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Decoding the Taste System of the Disease Vector Mosquito *Aedes Aegypti*

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Esto no haiga sido posible sin sus enormes sacrificios.
Los amo.

ABSTRACT OF THE DISSERTATION

Decoding the Taste System of the Disease Vector Mosquito *Aedes Aegypti*

by

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In the field of insect chemosensation, knowledge of basic principles has come from the model organism, *Drosophila melanogaster*. However, the different life histories of Culicids result in behaviors that are unique to mosquitoes. In hematophagous species, these behaviors facilitate the transmission of mosquito-borne diseases that pose grave threats to humans. Currently, we have a better understanding of the olfactory system despite many critical taste-driven behaviors such as blood-feeding. To better understand the role of the taste system and how it helps guide taste-driven behaviors, we aimed to characterize the neuronal responses of the mosquito labellum. A comprehensive survey was carried out on the labellar sensilla of the yellow fever mosquito *Aedes aegypti* using a panel of different categories of compounds (sweet, bitter, salt, water, and amino acids). The qualitative and quantitative differences observed across the different taste categories revealed five functional groups. Our survey showed that in addition to

sweet, bitter, water, and salt taste responses, mosquito labellar sensilla exhibit neuronal sensitivity to amino acids. Analysis of responses to mixtures suggests that amino acid sensitivity maps to a neuron distinct from those that respond to the other four taste categories. We then investigated whether the sensitivity of the peripheral taste organs is modulated by physiological changes that the female mosquito undergoes throughout the gonotrophic cycle using a diagnostic panel of tastants. We first measured and compared the sensitivity of non-mated females to that of mated females and found that sensillar sensitivity to sucrose correlates to the meal preference during both states. We then measured labellar sensitivity before a blood meal and 18-20 hours after obtaining a blood meal. Lastly, we measured the sensitivity of male mosquitoes and compared them to the female response. We found that the male sensitivities are higher than the mated females but more similar to non-mated female labellar sensitivities. Overall, this dissertation presents the first map of the functional organization of taste sensilla of the labellum for a major mosquito vector. Our results raise the possibility that alterations in peripheral taste sensitivity underlie shifts in feeding preference during a female's gonotrophic cycle.

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CHAPTER I

Introduction to the organization of the mosquito taste system

Portions of this chapter were submitted as a chapter to a book that is in preparation for publication. The book Sensory Ecology of Disease Vectors will be published by Wageningen Academic Publishers, 2022 – **AM Lomelí** and AA Dahanukar.

An introduction to the organization of the mosquito taste system

Overview: The insect taste system

The sense of taste is a chemosensory system used to evaluate non-volatile chemical cues at close range. For mammals, taste buds on the tongue will convey information about the palatability or toxicity of a substrate. G-protein coupled receptors detect different taste modalities such as sweet (T1R2+T1R2), bitter (T2Rs), and umami (T1R1+T1R3), while the responses to sour and salt are mediated by epithelium sodium channels (ENaC) and ion channels (OTOP), respectively (Chandrashekar *et al.,* 2010; Lewandowski *et al.,* 2016; Tu *et al.,* 2018; Zhang *et al*., 2003). The information gathered via peripheral taste cells will be used to drive feeding behaviors that will lead to the acceptance or avoidance of food.

In insects, a taste organ is recognized by the presence of uniporous trichoid sensilla that are innervated by up to four chemosensory neurons. Unlike vertebrates, insect taste organs are located throughout their bodies, such as the mouthparts, legs, ovipositor, and margins of the wings. The chemosensory cues gathered by the different taste organs can guide taste-driven behaviors such as locating appropriate food sources and mates, hosts, and suitable sites for oviposition. Chemical signals that drive these behaviors are detected via receptors expressed on the dendritic extensions of the chemosensory neurons that innervate the taste sensilla.

Studies in the model organism, *D. melanogaster,* have contributed to a fundamental understanding of the insect taste system. Taste is coded via the many receptor gene families expressed throughout the peripheral taste organs. The receptor gene families include gustatory receptors (Gr), ionotropic receptors (Irs), pickpocket channels (PPK), and transient receptor potential (TRP) channels. Combining single-sensillum extracellular tip recordings (SSR) and genetic tools available for *D. melanogaster* have made it possible to discern the identity of innervating neurons and the specific receptors that label each population. Classically, the neurons have been described as sweet-, bitter-, water- or salt-sensing neurons.

The Gr family consists of 68 Grs that primarily mediate responses to sweet and bitter compounds. Sweet taste is detected via a highly conserved clade of eight Grs expressed in the sweet-sensing neurons that mediate appetitive taste. The remaining 60 Grs are expressed in different combinations across the bitter-sensing neurons and mediate avoidance behaviors (Weiss *et al.,* 2011). The response to water is mediated by PPK28, a member of the ENaC/DEG family, which also labels the population of water neurons (Cameron *et al.,* 2010). Based on this information, one might infer that all taste modalities are mediated in the same straightforward manner. However, the Ir gene family mediates responses to several taste modalities, and their expression is not segregated to a specific neuronal population (Koh *et al.,* 2014; Sanchez-Alcañiz *et al.,* 2018). In addition, studies describing salt taste in *Drosophila* have shown that taste coding is complex

and a result of a concerted input spanning multiple neuronal populations (Jaeger *et al.,* 2018).

Taste information gathered by the peripheral taste organs is relayed to the subesophageal zone (SEZ), also known as the primary taste center of the brain. Neuronal projections from all taste organs converge in this region but are separated by taste organ and modality. Currently, two models exist to explain how the brain processes taste information. The labeled line model suggests that different taste modalities detected at the periphery will activate distinct pathways in the brain, as observed with the sweet and bitter taste modalities that give rise to opposing feeding behaviors. The second model, known as the across-fiber model, suggests that central neurons in the brain respond to multiple taste modalities. In this case, different tastes are recognized by the spatial or temporal pattern of the neuronal population activity. Recent studies support the labeled line model of processing (Harris *et al.,* 2015). However, several uncharacterized motor interneurons in the SEZ are involved in processing taste cues (Harris *et al.,* 2015; Scott *et al.,* 2018). Therefore, more detailed studies are needed to learn more about how processing of taste information leads to specific behavioral output.

Historically, insect taste-driven behaviors have posed threats to agriculture and human public health. Mosquitoes are considered one of the deadliest animals in the world. Collectively, mosquitoes contribute to the majority of vector-transmitted diseases, with *Aedes aegypti* being responsible for transmission of six alone (World Health Organization 2020). The female mosquito's need for a blood

meal to obtain protein for egg development facilitates the spread of infectious diseases. Consequently, millions of human lives are lost every year. A female mosquito relies heavily on its chemosensory system to find human hosts. The olfactory system allows them to hone in from afar by detecting exhaled carbon dioxide in combination with heat and odorants from human skin and sweat, which are detected at close range (Kwon *et al*., 2006; Melo *et al*., 2004; Riabinina *et al*., 2016). Upon landing, the female mosquito will employ the taste system to carry out the last phase of host-seeking behaviors to acquire a blood meal. However, very little is understood about the role of the taste system during this brief critical period.

Although the primary focus in the field has been to avoid blood-feeding behaviors via the mosquito olfactory system, several taste-driven behaviors are observed in both sexes of mosquitoes that remain elusive due to the overall lack of information about the mosquito taste system. Learning more about mosquito taste sensitivities will reveal more about how taste is coded across different taste organs and help discern the contribution of each organ to specific taste-driven behaviors. Systems-level analyses can reveal areas of mosquito biology that can be potentially exploited for mosquito control. This review will focus on the taste system of adult mosquitoes and its role in taste-guided behaviors. Here we summarized the current knowledge about the overall organization and function of the taste system at the periphery and the central nervous system.

Anatomical organization of the gustatory system in mosquitoes

The adult mosquito can detect taste cues via hair-like trichoid type 1 sensilla (T1) located throughout the taste organs. The tip of the tarsi, the labellum, and the female labrum make up the external taste organs (**Figure 1.1)**. Internally, the only taste organ is the cibarium, a structure located between the base of the labrum and the anterior end of the pharynx. Taste information gathered by the taste organs guides behaviors such as surveying food sources, blood-feeding, finding potential mates, hosts, and suitable sites for oviposition. T1 sensilla are also located on the margins of the wings. However, their role is not well understood.

In many dipteran species, the labellum is one of the primary taste organs. However, there are many distinctions between the sponging-sucking mouthparts of phytophagous dipterans like *Drosophila* or *Musca* and those of piercing-sucking mouthparts observed in Culicids. In mosquitoes, the proboscis is a complex elongated structure with a labial sheath, known as the labium, that runs the length of the organ with a pair of labellar palps at the distal end. The labium sheaths the six stylets (one labrum, one hypopharynx, a pair of mandibles, and a pair of maxillae) that come together to form the mosquito feeding tube in both male and female mosquitoes (**Figure 1.1)**. Of the six stylets, only the labrum in hematophagous female mosquitoes has chemosensory capabilities and is involved in blood-feeding. A mosquito can move its stylets and labium independently. Therefore, while blood-feeding, the labium is retracted to facilitate the insertion of the stylets into the host skin.

The change in dietary requirements seen in hematophagous female mosquitoes causes a shift in meal preference from sugar meals to blood meals. These meals are directed to a different part of the digestive tract: sugar meals go to the crop while blood meals go to the midgut (**Figure 1.1)**. The switching mechanism that determines a meal's destination is thought to be mediated by the cibarium because of its role in evaluating ingested fluids (Lee and Craig 1983; Lee and Craig 2009).

Gustatory sensilla

The T1 gustatory sensilla are characterized by a single pore at the apex of the hair, which differs from the highly porous olfactory sensilla found on olfactory appendages. The terminal pore on the taste sensilla necessitates contact to explore substrates. Most T1 sensilla will house one mechanosensory neuron and two to five chemosensory neurons whose dendrites extend through the shaft of the hair to reach the pore at the apex (Lee and Craig 2009; Pappas and Larsen 1975).

The number of gustatory sensilla on each taste appendage and their morphology and topography vary across the different taste organs and is species-dependent. Detailed descriptions of the functional organization of the mosquito taste sensilla do not exist as they do for the fly, *Drosophila melanogaster*. However, in species like *Aedes aegypti* and *Anopheles gambiae*, scanning electron micrographs (SEM) of the labellum reveal presence of about 23-30 larger trichoid-shaped sensilla (T1) and approximately 30 smaller trichoid-shaped

sensilla (T2) (Hill and Berry Smith 1999; Lee and Craig 2009; Kessler *et al.*, 2013; Saveer *et al.*, 2018). In *Ae. aegypti* and *A. gambiae*, the T1 sensilla on the labellar palps are stereotypically positioned among a field of smaller non-innervated microtrichia sensilla and are labeled based on their stereotypical topography (Hill and Berry Smith 1999; Kessler *et al.*, 2015).

Out of the six stylets that make up the mosquito mouthpart, the labrum is the only chemosensory structure. Due to the sclerotized nature of this stylet, the four sensilla found here are also sclerotized and lie flat at the distal end of the structure. The paired sensilla, known as the apical and sub-apical sensilla, are specific to the female stylet. Immuno-staining shows about 27 neurons found along the edges of the labrum (Jung *et al.,* 2015*;* Jové *et al.*, 2020). The group comprises a mix of chemosensory, mechanosensory, and support cells (Jung *et al.*, 2015). Those with chemosensory function extend their dendritic projections to either the apical or sub-apical sensilla at the tip, each of which is innervated by five dendrites (Liscia *et al.,* 1993). In addition to the four sensilla, a pair of campaniform sensilla is located at the structure's inner surface or floor. The campaniform sensilla are thought to detect the flow of imbibed liquids and the movement of the stylet (Jung *et al.*, 2015; Lee 1974).

After fluids are detected by either of the peripheral mouthparts and imbibed through the food canal, they pass to the cibarium. There are at least five different types of sensilla found among the ventral and dorsal surfaces of this structure; two to six are of the trichoid type, depending on the mosquito species (Lee 1974; Lee

and Craig 1983). The cibarium's location makes the sensilla inaccessible for meticulous electrophysiological experiments. However, behavior experiments suggest that the cibarium functions as the last checkpoint for feeding and determines whether a mosquito will reject or ingest fluids (Owen 1963; Salama *et al.*, 1966). A potentially toxic or harmful stimulus not detected by the labellum or tarsi can be rejected once it reaches this point. However, when it comes to appetitive stimuli like nectar and blood, the cibarium is thought to influence the meal destination, directing blood and nectar to the midgut and crop, respectively (Lee and Craig 1983; Lee and Craig 2009).

The sensilla located around the male and female genitalia are exclusively mechanosensors innervated by a single bipolar neuron (Rossignol and McIver 1977). Therefore, no chemosensory sensilla are present on the ovipositor, as in *Drosophila* (Stocker 1994). Males use their terminal hairs for copulatory behaviors (Rossignol and McIver 1977), and, contrary to belief, females do not use theirs for oviposition. Instead, female mosquitoes gather information about suitable sites for egg-laying by using their tarsi (Matthews *et al.*, 2019). In *Ae. aegypti*, there are five types of sensilla on the tarsi, four of which are mechanosensors (spines, type A, type B, and campaniform sensilla). The last type, known as type C, are chemosensory sensilla innervated by four to five dendrites. These are subdivided into C1, C2, and C3 (McIver and Siemicki 1978).

Like some of their Dipteran relatives, mosquitoes also have sensilla along their wing margins. In *Drosophila*, they respond to both sweet and bitter

compounds, but careful analysis of mosquito sensilla has yet to be done (Raad et al., 2016).

Receptor gene expression in adult mosquito chemosensory taste neurons

The Culicidae family of mosquitoes comprises more than 3,500 species separated by millions of years in divergence (Foster and Walker 2019). Genome data is available for only a handful of genera within the family. However, all have reported presence of three chemosensory gene families (Ionotropic receptors (Irs), Odorant receptors (Ors), and Gustatory receptors (Grs)), whose expression occurs in chemosensory neurons and other types of cells. A total of 61**,** 64, and 70-79 Grs have been found in the genomes of *Anopheles gambiae, Culex quinquefasciatus, and Aedes aegypti,* respectively (Kent *et al.,* 2008; Zhou *et al.,* 2014). Considerable variation can be seen in the number of Irs and Ors across the three species as well (*A. gambiae:* 46 Irs, 75 Ors; *C. quinquefasciatus*: 69 Irs, 178 Ors; *Ae. aegypti*: 95 Irs, 127 Ors) and improved genome annotation since has nearly doubled the number of Irs that have been identified, at least in *A. gambiae* and *Ae. aegypti* (Matthews *et al*., 2018). Chemosensory gene expression likely varies with the stage (larval or adult), tissue, sex, and fed state (sugar or blood). Several detailed descriptions exist in the literature, but the chemosensory repertoire for each condition has not yet emerged.

There is an overall reduction in the number of chemosensory genes (21 Grs, 87 Ors, 38 Irs) of the non-hematophagous mosquito, *Toxorhynchites*

amboinensis, when compared to the chemosensory gene repertoire of hematophagous mosquitoes (Zhou *et al.,* 2014). Given the fewer number of Grs and Irs found in *Toxorhynchites*, it could become easier to identify which chemosensory receptors are involved in sugar reception and nectar meal acquisition versus those specifically involved in host-seeking.

In addition to the three chemosensory gene families described above, additional chemosensory receptors encoded by the *odorant binding protein (OBP), pickpocket (ppk), and transient receptor potential (Trp)* gene families are also present in mosquito taste organs (Matthews *et al.*, 2016; Saveer *et al.*, 2018).

Sweet taste receptors

Sugar recognition was first attributed to members of a divergent subset of eight 7 trans-membrane Grs in *Drosophila* (Dahanukar *et al.,* 2007; Jiao *et al.,* 2008; Robertson *et al.,* 2003). Since then, Grs related to the putative sweet receptors have been found in many other insects, including mosquitoes, beetles, moths, bees, and wasps (Kent and Robertson 2009). Despite the large number of insects that rely on plant-derived sugary substances for energy, there is much variation in the number as well as amino acid sequence of sweet Grs found across different insect species. In most cases, it is difficult to identify one-to-one orthologs of the eight *D. melanogaster* sweet Grs outside of the drosophilids, and mosquitoes are no exception. *Anopheles*, *Aedes*, and *Culex* mosquitoes encode 7-13 functional sweet Grs, which all cluster with the *Drosophila* sweet Grs but show

mosquito-specific lineage expansions (Kent and Robertson 2009). Mosquitoes probe flowers with their labium, which is covered with taste hairs while the labrum remains retracted. Accordingly, transcripts of sweet Grs have are found in the labellum and tarsi. Sweet Gr expression was not found in the labrum of *Aedes aegypti* (Jové *et al.,* 2020), suggesting that the labrum may be merely involved as part of a feeding tube when it comes to nectar feeding. Consistent with these observations is the absence of nectar sugar-sensitivity in apical and subapical sensilla of the labrum (Jové *et al.,* 2020).

