito host-seeking circuits. We note that these advances were not trivial in *Drosophila melanogaster*, requiring efforts from multiple laboratories over the past decade (Datta *et al.*, 2008; Kohl *et al.*, 2013; Ruta *et al.*, 2010), and expect that the generation of these tools and the

subsequent characterization of the circuit will be significantly more challenging in the mosquito, a non-model organism.

6.3 *11211* is a novel sex-specifically spliced gene potentially controlling blood-feeding behavior

We identified a novel sex-specifically spliced gene, *11211*. We showed that the activity of *11211*-expressing neurons is specifically required for the initiation of blood-feeding behavior, suggesting that the expression of this gene marks a population of neurons that control blood feeding. The function of the *11211* gene and the role of sex-specific splicing in behavior remains under investigation.

11211 is conserved across the insects, and has conserved sex-specific splicing, with the notable exception of non-mosquito flies. However, the effect of the sex-specific splicing on sex-specific proteins differs between the more basal insects and mosquitoes, with a longer female protein isoform in bees, ants, bugs, and beetles, and a longer male protein isoform in mosquitoes. One hypothesis for why this gene has diverged is that it may control sex-specific traits that are also rapidly evolving, and thereby have undergone selection. For example, female and male bees show differences in social behavior (Beani et al., 2014). It is also possible that the evolution of this gene is non-adaptive and the result of genetic drift. The loss of sex-specific splicing in flies like *Drosophila* is intriguing. *Drosophila melanogaster 11211* does not show sex-differences in expression levels and has not duplicated into sex-biased paralogs,

suggesting that sex-specific splicing has not been replaced by another means of sex-biased regulation. Another intriguing finding is the inability to detect homologs of this gene in any Lepidopteran genomes, suggesting that it has been lost or rapidly diverged. While the role of sex-specific splicing of *11211* remains unclear, given the relative paucity of conserved sex-specifically spliced genes across the insects, we speculate that this gene is likely to play an important role in sex-specific traits in species where it is sex-specifically spliced.

What was the ancestral function of *11211*? In beetles and ants, this gene is more broadly expressed through the brain. Since we lack tissue-specific RNA-seq data or RNA *in situ* hybridization protocols to visualize gene expression body-wide, it is unclear how broadly expressed *11211* is throughout the body of these species. In contrast, in *Aedes aegypti*, *11211* expression is strongly enriched in the brain and antenna. Within the brain, it is enriched within subsets of neurons within the superior protocerebrum and the subesophageal zone. The difference in expression pattern between mosquitoes and ants hints at a difference in the function. It is possible that the ancestral function of this gene is not specific to behavior, and that it has a broader role in controlling sex-specific traits. The function of *11211* may then have been co-opted in the mosquitoes to specifically control behavior. The evolution of *11211* is in contrast to the relative conservation of *fruitless* and *doublesex*. As we described earlier, upstream genes in the sex-determination pathways tend to be more rapidly evolving. While it is not possible to determine the role of 11211 and if where in the sex-determination pathway it falls, we

speculate that this gene might face different selective pressures than the more conserved genes, perhaps related to its ancestral function.

We demonstrated that in the mosquito, *11211* encodes sex-specific proteins that localize to the nucleus in both sexes. It is possible that 11211 proteins similarly act to regulate transcription by binding to DNA. However, these proteins lack any predicted or annotated DNA binding domains, or indeed any other annotated protein domains. There are other possible functions for these proteins in the nucleus, including modulating nuclear architecture, DNA repair and replication, or RNA splicing. While the orthologs of *11211* in other species have not definitively been shown to localize to the nucleus, they all contain one or more nuclear localization sequences, suggesting that this nuclear function may be conserved. The nuclear localization of *11211* is in line with the localization of other well-known regulators of sexual dimorphism across the insects. Both *fruitless* and *doublesex* encode transcription factors that localize to the nucleus and bind to DNA to modulate the expression of downstream genes that specify sex-specific behavior and morphology. It has been hypothesized that certain classes of genes like transcription factors are more likely to underlie phenotypic differences between species (Bendesky and Bargmann, 2011; Martin and Orgogozo, 2013; Tosches, 2017). The conserved nuclear localization of *11211*, *fruitless*, and *doublesex* suggest that there may be constraints on the types of genes that regulate sex-specific behaviors.

