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Modulation of Host Learning in *Aedes aegypti* Mosquitoes

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SUMMARY

How mosquitoes determine which individuals to bite has important epidemiological consequences. This choice is not random; most mosquitoes specialize in one or a few vertebrate host species, and some individuals in a host population are preferred over others. Mosquitoes will also blood feed from other hosts when their preferred is no longer abundant, but the mechanisms mediating these shifts between hosts, and preferences for certain individuals within a host species, remain unclear. Here, we show that olfactory learning may contribute to *Aedes aegypti* mosquito biting preferences and host shifts. Training and testing to scents of humans and other host species showed that mosquitoes can aversively learn the scent of specific humans and single odorants and learn to avoid the scent of rats (but not chickens). Using pharmacological interventions, RNAi, and CRISPR gene editing, we found that modification of the dopamine-1 receptor suppressed their learning abilities. We further show through combined electrophysiological and behavioral recordings from tethered flying mosquitoes that these odors evoke changes in both behavior and antennal lobe (AL) neuronal responses and that dopamine strongly modulates odor-evoked responses in AL neurons. Not only do these results provide direct experimental evidence that olfactory learning in mosquitoes can play an epidemiological role, but collectively, they also

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AUTHOR CONTRIBUTIONS

C.V., C.L., and J.A.R. conceived the study. C.V. and C.L. participated in the execution and analysis of all aspects of the study. J.A.R. supervised and helped analyze the electrophysiology data presented in Figures 4 and 5. G.H.W. generated and processed the immunohistochemistry data and western blots presented in Figures 5 and S4. L.T.L. and J.E.L. helped carry out and analyze the behavioral assays presented in Figures 1–4. J.Z.P. helped design the RNAi assays. O.S.A. designed and generated the CRISPR mutant mosquitoes. M.H.D. designed the flight arena experiments presented in Figure 2. C.V., C.L., and J.A.R. wrote the paper, and all authors edited the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.cub.2017.12.015.

DECLARATION OF INTERESTS

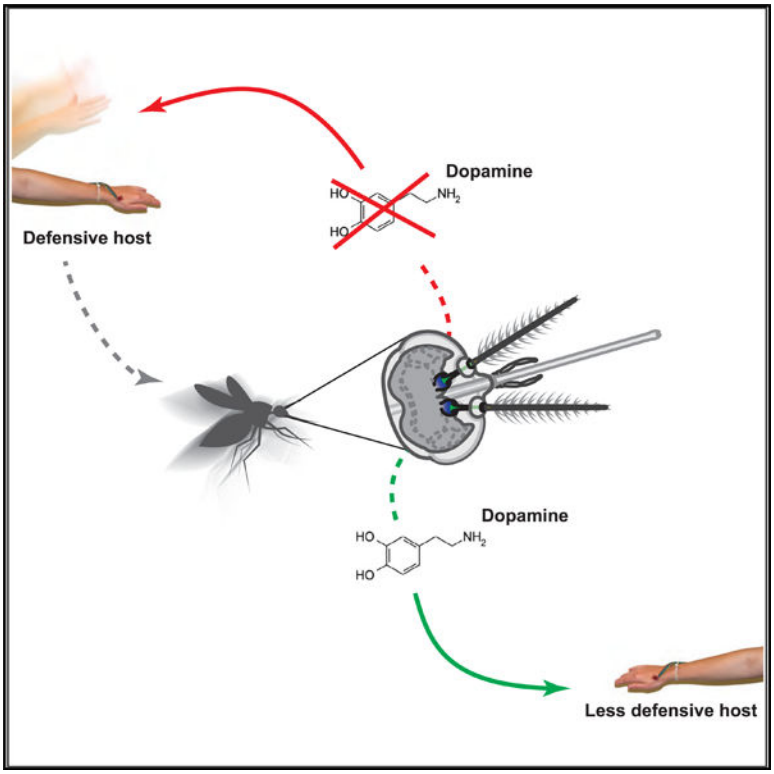
The authors declare no competing financial interests.

DATA AND SOFTWARE AVAILABILITY

Data and custom-built MATLAB code can be requested by email from the Lead Contact.

provide neuroanatomical and functional demonstration of the role of dopamine in mediating this learning-induced plasticity, for the first time in a disease vector insect.

Graphical Abstract



In Brief

Mosquitoes show an ability to avoid defensive hosts, but the mechanisms mediating these shifts in host preferences are unclear. Vinauger et al. show that *Aedes aegypti* mosquitoes rapidly learn host odors and that learning is dependent on the dopamine-1 receptor. Understanding the mechanisms of learning may provide new tools for mosquito control.

INTRODUCTION

Mosquitoes are notorious for their proclivity in host species preferences, and as some of us can attest, certain individuals are preferred over others [1–3]. In addition, many mosquito species can shift host species when their preferred blood resource is no longer present [4–6]. Although the abundance of certain hosts often determines mosquito choice (especially if the species is opportunistic), even mosquitoes with a clear host specialization may shift when their preferred host becomes less abundant [4, 5, 7]. For example, the generalist mosquito *Culex tarsalis* in California feeds primarily on birds in the summer but on both mammals and birds in the winter [5, 8]. This alteration is linked to fall migration of robins, the mosquitoes' preferred host. For the highly anthropophilic species *Anopheles gambiae sensu stricto*, in an environment where humans are not readily accessible, >80% of mosquitoes still

show an innate preference for human odor, even though the proportion of human feeds is low (<40%) [4]. Together, these results suggest that mosquitoes can feed on a readily available but less preferred host species as well as selectively bite certain individuals within a host population [2]

How do mosquitoes alter their preferences? Although genetic factors may be important (e.g., presence of conserved olfactory receptors to host odors), physiological factors and the mosquitoes' learning experiences using other blood hosts are likely mechanisms guiding these shifts [6]. Over the last decade, evidence of olfactory learning in blood-feeding insects has grown for mosquitoes [9–12] and kissing bugs [13–15]. In parallel, research in mosquito development and arousal to hosts have demonstrated the importance of biogenic amines [16, 17], like dopamine and octopamine, which in other insects, are also involved in learning and memory [18–22]. Despite these research foci, the links between how disease vector experience and learning influence host preferences, and the neurophysiological bases for learning, remain unclear.