A recent study designed a transgenic sweet Gr driver in *Ae. aegypti* (Jové *et al.,* 2020). Reporter expression of *AaegGr4-GAL4* was observed in single neurons innervating several labellar trichoid sensilla, consistent with the presence of a single sugar-sensing neuron in each sensillum. As mapped in flies, multiple sweet Grs are expected to be co-expressed in each sugar-sensing neuron, but Gr expression has not been investigated at this resolution yet.

Bitter taste receptors

Bitter-sensing neurons recognize many plant defense chemicals and their metabolites, and mosquitoes detect a range of bitter or aversive compounds (Dennis *et al.,* 2019; Kessler *et al.,* 2013; Sanford *et al.,* 2013; Sparks and Dickens 2016a; Sparks and Dickens 2016b). Not much is understood about specific receptors/receptor complexes that mediate these responses in mosquitoes. However, bitter-sensing neurons in *Drosophila* are known to co-express large

repertoires of Grs (Ling *et al*., 2014; Weiss *et al*., 2011). A few bitter Grs are widely expressed across most if not all bitter-sensing neurons and are typically required for all bitter responsiveness in these neurons. By contrast, other bitter Grs can be selective to varying degrees and may be expressed in very small subsets of bitter neurons. Investigations of the functional composition of a bitter receptor have found that ectopic expression of three or four Grs, including Gr66a and Gr33a of the commonly expressed Grs, is sufficient to confer responses to selected bitter tastants (Shim *et al*., 2015; Dweck and Carlson 2020). Comparison of the mosquito and fly Gr families reveals clades that are represented in both groups of organisms as well as clades that are unique to each (Hill *et al.,* 2002; Kent *et al.,* 2008).

Chemoreceptor expression has been evaluated via transcriptome analyses of chemosensory organs of different mosquito species. Gr expression is present in taste organs of both males and females, and the labellar palps of the proboscis express about 50-55% of the Grs in the genome (Matthews *et al*., 2016; Saveer *et al.,* 2018; Sparks *et al.,* 2013). Those with the highest expression levels are found in both sexes and include the sweet Grs as well as orthologs of a highly conserved internal fructose sensor, *Dmel* Gr43a (Jové *et al.,* 2020; Miyamoto *et al.,* 2012). In addition, there are several Grs related to bitter clades in *Drosophila*, as well as clades that are uncharacterized or that represent mosquito-specific expansions whose functions remain to be studied (Saveer *et al.,* 2018; Sparks *et al.,* 2013).

The few Grs expressed in tarsi are primarily found in the pro- and mesothoracic legs. Grs expressed at high levels in tarsi are also strongly

expressed in the labellum and include candidate bitter co-receptors with potentially broad expression (Sparks *et al.,* 2013). Comparison of Gr repertoires across taste appendages in *Aedes* shows that many are specific to the labellum, and only a few appear specific to the tarsi. The majority of Grs, including the sweet Grs, do not show expression levels that are sexually dimorphic, and only a handful in the labellum is either male-specific or female-specific (Saveer *et al.,* 2018; Sparks *et al.,* 2013). Not much is known about these sex-specific Grs in mosquitoes.

Amino acid and receptors

Irs are classified into ionotropic co-receptors, antennal ionotropic receptors, and divergent ionotropic receptors. The expression of two co-receptors, Ir25a and Ir76b, and a vast majority of the divergent receptors are found in taste neurons in *Drosophila* (Koh *et al*., 2014; Sanchez-Alcañiz *et al*., 2018; Stewart *et al*., 2015). Ir25a and Ir76b are required, in some cases together, in combination with divergent Irs for taste detection of various compounds in both appetitive and deterrent classes of taste neurons (van Giesen and Garrity 2017). Ir76b mediates amino acid taste in *Drosophila*, a function that is also likely conserved in mosquitoes (Ganguly *et al.,* 2017). Mosquito *Ir76b* is also expressed at high levels in taste tissues, and the *A. gambiae* ortholog can functionally substitute for the fly gene (Ganguly *et al.,* 2017; Saveer *et al.,* 2018). Recent reporter studies in *A. coluzzii* found that *Ir76b* is expressed at high levels in the labellum, labrum, and tarsi (Ye *et al.,* 2021-unpublished). However, in the labellum, it is mainly localized to the T2

olfactory sensilla, and unexpectedly, only a pair of the T1 trichoid taste sensilla. It will be interesting to uncover how the taste system encodes amino acids and which Irs are essential for detecting them.

Salt and water receptors

Two other types of taste neurons classically described in flies have also been identified in mosquitoes. Water-sensing neurons are activated by pure water and inhibited by sugar, salt, and bitter tastants. In *Drosophila,* the cellular and behavioral response to water has been attributed to *ppk28*, a member of the degenerin/epithelial sodium channel family expressed in the labellum (Cameron *et al.*, 2010). In *Ae. aegypti, ppk301*, the ortholog to *D.mel ppk28* is also expressed in the labellum and tarsi. Recent studies have shown that *ppk301* allows female mosquitoes to identify suitable freshwater sources containing low salt concentrations for oviposition (Matthews *et al.,* 2019). Calcium imaging data showed that *ppk301*-expressing neurons respond to water and high salt. Abolishing *ppk301* resulted in a near-complete loss of the water response while the salt response remained. This resulted in an overall lower number of eggs deposited in freshwater sources and a higher rate of eggs deposited in water with high salt concentrations.

Salt-sensing neurons are characterized by increased activity over a concentration range of salts, as seen from electrophysiological recordings taken from labellar T1 sensilla (Kessler *et al.,* 2013; Sanford *et al.*, 2013). Although

ppk301 plays an essential role in oviposition, mosquitoes use a separate pathway to detect high salt concentrations that has yet to be described. Based on the role of *ppk301* and its salt sensitivities, other ppk receptors are likely involved in salt detection. Comparisons across phytophagous and hematophagous insects show that the *ppk* gene family is highly conserved across many insect orders (Latorre-Estivalis *et al.,* 2021). Comparisons of the Culicid and fly genomes show that the number of *ppk* genes is similar for *Ae. Aegypti, A. gambiae*, and *D. melanogaster* (32 ppks in *Ae. Aegypti;* 26 ppks in *A. gambiae*; 31 ppks in *D. melanogaster*). The number of ppks in *Ae. albopictus* and *Cu. quinquefasciatus* was higher than the other three (49 *Ae. albopictus*; 48 in *C. quinquefasciatus*) (Latorre-Estivalis *et al.*, 2021; Matthews *et al.,* 2016; Matthews *et al.,* 2018; Zelle *et al.*, 2013).

In *D. melanogaster,* several chemosensory gene families are involved in salt detection. *PPK23* mediates responses to high-salt concentrations (Jaeger *et al.*, 2018). Additionally, the *Ir* gene family is also involved in salt detection. More specifically, *Ir76b* has been implicated in the cellular response and behavioral attraction to low-salt concentrations when expressed in the canonical sweet sensing neurons in the labellum (Zhang *et al.*, 2013). However, recent reports have found that it can also mediate cellular responses and behavioral aversion to high salt via its expression in the canonical bitter sensing neurons of the labellum (Jaeger *et al.*, 2018).

Aside from the salinity information that a female mosquito may gather for oviposition, high salt concentrations also depress the sugar response, and

mosquitoes reject mixtures of sucrose and high salt (Chen et al., 2019; Salama 1966). In the hematophagous insect, *Rhodnius prolixus*, high salt concentrations (greater than or equal to 300 mM) can interrupt feeding on mixtures of salt and ATP, a well-known phagostimulant found in animal blood that induces engorgement in hematophagous insects (Pontes *et al*., 2017). However, when the salt concentration in the mixtures was closer to the salt concentration found in human blood (145 mM), non-interrupted feeding that led to engorgement of the animal occurred (Pontes *et al.*, 2017).

Blood component receptors

Animal blood consists of various components that a mosquito may encounter in other food sources such as nectar. However, the specific ratios and combinations of the components and the organs used to detect it allow the female mosquito to distinguish its meals. As described in the previous section, the salt concentration found in human blood typically falls within the range of appetitive salt concentrations for insects like flies, mosquitoes, and kissing bugs. However, when we consider the mosquito taste organs, the labrum is the only taste organ that encounters host blood. In this context, salt could also be considered a human host cue since it is present in animal blood and is typically absent from floral nectars. When brought to the tip of the labrum, 140 mM sodium chloride (NaCl) elicits weak responses from chemosensory neurons. These responses were enhanced when

salt was combined with other blood components such as glucose (4.5 mM) and sodium bicarbonate (25 mM) (Jové *et al.*, 2020).

Arguably, one of the most critical components of animal blood is adenosine triphosphate (ATP), a well-known mosquito phagostimulant (Friend 1978; Galun and Rice 1971; Hosoi 1958; Hosoi 1959; Liscia *et al.,*1993). When added to saline female mosquitoes will feed until fully gorged as they do while blood-feeding (Jové *et al.,* 2020). When ATP is added to sugar solutions, the imbibed mixture is diverted to the midgut instead of the crop where sugar meals are typically stored (Friend 1981). ATP is not naturally present in the nectar sources that mosquitoes exploit, and, in fact, ATP does not elicit a response from labellar sensilla (Sanford *et al.*, 2013). The signal to imbibe ATP-laced substances until engorgement comes instead from the activation of sensory neurons within the apical and subapical sensilla found on the labrum (Jové *et al.*, 2020; Liscia *et al.*, 1993; Werner-Reiss *et al.,* 1999b).

Mosquito ATP receptors have been the subject of much speculation since the identification of ATP as a blood cue in the late 1950s. In mammals, P2X ligand-gated ion channels detect ATP. However, insects do not have P2X receptors. Recent transcriptome analysis showed that the labrum lacked expression of sweet taste receptors and identified four receptors specific to the female stylet, two of which belonged to the *Ir* gene family (Ir7a and Ir7f) (Jové *et al.*, 2020). Calcium imaging analysis showed that neurons labeled by both Ir7a and Ir7f responded to

blood but not ATP alone. Despite advances in genomic tools, identifying an ATP receptor has proved to be quite a challenge.

Receptors mediating attractive sensory cues used for host detection

CO_2 receptors

Exhaled carbon dioxide from human hosts is a highly attractive long-range cue for hematophagous female mosquitoes. Once a plume of CO_2 is detected, a female will fly upwind and use it as a guide to reach her host. Heteromeric receptors made up of three members of the *Gr* family (Gr1, Gr2, and Gr3 in *Aedes* and *Culex*; Gr22, Gr23, and Gr24 in *Anopheles)* are expressed in olfactory neurons within the capitate peg sensilla of the maxillary palps. The Gr activity-dependent response to CO_2 functions independently of Ors expressed in other neurons within the same sensillum as shown when odorant receptor co-receptor (orco*)* is silenced, and the response to CO_2 remains intact (DeGennaro *et al.*, 2013). In addition to CO_2, the receptor complex also mediates the response to a group of odorants independent of orco function (Tauxe *et al.*, 2013; Kumar *et al.*, 2020). Initial studies in *D. melanogaster* showed that mutating Gr63a resulted in the loss of the neuronal response to those odorants detected explicitly by the ab1C neuron (Tauxe *et al.*, 2013).

The detection of CO_2 is conserved across many insects, including *D. melanogaster*. In *Drosophila*, CO_2 is detected via the Gr21a/Gr63a receptor complex. However, unlike the female mosquito's attraction to CO_2, flies will actively

avoid it (Turner and Ray 2009). Mutant analyses show that the receptor is non-functional if one of the Grs is missing, which results in failure to avoid CO_2 (Turner and Ray 2009; Kumar *et al.*, 2020). Of the three Grs that make up the mosquito CO_2 receptor complex, only Gr2 is not orthologous to either *D. mel*Gr21a (Gr1 ortholog) or *D. mel*Gr63a (Gr3 ortholog) (McMeniman *et al.*, 2014).

Before CRISPR technology, making any genetic manipulation in mosquitoes was difficult and expensive. Therefore, only a Gr3 mutant was used to evaluate the role of the CO_2 receptor complex in *Aedes*; without Gr3, the response to CO_2 is lost (McMeniman *et al.*, 2013). A later study took advantage of the empty ab1C neuron that resulted in the Gr21a/Gr63a double mutant in *D. melanogaster* and heterologously expressed the mosquito CO_2 Grs (Kumar *et al.*, 2020). Expressing Gr2 +Gr3 in the empty neuron was sufficient to restore the response to CO_2. Including Gr1 with the other two Grs increased the response to CO_2 and decreased the response to pyridine, one of the few non-CO2 agonists that activate the CpA neuron in mosquitoes (Kumar *et al.*, 2020; Tauxe *et al.*, 2013). Therefore, Gr1 modulates the responses produced by Gr2 +Gr3.

Heat and humidity

Heat and humidity are additional close-range cues that female mosquitoes use to locate a host during the host-seeking phase (Brown *et al.*, 1966). Individually, neither cue is enough to induce host-seeking behaviors in the females. The presence of CO2 induces the mosquito host-seeking phase, a continuous

phase that can last upwards of 15 minutes until additional cues that are indicative of a human host such as heat, humidity, and skin odors are detected (Liu *et al.,* 2019; McMeniman *et al.,* 2014; Sorrells and Vosshall, 2021).

Early ablation experiments in *Anopheles* suggested that thermal sensors were in the antennae (Ismail 1962). Electrophysiological studies done in *Ae. aegypti* found a pair of heat sensing neurons in antennal small coeloconic sensilla (SC) that responded to both hot and cold temperatures (Davis and Sokolove, 1975; Gingl *et al.,* 2005). However, much of what is known about specific genes that mediate heat and humidity come from studies done in *Drosophila*. For instance, detection of heat has been attributed to the *transient receptor potential (TRP)* gene family (Caterina *et al.,* 1997; Lee *et al.,* 2005; Montell 2005; Neely *et al.,* 2011). One study in *Anopheles* confirmed that temperature responses come from SC at the distal end of the antennae and attributed the response to *AgTRPA1*, an ortholog of *Drosophila* TRP channels (Wang *et al.,* 2009). More recent transcriptome analyses have reported that, in addition to the antennae, TRP channels are found in the labellum, maxillary palps, and tarsi (Sparks *et al.,* 2013; Matthews *et al.,* 2016).

In *Drosophila*, hygrosensation has been attributed to activity of Irs via two separate pathways: detection of dryness and the detection of moisture via cells located in the antennal sacculus. Three Irs are required for dry sensing: *Ir25a*, *Ir93a,* and *Ir40a* (Enjin *et al.,* 2016). Moisture sensing requires activity of Ir25a, along with *Ir93a* and *Ir68a* (Knecht *et al.,* 2017). Although work to identify

hygrosensing pathways comes from *Drosophila*, identifying homologous receptors in mosquitoes will help pinpoint specific receptors involved. Transcriptome analysis could also shed light on how gene expression may change across physiological conditions, such as before and after a blood meal (Matthews *et al.,* 2016).

Receptors involved in detection of volatile compounds on taste appendages

Given presence of T2 olfactory sensilla on the mosquito labellum, it is not surprising to find Ors and OBPs expressed in this organ (Matthews *et al.*, 2016; Matthews *et al.*, 2018; Saveer *et al.*, 2018). Electrolabellograms (ELG) and single sensillum recordings (SSR) from T2 labellar hairs in female *Anopheles* show that the labellum is sensitive to various classes of odorants (alcohols, aldehydes, acids, amines, esters, indoles, ketones, lactones, sulfides, terpenes, and thiazoles), some of which are present in human odor (Kwon *et al.*, 2006; Saveer *et al.*, 2018). Of these, acids and amines are detected by the *Ir* gene family (Benton *et al.*, 2009; Min *et al.*, 2013; Silbering *et al.,* 2011). Therefore, expression of Ir co-receptors *Ir25a* and *Ir76b* in the mosquito labellum is no coincidence. Recent studies in *Anopheles* reported that expression of *Ir76b* is mainly localized to T2 sensilla except for a pair of T1 sensilla (Ye *et al.*, 2021). This expression pattern supports likely involvement of the *Ir* gene family in mediating the responses to the odorants that fall within these groups (Saveer *et al.*, 2018*)*.

Immunohistochemistry analysis reported presence of *orco, Or8,* and *Or49* in neurons innervating apical and sub-apical sensilla in the labrum of *Ae. aegypti* (Jung *et al.,* 2015). Expression of *AaOr8* and *AaOr49* in cells resulted in dose-dependent calcium activity in response to blood volatiles (Jung *et al.,* 2015). However, a more recent transcriptome analysis could not reliably detect *orco* or other Ors in this structure (Jové *et al.,* 2020). Instead, the Ir co-receptors, *Ir25a* and *Ir76b*, and some labrum-specific Irs, *Ir7a,* and *Ir7f* were present. The same study reported the involvement of the labrum-specific Irs in blood detection. However, it is possible that any additional Irs in this structure could be mediating the response to any other amine or acidic volatiles.

Although the labellum of *Drosophila* lacks Ors and OBPs, members of the *Ir* gene family are expressed in the organ. However, the same panel of odorants tested on mosquitoes failed to elicit a response from labellar sensilla of *D. melanogaster* (Kwon *et al.*, 2006). Lack of olfactory sensitivity shows that the *Drosophila* labellum is a true taste organ that lacks olfactory sensilla. This is supported by the fact that labellar neural projections only reach the taste region of the brain. Thus, expression of odorant receptor genes in taste organs is a unique arrangement found in mosquitoes.