6.4 Modular genetic and neural control of mosquito host-seeking and blood-feeding behaviors

Host seeking is the first step in a complex sequence of behaviors that lead to blood feeding. After detecting and flying toward a human host, the female mosquito must land on the human, pierce the skin, and ultimately engorge on blood (Bowen, 1991). We have shown that *fruitless* has evolved to control sexual dimorphism in one module of this specialized behavior, the ability to host-seek. Sexual dimorphism in subsequent feeding behaviors does not appear to be controlled by *fruitless* in the male mosquito, since neither wild-type nor mutant *fruitless* males will drink warm blood. In contrast, *11211*-expressing neurons are required specifically for female blood-feeding behavior and not host seeking. The modular organization of mosquito behavior sparks intriguing parallels to other complex sexually dimorphic behaviors like mouse sexual behavior and parenting (Kohl et al., 2018; Xu et al., 2012). To be effective parents, female mice must build nests, and then retrieve, groom, and nurse their pups. In the deer mouse *Peromyscus,* the conserved peptide vasopressin has evolved to specifically control nest building, while other unidentified genes control other steps of parenting behavior (Bendesky *et al.*, 2017).

Our work suggests that *fruitless* has evolved the novel function of enforcing female-specific host seeking while maintaining its presumably ancestral male mating function. How might *fruitless* have evolved to control host seeking? One possibility is that non-sex-specific host-seeking neural circuits first emerged in the ancestral mosquito, and

then secondarily began to express *fruitless* to suppress the development or adult function of host-seeking circuits specifically in males. Another possibility is that mosquitoes duplicated and co-opted the ancestral *fruitless*-expressing mating neural circuits and retuned the inputs and outputs into this circuit to drive host seeking. Circuit duplication is one of the mechanisms by which neural circuits are proposed to evolve (Tosches, 2017), and has been demonstrated in the case of vocal learning (Chakraborty and Jarvis, 2015) and in the evolution of cerebellar nuclei (Kebschull et al., 2020). In these duplicated mosquito circuits, *fruitless* function would have switched from promoting mating to inhibiting host seeking in males. We speculate that both possibilities allow for *fruitless* to control both sex-specific host-seeking and mating behaviors, and identification and molecular profiling of the *fruitless* cells controlling host seeking and mating will help distinguish between these models.

The ancestor of mosquitoes likely had pre-existing circuits that allowed for persistent attraction to an odor, given that this is a conserved behavior that allows for animals to feed or mate. How has blood feeding, a relatively more complicated behavior, evolved? The mosquito has two feeding programs: nectar feeding, which is performed by both males and females, and blood feeding, which is female-specific (Jové *et al.*, 2020). It is possible that these two behaviors evolved from a shared ancestral feeding behavior, and blood feeding acquired sex-specific regulation. Alternatively, the ancestor of mosquitoes may already have had sex-specific feeding programs, one of which evolved into blood feeding. Female *Drosophila melanogaster* show increased protein and salt appetite after mating, while sugar-seeking remains unchanged (Ribeiro and Dickson,

2010; Walker et al., 2015). Given that females have different physiological needs during reproductive states, such differences in appetite are found across the animal kingdom (Cohen and Woodside, 1989). Therefore, it is possible that the neural circuit controlling blood feeding evolved from a pre-existing sexually dimorphic protein-seeking circuit. We speculate that during the course of mosquito evolution, either a novel circuit or a pre-existing circuit would have selectively gained or enhanced the expression of *11211*.

CHAPTER 7. Methods

Human and animal ethics statement

Blood-feeding procedures and behavioral experiments with live hosts were approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee (IACUC protocol 17018) and Institutional Review Board (IRB protocol LV-0652), respectively. Human subjects gave their written informed consent to participate.

Mosquito rearing and maintenance

Aedes aegypti wild-type laboratory strains (Liverpool-IB12) were maintained and reared at 25 - 28°C, 70-80% relative humidity with a photoperiod of 14 hr light: 10 hr dark (lights on at 7 a.m.) as previously described (DeGennaro *et al.*, 2013). All behavioral assays were performed at these conditions of temperature and humidity. Adult females were blood-fed on mice for stock maintenance and on human subjects for initial stages of mutant generation. *Anopheles gambiae* (G3 strain), *Wyeomyia smithii* (PB strain), *Toxorhynchites amboinensis*, and *Culex quinquefasciatus* (JHB strain) were reared in similar conditions, following previously described protocols for each species (Bradshaw *et al.*, 2018; Werling et al., 2019; Zhou *et al.*, 2014). Adult mosquitoes of each species were provided constant access to 10% sucrose.