For mosquitoes, hosts serve as both prey (source of food, i.e., blood) and predator. The host's anti-parasitic and defensive behaviors are a major source of mortality for adult female mosquitoes [23]. Here, we take advantage of host defensive behaviors to examine the ability of mosquitoes to learn the association between host odors and aversive stimuli. We specifically asked (1) whether mosquitoes can aversively learn non-human hosts and the scent from individual humans; (2) whether the mosquito's dopaminergic system, similar to what is found in other insect species, is involved in aversive learning; and (3) how biologically important odors are represented in the mosquito brain and how dopamine modulates those responses. Our results show that dopamine is necessary for aversive learning in mosquitoes and plays an important role in modulating olfactory responses, allowing for an increased ability to discriminate between odors and hosts.

RESULTS

Mosquitoes Learn to Avoid Host Odors

When encountering a defensive host, mosquitoes are exposed to mechanical perturbations (e.g., swatting, shivering) that can be perceived as negative reinforcement by the insect when paired with other host-related cues such as host odors. Learning the association between host odor and mechanical perturbation would allow mosquitoes to use information gathered during previous host encounters. To determine whether mosquitoes can aversively learn human body odor, 6-day-old mated *Aedes aegypti* females were trained in small individual chambers to associate host-related odorants (conditioned stimulus [CS]) with an aversive stimulus consisting of mechanical shocks/vibrations (unconditioned stimulus [US]) mimicking host defensive behaviors (Figures 1A and 1B). 24 hr post training, the behavioral response of mosquitoes was assessed in a Y-maze olfactometer in which the insects had to fly upwind and choose between one arm delivering the test odor (i.e., the CS odor) and a control arm carrying only the solvent control (Figures 1C and 1D).

Ae. aegypti mosquitoes have a strong preference for human hosts [24, 25] but also show strong variation in their attraction to the odor of different individuals [26, 27]. The body

odors of individual human subjects (five males, five females) were collected with nylon sleeves (Figure S1), and mosquito responses were tested in the Y-maze olfactometer. Whereas naive mosquitoes were strongly attracted to human body odors (Figure 1F), trained mosquitoes suppressed their attraction and treated the human body odor the same as the no-odor control. This loss of attraction shown by trained mosquitoes was not a function of the physiological stress or number of active individuals but rather was an active decision in response to the previously experienced odor and fly into the control arm ($p > 0.05$, Student's t test comparisons of flight velocities and activity levels, n = 24–39; $t > 3.8$; for all treatments depicted in Figures 1 and S2). As an important control, we exposed mosquitoes to the CS and US in an unpaired way, thereby preventing the temporal contingency between the stimuli. These mosquitoes were not significantly different from naive ($p > 0.05$) and were still significantly attracted to human odors. Interestingly, not all human subjects elicited the same levels of attraction in naive mosquitoes, and learning performances differed between groups of trained mosquitoes as a function of the individual human body odor used as a CS (Figure S1). These responses were not correlated with the concentration of an individual's body odor ($R^2 = 0.12$; $p = 0.32$).

To test whether associative learning could also affect host selection processes at interspecific levels, rat and chicken body odors were collected using similar nylon sleeves and used in training. The preference of mosquitoes for one of the two host species was tested in the Y-maze olfactometer 24 h after training. In this experiment, one arm delivered the rat odor, while the other delivered the chicken odor. Whereas naive mosquitoes and mosquitoes from the unpaired group were equally attracted to the scent of the two host species, mosquitoes trained against the rat odor were significantly more likely to avoid the rat arm and flew preferentially into the arm delivering the chicken odor (Figure 1G). Conversely, training did not affect mosquito choice when the chicken odor was used as a CS. These results mirror those obtained in the triatomine bug *Rhodnius prolixus*, where bugs successfully learned the association between rat body odors and a mechanical shock but did not learn as well when bird odor was used as a CS [15].

The scents emitted by humans and other hosts are complex mixtures of hundreds of odorants, making it difficult to identify which features the mosquitoes might be using to learn the association. We therefore examined the learning capabilities of mosquitoes to single odorants, several of which are emitted from hosts. One that elicited clear learning responses was 1-octen-3-ol (octenol), a common odorant found in the headspace of mammals [11, 28, 29] but missing in birds (Figure 1E). We therefore used octenol to more fully explore the ability of mosquitoes to learn the association between the shock and a single host-related odorant. 24 hr after training, mosquitoes remembered the association between the mechanical shock and octenol (Figure 1H), and their aversive response was comparable to the responses of naive mosquitoes to 40% DEET (N,N-diethyl-meta-toluamide), a concentration corresponding to commercially available doses of this common insect repellent. Again, mosquitoes from the unpaired group, or mosquitoes that were exposed to either the CS or the US only, did not show learned responses to octenol, clearly demonstrating the associative nature of their learning.