Tarsi in mosquitoes were deemed taste appendages based on behavior assays that showed they could detect chemosensory stimuli via touch (Clements 1992; Pappas and Larsen 1978). Expression of Grs and Irs in these tissues is, therefore, no surprise given the roles of both gene families in taste transduction.

However, not only are Grs expressed at lower levels compared to Irs, but fewer Grs are expressed overall (Matthews *et al.,* 2016; Sparks *et al.,* 2013). Transcriptome analyses in *Aedes* show the expression of *AaegOr7/orco* along with a handful of additional Ors across the tarsi, albeit at low levels. However, tarsal expression of OBPs in male and female *Aedes* is higher than expression levels of Ors. Olfactory responses of tarsi have not been investigated, and taste responses have been based solely on behavioral experiments. Nevertheless, it would be interesting to see if expression levels of the different receptor gene families are indicative of tarsi being primarily used to carry out olfactory-driven behaviors.

Sensory projections from taste organs to the brain

Afferent projections of the gustatory receptor neurons (GRNs) in mosquito taste organs terminate in regions of the brain known as subesophageal zone (SEZ) and tritocerebrum (Ignell *et al.,* 2005). Within these regions, GRNs from the labellum, labrum, and cibarium project to seven distinct areas that likely help the animal distinguish chemosensory information gathered by each organ. Immunohistochemistry experiments localized *orco* to apical and sub-apical sensilla of the labrum in *Ae. aegypti* and reported that neuronal projections from this structure also projected to the antennal lobe (AL) (Jung *et al.,* 2015). Recent double-labeling experiments of the labium and labrum show that labial projections reach the posterior region of the SEZ and do not overlap with the neuronal projections of the labrum (Jové *et al.,* 2021). Together, segregation of organ

projections and lack of sweet-sensing Grs in the labrum shows that signals for blood meals and nectar meals are processed in segregated regions of the SEZ. Currently, no information on neural projections of the tarsi of mosquitoes exists.

As mentioned in the previous section, olfactory receptors are found in multiple mosquito taste organs. Early dye-tracing experiments showed that in addition to the SEZ, axonal projections from the labellum also targeted ventroposterior regions of the AL (Kwon *et al.*, 2006). In recent years, specific GFP labeling of *orco* positive ORNs from the labellum were shown to converge in the SEZ (Riabinina *et al.*, 2016). A total of eight glomerular structures in the SEZ were identified and confirmed by neurobiotin backfills of the proboscis of both males and females. Although these neurobiotin experiments did not show any labeling of the AL as previously reported, they did report labeling of a broader SEZ region. This is not entirely unexpected given the fact that SEZ glomeruli were identified based solely on *orco* positive neurons from the proboscis, while gustatory and mechanosensory neurons remain uncharacterized.

Concluding remarks

There have been many advances in our understanding of the mosquito chemosensory system. However, our current knowledge of the olfactory system far exceeds that of the taste system. This dissertation describes the first functional map of a mosquito taste organ. This map results from a comprehensive survey of labellar T1 sensilla in *Ae. aegypti*. Use of a broad panel of tastants that

encompasses various taste categories revealed mosquito sensitivity to amino acids, which had previously been described only in the context of behavioral responses. The amino acid response maps to a neuron distinct from the sweet-, bitter-, salt-, and water-sensing neurons that innervate T1 sensilla. Following initial characterization, a diagnostic panel of tastants was used to test if responses are modulated across different physiological states of the gonotrophic cycle (mating and blood-fed state of the female) and to compare responses of male and female mosquitoes.

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Figure 1.1 Mosquito taste organs and digestive tract

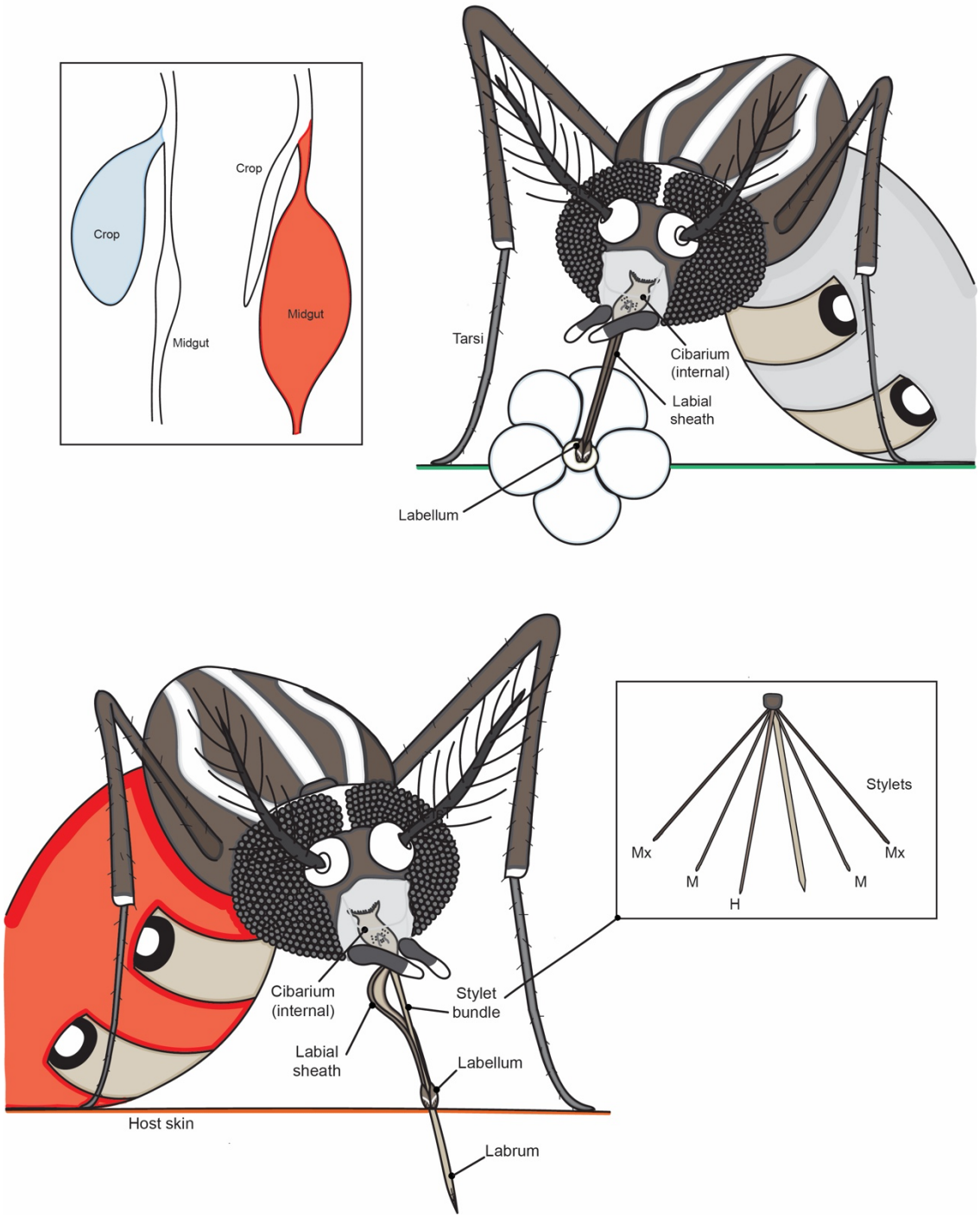

Figure 1.1 Mosquito taste organs and digestive tract

Schematic showing the mosquito's taste organs while feeding on floral nectar (top) or blood (bottom). Upon landing, a mosquito can use the **tarsi** and **labellum** to survey the plant//flower or surface of the host skin. Once the nectar sugars stimulate the T1 sensilla on the labellum, the mosquito will begin to feed. The labial sheath covers the six neatly stacked stylets that form a feeding tube, which gives the appearance of a single structure through which the fluids are imbibed. The inlet on the bottom right shows the individual stylets (maxillae (Mx), mandibles (M), hypopharynx (H), and the labrum). During a blood meal, the mosquito will use its stylets to cut through the host skin, but only the **labrum** is inserted through the skin to locate host blood. When a meal is imbibed, it passes through the feeding tube formed by the stylets to the **cibarium**; an internal structure considered the last checkpoint before a meal is diverted to the appropriate part of the digestive tract. The inlet on the top left shows the crop as the destination for sugary meals, whereas blood meals are diverted to the midgut. Note: mid and hindlegs have been omitted for clarity.

Chapter 2

A functional map of the labellar taste sensilla of *Aedes aegypti*

Overview

The mosquito taste system guides several taste-driven behaviors, many of which contribute to a female mosquito's reproductive fitness. However, information about the underlying organization of the taste organs and how they detect taste signals is not well understood. We aimed to characterize the responses of the trichoid type 1 (T1) sensilla on the labellum. A comprehensive survey of the labellar sensilla was performed with a broad panel of tastants from different taste categories. Our survey revealed that most sensilla are innervated by neurons responsive to each category of tastants that was tested. However, cluster analysis of the sensillar responses identified five functional groups based on differences in their activation profiles. Notably, our panel included five amino acids whose responses varied across the five sensillar classes. Until now, neuronal responses to amino acids had not been characterized in mosquitoes. Recordings with mixtures of amino acids and compounds diagnostic of each of the other taste categories mapped amino acid sensitivity to a distinct neuron. We describe the first functional map of a mosquito taste organ generated from our comprehensive survey.

Introduction

As vectors of disease, mosquitoes are a significant threat to global public health and result in the loss of millions of human lives every year. Efforts to reduce disease transmission have expanded knowledge of the mosquito olfactory system to understand the sensory basis of host-seeking behavior. However, anosmic mosquitoes can obtain blood meals successfully, thereby demonstrating that mosquitoes rely on the integration of many chemosensory cues to navigate their environments and find a host (DeGennaro *et al.*, 2013; Liu *et al.,* 2019; McMeniman *et al.,* 2014; Sorrells and Vosshall, 2021).

The gustatory system, in particular, is responsible for guiding several mosquito taste-driven behaviors: the feeding on nectar during the first few days of life followed by the blood-feeding phase where female mosquitoes survey the surface of the host's skin right before probing and obtaining a blood meal, and lastly oviposition. The swiftness with which a female mosquito uses her taste organs to execute the series of behaviors that precede a blood meal, and the risk that her feeding behavior poses to human health, warrants more detailed studies of the gustatory system.

Taste organs in insects are coated with uniporous sensilla, otherwise known as trichoid type-1 sensilla (T1) in mosquitoes and require contact to detect non-volatile stimuli. The T1 sensilla are stereotypically arranged across mosquito taste organs such as the labellum, labrum, tarsi, and wing margins (Hill and Smith, 1999; Liscia *et al.,* 1993; McIver and Siemicki, 1978; Pappas and Larsen, 1976; Sparks

and Dickens, 2017). Still, in *Aedes aegypti,* only the topography of the labellar sensilla has been carefully described (Hill and Smith, 1999). However, the nomenclature based on morphology and location is not necessarily indicative of any functional organization, which can only be accomplished by surveying individual sensillum responses (Weiss *et al.*, 2011).

The labellar sensilla of *D. melanogaster* were initially classified by their morphology as long (L), intermediate (I), and small (S) (Hiroi *et al.*, 2002). Functional analyses further separated the sensilla into different functional classes for the I and S types (I: I-a, I-b; S: S-a, S-b, S-c) (Weiss *et al.*, 2011), each of which is innervated by the same number of chemosensory neurons but may differ from the other types (I-type innervated by two neurons, L- and S-type innervated by four neurons). Ultimately, the underlying sensillar organization is stereotypical, and the number of innervating chemosensory neurons and the unique receptor combinations that they express is what gives rise to the taste coding differences seen across sensilla of the fly labellum (Dethier 1976; Falk *et al.*, 1976; Fujishiro *et al.*, 1984; Hiroi *et al.*, 2004; Nayak and Singh, 1983; Rodrigues and Siddiqi, 1978). In mosquitoes, the T1 sensilla are innervated by up to five chemosensory neurons (Lee and Craig 2009; Pappas and Larsen, 1976), which differs from the maximum of four typically found in *Drosophila* taste sensilla. The possibility of a fifth neuron innervating any given sensillum would suggest that the functional organization of the taste systems of the two dipteran species may be different. In addition, many distinct life strategies and host preferences can be seen within the

Culicidae family of mosquitoes, suggesting that the taste system's functional organization is likely unique to each mosquito species.

Our study aims to characterize the taste system of *Aedes aegypti* by focusing on the labellum of female mosquitoes. The labellum has 30 T1 sensilla that are easily accessible for electrophysiological analysis. Using a panel of 23 compounds (comprised of sugars, salt, water, bitter compounds, a skin compound, and amino acids), we surveyed the responses of all the sensilla located on a single labellar palp (15). We found qualitative and quantitative differences in sensillar responses, which revealed five functional groups of sensilla. Additionally, our survey revealed the female mosquito's robust response to amino acids, contrasting with weaker labellar responses described in *Drosophila melanogaster* (Park and Carlson, 2018). Our results suggest that a previously uncharacterized neuron that is not activated by water, sugar, salt or bitter compounds is the one activated by amino acids. This work provides a first map of the functional organization of the labellar sensilla for a major hematophagous mosquito species. Overall, our study highlights both similarities and differences in the functional organization of the mosquito labellum and the well-characterized taste system of *Drosophila melanogaster*.

RESULTS

Topography and neuronal sensitivities of Aedes aegypti labellar sensilla

Before beginning our survey, scanning electron micrographs of the dorsal and ventral surface of the *Ae. aegypti* labellum were taken. Stereotypy of the labellar sensilla is observed in both sexes. The observed topography of labellar sensilla matches published descriptions and is therefore labeled using the previously described naming system (**Figure 2.1 A, B) (**Hill and Smith, 1999). To distinguish between sensilla located on dorsal and ventral sides of the labellum, we included the letter D or V preceding the sensillum number (**Figure 2.1 A, B)**.

To perform single sensillum recordings (SSR), we immobilized non-blood-fed, mated female mosquitoes by removing all legs and taped them to a microscope slide (**Figure 2.2A).** Trichoid type 1 (T1) sensilla in mosquitoes are said to be innervated by up to five chemosensory neurons (**Figure 2.2B**) (Lee and Craig, 2009). Therefore, we carried out an initial screen with tastants from the human-described taste categories: sweet, bitter, water, and salt (TCC is the recording electrolyte). Sample traces depict the female mosquito's robust responses for each category tested (**Figure 2.2C)**. An amino acid was included in the initial survey since they are found in sweat and, therefore, on the surface of human skin where the female labellum is likely to encounter them. We found that female mosquitoes showed a robust response to amino acids. Apart from the amino acids, which hadn't been tested before, our initial screen showed that

female mosquitoes had responses similar to what was previously reported for a single-sensillum survey of the *Ae. aegypti* (Sanford *et al.,* 2013).

Labellar sensilla show diverse responses across different taste categories

To characterize the mosquito taste system, our panel included 10 sugars at 100 mM, four bitter compounds at 10 mM (Lobeline was tested at 1 mM), five amino acids at 10 mM, one skin compound: 1% lactic acid, sodium chloride (50 mM NaCl in 1 mM KCl), water (in 1mM KCl) and 30 mM TCC (the recording electrolyte used for all tastants except NaCl and water). The 15 sensilla found across the dorsal and ventral sides of one labellar palp were surveyed with all 23 tastants. This resulted in 15 X 23 = 345 sensillum-tastant combinations. A minimum of seven sensilla from at least 7 animals were tested for each sensillum-tastant combination.

A heat map of sensillar responses showed observable qualitative and quantitative differences across all taste categories (**Figure 2.3)**. All sensilla were sensitive to sugars, albeit not all responded to the same sugars or the same number of sugars. Typically, sucrose, maltose, and maltotriose elicited the highest responses, whereas glycerol did not elicit a response from any sensillum. As seen for sucrose, the degree of sensitivity to sugars also differed across all sensilla. A given sensillum could be sensitive to about 3-8 of the 10 sugars used in our panel.

Bitter tastants elicited responses from only a handful of sensilla, with the exception of denatonium, which produced a robust response from all 15 sensilla.

Denatonium exhibited unique temporal dynamics not otherwise seen from other bitter compounds (**Figure 2.4A, C)**. A delay of about 2-8 seconds was observed before a train of action potentials appeared in response to denatonium (**Figure 2.4B**), which was captured by expanding the recording window to 10 seconds. A second train of larger action potentials appeared in the seconds that followed the initial train of action potentials. When analyzed closely, 2-3 neurons appear to be firing in response to denatonium, consistent with which is the presence of large spikes that represent summations (**Figure 2.4B**). Although the response latency was variable, the strength of the neuronal response appeared similar across all sensilla.

Salt and water elicited robust responses from most of the dorsal sensilla and about half of the sensilla on the ventral side of the labellum. Four sensilla (D2, V7, V8, V9) showed responses to either salt or water. Sensilla D5 and V6 were the only ones that did not respond to either.