RNA-sequencing

7 to 14 day-old mosquitoes of each species were cold-anesthetized and kept on ice for up to 1hr or until dissections were complete. Brains were dissected in ice-cold RNase-free phosphate-buffered saline (PBS) (Invitrogen AM9625) on ice, moved into a

microfuge tube with forceps, and immediately snap frozen in a cold block (Simport S700-14) chilled to -80ºC on dry ice. Each sample group was dissected in parallel to avoid artefacts and batch effects, and five brains were used per sample. Dissected tissue was stored at −80°C until RNA extraction. RNA extraction was performed using the PicoPure Kit (ThermoFisher #KIT0204) following the manufacturer's instructions, including DNase treatment. Samples were run on a Bioanalyzer RNA Pico Chip (Agilent #5067-1513) to determine RNA quantity and quality. Libraries were prepared using the Illumina TruSeq Stranded mRNA kit #20020594, following manufacturer's instructions. Library quantity and quality were evaluated using High Sensitivity DNA ScreenTape Analysis (Agilent #5067-5585) prior to pooling. Bar-coded samples from all non-*Aedes* tissues were pooled in an equal ratio before distributing the pool across 2 sequencing lanes. Sequencing was performed at The Rockefeller University Genomics Resource Center on a NextSeq 500 sequencer (Illumina). All reads were 2 x 150 bp. Data were de-multiplexed and delivered as fastq files for each library. Sequencing reads have been deposited at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA612100.

Bioinformatic analysis

Reads from individual libraries were mapped to the respective genomes downloaded from NCBI using STAR version 2.5.2a with default settings (Dobin et al., 2013). Raw counts were used for differential splicing analysis using DEXSeq version 1.32.0 (Anders *et al.*, 2012) as per author instructions. For genome-guided differential expression analysis, aligned genomic reads were quantified using featureCounts version 2.0.2

(Liao et al., 2014), and differential expression analysis was carried out with DESeq2 version 3.14 (Love *et al.*, 2014). For *de novo* transcriptome analysis, we assembled sex-specific *de novo* transcriptomes using Trinity version 2013-03-25 with default settings (Grabherr *et al.*, 2011). In all cases, homologs between each species were identified using BLAST 2.6.0 (Altschul et al., 1990).

Generation of mosquito mutant and transgenic strains

The *fruitless* and *11211* genes were targeted using CRISPR-Cas9 methods as previously described (Kistler *et al.*, 2015). Gene-targeting reagents were injected into wild-type Liverpool-IB12 embryos at the Insect Transformation Facility at the University of Maryland Institute for Bioscience & Biotechnology Research. For each line, either 2000 or 1000 embryos were injected with 600 ng/μL plasmid or 125 ng/μL ssODN, 300 ng/μL Cas9 protein (PNABio CP01-200), and 40 ng/μL sgRNA. Proper integration was confirmed in each strain using polymerase chain reaction (PCR) and sequencing. Animals were then back-crossed to wild-type Liverpool-IB12 for at least four generations.

All homology arms for homology-directed integration were isolated by PCR using Liverpool-IB12 genomic DNA. sgRNA DNA template was prepared by annealing oligonucleotides as previously described (Kistler *et al.*, 2015). *In vitro* transcription of sgRNA template was performed using HiScribe Quick T7 kit (New England Biolabs #E2050S) following the manufacturer's directions and incubating for 4 hr at 37°C. Following transcription and DNAse treatment for 15 min at 37°C, sgRNA was purified

using Ampure RNAse-free SPRI beads (Beckman-Coulter #A63987) and eluted in Ultrapure water (Invitrogen #10977–015). For all plasmids, fragments were generated by PCR from the indicated template with the indicated primers and assembled using NEBuilder HiFi DNA Assembly (NEB E5520S). Plasmids were transformed into NEB competent cells (NEB C2987I), purified with the NucleoBond Xtra Midi Endotoxin Free kit (Clontech 740420.50), and sequence verified.

The *fruitless*$^{\Delta M}$ mutant was generated in the course of attempting to generate a *fruitless* QF2 knock-in mutant (Table 3.1). One of the families had viable 3xP3-dsRed positive offspring and an out-of-frame QF2 insertion, which was predicted to produce a truncated fruitless protein in males. This was the *fruitless*$^{\Delta M}$ mutant allele we used in the study.

The *fruitless*$^{\Delta M\text{-}tdTomato}$ knock-in/knock-out strain was generated by inserting a cassette containing T2A followed by CsChrimson fused to the fluorescent protein tdTomato and the 3xP3-EYFP strain marker. We obtained 2 independent viable lines and selected one for use in this study. We used the CsChrimson:tdTomato protein expressed from the *fruitless* locus in *fruitless*$^{\Delta M\text{-}tdTomato}$ animals as a marker for *fruitless* expression in these studies.