Aversive Learning Modifies Odor-Guided Feeding Preferences and Tethered Flight Responses

Evidence that learning modifies mosquito olfactory flight preference does not necessarily mean that biting and landing preferences might also be modulated. To examine this, we trained groups of mosquitoes using our aversive learning paradigm (Figures 1A and 1D) and released them into a cage in which they had access to two artificial feeders filled with heparinized bovine blood (37°C); one feeder was scented with octenol, while the other was unscented (Figure 2A). Significantly fewer trained mosquitoes landed on the octenol feeder compared to the control feeder ($p < 0.0001$, binomial test; Figure 2B). Once they landed, an equal proportion of trained mosquitoes initiated probing on the two feeders ($p = 0.32$, paired Student's t test, $n = 10$; $t = -1.03$; Figure 2C), although we did observe a tendency for the mosquitoes to feed more on the control feeder than the octenol feeder (24.6% and 15.6% of mosquitoes that landed initiated feeding, respectively; $p = 0.057$, binomial test; Figure S3). By contrast, naive mosquitoes demonstrated no preference in their landing and biting responses to the two feeders ($p = 0.22$, binomial test). The unpaired group showed a slight but significant increase in the proportion of mosquitoes that landed on the scented feeder ($p = 0.002$, binomial test), suggesting that prior exposure to octenol modified their responses in this context. Together, these results suggest that olfactory learning mediates long- (>1 m) and short-range (~0.1 m) discrimination by the mosquitoes, but once the mosquitoes land, other cues (e.g., heat, water vapor) may partially override these responses [32, 33].

To better understand how learning modulates flight responses and to determine whether mosquitoes fly while tethered (thereby allowing simultaneous behavioral analysis and electrophysiological recordings from the antennal lobe [AL]), we positioned mosquitoes in the center of a virtual LED arena where they were tethered by the thorax and maintained in a laminar airflow (Figure 2D). An infrared (IR) light and a two-sided IR sensor allowed real-time measurements of the mosquitoes' wingstroke frequency, amplitude, and turning tendency. Results showed that naive and unpaired mosquitoes exhibited a frequency increase in response to a brief octenol pulse, similar to a ''surge'' exhibited by free-flying mosquitoes when encountering an odor plume. By contrast, trained mosquitoes significantly decreased their flight frequency in response to the same stimulus ($p = 0.013$, Student's t test, $n = 34$; $t = 2.67$; Figures 2E, 2F, and S3).

Dopamine Is Critical for Aversive Learning

Classical insect models for studying learning and memory have shown that dopamine is a key neuromodulator involved in aversive learning [19–22]. To test whether dopamine is also implicated in aversive learning in mosquitoes, we used several ways to manipulate dopamine receptors, including dopamine receptor antagonist injections (Figure 3A, top left), gene knockdown via RNAi (Figure 3A, top center), and CRISPR/Cas9 gene-editing methods (Figure 3A, top right). After aversive training to octenol, mosquitoes were tested in the Y-olfactometer (Figure 1C), allowing us to quantify their flight velocities and behavioral preferences. First, adult female mosquitoes that received dopamine receptor antagonist injections showed significant deficits in their learning abilities compared to uninjected and saline-injected mosquitoes, which showed robust learning responses (Figure 3B). Similarly, female mosquitoes that were injected with dsRNA targeting the *Dop1* gene and CRISPR

mutants with a 6-amino-acid deletion of the Dop1 receptor (Figure S4) showed significant learning deficits compared to the uninjected, non-target dsRNA injected and saline-injected control groups ($p < 0.05$, binomial test compared to control groups; Figures 3B and S4). There were no significant differences in the responses of mosquitoes in treatment groups in which the dopamine receptor was manipulated (i.e., antagonist injected, dsRNA injected, CRISPR edited; $p > 0.64$, binomial test). To evaluate the effects of dopamine receptor manipulation on flight responses, we quantified the mosquito flight trajectories in the olfactometer. Results showed that there was no significant difference in flight velocity between dopamine-impaired treatment groups or between those groups and the saline-injected and uninjected controls ($p > 0.05$, Student's t test, pairwise comparisons Holm p value adjustment, n = 17–29; $t < 2.03$; Figure S2), suggesting that dopamine receptor manipulation did not affect mosquito flight-motor responses. However, it is worth noting that dsRNA-injected mosquitoes and *Dop1* mutants were significantly less aroused to the odors than the other treatment groups ($p < 0.05$, binomial test; Figure S2C). Nonetheless, when these dopamine-impaired mosquitoes were tested against CO_2 or human host odors, they all showed significant attraction ($p < 0.05$, binomial test; Figures 3C and 3D), revealing that manipulating the dopamine receptors impaired their ability to learn aversive information but did not affect their innate olfactory behavior.

Given the inability to learn octenol by the *Dop1* mutants, how might they respond to human scent that contains hundreds of volatiles that are highly attractive to mosquitoes? Results showed that naive *Dop1* mutants were significantly attracted to the scent of human hosts that were also attractive to wild-type mosquitoes ($p < 0.05$, binomial test; Figures 3D and S1). Trained *Dop1* mutants failed to learn the association between the shock and human odors, exhibiting similar behavioral responses to the naive mosquitoes ($p = 0.79$ when compared to the naive CRISPR tested against human odors, binomial test; Figure 3D). Moreover, responses by the trained *Dop1* mutants contrasts those of the trained wild-type mosquitoes, which showed learned aversive responses to those same hosts (Figure 1F).