Lactic acid is a highly attractive volatile compound when paired with CO_2 and is detected via the Ir8a pathway expressed in mosquito antennae (Acree *et al.*, 1968; Raji *et al.*, 2019). Since lactic acid is found in sweat and likely encountered by the taste system as well, as shown recently for the *Drosophila* labellum, we continued surveying T1 sensilla (Stanley *et al.,* 2021). However, lactic acid failed to elicit a response from labellar T1 sensilla.

Neuronal responses to amino acids had only been reported in tarsal sensilla in mosquitoes, despite their presence in ecologically relevant food/host sources of

nectar and sweat, where labellar sensilla are likely to encounter them (Elizarov and Sinitsina, 1974). The mosquito labellum has both olfactory and taste capabilities. However, amino acids have little to no volatile properties and are likely detected primarily through taste sensilla. Binary choice feeding assays done with *Ae. aegypti* found that specific sucrose-amino acid mixtures elicited enhanced preference over sucrose alone (Ignell *et al.*, 2010), suggesting that mosquitoes have the capacity to sense and discriminate amino acids. Of the five amino acids used in our survey, methionine, valine, and leucine elicited the strongest responses from a subset of sensilla. Our findings show that mosquito labellar T1 sensilla exhibit a range of sensitivities to amino acids; the differences in sensillar responses raise the possibility that mosquitoes may be able to discriminate between amino acids as well.

Overall, our survey shows that labellar sensilla respond to different categories of compounds. Based on comparisons of sensillar anatomy and function in flies, we infer that presence of water, sugar, salt, and bitter-sensing neurons in the mosquito sensilla. Moreover, we found considerable heterogeneity in responses to compounds of the same taste categories (i.e. differences between sugar-sensing neurons, for example), predicted to result from differences in chemoreceptor expression across these neurons. Not all sensilla may be innervated by the same number of neurons, as seen by the lack of water and salt responses of sensilla D5 and V6.

Functional organization of labellar T1 sensilla

Given the apparent differences in the number of innervating chemosensory neurons, our next step was to evaluate the functional organization of the T1 labellar sensilla. Our hierarchical cluster analysis gave rise to five different functional groups of sensilla (**Figure 2.5A, B**). Group #5 separates from the others due to strong responses to sugars and not much of anything else. This group contains sensilla that lack water and salt responses or only one of the two. Group #4 comprises one sensillum, V3, and is the most broadly tuned. Not only was V3 sensitive to all taste categories, but multiple tastants within each category also elicited robust responses. The response profiles of groups #1-3 look very similar, but these three groups can be distinguished based on differences in their sensitivities to the tastants.

Each group comprises 3-4 sensilla from both dorsal and ventral sides of the labellum. The only exception is group #4, which contains a single sensillum, V3. Once the sensilla were sorted into their respective groups, we could see that they fit nicely based on the small standard error of the mean across groups #1-3 and #5 (**Figure 2.5B)**. Error bars in group #4 were larger, since it comprised only one sensillum and yielded overall fewer replicates (n=7-11, and n=43 for sucrose compared to a range of n=21-38, and n=128-196 for sucrose in other groups).

Mapping amino acid responses to identified neurons

Early studies in flesh flies postulated that amino acid sensitivity in insects is limited and that such responses were detected via sugar receptors (Shimada 1975). In mosquitoes, careful characterization of the neuronal response to amino acids has not been accomplished prior to this study. A behavioral study in *Ae. aegypti* put forth two theories to explain enhanced feeding preference observed for specific sucrose-amino acid mixtures. The first suggested a synergistic interaction between amino acids and the sweet neuron, and the second was that amino acids enhanced the "sweetness" of sugars (Ignell *et al.*, 2010). Our survey of amino acids showed that *Ae. aegypti* is sensitive to all five of the amino acids we tested. However, due to the possible innervation of a fifth neuron in the T1 sensilla, it was difficult to discern whether the amino acid response is mediated by one of the four neurons of canonical identity (sweet-sensing, bitter-sensing, water-sensing, or salt-sensing neuron) or a possible fifth undescribed neuron.

To address this, we performed SSR with mixtures of 10 mM methionine and a compound that represented each taste category known to elicit responses from previously described neurons (sweet-sensing, bitter-sensing, water-sensing, or salt-sensing neuron). Recordings with mixtures could allow us to map the neuronal response to amino acids based on spike amplitude, should those originating from different neurons be distinguishable. A mixture of Met + 100 mM sucrose resulted in spike amplitudes of two sizes, along with occasional larger summation spikes characteristic of a coincident response from two neurons (**Figure 2.6A)**. Our

results show that amino acids are not detected via the sugar-sensing neuron in *Ae. aegypti*.

The strategy that was used for sucrose and methionine was less effective for the other modalities because two spikes were not always visible or easily discerned in the traces. To rule out the contribution of the bitter-sensing neuron a mixture of Met + 10 mM strychnine was tested. The response to the mix resulted in a train of action potentials with a gradual increase in amplitude size (**Figure 2.6B**). One spike was larger than the others, but otherwise, it was difficult to discern whether the remaining action potentials resulted from signals coming from one or two separate neurons. However, a fraction of the recordings showed the presence of a smaller spike towards the end of the trace when the frequency of the large spikes began to dissipate (**Figure 2.6B, 2.7A**). This suggests that amino acids and bitter compounds are being detected via separate neurons. Individual responses elicited by methionine and strychnine show apparent differences in amplitude size, frequency, and temporal dynamics of the two (**Figure 2.6B**). These patterns persisted when re-tested individually after the mixed stimulus. The larger spike in the trace could be a summation spike indicative of two active neurons.

The following mixture was Met + 50 mM NaCl (**Figure 2.6C**). For the original survey, 1 mM KCl had been used as the recording electrolyte to survey the response of NaCl. Therefore, we used KCl as the recording electrolyte for all stimuli used for this set of experiments. The response to 1 mM KCl alone is the first trace at the top of the column. The marked action potentials are those of the

water neuron since it is no longer silenced due to the absence of TCC. Individual spike responses to Met and NaCl were uniform in amplitudes, but distinct from each other. Responses to NaCl appeared to be smaller in size. A higher frequency of the larger Met spikes was observed when the mixture was tested, and only a few smaller spikes corresponded to NaCl. Individual traces that followed further supports the notion that the salt neuron does not mediate the response to amino acids, but we could not confidently eliminate the contribution of the salt neuron.

To assess the role of the water neuron, we compared responses of water and Met (both used KCl as the recording electrolyte) (**Figure 2.6D**). Both traces exhibited very similar amplitude size, frequency, and temporal dynamics, making it difficult to discern whether this was the activity of one or two neurons or whether the response to Met had dampened out the activity of the water neuron. However, responses from our comprehensive survey (**Figure 2.3**) show that sensilla such as V7 and V9 that lack a response to water still exhibit a robust response to MET. Conversely, sensillum D4 has a water response but has little to no response to amino acids. Taken together, this data could suggest that a water-sensitive neuron does not mediate the amino acid response. However, as the panel of compounds were tested for each mixture there were a fraction of mosquitoes that had neurons that died in the process, which provided compelling evidence for the mapping experiments.

While testing mixtures of Met and strychnine there were two mosquitoes that showed responses to Met in the absence of the strychnine response and a

separate two mosquitoes that showed responses to strychnine in the absence of a response to Met (**Figure 2.7B**). Similar examples were seen in three mosquitoes for mixtures of Met and NaCl, which resulted in a Met response in the absence of the salt response (**Figure 2.7C**). In five different mosquitoes, mixtures of Met and water showed a response to water in the absence of a response to Met (**Figure 2.7D**).

Overall, our mapping experiments along with the neuronal death data point to amino acid sensitivity being mediated by an uncharacterized neuron within T1 sensilla separate from the sugar-sensing, bitter-sensing, salt-sensing, and water-sensing neurons (**Figure 2.6E**). However, this does not mean that all T1 labellar sensilla are innervated by a fifth uncharacterized neuron. This innervation pattern likely only applies to sensillum V3, which is not only broadly tuned but also shows robust responses to tastants across the five taste categories. Sensilla across the other four groups are innervated by 2-4 chemosensory neurons, one of which may be this uncharacterized neuron that mediates amino acid sensitivities. Ultimately, mapping different chemosensory receptors to the neurons innervating the T1 labellar sensilla will help determine the number of neurons and their specific identities.

Discussion

Our study provides the first functional map of labellar T1 sensilla for *Ae. aegypti*. Previous electrophysiological studies performed on both *Aedes* and

Anopheles mosquitoes showed that sweet-, bitter-, salt- and water-sensing neurons innervate the labellar T1 sensilla (Kessler *et al.,* 2013; Kessler *et al.,* 2015; Sanford *et al.,* 2013; Sparks and Dickens, 2016). Our initial electrophysiological screen generated results comparable to earlier studies. However, surveying all T1 sensilla with a broad panel of compounds allowed us to take it a step further and reveal that T1 sensilla are organized into five functional groups.

The response profiles for each functional group suggest that most T1 sensilla are innervated by 4-5 neurons tuned to different taste modalities, except for group #5, which appear to be narrowly tuned and only innervated by 1-2 neurons. However, we still find qualitative and quantitative differences across all groups. Transcriptome analyses in mosquitoes show that several chemosensory gene families are expressed in the labellum (Matthews *et al.,* 2016; Saveer *et al.,* 2018*;* Sparks *et al.,* 2013). More recently, driver lines for the sweet clade of Grs mapped their expression specifically to the labellum and not the labrum of female mosquitoes (Jové *et al.,* 2020). The next step would be to generate a receptor-to-neuron map of different chemosensory gene families expressed in the labellum. Doing so would help map combinations of receptors in neurons with specific response profiles seen across the various functional groups.

Amino acids are known to play a crucial role in the internal physiology of the female mosquito, particularly during vitellogenesis (Attardo *et al.,* 2006). Only one other study done in the hematophagous tsetse fly described the neuronal response to amino acids from a peripheral taste organ (tarsi) (Van der Goes van Naters and

Den Otter, 1998). Including amino acids on our panel allowed us to determine that they elicit responses of various strengths across the different sensillar groups. Although the responses were not as strong as those reported for the hematophagous tsetse fly, they were much stronger than those reported for *Drosophila* (Park and Carlson, 2018). These three dipteran species have very different life strategies, which likely explain the differences in sensitivity. One could also expect to find differences among other mosquito species, which opens many research avenues that could be pursued to gain a better understanding of taste detection in mosquitoes.

Our recordings with mixtures found that the response to amino acids does not map to a neuron of known identity across the labellar T1 sensilla. Chemosensory receptor maps and calcium imaging experiments would help identify the neuron mediating this response. Although our panel of amino acids is limited in scope, it allowed us to discern apparent differences in the neuronal responses to amino acids coming from a peripheral taste organ in mosquitoes. However, expanding the panel to include all 20 amino acids will help identify those that elicit the most robust responses. This data can later be used to learn more about the information gathered upon detecting amino acids and their contribution to a female mosquito's behavioral response.

When considering mosquito behavior, it is important to remember that any behavioral output results from integrating information collected from different peripheral appendages. Our study characterized only one taste organ and should

therefore not be interpreted as conclusive. More details about the functional groups would likely arise if our panels were expanded to include more compounds for each taste category. This information could lead either to generation of sub-classes of sensilla or complete re-arrangement of sensillar functional groups. We are still referring to the labellum in this scenario and not yet considering tarsal sensitivities.

Uncovering sensitivities of the tarsal sensilla as well as those along the wing margins will yield a complete picture of the mosquito taste system. Despite this knowledge gap, we have learned about a mosquito's general sensitivities and have a basic understanding of taste modalities that a female mosquito can detect. Behavior assays with ablated tarsal sensilla can be done like those performed in tsetse flies (Van der Goes van Naters and Rinkes, 1993) to tease apart the contribution of the labellum and tarsi.

A superficial comparison of the labellum of *Ae. aegypti and D. melanogaster* may highlight many similarities, such as having a similar number of chemosensory neurons innervating the trichoid sensilla and the expression of several orthologous genes. However, a peek beneath the surface will reveal that the functional organization of the two organs and their taste sensitivities are very different.

Methods and Materials

Mosquito rearing

Aedes aegypti (Liverpool) mosquitoes were raised at 27°C and 65-80%humidity on a 14:10 light-dark cycle. Females used for the initial survey were raised in cages

with males and were presumed to be mated but were not blood-fed. Larvae were raised in plastic Sterilite trays with distilled water and were fed TetraMin tropical tablets. Upon emergence, adults were placed in cages with access to 10% sucrose solution *ad libitum*.

Scanning electron micrographs (SEM)

Mosquitoes were immobilized on a Petri plate placed on ice. The whole proboscis was dissected from the base right before imaging, and the sample tissue was not subject to chemical fixation. The whole mouthpart was placed on a piece of double-sided sticky tape and secured with a few thin-cut strips of tape placed across the rostrum. Separate sample tissues were used for SEM of the dorsal or ventral view of the labellum.

Electrophysiology

Female mosquitoes aged 4-10 days old were used for electrophysiology experiments. Mosquitoes were immobilized on ice, and all six legs were removed. The mosquito was then placed on a small square of (foamy) double-sided sticky tape set on the short edge of a glass slide. In this position, only the labellar palps protrude from the edge of the tape. Four thin strips of double-sided sticky tape were cut and placed over the tip of the abdomen, thorax, base of the labium, and base of the labellum to hold the mosquito in place. A reference electrode filled with Beadle-Ephrussi Ringer solution was used to pierce the mosquito's thorax (Cold

Spring Harbor Laboratory Press, 2011). The recording electrode was filled with the test solutions dissolved in 30 mM Tricholine Citrate (TCC) and was then brought to the tip of individual sensilla. Each tastant was tested a minimum of seven times, which meant that seven individual mosquitoes were used to complete the set of replicates for each tastant.

Experimental stimuli

We used a panel comprised of 10 sugars at 100 mM (except for glycerol which was at 10%), 4 bitter compounds at 10 mM (except for Lobeline, which was tested at 1 mM), 5 amino acids at 10 mM, 1 skin compound: lactic acid, at 1%, 50 mM NaCl and water. The salt and water solutions were made with 1 mM KCl as the recording electrolyte. Most chemicals used were purchased from Sigma-aldrich: sucrose (S0389), fructose (F0127), trehalose (T9531), maltose (M9171), methyl-a-glucoside (M9376), maltotriose (M8378), melezitose (M5375), raffinose (R0514), glucose (G6152), glycerol (G7893), caffeine (C8960), denatonium (D5765), lobeline (141879), strychnine (S8753), valine (94619), methionine (64319), arginine (A8094), leucine (61819), phenylalanine (P5482), lactic acid (L1750) and tricholine citrate (T0252). Macron fine chemicals: NaCl (7581-06). Mallinckrodt AR (ACS): KCl (6858).

Statistical Analysis

Hierarchical cluster analyses using Ward's method were performed by using the statistics program PAST (https://www.nhm.uio.no/english/research/infrastructure/past/) (Hammer et al., 2001). All error bars represent standard errors of the mean (SEM).

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Figure 2.1 Topographical map of the labellar sensilla of *Aedes aegypti*

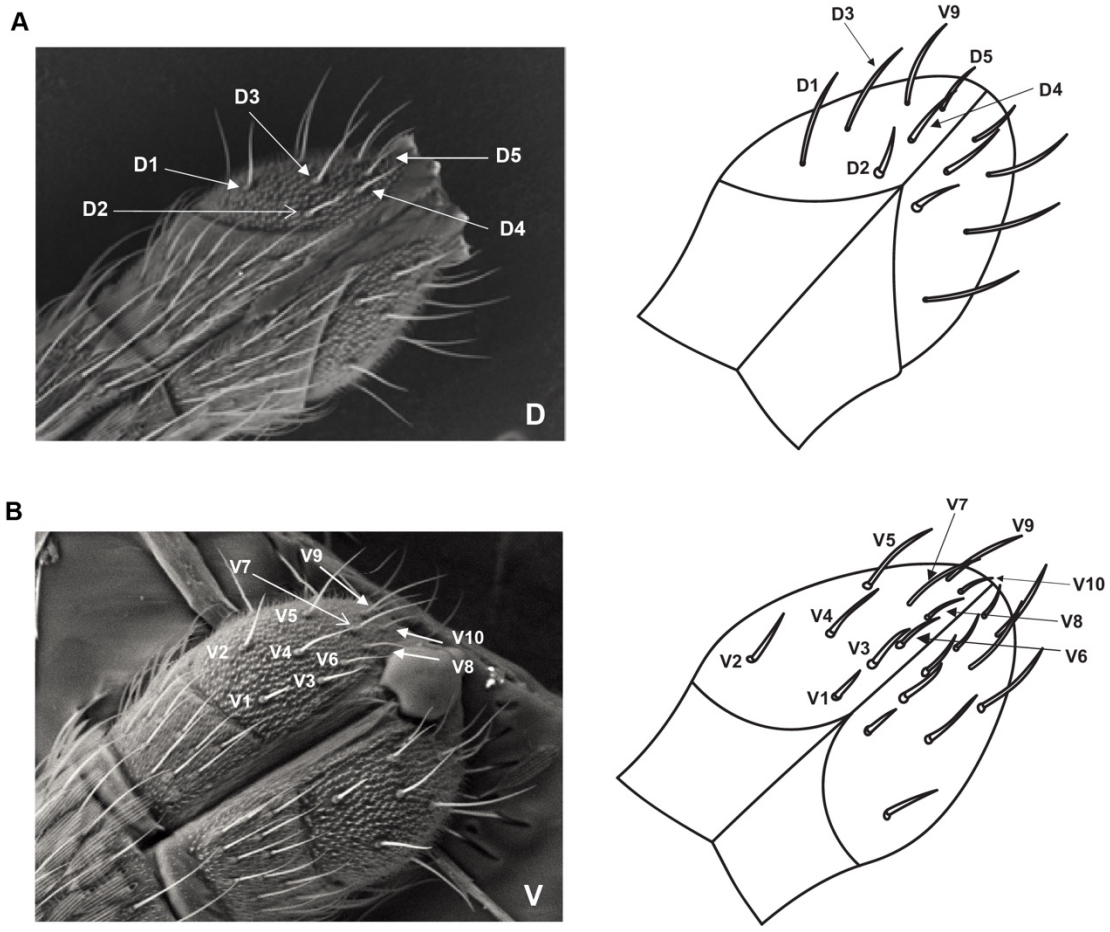

Figure 2.1 Topographical map of the labellar sensilla of *Aedes aegypti*

Scanning electron micrograph (left) and schematic (right) of the (**A**) dorsal and (**B**) ventral view of the *Aedes aegypti* female labellum. The sensilla are arranged stereotypically and match the previously described topography for this species. Established nomenclature is used (Hill and Berry Smith, 1999).