For the *fruitless*$^{\Delta F}$ lines, embryos were injected with 300 ng/μL Cas9 protein, 125 ng/μL oligonucleotide with template repairing the splice site, 40 ng/μL each of two sgRNAs targeting the beginning and end of the female-specific exon. We recovered multiple G1

animals with the correct integration, as verified by PCR and sequencing. Male *fruitless* splicing in *fruitless*$^{\Delta}$ females was verified with reverse-transcription PCR (data not shown).

The *11211*^{QF2} knock-in/knock-out strain was generated by inserting a cassette containing T2A followed by QF2, and the 3xP3-dsRed fluorescent marker. We obtained 2 independent viable lines and selected one for use in this study.

The *11211*^{ie1} knock-out strain was generated by inserting a cassette containing a stop codon followed by the ie1-dsRed fluorescent marker. We obtained 2 independent viable lines and selected one for use in this study.

The *11211*$^{F\text{-}HA}$ *11211*$^{M\text{-}HA}$ knock-in strains were generated by injecting embryos with 300 ng/µL Cas9 protein, 125 ng/µL oligonucleotide with template encoding 2x-HA and 60 bp homology arms, and 40 ng/µL of an sgRNA targeting the end of either the female-specific exon 6 or male-specific exon 9.

Antibody staining – brain whole mounts

Dissection of adult brains and immunostaining was carried out as previously described (Jové *et al.*, 2020; Matthews et al., 2019b). 6 to 14 day-old mosquitoes were anesthetized on ice and decapitated. Heads were fixed in 4% paraformaldehyde (Electron Microscopy Sciences 15713-S), 1X Ca^{+2}, Mg^{+2} free PBS (Thermo 14190144), 0.25% Triton X-100 (Sigma 93443), and nutated for 3 hr at 4ºC. Brains were then

dissected and placed in cell-strainer caps (Falcon #352235) in a 24 well-plate. All subsequent steps were performed on a low-speed orbital shaker. Brains were washed for 15 min at room temperature in 1x PBS with 0.25% Triton X-100 (0.25% PBT) at least 6 times. Brains were permeabilized with 4% Triton X-100 with 2% normal goat serum (Jackson ImmunoResearch #005-000-121) in PBS at 4ºC for 2 days. Brains were washed for 15 min at least 6 times with 0.25%PBT at room temperature. Brains were incubated in 0.25%PBT plus 2% normal goat serum with primary antibodies at the following dilutions: rabbit anti-RFP (Rockland 600-401-379) 1:200 and mouse anti-*Drosophila melanogaster* Brp (nc82) 1:5000. The nc82 hybridoma developed by Erich Buchner of Universitätsklinikum Würzburg was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Primary antibodies were incubated for 2 nights at 4ºC degrees then washed at least 6 times for 15 min with 0.25% PBT at room temperature. Brains were incubated with secondary antibody for 2 nights at 4ºC with secondary antibodies at 1:500 and 2% normal goat serum. Secondary antibodies used were goat anti-rabbit Alexa Fluor 555 (Thermo A32732) and goat anti-mouse Alexa Fluor 647 (Thermo A-21235). Brains were then washed for 15 min at least 6 times with 0.25% PBT at room temperature and mounted in Slowfade Diamond (Thermo S36972) using #1.5 coverslips as spacers before confocal imaging.

Antibody staining – antennal whole mounts

This protocol was adapted from a *Drosophila* embryo staining protocol (Manning and Doe, 2017). 6 to 10 day-old mosquitoes were anesthetized, decapitated, and placed in