Odor Stimuli Are Learned and Represented Distinctly in the Mosquito Brain

Given the differences in mosquito olfactory preferences between human and vertebrate hosts and previous work showing that only certain odor stimuli can be learned [12], we next examined how mosquitoes learn different odorants and how odor stimuli are represented in the brain. 24 hr after training, behavioral responses showed that mosquitoes did not learn all odorants equally. For example, whereas responses to nonanol were not influenced by aversive training, those to octenol showed learned aversive responses and L-(+)-lactic acid caused significant attraction (Figures 1H and 4A). To evaluate how different host- and plant-associated odorants are represented in the mosquito brain, we performed extracellular recordings of projection neurons (PNs) and local interneurons (LNs) in the antennal lobe (AL), simultaneous with behavioral recordings (Figure 4B). The extracellular recording method did not allow us to distinguish between PNs and LNs, but it did provide stable recordings (>1 h) of multiple neural units (Figure S5) while allowing us to simultaneously quantify odor-evoked changes in wingbeat amplitudes. Whereas the mineral oil (no odor) control elicited no change in behavioral and neural responses, stimulation with octenol and ammonia elicited strong firing-rate responses in single units (Figure 4C). Interestingly,

whereas ammonia elicited a 1–2 s change in wingstroke activity, stimulation with octenol elicited much longer behavioral responses that lasted many seconds beyond the duration of the stimulus (400 ms) (Figure 4C). Examining single-unit responses across the odor panel, we found that the majority of units (~65%) showed strong odor-evoked responses, with the remaining units showing no significant change in activity (Figures 4D and S5). Moreover, some units (19%) were broadly responsive to different odorants, including units that were responsive to aromatics (e.g., benzaldehyde) and aliphatic compounds (e.g., octenol), as well as monoterpenes (e.g., D-limonene) (Figure S5). By contrast, others (27%) were more narrowly tuned, including units that only responded to one chemical class. In these experiments, hexanol, hexanal, butyric acid, cresol, DEET, ammonia, and breath evoked behavioral responses that were significantly higher than observed for the control ($p < 0.05$, pairwise Student's t tests with Holm correction for multiple comparisons, n = 10–16; $t > 2.38$). Interestingly, the behavioral state (i.e., flying or non-flying) had a significant effect for units that showed suppressed firing activity when stimulated with an odor ($p < 0.01$, Kruskal-Wallis rank sum test, $\chi^2 = 6.95$) but not for units that showed excitatory responses ($p = 0.51$, Kruskal-Wallis rank sum test, $\chi^2 = 0.44$). It is also worth noting that the spontaneous activity of units was slightly (but not significantly) higher when the mosquitoes were flying ($p = 0.083$, Kruskal-Wallis rank sum test, $\chi^2 = 3.01$).

At the neural population level, ensemble responses showed distinct clustering in the multivariate (principal component analysis) space based on the type and chemical class of the olfactory stimuli ($p < 0.001$, ANOSIM; Figure 4E). For example, monoterpenes and aromatics like D-limonene, β-myrcene, benzaldehyde, and cresol occupied a distinct region of the olfactory space relative to the aliphatic acids, alcohols, and aldehydes. By contrast, odor stimuli that evoked strong responses across the ensemble (DEET, ammonia, and breath) were grouped together and were significantly different from the other odorants ($p < 0.001$, ANOSIM), demonstrating that the AL neural ensemble can generalize among and discriminate between olfactory stimuli.

Dopamine Selectively Modulates AL Neurons

To examine how dopamine modulates the processing of olfactory information, we first used immunohistochemistry to examine dopaminergic innervation (via tyrosine hydroxylase, a dopamine precursor) in the mosquito brain. We found extensive dopaminergic innervation across the brain but particularly concentrated in the ALs and lateral protocerebrum, including the mushroom bodies (Figures 5Aand 5B), which are centers that mediate olfactory learning and memory in insects [35, 36]. Dopaminergic innervation is heterogeneous in the AL (Figures 5B and 5C), with some glomeruli being more innervated than others, including the MD2 glomerulus that receives input from the octenol-sensitive aB2 neuron in the maxillary palp. Antisera against the D1-like dopamine receptor Dop1 reveal staining of cell bodies around the ALs, as well as enrichment in the lateral protocerebrum surrounding the mushroom bodies (Figure 5D). We therefore sought to determine the effects of dopamine on odor-evoked responses of mosquitoes' AL neurons.

To test for the neuromodulatory role of dopamine in mosquitoes, we simultaneously recorded the electrophysiological and behavioral responses evoked by a sub-panel of

odorants comprised of octenol, L-(+)-lactic acid, b-myrcene, benzaldehyde, and ammonia before, during, and after superfusion of dopamine (1 μM) over the brain. Dopamine application increased odor-evoked firing-rate responses (Figures 6A and 6B) in 69.6% of responsive AL units, decreased responses in 21.7% of units, and had no effect in 8.7% of units. Dopamine also increased the sensitivity of ~17% of the recorded units, leading to a higher number of cells responding to olfactory stimuli. These effects could be washed out in approximately 50% of units, and in contrast to preparations that were superfused with dopamine, additional control experiments with mosquitoes that were continuously superfused with saline showed no change in spontaneous responses ($p > 0.05$, pairwise comparisons using t tests with pooled SD, $t < 1.52$; Figures S6B and S6C). Moreover, at the level of the neural ensemble, odorant representation significantly changed during dopamine application compared to the pre- and wash-phases of the experiment ($p < 0.05$, ANOSIM) causing stimuli—in particular, octenol—to become more separated in the olfactory space (Figure 6C). Interestingly, the degree of modulation was not the same for all odorants, suggesting that the observed differences in dopaminergic innervation of glomeruli may be functionally linked to glomerular response modulation (Figures 5B and 6C).