Figure 2.2 The labellar sensilla of *Aedes aegypti* respond to various taste categories

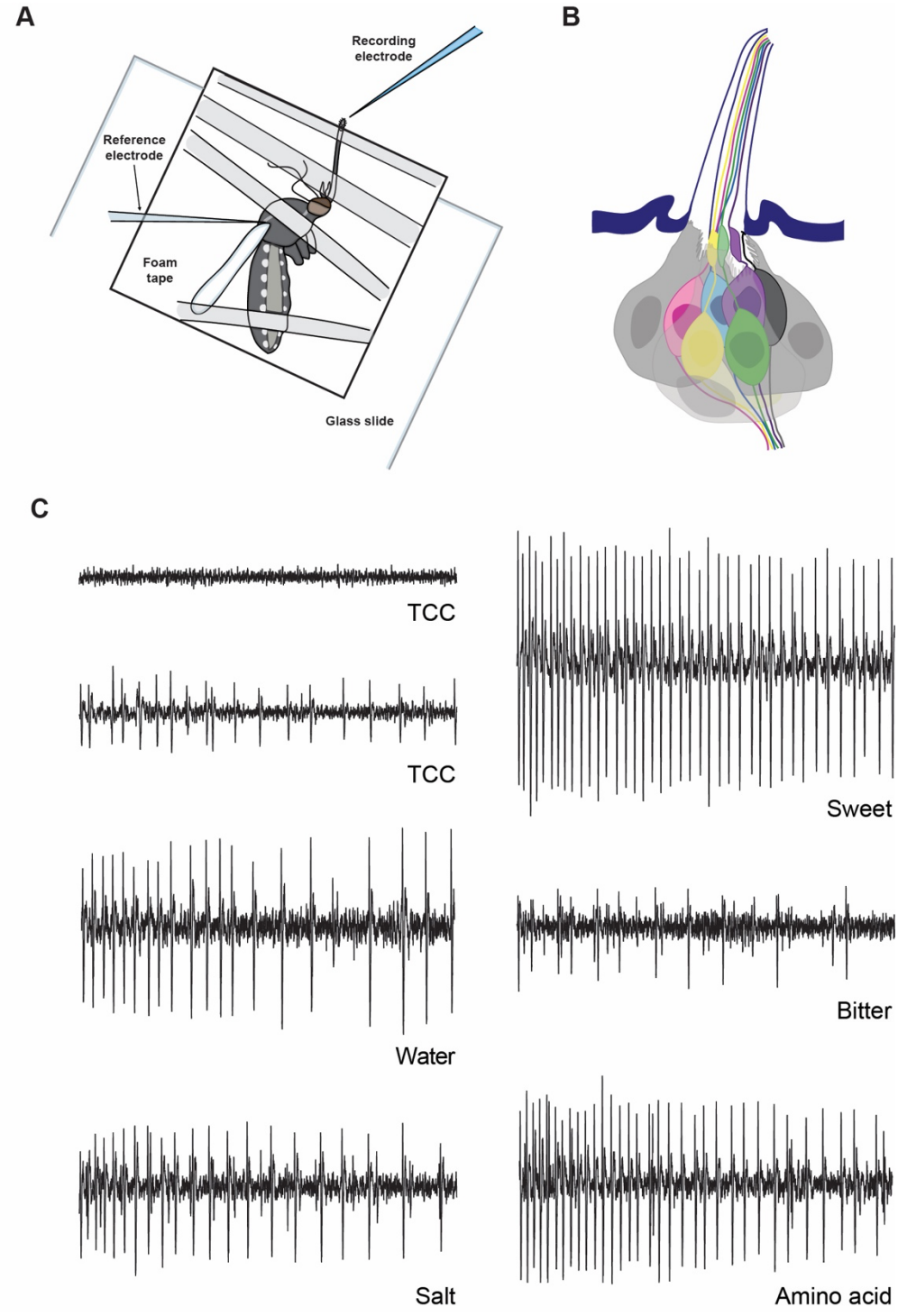

Figure 2.2 The labellar sensilla of *Aedes aegypti* respond to various taste categories

(A) Schematic of the mosquito preparation for single sensillum recordings (SSR) used to survey the labellar trichoid sensilla.

(B) Schematic of a single T1 sensillum that can be innervated by up to five chemosensory neurons, each of which responds to a different taste category with unique temporal dynamics.

(C) Sample traces for the recording electrolyte, tricholine citrate (TCC), and a compound from the taste categories of bitter, sweet, salt, water, and amino acids. Two sample traces are used for TCC because, in some cases, it elicits a train of action potentials from some of the labellar sensilla. The sample traces depict the difference in amplitude size of action potentials and spike frequency across the different taste categories. Each taste category shows a trace with action potentials of the same size. This pattern indicates that only one neuron is firing in response to a stimulus. These features allow us to identify the neuron that mediates a response to a stimulus. The T1 labellar sensilla are also sensitive to amino acids, but the neuron(s) mediating this response is unknown. Water and salt were applied with 1 mM KCl as the recording electrolyte.

Figure 2.3 Activation profiles of *Aedes aegypti* labellar sensilla show qualitative and quantitative differences

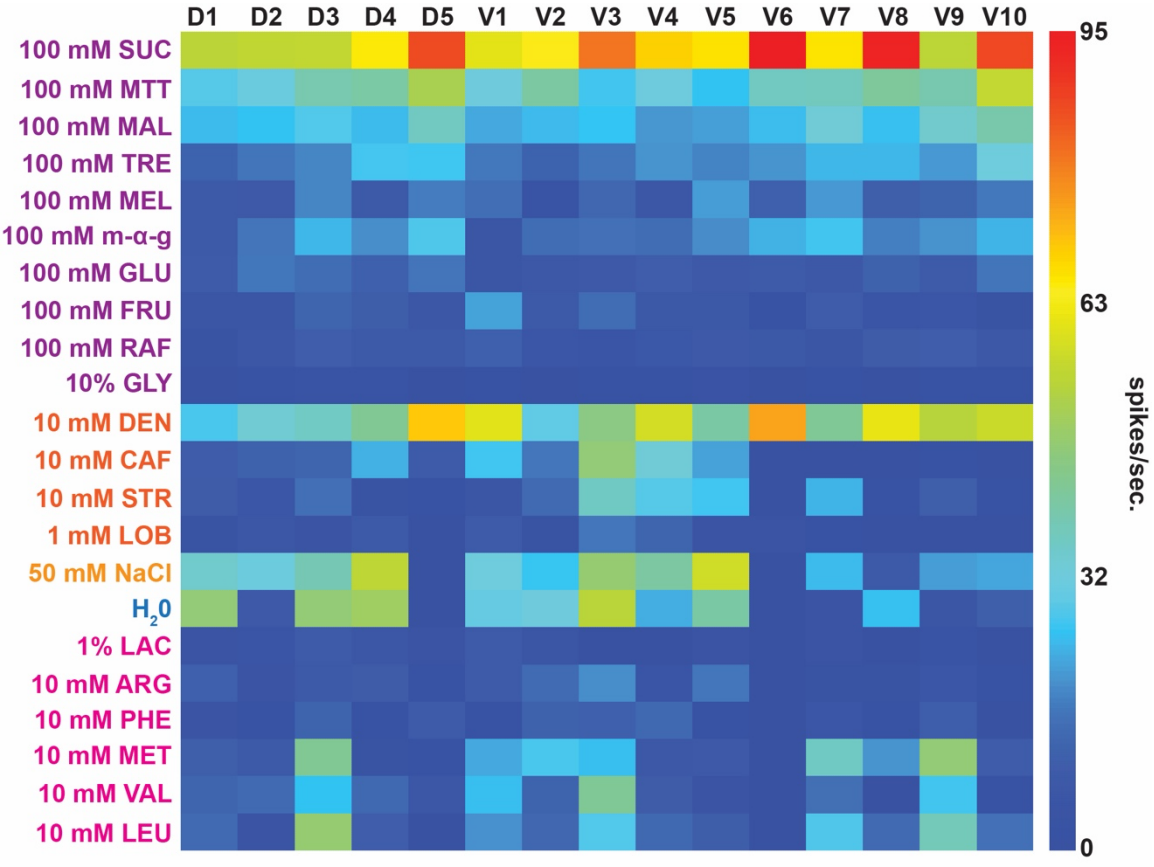

Figure 2.3 Activation profiles of *Aedes aegypti* labellar sensilla show qualitative and quantitative differences

The heat map shows the activation response profiles for each sensillum as the number of spikes/ second in response to the panel of 22 tastants (TCC not included). The colored scale on the right represents the strength of the response, red being the strongest at 95 spikes/sec and the darkest blue being the weakest response at 0 spikes/sec. The sensilla are listed across the top of the heat map as D1-5 and V1-10. The panel of tastants is made up of compounds from different taste categories: sugars (purple), bitter compounds (orange), salt (yellow), water (blue), a skin compound, and amino acids (pink). Each tastant per sensillum, n =7-14; sucrose had the most replicates because it was used as a positive control every time a new mosquito was tested, n=38-57.

Figure 2.4 Denatonium exhibits unique temporal dynamics

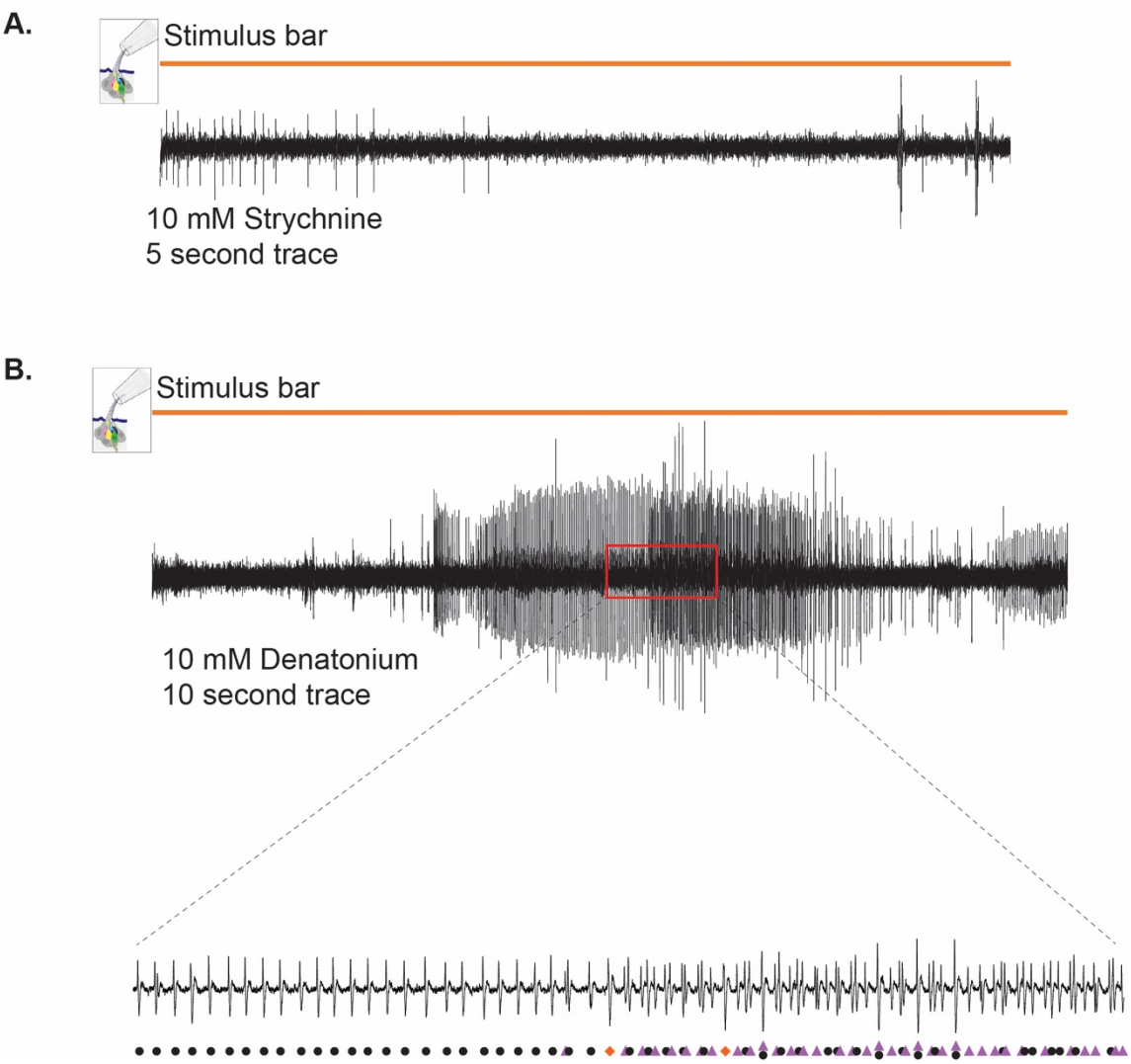

Figure 2.4 Denatonium exhibits unique temporal dynamics

(A) A five-second trace for strychnine shows the typical phasic/tonic temporal pattern of firing that eventually dissipates. Orange bar is used to show the duration of the stimulus.

(B) To visualize the response to denatonium, the recording window was increased to 10 seconds. The initial delay can range between 2-8 seconds before the neuron fires. After the action potentials appear, the frequency seems to increase after only a few seconds. A closer look into the region marked by the red rectangle reveals a second neuron that begins to fire in addition to the first. The action potentials of the first neuron have been marked with the black circles, and the smaller ones produced by the second neuron are marked with purple triangles. The stacked circle and triangle mark the larger action potentials that indicate summation, where the response of multiple neurons results in a larger spike. Two additional action potentials are marked with an orange diamond shape because the amplitude size is between neurons 1 and 2. Still, it is difficult to discern if this is, in fact, a third neuron that is firing. Orange bar is used to show the duration of the stimulus.

Figure 2.5 Cluster analysis reveals five major functional groups of sensilla

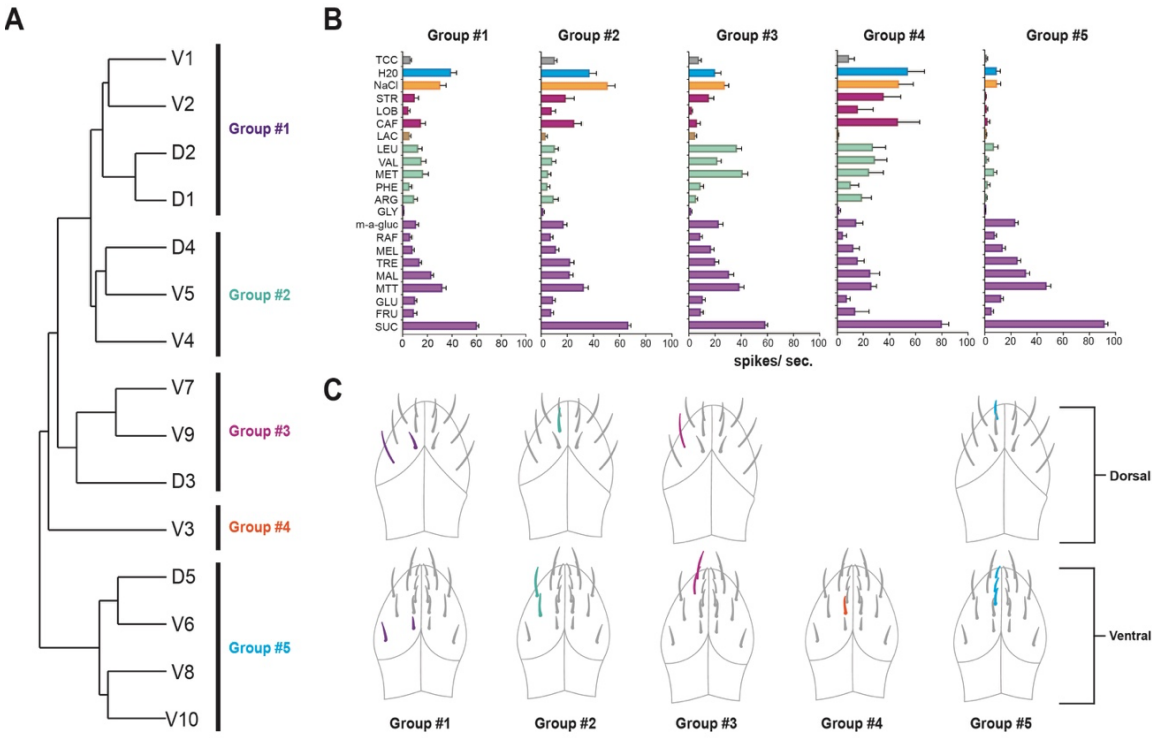

Figure 2.5 Cluster analysis reveals five major functional groups of sensilla

(A) Cluster analysis showing the five different functional groups.