1.5 mL 5 U/mL chitinase (Sigma C6137) and 100 U/mL chymotrypsin (Sigma CHY5S) in 119 mM NaCl, 48 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25mM HEPES buffer on ice. Male heads were incubated for 5 min on a ThermoMixer (Eppendorf 5382000023), and 25 min in a rotating hybridization oven, and female heads were incubated for 10 min on the ThermoMixer and 50 min in rotating oven, all at 37ºC. Heads were then rinsed once and fixed in 4% paraformaldehyde, 1X Ca^{+2}, Mg^{+2} free PBS, and 0.25% Triton X-100 for 24 hr at room temperature on a rotator. All subsequent 4ºC steps used a nutator, and room temperature steps used a rotator. Heads were washed for 30 min at room temperature at least 3 times in 1X PBS with 0.03% Triton X-100 (0.03% PBT). Antennae were then dissected into 0.5 mL microfuge tubes and dehydrated in 80% methanol / 20% DMSO for 1 hr at room temperature. Antennae were washed in 0.03% PBT for 30 min at room temperature, and blocked/permeabilized in 1X PBS, 1% DMSO (Sigma 472301), 5% normal goat serum, 4% Triton X-100 for 24 hr at 4ºC. Antennae were washed for 30 min at least 5 times with 0.03% PBT, 1% DMSO at room temperature, and then moved to primary antibody in 1X PBS, 1% DMSO, 5% normal goat serum, 0.03% Triton X-100 for 72 hr at 4ºC. Primary antibodies used were mouse anti-*Apocrypta bakeri* Orco monoclonal antibody #15B2 (1:50 dilution, gift of Joel Butterwick and Vanessa Ruta), and rabbit anti-RFP (1:100, Rockland 600-401-379). Orco monoclonal antibody specificity was verified in *Aedes aegypti* by staining *orco* mutant antennae, which showed no staining (data not shown). Antennae were washed for 30 min at least 5 times with 0.03% PBT, 1% DMSO at room temperature, and then washed overnight in the same solution. Antennae were then moved to secondary antibody (1:200) and DAPI (1:10000, Sigma D9542) in 1X PBS, 1% DMSO, 5% normal

goat serum, 0.03% Triton X-100 for 72 hr at 4ºC. Secondary antibodies used were goat anti-mouse Alexa Fluor 488 (Thermo A-11001) and goat anti-rabbit Alexa Fluor 555 Plus (Thermo A32732). Antennae were washed for 30 min at least 5 times with 0.03% PBT, 1% DMSO at room temperature, and then washed overnight in the same solution. Antennae were rinsed in 1X PBS, rinsed 3 times in Slowfade Diamond (Thermo S36972), and mounted in Slowfade Diamond.

RNA *in situ* hybridization

RNA was detected in whole mount antenna and maxillary palp using the hybridization chain reaction (HCR) technique as previously described (Choi et al., 2018) with modifications. Probes, amplifiers, Probe Hybridization Buffer, Amplification Buffer, and Probe Wash Buffer were purchased from Molecular Instruments. 7-14 day-old mosquitoes were cold anesthetized, manually decapitated with forceps, and heads were fixed in 4% paraformaldehyde, 1X PBS, 0.25% Triton X-100 on a rotator at 4^{o}C for 3 hours. Heads were washed 4 times for 10 min each in 0.25% PBS- Triton X-100. Brains were dissected in 0.1% PBS-Tween-20 on ice and dehydrated with a graded series of methanol/0.1% PBS-Tween: 25% methanol in 0.1% PBS-Tween-20 for 10 min on ice, 50% methanol in 0.1% PBS-Tween-20 for 10 min on ice, 75% methanol in 0.1% PBS-Tween-20 for 10 min on ice, and two washes of 100% methanol for 10 min on ice. Tissues were incubated overnight in 100% methanol at -20oC and were subsequently rehydrated with a series of graded methanol/0.1% PBS-Tween-20: 75% methanol in 0.1% PBS-Tween-20 for 10 min on ice, 50% methanol in 0.1% PBS-Tween20 for 10 min on ice, 25% methanol in 0.1% PBS-Tween-20 for 10 min on ice, and two washes of

0.1% PBS-Tween-20 for 10 min each on ice. Tissue was digested in 20 µg/mL Proteinase-K (Thermo Fisher AM2548) in 0.1% PBS-Tween for 30 min at room temperature and washed twice with 0.1% PBS-Tween-20 for 10 min each at room temperature. Tissue was fixed in 4% paraformaldehyde in 0.1% PBS-Tween-20 for 20 min at room temperature and washed 3 times for 10 min each in 0.1% PBS-Tween-20 at room temperature. Tissue was incubated in Probe Hybridization Buffer at room temperature for 5 min and then in 37^{o}C pre-warmed Probe Hybridization Buffer rotating in a hybridization oven for 30 min. 4 pmol of each probe set was prepared in 37oC prewarmed Probe Hybridization Buffer and tissue was incubated in probe solution at 37^{o}C in a hybridization oven for 2 nights. Tissues were washed in 37oC pre-warmed Probe Wash Buffer 5 times for 10 min each at 37oC. Tissues were washed twice in 5X SSC-0.1% Tween-20 at room temperature for 10 min each. Tissues were pre-amplified in room temperature Amplification Buffer for 10 min. 9 pmol hairpins were separately prepared by heating 3 µl of 3 µM stock of hairpins H1 and H2 at 95oC for 90 sec on an Eppendorf Mastercycler and allowing to cool to room temperature in a dark drawer for 30 min. Hairpins were resuspended in 100 µl amplification buffer and tissues were incubated in this hairpin solution in the dark on a rotator at room temperature overnight. Tissues were washed 5 times for 10 min each in 5X SSC 0.1% Tween-20 and mounted in SlowFade Diamond (Thermo Fisher S36972) on glass slides with coverslips for confocal imaging.