DISCUSSION

Heterogeneity in mosquito biting and consequently host infection plays an important role in the spread of vector-borne diseases [37, 38], and previous studies have documented interindividual differences in attractiveness to mosquitoes [2], as well as an ability for mosquitoes to shift species when their preferred host is no longer available [4, 39]. Despite these studies, the processes mediating these mosquito behaviors have remained unclear [40]. Here, we show that learning can contribute to these host shifts and that their direction seems to be driven by the composition of the host odor. One interesting note is that our results suggest that human individuals that are highly attractive to mosquitoes are the ones that mosquitoes can learn. These responses were not a function of the concentration of an individual's scent but rather may be associated with the composition, or ratio, of compounds in the odor. Mosquito learning may thus partially explain host preference heterogeneity and flexibility, and it may also elucidate which olfactory channels mediate these changes.

In this study, we employed an integrative approach to demonstrate that mosquito learning can influence both specificity for individual hosts and their flexibility in olfactory preferences. The ability of mosquitoes to aversively learn depended on odorant type, for instance, L-(+)-lactic acid, an odorant emitted by hosts, could be learned in an appetitive but not aversive context [12], whereas octenol—another odorant emitted by both plants [41] and blood hosts [28, 29]—could be appetitively and aversively learned, suggesting that certain odorants may be encoded by specific olfactory channels that allow rapid learning of attractive or defensive hosts or other important odor sources (e.g., carbohydrates). In concert with these findings, our results indicate that dopaminergic neurons are heterogeneous in their innervation of glomeruli in the AL, which could provide a means for mosquitoes to process, and selectively learn, specific odorants from a complex bouquet emitted from individual hosts. Indeed, our electrophysiological recordings revealed that the AL represented the odorants by chemical class and activity level, and dopamine—a critical neuromodulator involved in learning and arousal [42]—further increased the separation of

certain odorants in the AL encoding space. Dop1 is critical for mediating this plasticity in AL responses and learning abilities, with CRISPR mutants for this receptor showing an inability to learn. The tethered preparation and results in this study provide motivation for future studies to examine how odor representations change during training, thereby allowing direct coupling of learning with how odor information is modulated in the AL and higher-order brain regions.

Host defensive behavior is a major source of mortality for mosquitoes [23], with hosts operating as both predator and prey. In addition, within a host species, there is strong variation in which individuals are bitten [1–3], and this heterogeneity in mosquito biting is thought to play an important role in disease transmission and epidemiology [2]. The ability by mosquitoes to possibly learn which individuals are more, or less, defensive, will have strong fitness consequences for the mosquitoes. However, despite obvious epidemiological relevance, a characterization of the neuroanatomical and neural processes that allow learning-induced plasticity was hitherto missing in disease vector insects. The present work therefore closes a critical knowledge gap in mosquitoes by demonstrating the role of dopamine in olfactory learning and processing. Finally, CRISPR has been highlighted as an important tool in the fight against vector-borne disease [43, 44]. Notably, these mutants have allowed us to target the dopaminergic pathway and impair mosquitoes' ability to use their experience to fine-tune their responses to host signals. Identifying the mechanisms and pathways enabling flexibility in mosquito behavior may provide tools for more effective mosquito control.

STAR★METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeff Riffell (jriffell@uw.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mosquitoes rearing and colony maintenance—Multiple strains of *Aedes aegypti* mosquitoes were used for the experiments: Rockefeller (ROCK), Liverpool (LVP-IB12) and CRISPR transgenic line from the Liverpool strain. Mosquitoes were maintained in a climatic chamber at 25 ± 1°C, 60 ± 10% relative humidity (RH) and under a 12–12h light-dark cycle. Mosquitoes were fed weekly using an artificial feeder (D.E. Lillie Glassblowers, Atlanta, GA, USA; 2.5 cm internal diameter) supplied with heparinized bovine blood (Lampire Biological Laboratories, Pipersville, PA, USA) and heated at 37°C using a water-bath circulation (HAAKE A10 and SC100, Thermo Scientific, Waltham, MA, USA). Cotton balls soaked with 10% sucrose were continuously provided to the mosquitoes. Eggs were hatched in deionized water that contained powdered fish food (Hikari Tropic 382 First Bites - Petco, San Diego, CA, USA), and larvae were cultured and maintained in trays containing deionized water and the fish food. For the experiments, groups of 100 to 120 pupae (both males and females) of the same age were isolated in individual containers and maintained exclusively on 10% sucrose after emergence (i.e., no blood-feeding). Six-day-old female mosquitoes were individually isolated in 15 mL conical Falcon tubes (Thermo Fisher

Scientific, Pittsburgh, PA, USA) covered by a piece of fine mesh that permitted odor stimulation during training. Experiments were conducted when the mosquitoes were the most active and responsive to host related cues: 2 hr before their subjective night [12, 45].