(B) Mean responses of all sensilla that make up each functional class. The different colors separate the different taste categories on the panel: Grey-TCC, blue-water, yellow-salt, magenta-bitter compounds, green-amino acids, purple-sugars. Error bars represent the SEM

(C) Images showing the spatial distribution of labellar sensilla for each group. Each group of sensilla are highlighted in a different color.

(D) The sensillar distribution across the dorsal and ventral sides of the labellum for each functional group of sensilla.

Figure 2.6 The amino acid response is mapped to a neuron of unknown identity.

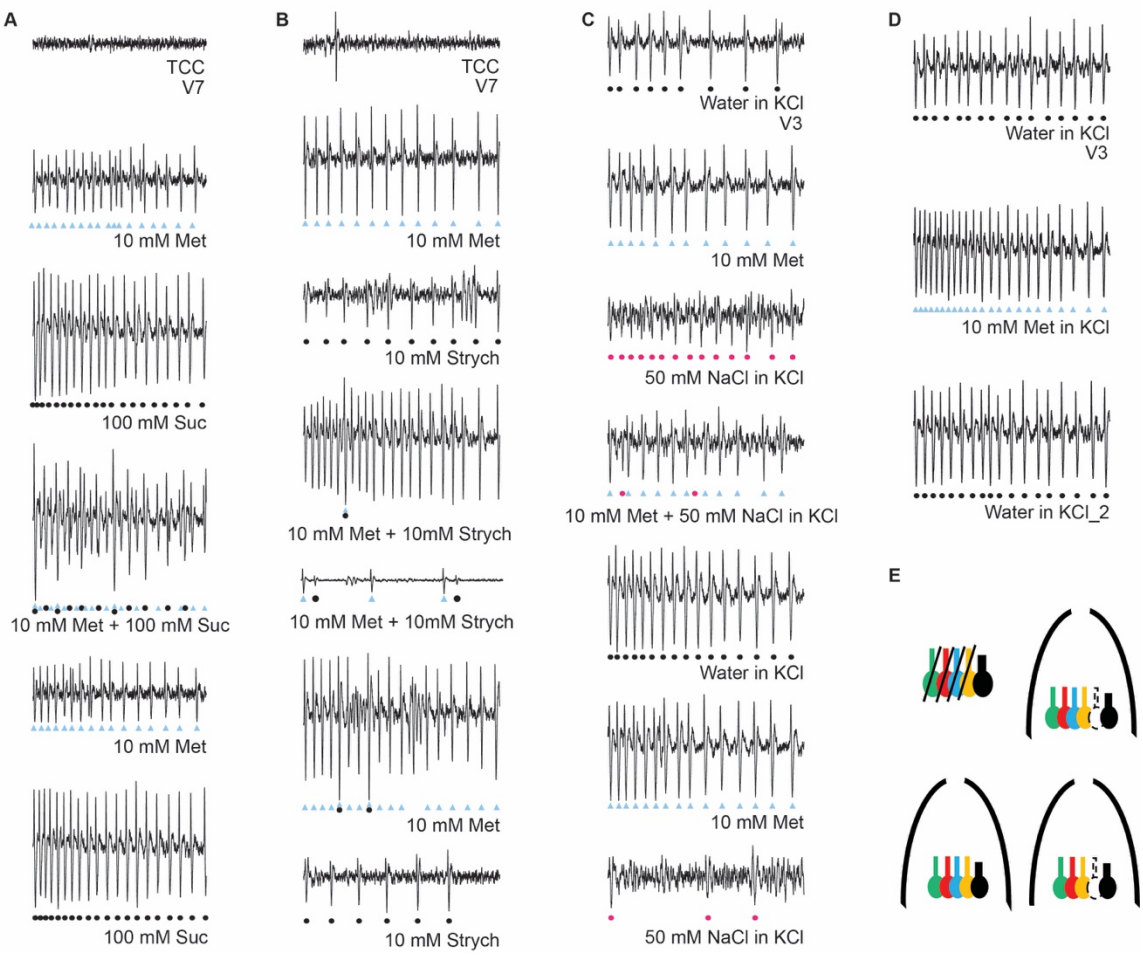

Figure 2.6 The amino acid response is mapped to a neuron of unknown identity

(A) Sample traces of TCC, 10 mM methionine, 100 mM sucrose. Both methionine and sucrose elicit a clean trace with only one amplitude present. The mixture of the two clearly shows two different amplitudes in addition to a few larger summation spikes indicative of two active neurons. Methionine and sucrose tested after the mixture again result in a clean trace of only one spike amplitude. Therefore, the response to methionine is not mediated by the sugar-sensing neuron.

(B) Sample traces of methionine and 10 mM strychnine show clean traces with only one spike amplitude present—the mixture of the two showed a gradual increase of action potentials and one large summation spike. However, in a fraction of mosquitoes there are two different spike amplitude sizes that can be visualize towards the end of the traces after the frequency of the larger action potential begins to dissipate. Methionine and strychnine tested after the mixture again result in clean traces of only one spike amplitude with different spike frequencies, further supporting the fact that the bitter-sensing neuron does not mediate the response to methionine.

(C) Sample trace of water in 1 mM KCl is shown at the top since 1 mM KCl is the recording electrolyte used for the salt stimulus. The response to water, methionine and 50 mM NaCl results in clean traces with only one spike amplitude. The mixture of methionine and NaCl resulted in a uniform trace with two smaller visible spikes,

labeled as the salt neuron. The methionine and NaCl stimulus following the mixture result in traces with very different spike frequencies, suggesting that the salt-sensing neuron does not mediate the response to methionine.

(D) Sample trace of water in 1 mM KCl followed by a sample trace of 10 mM methionine. Both stimuli result in clean traces of only one spike amplitude. Discerning whether the water neuron mediated the response to methionine was challenging to do by eye. However, data from our comprehensive survey has examples of sensilla that have a methionine response in the absence of a water response (V9) and vice versa (D4). Furthermore, as the mapping experiments were being carried out, there were instances of a response to methionine in the presence of a non-responsive water neuron. Our data would suggest that the water neuron does not mediate the response to methionine.

(E) Schematic showing the possible arrangement of chemosensory neurons that innervate the T1 labellar sensilla. The image on the top left shows that the sweet- (green), bitter- (red), water- (blue), and salt-sensing (yellow) neurons do not mediate the response to amino acids. Based on the amino acid responses across the different functional groups, it is possible that the neuron mediating the amino acid response (dashed neuron) is not present in all sensilla. Only in a few sensilla would there be a total of five innervating neurons (top right). The bottom two images show the possible neuron arrangement for those sensilla that have either a response to water and not amino acids or (bottom left) or a response to amino acids and not water (bottom right).

Figure 2.7 Response to amino acids is visible in the absence of neuronal activity from additional innervating neurons

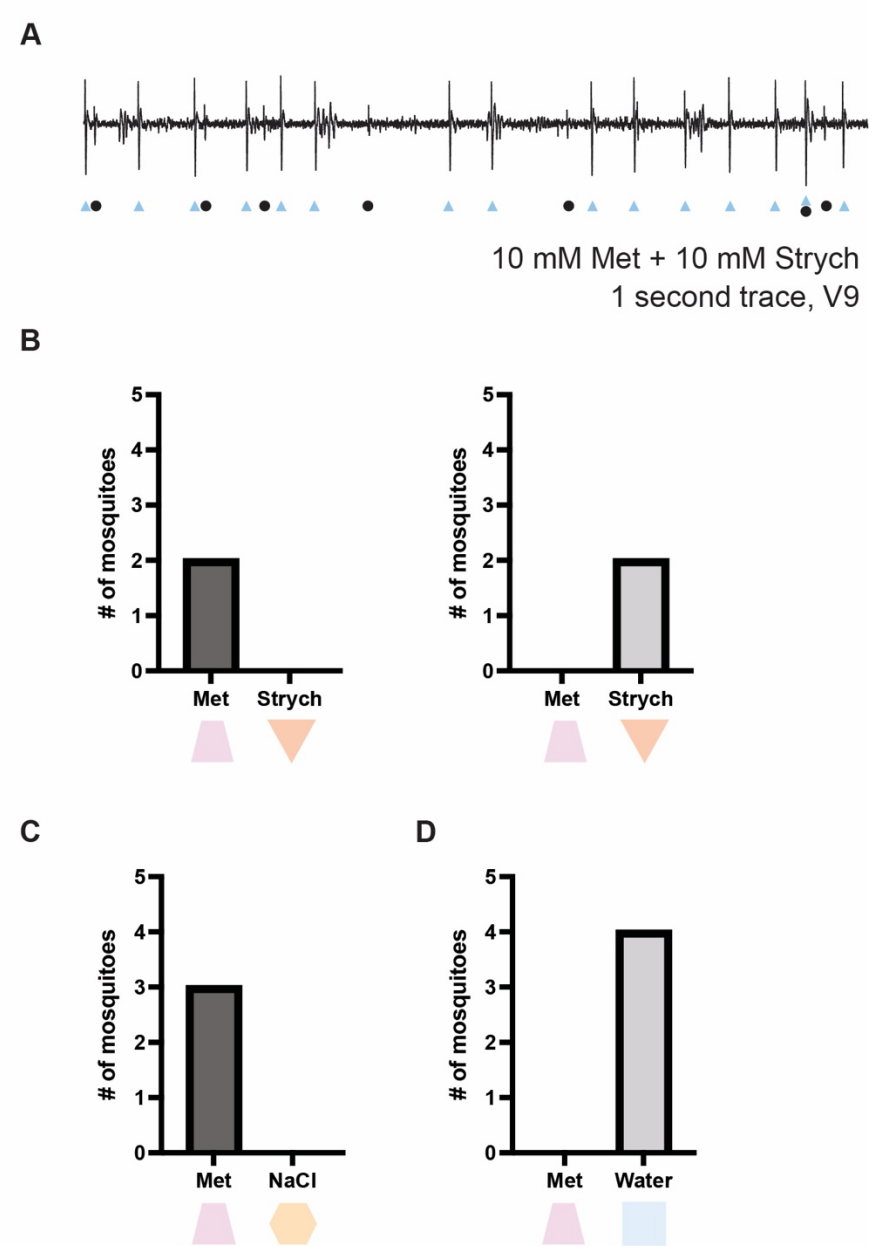

Figure 2.7 Response to amino acids is visible in the absence of neuronal activity from additional innervating neurons

(A) One second sample trace from sensillum V9 of the Met and strychnine mixture to show the presence of a second action potential of smaller amplitude size. Blue triangles mark the larger action potentials that correlate with the Met response. The black circles mark the smaller action potential that correlates to strychnine response.

(B) Two mosquitoes showes responses to Met in the absence of a strychnine response while a different two mosquitoes showed a response to strychnine in the absence of the Met response.

(C) Three mosquitoes showed a response to Met in the absence of a response to NaCl.

(D) A total of four mosquitoes showed a response to water in the absence of a Met response.

Chapter 3

Labellar sensitivity of *Aedes aegypti* across sexes and the gonotrophic cycle

Overview

A female mosquito undergoes many physiological changes during its life. These changes shift meal preference from nectar to blood. During this time, female mosquitoes become sensitized to host cues such as, heat, and human skin odors, all of which are detected via olfactory appendages. Thus, we became interested in the role of the taste system during different phases of the gonotrophic cycle and whether any observable differences in sensitivities of the peripheral taste organs exist. To address this, we used a diagnostic panel of tastants to measure responses of labellar sensilla of non-mated females or those in the post-blood-fed condition and compared them to the responses obtained from mated non-blood-fed female mosquitoes from our comprehensive screen. Response of male mosquitoes were also measured and compared to non-mated and mated females to examine sexual dimorphism in their sensitivities.

Introduction

During the first few days of life, both male and female mosquitoes feed on sugar-rich meals to fuel the energy-expensive process of flight. A few days later, the reproductive demand of a female mosquito will warrant a switch to vertebrate blood, a more nutrient-rich meal that provides protein for egg development. Morphologically speaking, differences in the digestive tract of the female mosquito allow for the efficient intake and digestion of a blood meal (Godoy *et al.,* 2015). However, these shifts in meal preference do not occur simply because the female mosquito has the machinery to break down complex meals. Instead, many underlying physiological changes occur to prime the female mosquito for the ingestion of both meals, the timing of which coincides with fluctuating hormone levels across the gonotrophic cycle (Zhu *et al.,* 2000).

Examples of this are seen in transcriptome analyses of female *Aedes aegypti* fat body, which show that priming of metabolic pathways accommodates the switch between food sources throughout the gonotrophic cycles (Hou *et al.,* 2015). Carbohydrate metabolism transcripts that were elevated 24 hours post-eclosion, declined after 72 hours, and fluctuated once again after the blood meal. These transcript fluctuations were synchronous to the mosquito's energetic requirements. Additionally, these transcript fluctuations occurring throughout the gonotrophic cycle were found to be dependent on hormonal fluctuations of juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Hou *et al*., 2015).

Myriad underlying physiological changes that prepare a female mosquito for consuming a blood meal give rise to a series of behavioral changes. While a female mosquito feeds on nectar meals, it is largely unaffected by any host-associated cues such as CO_2, heat, humidity, or human skin odors. However, about 4-5 days post-emergence, the presence of CO_2 triggers the host-seeking response in the females, and she is primed to respond to additional cues like heat and human skin odors, which are vital for locating a host (McMeniman *et al.,* 2014; Sorrells *et al.,* 2021). Transcriptome analyses have reported that changes in the expression of chemosensory receptors in the antennae occur before and after a blood meal is taken and are accompanied by changes in olfactory sensitivities (Rinker *et al.*, 2013; Hill *et al.,* 2021). Recent transcriptome analysis reported that several genes are differentially expressed in mosquito taste appendages after a blood meal (Matthews *et al.,* 2016). Thus, we became interested in potential changes in the physiology of taste organs during these different phases of the female mosquito's gonotrophic cycle.

For this study, we generated a sub-panel of tastants to carry out single-sensillum recordings (SSR) of the labellar sensilla of female mosquitoes at different points of the gonotrophic cycle. We surveyed responses from non-mated females and mated post-blood fed (PBF) females and compared them to the mated (non-blood-fed) female responses obtained from the initial comprehensive screen. Labellar sensilla of male mosquitoes were also surveyed and compared to mated mosquitoes to detect any sex differences in the responses.

Results

To begin our study, we generated a sub-panel of tastants to survey labellar sensilla of the previously identified functional groups. The panel comprised five tastants, each of which represented taste categories tested in our initial comprehensive screen: sucrose-sweet, strychnine-bitter, water, sodium chloride (NaCl)-salt, and leucine-amino acid. The skin compound lactic acid was not a part of the sub-panel, since expression of *Ir8a* is localized to the olfactory sensilla and therefore does not elicit a response from T1 labellar sensilla.

Labellar sensitivity during the different mating states of a female mosquito

The first part of the female mosquito gonotrophic cycle is the previtellogenic period. This phase is governed by a gradual increase and decrease of JH that spans a few days and terminates when a blood meal is ingested (Zhu *et al.,* 2000). At this stage, female mosquitoes feed on carbohydrate-rich meals that support basic metabolic needs like flight and the beginning of egg development (Foster 1995). Female mosquitoes will mate during the previtellogenic phase and undergo several physiological changes that prime them for ingesting a blood meal, which always results in behavioral output. Many changes in olfactory sensitivity and the genes that mediate the responses have been described. However, given the many behaviors that female mosquitoes exhibit during this phase, many of which involve the taste system, we postulated that there would also be changes in taste sensitivities. Thus, we used our sub-panel of tastants and surveyed the sensillar

responses of non-mated females in the previtellogenic phase that had not yet been blood-fed. The responses were compared to responses of the mated females (non-blood-fed) surveyed in our initial comprehensive screen (**Figure 3.1)**.