Stylet and labium dye fills

Dye fills were performed as previously described (Jové *et al.*, 2020). 7 to 14 day-old *11211^{QF2}/+;QUAS-CD8-GFP/+* mosquitoes were anesthetized on ice. The labium was separated from the stylet using forceps. Mosquitoes were affixed on their side to a plastic dish (Falcon #353001) using UV-curable glue (Bondic, Amazon #B0181BEHQU) or double-sided tape so that the stylet and labium were flat on the dish and distal tips were separated. The labium was cut at the base of the labellar lobes using a scalpel and 1 µL of Dextran, Texas Red diluted to 1 mg/10 µL in External Saline was added immediately. The mosquito was left on ice and covered for approximately 3-5 min before excess dye was removed by pipette. The stylet was cut approximately 300 – 750 µm away from the distal tip and 1 µL of Dextran, Biotin, 3000 MW, Lysine Fixable (ThermoFisher #D7156) diluted to 1 mg/10 µL in External Saline was immediately added. The External Saline recipe (Matthews *et al.*, 2019) is based on *Drosophila melanogaster* imaging saline: 103 mM NaCl, 3 mM KCl, 5 mM 2-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 1.5 mM CaCl2, 4 mM MgCl2, 26 mM NaHCO3, 1 mM NaH2PO4, 10 mM trehalose, 10 mM glucose, pH 7.3, osmolality adjusted to 275 mOsm/kg. The mosquito was left on ice and covered for approximately 3-5 min before excess dye was pipetted up. Mosquitoes were left at 4°C overnight with a moist Kimwipe to prevent desiccation. Heads were then removed and fixed prior to tissue dissection according to the tissue fixation protocol.

Fixed heads of both single and double dye-fill preparations were then dissected and brains were placed in cell-strainer caps (Falcon #352235) in a 24 well-plate. Brains

were stained using a modification of previously published methods (Matthews *et al.*, 2019). All subsequent steps were performed on a low-speed orbital shaker. Brains were washed at room temperature in PBT for at least 4 times 15 min. Brains were permeabilized with 4% Triton X-100 with 2% normal goat serum (Jackson ImmunoResearch #005-000-121) in PBS at 4°C for 2 days. Brains were washed at least 5 times 15 min with PBT at room temperature before being incubated in PBT plus 2% normal goat serum for 1 day at 4°C degrees. The following dyes at the following dilutions were used: Phalloidin-Alexa Fluor 405 1:100 (ThermoFisher # A30104) and Steptavidin-Alexa Fluor 647 1:100 (ThermoFisher #S21374). Following dye incubations, brains were washed at least 5 times 15 min with PBT at room temperature. Brains were then washed in PBT and mounted in SlowFade Diamond.

Confocal image acquisition

Images were acquired with a Zeiss Axio Observer Z1 Inverted LSM 880 NLO laser scanning confocal microscope (Zeiss) with either 25x/0.8 NA (whole brains) or 40x/1.4 NA (antennal lobes, antennae) immersion-corrected objective at a resolution of 1024 x 1024 or 2048 x 2048 (brains) or 3024 x 1024 (antennae) pixels. Confocal images were processed in ImageJ (NIH).

Arm-next-to-cage assay

This assay was performed as described previously (DeGennaro *et al.*, 2013). Briefly, for each trial, 20 adult mosquitoes were sorted under cold anesthesia (4ºC) and placed in a cage and allowed to acclimate for 30 min. A human arm was placed 2.5 cm from one

side of a standard 28 x 28 x 28 cm cage. Mosquitoes could not directly contact the human arm. A Logitech C920s HD Pro Webcam was positioned to take images of mosquitoes responding to the human arm. Trials ran for 10 min and images were acquired at a rate of 1 frame per sec. To quantify mosquito responses, we manually counted the number of mosquitoes resting on the lower portion of the screen closest to the human arm.