***Dop1 Ae. aegypti* mutant generation—**sgRNAs that were specific for *Dop1* were generated using *in vitro* transcription by combining primer pairs (primers 3 and 5) to make sgRNA-Target 1 and combining primers pairs (primers 4 and 5) to make sgRNA-Target 2 (Table S1). We then combined these sgRNAs (40 ng/μl) with purified Cas9 protein (300 ng/ml) purchased from PNA-bio (Newbury Park, CA, USA) and pre-blastoderm embryonic microinjections (n = 300) were performed following previously established procedures [43]. Following microinjection we individually isolated all surviving females (n = 68), mated, blood fed, and allowed them to lay eggs. After egg laying, we isolated genomic DNA (QIAGEN DNeasy Blood and Tissue Kit (Hilden, Germany)) from these females (focusing only on females that laid eggs (n = 29)) and confirmed mutations in target sequences via PCR (standard techniques) with a primer pair that spans the cleavage sites amplifying 242bp of genomic DNA (primers 1 and 2). We discovered mutations in 68% (n = 20/29) of the injected G0 females that laid eggs. Mosquitoes were individually outcrossed, using individual female to wild-type male crosses every generation for 6 generations. We then selected a mutant line (that stably transmitted the mutation to the offspring) that generated an 18 nucleotide – 6 amino-acid deletion (LRRIGN) in the conserved 7tm-4 superfamily domain and homogenized them for 9 generations. Mutations were verified using PCR/sequencing every generation (100% mutants). Mutants from this line exhibited normal circadian rhythms, blood feeding, and oviposition rates – one of the reasons for selecting this line –, as well as exhibiting robust flight responses in our wind tunnel bioassays. As additional controls, randomly selected mutant mosquitoes used in behavioral and electrophysiological assays were verified using PCR/sequencing after testing (100% were mutants), and electrophysiological AL recordings from *Dop1* mutants showed no significant changes in neuronal odor-evoked responses and spontaneous activity during dopamine superfusion (Figure S6), verifying the efficacy of the CRISPR *Dop1* mutants. Primers and sgRNA sequences can be found in Table S1; see also Figure S2.

Rats used in scent collections—Rat (*Rattus norvegicus*) scent collections were conducted using 4 male Sprague Dawley rats (350–420 g; Simonsen Laboratories). The rats were maintained on a 12 h L/D cycle (lights on at 7:00 a.m.) and all scent collections were performed during the light phase. Each rat was allowed access to water and food *ad libitum*. All animal care and use were conducted in accordance with University of Washington's Institutional Animal Care and Use Committee guidelines, and all procedures were approved by the Animal Care and Use Committee of the University of Washington (IACUC Protocol # 4385–01) and conformed to principles enunciated in the NIH guide for the use and care for laboratory animals.

Chicken hatchlings used in scent collections—Chicken (*Gallus gallus*) scent collections were from 12 unsexed White Leghorn 3–7 day old hatchlings, which were hatched from fertile eggs (Featherland Farms, Coburg, OR) and maintained in vivarium facilities at the University of Washington. Hatchlings were not sexed because of the

difficulty in accurate identifying the sex at this age. Post-hatch chickens were maintained in heater brooders with ample food and water under a 12 h L/D cycle (lights on at 8:00 a.m.), and all scent collections were performed during the light phase. Animal care and experimental protocols involving the chicken hatchlings were approved by the University of Washington Animal Care Committee (IACUC Protocol # 4385–01), and conformed to principles enunciated in the NIH guide for the use and care for laboratory animals.

Humans used in scent collections—Human scent collections were collected from 5 males and 5 female wild-type individuals on the University of Washington (Seattle) campus (ages 23–43 years old). Volunteers were from various backgrounds: Hispanic (one male), white (three males and three females), and Asian (one male and two females). The number of individuals and samples (3–6 per volunteer) was sufficient for statistical comparison between individuals ($\alpha = 0.05$). Protocols were reviewed and approved by the University of Washington Institutional Review Board, and all human volunteers gave their informed consent to participate in the research.

METHOD DETAILS

Host odor collection and GCMS analysis—Host body odors were collected using nylon sleeves (Ililily Inc., Irvine, CA, USA) that were worn for 3.5 hr. For human scent collection, volunteers wore a nylon sleeve around each ankle and arm. For each human individual, a sleeve from an ankle and arm were paired, and one pair was used to train the mosquitoes, and the other ankle+arm pair were used to test the mosquitoes. Volunteers used fragrance-free detergents and soaps to prevent bias in mosquito behavior, and prior to wearing the sleeve individuals rinsed their arm under water for 5 minutes. In addition, we also collected headspace volatiles from adult human volunteers as previously described [46] by wrapping a volunteer's arm in aluminum and piercing the aluminum with a 75um CAR/PDMS SPME fiber (57344-U; Supelco, Bellefonte PA USA). Scent from rats and chicken hatchlings (from < 2 years old male rats and < 10-day-old chicken hatchlings; both approximately the same mass) were collected by placing a nylon sleeve around the abdomen for 3.5 hr (IACUC Protocol # 4385–01). To discriminate between endogenous and exogenous volatiles, controls were performed by keeping clean nylon sleeves in clean, unoccupied rearing containers for the same duration as for the odor collection procedure. Host odors were collected by either the SPME method or by dynamic sorption. The latter method involved enclosing the nylon socks in a nylon oven bag (Reynolds Kitchens, USA). Air was withdrawn from the bag via a diaphragm vacuum pump (400–1901, Barnant Co., Barrington, IL, USA) and passed through a headspace trap comprised of a Pasteur pipette with 50 mg of Porapak powder Q 80–100 mesh (Waters Corporation, Milford, MA, USA) packed between two plugs of glass wool (Restek, Belfonte, PA, USA); air was returned to the bag through a charcoal-filter. Headspace collections lasted for 24 hr. Volatiles were eluted from the traps with 600 μL of 99% purity hexane (Sigma Aldrich, St. Louis, MO, USA), and samples were stored in 2 mL amber borosilicate vials (VWR, Radnor, PA, USA) with Teflon-lined caps (VWR, Radnor, PA, USA) at −80°C until they were run on a Gas Chromatograph coupled to a Mass Spectrometer (GCMS). Fibers were exposed to host volatiles for 1 hr before being run on the GCMS.