Our results showed that non-mated female responses to sucrose and water were higher than those of mated females. The resulting sensitivities coincide with metabolic requirements of a female mosquito in the early previtellogenic stage that still feeds on a carbohydrate-rich diet. Although comparison of sucrose and water was the only one that was statistically significant, mated female responses to NaCl were consistently higher than those of non-mated females, which could indicate the beginning of the switch in meal preference. The responses to leucine and strychnine in both conditions did not exhibit any observable trends. Overall, our experiment shows that the responses correlate with sugar feeding preference in the mated and non-mated states (Foster and Walker, 2019).

Labellar sensitivity after a blood meal

After a female has obtained a blood meal, vitellogenesis begins, and there is now a rise in 20E levels (Zhu *et al.,* 2000). The level of 20E peaks at about 18-24 hours after a blood meal. During this time, the female rests while the eggs undergo the rest of their development, and all host-seeking behaviors are halted (Klowden and Lea, 1979a; Klowden, 1981; Liesch *et al.,* 2013). A recent study showed that differentially expressed genes in the antennae could be seen soon after ingesting a blood meal and persisted for several days (Hill *et al.,* 2021).

Therefore, we chose to survey the T1 sensilla 18-20 hours after a blood meal to coincide with the peak in 20E. Overall the sensillar responses of the PBF condition were similar to that of the NBF state (**Figure 3.2)**. There was considerable variation in the responses across the functional groups without any probable patterns. However, the PBF state had a slightly lower response to sucrose across four groups and a lower response to NaCl in group #2.

Labellar sensitivity of males and females

Given that males are obligate nectar feeders whereas females alternate between nectar and blood meals, we were also interested in determining if there is any sexual dimorphism in the functional organization of labellar sensilla. Historically, female mosquitoes have been studied in more detail due to their threat to humans leaving the male taste system largely unexplored. Therefore, we used the same sub-panel to survey labellar T1 sensilla of male mosquitoes (**Figure 3.3**). The topography of the labellar sensilla of males is identical to that of the female mosquito, which facilitate comparison of the same sensillar groups between the two sexes (Hill and Berry Smith, 1999). The most notable differences are seen in sucrose and water responses. Like the non-mated females, the male response to sucrose and water was much higher than the response of the mated females.

Responses to NaCl and leucine were very similar between the two sexes across all functional groups. When comparing responses to strychnine, it was only significantly different in group #2, where the male response was higher than the

female response. Due to apparent similarities, we decided to compare responses of the non-mated NBF female mosquitoes to the males (**Figure 3.4)**. The only significant differences observed were for sucrose and strychnine in group #2 and the water response in group #3, where male sensitivity was higher for all three. Thus, it appears that non-mated females in the previtellogenic phase of the gonotrophic cycle are most like the males.

Given that males rely on sugar meals for their survival, it is not surprising to see that there are sex-dependent differences in the labellar sensitivities of male and female mosquitoes. This result is supported by transcriptome analysis and reporter expression that show that the clade of gustatory receptors that mediate the responses to sweet compounds are localized to the labellum and not the labrum (Jové *et al.,* 2020).

Discussion

Our study showed that labellar sensitivity of the female mosquito changes depending on the physiological state. We found that feeding preferences of the female mosquito correlate to sensitivities observed for the mated and non-mated states. Comparisons between male and female responses showed that males exhibited higher sensitivities to sucrose and water. Overall, male sensitivities resembled those of non-mated females. This result suggests that when considering sensitivities, the base states are likely those of the non-mated females or the males.

Our initial comprehensive survey was carried out using mated females. However, given our results, there is a possibility that a new functional organization would arise if non-mated females were surveyed with an extended panel of compounds. Nonetheless, our original survey still provides valuable information for female mosquitoes during the critical host-seeking phase, but careful consideration should be given when designing future experiments.

Previous studies in the literature have described internal physiological changes that a female mosquito undergoes after obtaining a blood meal. While the process of vitellogenesis is taking place, the female is in a state of rest and is no longer in an active host-seeking phase. This behavior correlates with the reduced olfactory sensitivity and transcriptional changes reported for *A. gambiae* and *Ae. aegypti* (Rinker *et al.,* 2013; Siju *et al.,* 2010; Qiu *et al.,* 2004). However, our survey of the labellar sensilla showed that there were no significant differences in labellar sensitivities 18-20 hours after a blood meal. Only the reduced salt response of the PBM condition in group #2 had been statistically significant.

Given the length of the vitellogenic period of a female mosquito's gonotrophic cycle, it is not surprising to find that we did not observe any significant changes in the labellar sensitivity after only the early portion of this phase. To get a better sense of whether the sensitivity of the taste organ changes after a blood meal, the labellar sensitivity could be measured a second time at the 72-hour time point, which is closer to the end of the cycle. At this time, a female mosquito is ready to oviposit her eggs and has still not entered the host-seeking phase.

Therefore, we expect a change in behavior to help her achieve oviposition, and the cues she relies on are probably different than those used during other phases of the gonotrophic cycle.

Another important consideration is that we have only looked at one taste organ while using a limited panel of tastants. Therefore, we only have a snapshot of the changes that the taste system may undergo throughout the entirety of the gonotrophic cycle. Transcriptome analyses have reported differentially expressed genes in the hindlegs of *Aedes* mosquitoes after a blood meal and while the female is gravid (Matthews *et al.,* 2016). This information suggests that the labellum is not the only taste organ capable of mediating taste throughout these different phases and that the role of the taste system should not be dismissed. However, it will be difficult to discern the precise location of any differentially expressed genes in organs like the labellum without detailed receptor-to-neuron maps since both olfactory and taste sensilla are present. The taste organs' unique morphology will thereby add to the difficulty of attributing differentially expressed genes to olfactory- or taste-mediated behaviors and sensitivities.

Methods and Materials

Mosquito rearing

Aedes aegypti (Liverpool) mosquitoes were raised at 27°C and 65-80% humidity on a 14:10 light-dark cycle. Females from the mated condition were raised in cages with males and were presumed to be mated but were not blood-fed.

Larvae were raised in plastic Sterilite trays with distilled water and were fed TetraMin tropical tablets. Upon emergence, adults were placed in cages with access to 10% sucrose solution *ad libitum*.

Collection of non-mated female mosquitoes

Female mosquitoes were collected within 1-2 hours of emergence to avoid mating. Females were placed in a separate cage with 10% sucrose until they were the appropriate age for recordings (4-10 days old).

Post-blood-feeding experiments

Female mosquitoes were raised with males until it was time for a blood meal. A day before the experiment, a few female mosquitoes were placed into a new cage and exposed to the artificial blood feeder. After 2-3 mosquitoes had fed on blood, the feeder was removed. Additional female mosquitoes that had not been blood-fed were also placed in this cage to serve as a control for the handling of mosquitoes during the transfer from one cage to the other. One of these non-blood-fed female mosquitoes was tested during every recording session with the blood-fed females. Recordings were done 18-20 hours after the blood meal so that they coincided with the peak of ecdysone that occurs 18-24 hours after a blood meal is taken.

Electrophysiology

Female mosquitoes aged 4-8 days old were used for electrophysiology experiments. Mosquitoes were immobilized on ice, and all six legs were removed. The mosquito was then placed on a small square of foamy double-sided sticky tape and placed on the short edge of a glass slide. In this position, only the labellar palps protrude from the edge of the tape. Four thin strips of double-sided sticky tape were cut and placed over the tip of the abdomen, thorax, base of the labium, and base of the labellum to hold the mosquito in place. A reference electrode filled with Beadle-Ephrussi Ringer solution was used to pierce the mosquito's thorax (Cold Spring Harbor Laboratory Press, 2011). The recording electrode was filled with the test solutions dissolved in either 30 mM Tricholine Citrate (TCC) or 1 mM KCl and was then brought to the tip of individual sensilla.

Experimental stimuli

A sub-panel made up of five tastants was used to perform electrophysiological recordings. These compounds were representative of each taste category tested in the original comprehensive screen described in chapter 2: 100 mM sucrose, 10 mM leucine, 50 mM NaCl in 1 mM KCl, 10 mM strychnine, and water in 1 mM KCl. All chemicals used were purchased from Sigma-Aldrich: sucrose (S0389), leucine (61819), strychnine (S8753), and tricholine citrate (T0252). Macron fine chemicals: NaCl (7581-06). Mallinckrodt AR (ACS): KCl (6858).

Comparisons to mated female conditions

A minimum of six mosquitoes was used for the comparison experiments for each tastant. Data points from the original screen with mated females were pulled at random using an online randomization tool (https://miniwebtool.com/random-picker/) in order to match the sensilla that had been tested for each tastant in order to make comparisons with mated females for each condition (i.e., mated females vs. non-mated females, mated females vs. males, non-blood-fed (mated females) vs. post-blood-fed females).

Statistical analysis

All error bars are reported as the standard errors of the mean (SEM). Two-way ANOVA multivariate comparisons with a Bonferroni test were used to analyze mated vs. non-mated, post-blood-fed vs. non-blood-fed, and male vs. female conditions.

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Figure 3.1 Sensillar responses can be correlated to feeding preferences of mated and non-mated states

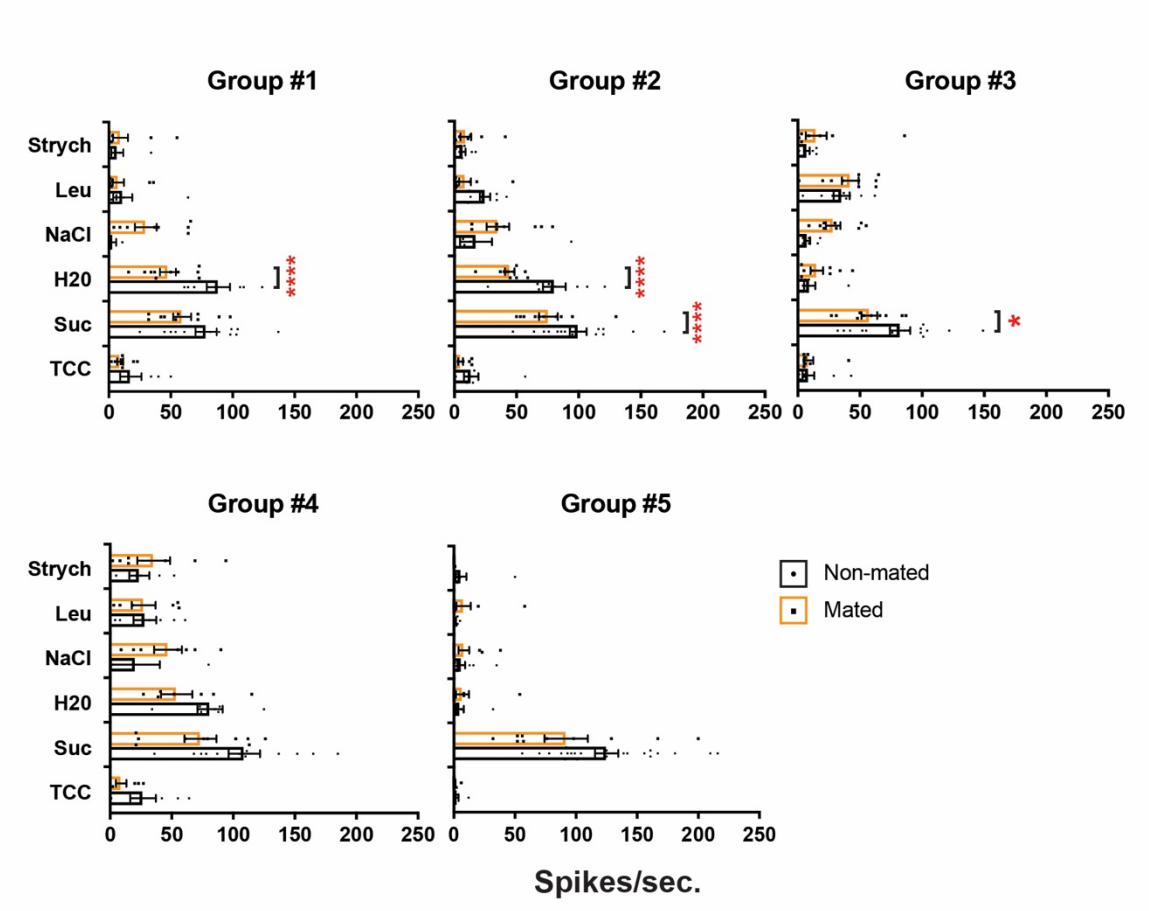

Figure 3.1 Sensillar responses can be correlated to feeding preferences of mated and non-mated states

Activation response profiles from the five functional groups of mated females compared to non-mated female mosquitoes in response to a sub-panel of compounds representing each of the categories tested in the initial comprehensive screen. Although not significant for all groups, sucrose and water responses of non-mated females were higher than mated females. Sensilla were pooled for each group for both conditions: group #1 (D1, D2, V1, V2) n=7-16 sensilla across an n=6-16 mosquitoes, group #2 (D4, V4, V5) n=7-18 sensilla across an n=6-18 mosquitoes, group #3 (D3, V7, V9) n=8-16 sensilla across an n=6-16 mosquitoes, group #4 (V3) n=7-15 sensilla across an n=7-15 mosquitoes, group #5 (D5, V6, V8, V10) n=7-22 sensilla across an n=6-14 mosquitoes. Two-way ANOVA was carried out using the N's for sensilla, Bonferroni test, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Figure 3.2 Sensitivity of labellar sensilla 18-20 hours after a blood meal

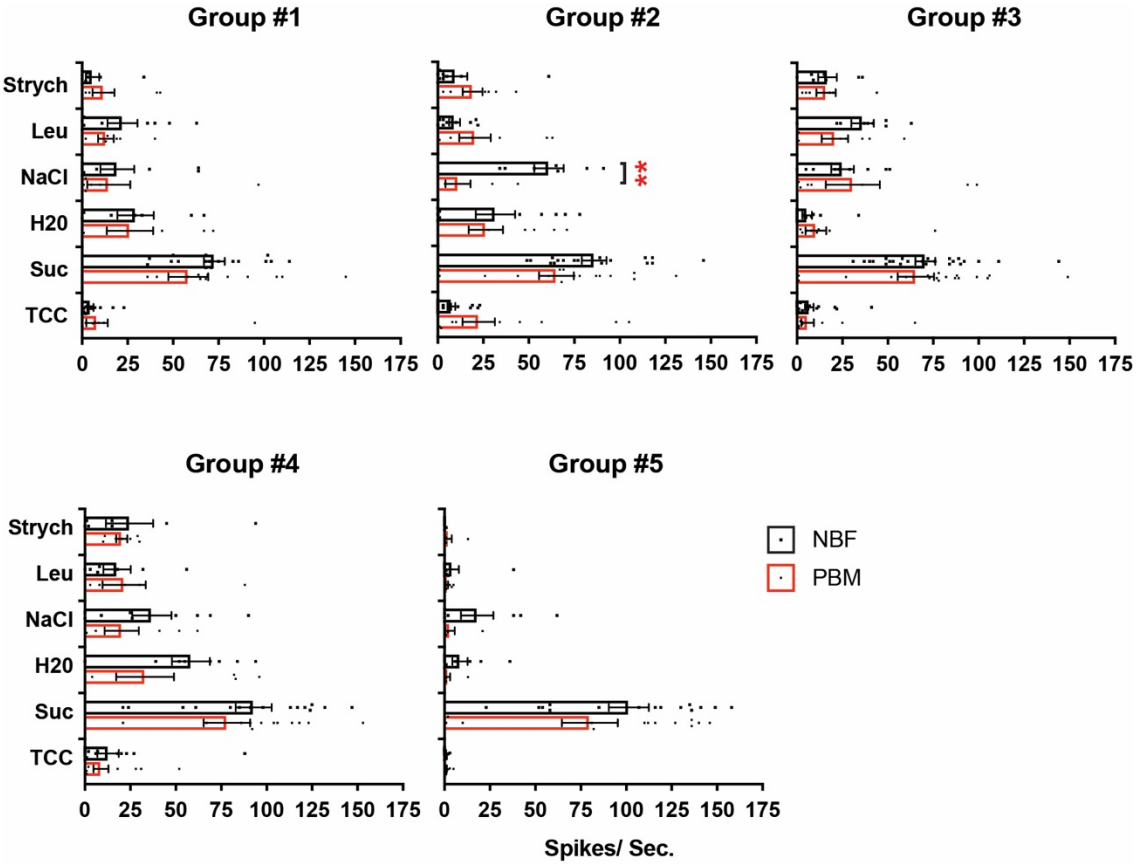

Figure 3.2 Sensitivity of labellar sensilla 18-20 hours after a blood meal

Activation response profiles from the five functional groups of mated non-blood-fed (NBF) females compared to those of mated females 18-20 hours post-blood meal (PBM) in response to a sub-panel of compounds representing each of the categories tested in the initial comprehensive screen. Sensitivities were very similar for both conditions except for the reduced response in the PBM condition to NaCl in group #2, which was statistically significant. Sensilla were pooled for each group for both conditions: group #1 (D1, D2, V1, V2) n=7-17 sensilla across an n=8-17 mosquitoes, group #2 (D4, V4, V5) n=7-17 sensilla across an n=6-17 mosquitoes, group #3 (D3, V7, V9) n=7-24 sensilla across an n=6-24 mosquitoes, group #4 (V3) n=7-15 sensilla across an n=7-15 mosquitoes, group #5 (D5, V6, V8, V10) n=7-15 sensilla across an n=6-15 mosquitoes. Two-way ANOVA was carried out using the N's for sensilla, Bonferroni test, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Figure 3.3 Labellar sensilla of males exhibit higher sensitivities than mated females