Feeding assay

Mosquitoes were cold-anesthetized, and 20 mosquitoes were sorted into 250 mL bottles covered with a taut net secured with rubber bands. They were allowed to acclimate for 24 hr with access to water through cotton balls. The following meals were presented: water, 10% sucrose, or sheep's blood (Hemostat DSB100) supplemented with 1mM ATP (Sigma A6419). Meals were warmed to 45ºC before being used in the assay. 10 mL of a given meal was pipetted into the bottle caps, animals were activated with a 30 sec pulse of 4% CO_2, and bottles were inverted on top of the caps. Mosquitoes were allowed to feed on each meal through the net for 10 min and were then anesthetized at 4ºC and scored as fed if any level of feeding was observed, as assessed by visual inspection of the abdomen of the animal.

Insemination assay

Mosquitoes were separated by sex at the pupal stage and sex was confirmed within 24 hr of eclosion. For each trial, 10 female Liverpool-IB12 virgin mosquitoes were crossed to 11 virgin male mosquitoes of either Liverpool-IB12 or *fruitless*$^{\Delta M/}$*fruitless*$^{\Delta M\text{-}tdTomato}$

genotype in a bucket cage for 24 hr, with access to 10% sucrose. Mosquitoes were then anesthetized at 4ºC, females separated from males, and female spermathecae were dissected to score for insemination as a sign of successful mating (Degner and Harrington, 2016). Control virgin females were dissected in parallel to verify absence of insemination.

Quattroport olfactometer

Details of Quattroport fabrication and operation are available at https://github.com/VosshallLab/Basrur_Vosshall2020. Briefly, the Quattroport consists of four tubes, each with its own stimulus box, trap, and mosquito start chamber. There are adjustable gates between each chamber. The stimulus was placed upstream of a trap, and mosquitoes are prevented from contacting the stimulus by a mesh barrier. In each trial, four stimuli were run in parallel, with the positions of stimuli randomized and rotated between each trial. Air was filtered and pumped into each box, either at a final concentration of 1% CO_2 (for host-seeking assays) or at ambient CO_2 (honey-seeking assays). For all assays, ~20 mosquitoes were sorted and placed into canisters the day of behavior. Mosquitoes were allowed to acclimate in the assay for 10 min, then exposed to the stimulus for 30 sec, after which gates were opened and animals allowed to fly for 5 min. After this time, gates were closed and mosquitoes were counted to quantify the percent of mosquitoes in the trap.

For honey assays, 3 to 7 day-old mosquitoes were fasted for 24 hr before the experiment by replacing 10% sucrose with a water source. CO_2 was not added for

honey assays. Either 1 g of leatherwood honey (Tasmanian Honey Company, Tasmania, Australia) or glycerol (Sigma G5516) was applied to a 55 mm diameter Whatman filter paper circle (GE Healthcare, Buckinghamshire, UK) and placed in a Petri dish.

For host-seeking assays, mosquitoes were allowed access to sucrose before the experiment. A final concentration of 1% CO_2 was supplied in the airstream for the duration of the 5 min 30 sec assay in all host-seeking assays (for both human forearm/odor stimuli and blank/unworn nylon controls). For live human host-seeking assays, a human subject placed their forearm on an acrylic box, exposing a 2.5 x 5 cm rectangle of skin to the airstream.

For human odor host-seeking assays, the same human subject wore a tan nylon sleeve (L'eggs Women's Comfortable Everyday Knee Highs Reinforced Toe Panty Hose, modified with scissors to remove the toe area) on their forearm. A second black nylon sleeve was worn on top of the tan nylon odor sampling sleeve to protect it from external odors. After 6 hr of continuous wear, the black nylon sleeve was discarded, and the tan nylon sleeve was frozen at -20ºC. Nylons were used within one week of being worn. On the day of the assay, nylons were thawed for at least 1 hr at room temperature. A 10 x 14 cm piece of the sleeve was presented with the skin-contacting surface facing upward in the stimulus box along with CO_2. Unworn nylons were similarly frozen, thawed, and cut to serve as negative controls.

Heat-seeking assay

Experiments were performed as previously described (Corfas and Vosshall, 2015; McMeniman *et al.*, 2014). Briefly, 45-50 mosquitoes were fasted for 3 hr before the experiment by replacing 10% sucrose with a water source and were then transferred into a custom-made Plexiglass box (30 x 30 x 30 cm), with carbon-filtered air pumped continuously into the box via a diffusion pad installed on the ceiling of the enclosure. All stimulus periods lasted 3 min and were presented on a single Peltier element (6 x 9 cm, Tellurex) covered with a piece of standard white letter-size printer paper (NMP1120, Navigator) cut to 15 x 17 cm and held taut by a magnetic frame. CO_2 pulses (20 sec, to >1000 ppm above background levels) were added to the air stream and accompanied all stimulus period onsets. Mosquito landings on the Peltier were monitored by fixed cameras (FFMV-03M2M-CS, Point Grey Research) with images acquired at 1 Hz. Images were analyzed using custom MATLAB scripts to count mosquito landings within a fixed target region. Mosquito occupancy on the Peltier was quantified during seconds 90-180 of each stimulus period.