Liquid samples were injected (or SPME fibers were exposed) into an Agilent 7890A gas chromatograph (GCMS) with a 5975C Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA, USA). A DB-5 GC column (J&W Scientific, Folsom, CA, USA; 30 m, 0.25 mm, 0.25 μm) was used, and helium was used as the carrier gas at a constant flow of 1 cc.min−1. The oven temperature was 45°C for 3.75 min, followed by a heating gradient of 10 degrees.min^{-1} to 250°C, which was then held isothermally for 10 min. Chromatogram peaks were manually integrated using the ChemStation software (Agilent Technologies), tentatively identified by the NIST library before verification using Kovats Indices and synthetic standards (Figure S1).

Mosquito training protocol and control groups—A total of 2524 individual female mosquitoes were used in the behavioral experiments. Before each training session, individual mosquitoes were allowed to acclimate for 1 min in the absence of stimulation, except for the delivery of a clean air at 30 cm.s^{-1}, room temperature (23°C) and relative humidity (50%). Mosquitoes were then simultaneously exposed to the olfactory stimulus (e.g., octenol at 140 mM; equivalent to the concentrations used in other mosquito training experiments [12]) and a mechanical shock that was delivered for 30 s by a vortexer (Thermo Fisher Scientific, Waltham, MA, USA) at 1.65 g at 44 Hz. Forces were scaled to host defensive behaviors that occur when a human slaps his/her arm to drive off biting mosquitoes (Figures 1A and 1B) as well as exposing mosquitoes to a strong mechanical perturbation without damaging their wings or causing apparent physiological and/or physical damage. Mosquitoes were exposed to ten training trials, each separated by a 2 min interval. During this inter-trial interval (ITI), mosquitoes were maintained in the same experimental room and exposed to a filtered air flow. A vacuum line was used throughout the training session to remove environmental contaminants and olfactory stimuli from the container during the ITI. After conditioning, mosquitoes were placed in a humidified climatic chamber (25°C; 60% RH; 12–12 h L:D) and tested in the Y-olfactometer 24 hr post-training. Four types of control groups were used to test for the effects of aversive learning: a ‘‘naive’’ untrained group; a CS only, a US only, and an ‘‘unpaired’’ group (Figure 1D). The CS and US only control groups controlled for the effect of pre-exposure to either the odor or the mechanical shock, respectively. The ‘‘unpaired’’ group controlled for the associative nature of the learning, by exposing mosquitoes to the odor and the mechanical shock in a pseudo-random, unpaired sequence, i.e., in the absence of temporal contingency [47]. Each of the control groups was tested 24 hr later.

Behavioral testing in the olfactometer—We used a custom-made, Plexiglas Y-maze olfactometer to evaluate and compare mosquito responses to different odor stimuli, as previously described [12] (Figure 1C). Briefly, the olfactometer comprised of a starting chamber, allowing mosquito release, an entry tube (30 cm long, 10 cm diameter) connected to a central box where two ‘‘choice’’ arms were attached (both 39 cm long, and 10 cm diameter). Charcoal filtered air entered as a uniform laminar flow at 20 cm.sec^{-1} into the arms of the olfactometer (Figure 1C). Odor stimuli were delivered to each choice arm via teflon tubing connected to one of two 20mL scintillation vials containing either the tested odor or the control solution (mineral oil) (Figure 1C). Each line was connected to the corresponding choice arm of the olfactometer and placed centrally in the olfactometer arm.

All the olfactometer experiments were conducted in a well-ventilated climatic chamber (Environmental Structures, Colorado Springs, CO, USA) at 25°C and 50% RH. After each experiment, the olfactometer, tubing and vials were cleaned up with water followed by 70% and then 100% ethanol to avoid any contamination between experiments. Finally, to avoid any biases, the side of the stimulus and control arms was randomized daily.

Testing sessions began when one single mosquito was placed in the starting chamber. The mosquito then flew along the entry tube and, at the central chamber, could choose to enter one of the olfactometer arms, one emitting the trained stimulus and the other the ‘‘clean air’’ (solvent only) control [12]. We considered the first choice made by mosquitoes when they crossed the entry of an arm. Mosquitoes that did not choose or did not leave the starting chamber were considered as not responsive and discarded from the preference analyses. Overall, 68.5% of the females were motivated to leave the starting chamber of the olfactometer and choose between the two choice arms. In addition, four treatments were used to ensure that contamination did not occur in the olfactometer and to test mosquitos' responses to innately attractive or aversive stimuli. Untrained ‘‘naive’’ mosquitoes were placed in the olfactometer and exposed to either: (1) two clean air currents (neutral control); (2) a clean air stream versus CO_2 (positive control, $[CO_2]$ = 2300 ppm above ambient level) [48]; (3) a clean air stream versus 40% DEET (an innately aversive control); or (4) a clean air versus octenol (i.e., naive control). Mosquito trajectories were captured with a video camera (Model C615, Logitech, Newark, CA, USA) (Figures 1F and S2) and mosquito flight speeds were calculated for each individual.