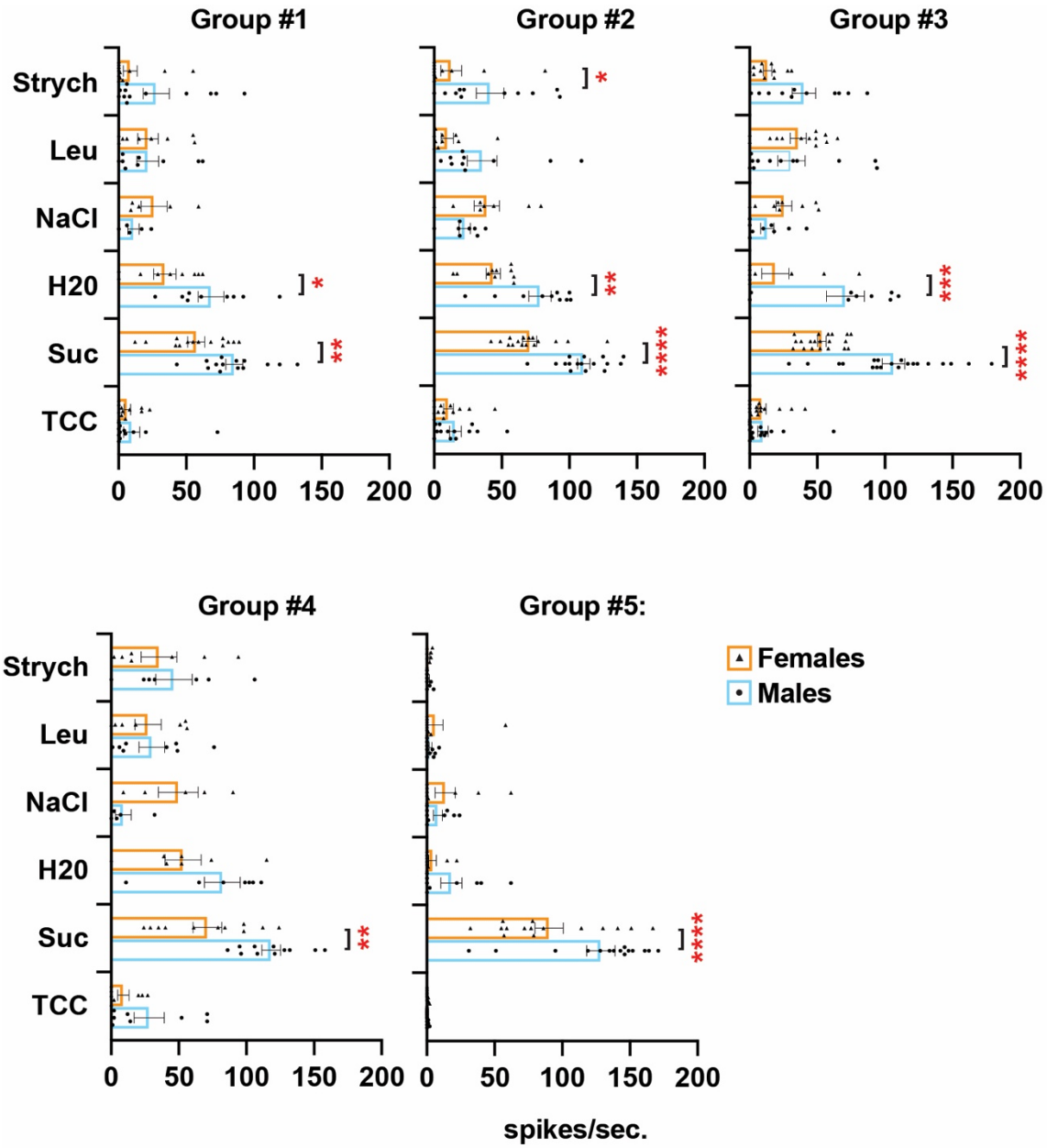

Figure 3.3 Labellar sensilla of males exhibit higher sensitivities than mated females

Activation response profiles from the five functional groups of mated females compared to males in response to a sub-panel of compounds representing each of the categories tested in the initial comprehensive screen. Overall, the males' response to sucrose and water was higher than the females' response. Only for group #2 was the male response to strychnine higher than the female response. Sensilla were pooled for each group for both conditions: group #1 (D1, D2, V1, V2) n=5-14 sensilla across an n=5-14 mosquitoes, group #2 (D4, V4, V5) n=8-16 sensilla across an n=8-16 mosquitoes, group #3 (D3, V7, V9) n=9-20 sensilla across an n=9-20 mosquitoes, group #4 (V3) n=5-11 sensilla across an n=5-11 mosquitoes, group #5 (D5, V6, V8, V10) n=8-15 sensilla across an n=8-15 mosquitoes. Two-way ANOVA was carried out using the N's for sensilla, Bonferroni test, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Figure 3.4 Labellar sensitivity of males and non-mated females are more similar to each other and higher than the mated female response

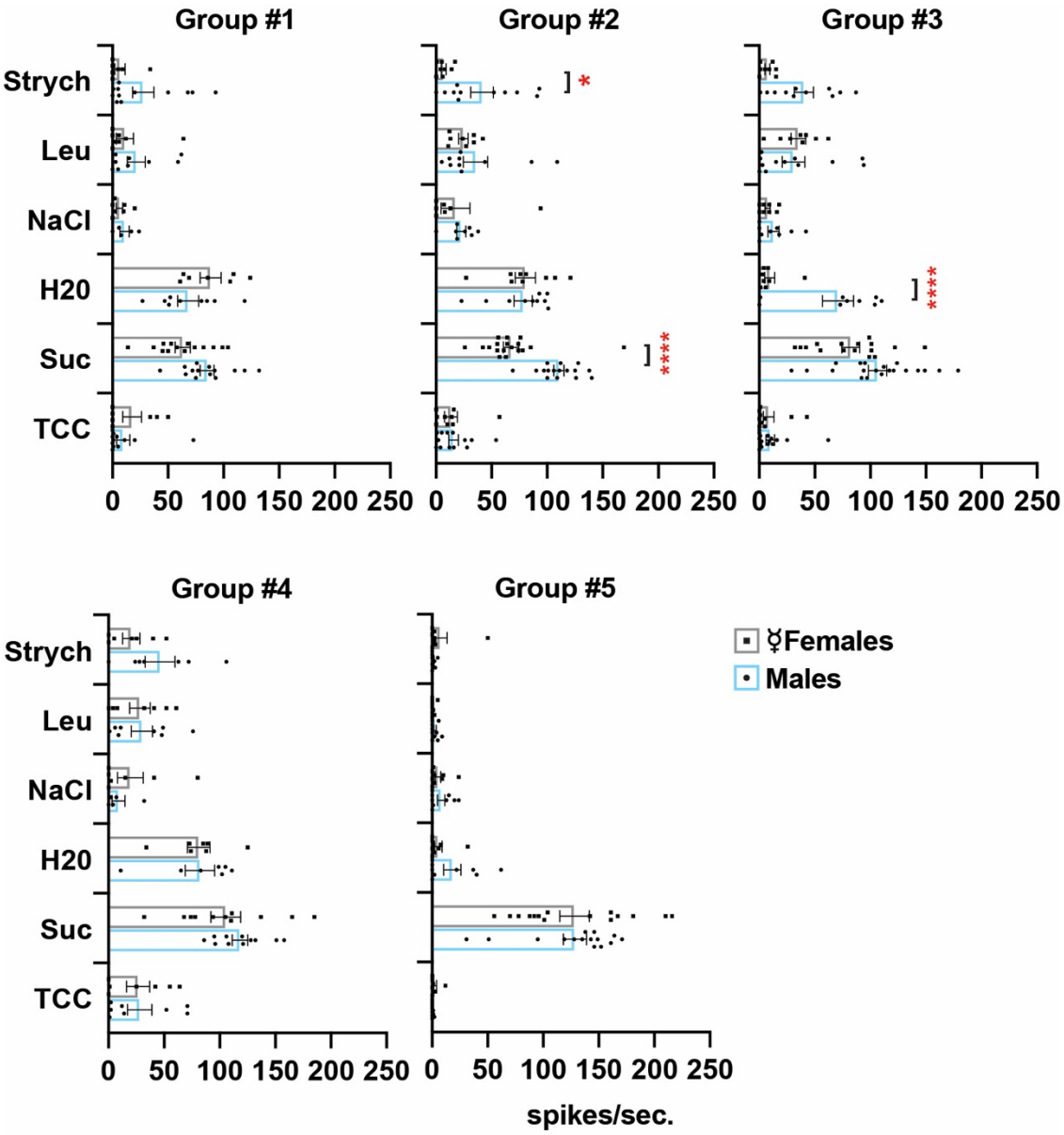

Figure 3.4 Labellar sensitivity of males and non-mated females are more similar to each other and higher than the mated female response Activation response profiles from the five functional groups of non-mated females compared to males in response to a sub-panel of compounds representing each of the categories tested in the initial comprehensive screen. The responses between the males and non-mated females were very similar across all sensillar groups. The only statistically significant differences were seen in group #2 where the male response to sucrose and strychnine was higher than that of the non-mated females, and in group #3, the male water response was higher than the response of the non-mated females. Sensilla were pooled for each group for both conditions: group #1 (D1, D2, V1, V2) n=5-14 sensilla across an n=7-14 mosquitoes, group #2 (D4, V4, V5) n=7-18 sensilla across an n=7-18 mosquitoes, group #3 (D3, V7, V9) n=8-20 sensilla across an n=8-20 mosquitoes, group #4 (V3) n=5-11 sensilla across an n=5-11 mosquitoes, group #5 (D5, V6, V8, V10) n=7-15 sensilla across an n=7-15 mosquitoes. Two-way ANOVA was carried out using the N's for sensilla, Bonferroni test, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Chapter 4

Concluding remarks and future directions

The beginnings of mapping the mosquito taste system

Our understanding of the mosquito chemosensory system is mainly made up of knowledge about the olfactory system. The breadth in knowledge comes from many years dedicated to preventing the spread of mosquito-borne diseases. In contrast, very little is understood about the taste system despite the female mosquito's shifts in energetic requirements that guide her meal preference and the dependence on the taste organs to obtain a blood meal. Behavior experiments have shown that taste organs mediate various behaviors, but the specific cues used to guide them remain elusive. The presence of gustatory and olfactory sensilla on the same organ, like the labellum, makes it challenging to parse out signals that mediate behaviors. In-depth analysis of mosquito taste organs sensitivities would significantly contribute to our understanding of signal processing towards behavioral output.

Electrophysiological data collected from taste sensilla (T1) of the labellum have shown that mosquitoes are sensitive to different taste modalities: sweet, bitter, water, and salt (Kessler *et al.* 2013; Kessler *et al.* 2015; Sanford *et al.,* 2013; Sparks and Dickens, 2016a; Sparks and Dickens, 2016b). Although important, this information is a compilation of piecemeal contributions across different mosquito species and select sensilla. Therefore, we characterized responses of all T1

labellar sensilla of *Aedes aegypti* using a broad panel of tastants. Our data confirmed that the mosquito labellum is sensitive to the previously mentioned taste categories and showed that mosquitoes are sensitive to amino acids. Amino acid sensitivity had only previously been described in terms of behavior and a brief neuronal response of tarsal sensilla. Here we showed that there was variation in the responses to amino acids across all T1 sensilla.

Qualitative and quantitative differences are seen across all responses of the T1 labellar sensilla for every taste category that was tested. These differences could partly be attributed to the varying number of chemosensory neurons that innervate the T1 sensilla. Mapping the response of amino acids suggests that there is a neuron specifically tuned to detect amino acids and is different from the sweet-, bitter-, water- and salt-sensing neurons. The presence of this neuron meant that some sensilla could house up to five chemosensory neurons, more than what is reported for the well-described *Drosophila* taste system. Surveying all T1 sensilla uncovered heterogeneity in the organization of taste sensilla, which gave rise to five previously undescribed functional groups. Thus, our study contributes the first functional map of a taste organ in mosquitoes.

Having a functional map of the labellum gives us a better understanding of how taste is coded across the T1 sensilla. However, variations in sensitivities and the number of innervating neurons can be seen within some functional groups. Increasing the number of tastants for each taste category may help discern any additional sub-classes of sensilla, as seen for the bitter class of sensilla in

Drosophila melanogaster (Weiss *et al.,* 2011). The results could give rise to a more comprehensive functional map.

Naturally, the next step would be to generate a receptor-to-neuron map for the T1 labellar sensilla. The development of reporter lines in mosquitoes makes it possible to map out the expression of the many chemosensory receptor gene families across taste organs. Combining the functional map with a receptor-to-neuron map would shed more light on receptors that mediate the diversity of responses seen across the T1 sensilla. From here, it would be possible to identify regions of the subesophageal zone (SEZ) that process signals from specific taste categories. However, careful consideration should be given since we have only characterized one mosquito taste organ. A more comprehensive view of the taste system will include analyzing and characterizing other major taste organs, such as the tarsi.

The mosquito taste system across different gonotrophic stages

A female mosquito undergoes many physiological changes during the gonotrophic cycle that will ultimately influence behavioral output. Transcriptional studies of internal organs have shown that these changes coincide with different stages of the gonotrophic cycle (Camargo *et al.,* 2020; Hou *et al.,* 2015). Behavioral changes, as well as changes in the cellular response to olfactory stimuli observed after a blood meal, are attributed to the differential expression of chemoreceptors in the antennae (Hill *et al.,* 2021; Rinker *et al.,* 2013; Saveer *et*

al., 2018; Siju *et al.,* 2010). However, differentially expressed genes can also be seen in taste organs after a blood meal (Matthews *et al.,* 2016).

To date, only the olfactory sensitivity of the labellum has been documented before and after a blood meal, leaving the taste system's contribution largely unexplored (Saveer *et al.,* 2018). During the second half of our study, a sub-panel of compounds was used to survey the responses of labellar T1 sensilla of female mosquitoes during different times of the gonotrophic cycle. We found that sensillar sensitivities correlate to the female's meal preference for mated and non-mated states. However, comparisons of labellar sensitivity of pre- and post-blood-fed states did not correlate to any meal preferences or behaviors observed during these states. Given the use of a limited panel, we may have missed other observable changes in sensitivity for both comparisons. Expanding the panel to include more amino acids or other non-volatile skin or sweat compounds would make for a more thorough analysis.

For the pre- and post-blood meal comparison, more time points after the blood meal must be added to cover the duration of the gonotrophic phase leading up to oviposition. Transcriptome analysis like those performed by Hill *et al.,* 2021 for the antennae of *Ae. aegypti* at different time points after the blood-meal are needed in parallel for all peripheral taste organs. Behavioral observations have shown that the female mosquito uses the labellar palps and the labrum to sense and feed on sugary meals and blood, respectively. A recent study reported that the expression of the clade of sweet-sensing gustatory receptors is restricted to the

labellar palps and is absent from the labrum (Jové *et al.,* 2020). Therefore, there is a possibility that changes in labellar sensitivities will not be observed throughout the gonotrophic cycle since feeding on the two primary food sources is carried out by separate organs. Conversely, the tarsi were recently shown to be the primary organs used to identify suitable sites for oviposition (Matthews *et al.,* 2019). Therefore, tarsal sensitivity must be analyzed before and after a blood meal.

The last comparison made was between the two sexes. We found that labellar sensitivity of males was higher for a few of the compounds on the sub-panel. Overall, responses of non-mated females were more like that of males, which suggests that the base state could be considered that of males or non-mated females. In this case, careful consideration should be given to the functional organization presented and should mainly be attributed to the mated non-blood-fed state of the female mosquito. Performing a comprehensive survey in males and non-mated females could result in different functional organizations. This information would be valuable since the males carry out a separate set of behaviors, and it would be interesting to delve deeper into the required chemosensory cues.

Summary

This map of the mosquito labellum contributes a comprehensive summary of the organ's sensitivity and functional organization of T1 sensilla. This map will help parse out signals used for olfactory and taste-driven behaviors, which would

allow for a more informed design of experiments and open many avenues for exploration. One such example is the previously undescribed cellular response to amino acids in males and females. It is easy to postulate that amino acids may be used as host cues since nectar is typically devoid of amino acids but can be found in human sweat. However, amino acid sensitivity in males is particularly interesting since males do not engage in host-seeking behaviors where they would encounter them on the surface of human skin. Ultimately, this body of work contributes new possibilities for mosquito control.

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