Blood-feeding glytube assay

This assay was performed as previously described (Costa-da-Silva *et al.*, 2013; Duvall *et al.*, 2019; Jové *et al.*, 2020). 7 to 21 day-old female mosquitoes were anesthetized at 4°C and sorted into groups of 15-20 females, and placed into a 32 oz. HDPE plastic cup (VWR #89009-668). The cup was prepared by cutting a 10 cm hole in the lid with a razor blade, covering the cup with a 20 cm x 20 cm piece of white 0.8 mm polyester mosquito netting (American Home & Habit Inc. #F03A-PONO-MOSQ-M008-ZS) and

securing the mesh to the cup by snapping on the modified lid. Animals recovered overnight at 25 - 28°C, 70%–80% relative humidity with access to water. The assay chamber was a modification of previously published methods (McMeniman *et al.*, 2014) and used a translucent polypropylene storage box 36 cm L x 31 cm W x 32 cm H with a removable lid. One 1.5 cm hole was made on the chamber wall and was used to introduce silicone tubing for CO2 delivery. The CO2 diffusion pad (8.9 cm x 12.7 cm; Tritech Research) was affixed to the inner center of the lid to allow delivery of purified air and CO2 to condition the chamber atmosphere during the trial. Up to 4 cups were placed in the chamber per trial and feeding positions were randomized according to meal during assays. Females were fed sheep blood using Glytube membrane feeders exactly as described, except the Parafilm feeding surface was not rubbed on human skin prior to offering the Glytube to mosquitoes to avoid introducing contact chemosensory cues as secondary stimuli in our experiments. All meals and Glytubes were preheated for at least 15 min in a 45°C water bath and ATP was added to meals immediately before feeding and mixed by vortexing. At the start of each trial, cups were placed in the assay chamber and allowed to acclimate for 5 min before 1 Glytube containing 1.5 mL of a given meal was placed on top each cup and CO2 was turned on for 15 min. Fed females were scored by eye for engorgement of the abdomen. In the rare cases that females partially fed they were counted as non-fed and discarded.

Nectar-feeding assay

This assay was performed as previously described (Jové *et al.*, 2020). Animals were prepared exactly as described for the Glytube assay. A cotton ball (Fisher Scientific

#22456880) was soaked in 10% sucrose mixed with a green food-dye, and the cotton ball was briefly dabbed on a Kimwipe to prevent excess liquid from dripping through the mesh, and placed on top of the mesh covering the cup. Animals were allowed to feed for 4 hours. After feeding, sugar feeding was scored manually.

Quantification, statistical analysis, and reproducibility

All statistical analysis was performed using GraphPad Prism Version 8. Data collected as percent of total are shown as mean±s.e.m. Details of statistical methods are reported in the figure legends. Preliminary experiments were used to assess variance and determine sample sizes before carrying out experiments. Typically, sample sizes were n=6-14 in behavioral assays. We used similar sample sizes for all experiments where the same variable was being compared. No data were excluded from this study. Since mosquito behavior is variable, all olfactometer experiments with a human arm were carried out repeatedly to assess the effect of external environmental conditions on behavior. No experiments were performed on days when <40% of wild-type females responded to a live human arm. No data met these exclusion criteria. All attempts at replication over multiple days were successful. We carried out all experiments with different groups of animals hatched up to 4 weeks apart, and over multiple days. Several experiments were carried out repeatedly over the course of this study, namely the wild-type female response to a live human arm in Quattroport olfactometer assays. These results were robust and reliable over the course of the many years it took to complete this study. For all experiments, mosquitoes from a cage were randomly selected and sorted by sex into groups for behavioral assays. All stimuli and genotypes

were interspersed, and positions were randomized when possible. Every experiment involves replicates collected over multiple days, to ensure that there is no effect of daily environmental or experimental conditions. We also collected a similar sample size for each variable every time the experiment was run, to ensure no effect of external conditions. Blinding to genotype was performed in the heat-seeking assays. The experimenter was not blinded to genotype in the host-seeking assays. This is because the mutant phenotype we describe is so robust it is impossible to be blinded in these assays.

CHAPTER 8. References

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