Behavioral testing with the artificial feeder—In order to test whether mosquitoes could use learned information in the context of blood-feeding, groups of 17 female mosquitoes were released in a cage (30.5 × 30.5 × 30.5, Bioquip, Rancho Dominguez, CA, USA) on top of which two artificial feeders containing heparinized bovine blood, warmed up to 37°C, were positioned. One feeder was treated with the CS odor (pipetted onto a Kimwipe (Kimberly-Clark professionals, Roswell, GA, USA) surrounding the feeder), while the control feeder (odorless) was treated with the solvent only (i.e., MilliQ water). Two video cameras (Model C615, Logitech, Newark, CA, USA) were used to record mosquitoes' activity at each feeder over the course of the experiment (25 min duration) (Figures 2A) and the total number of landing, piercing and feeding events was counted for each feeder. The position of the feeder associated with the CS odor was randomized in order to avoid any potential spatial bias.

Behavioral testing in the flight arena—Tethered flight responses by mosquitoes to the trained odor were tested in an LED-based arena [30]. Mosquitoes were cold anesthetized on ice and tethered to a tungsten wire using UV-activated glue (Loctite 3104 Light Cure Adhesive, Loctite, Düsseldorf, Germany) applied on the thorax. The main body axis was positioned at a 30° angle from the tether. Mosquitoes were then stored at room temperature in a closed container for an approximate 30 minutes recovery period. Tethered mosquitoes were centered in a hovering position within an arena composed of 12 columns of 2 panels each [30], which were arranged into a regular dodecagon and produced a display resolution of 96 × 16 pixels (Figure 2D). Mosquitoes were placed directly under an infrared (IR) diode

and situated above an optical sensor coupled to a wingbeat analyzer [30, 49, 50] (JFI Electronics, University of Chicago). The beating wings cast a shadow onto the sensor, allowing the analyzer to track the motion of both wings and measure the amplitude and frequency of each wing stroke. Measurements were sampled at 5 kHz and acquired with a National Instrument Acquisition board (BNC −2090A, National Instruments, Austin, Texas, USA). The mosquito was centered between an air inlet and a vacuum line aligned diagonally with one another, 30o from the vertical axis (Figure 2D). The air inlet was positioned 12 mm in front of and slightly above the mosquito's head, targeting the antennae from an angle of 15°. The vacuum line was positioned behind the mosquito 25 mm away from the tip of the abdomen. Two different airlines independently controlled by a solenoid valve intersected this main air inlet, either delivering clean air or an odor. A static visual pattern of alternating stripes comprised of either inactive or fully-lit LEDs, each 16 × 6 pixels in size (22.5°) was used in conjunction with the odor stimulus. Odor stimuli consisted of a 400 ms pulse followed by a 60 s inter-trial-interval, after which the sequence was repeated for a total of 10 pulses. Three groups of mosquitoes were tested: naive (n = 16), octenol-trained (n = 17), and unpaired (n = 18). A clean air control (n = 23) was also performed.

The wingbeat frequency and amplitude were used as proxy for changes in the flight speed of the insect, as an increase in these signals is an indicator of ''surge'' behaviors to an odor plume in free-flight [51]. For each odor pulse, a baseline wingbeat frequency was determined by averaging the frequency across a 1 s time window preceding the odor delivery and then subtracting this value from the frequency values following the pulse. Trials were discarded in which mosquitoes had frequency fluctuations greater than 5 Hz in this 1 s window or frequency changes greater than 30 Hz that did not begin within the four seconds following the octenol stimulation (as they presumably were not in response to the stimulus). On average, 5 out of the 10 trials were discarded per mosquito.

Interrogation of dopamine pathways in the mosquito brain—To evaluate the impact of dopamine on mosquito olfactory learning, we used three different approaches: 1) dopamine receptor antagonist injections; 2) knockdown of *Dop1* using RNA interference and 3) modification of *Dop1* using the CRISPR/Cas9 method.

Pharmacological suppression of learning using dopamine receptor antagonists: To evaluate the impact of dopamine on mosquito olfactory learning, we used dopamine receptor antagonists that targeted different dopamine receptor types: Bulbocapnine (Dop1 receptor antagonist - D046–25MG), SCH-23390 (Dop1 receptor antagonist - D054–5MG), Fluphenazine (Dop1 and Dop2 receptor antagonist - F4765–1G) and Flupentixol (Dop1 and Dop2 receptor antagonist - Y0000054) (Sigma Aldrich, St. Louis, MO, USA). All antagonists were dissolved in saline and used at a concentration of 10^{-6} M.

Six-day-old mated adult females, that had never been blood fed, were placed on ice to immobilize them before injection. Borosilicate capillaries (Sutter instrument, Novato, CA, USA - #BF100–78-10,) were pulled to form micropipettes using a P-2000 laser puller (Sutter Instrument Co., Novato, CA, USA) and filled with either the dopamine receptor antagonist or saline as an injection control. A micropipette was then connected to a Picospritzer (Picospritzer III, Parker Hannifin, NJ, USA). In order to calibrate injection

volumes between micropipettes, the diameter of a drop of solution in mineral oil over a micrometre was measured before each injection session. Two drops of a diameter of 500 ± 10 μm (65 nL) of the solution containing the receptor antagonist or saline (control) were injected on the side of the thorax. After injection, mosquitoes were able to recover for 30 minutes before being trained. A group of naive mosquitoes (injected but not trained) was used in parallel to determine whether antagonist or saline injections influenced innate responses to carbon dioxide. The injected females were tested 24 h post-treatment in the olfactometer as described above.

In a separate experiment, we fed mosquitoes with dopamine receptor antagonists, trained them and tested their behavioral response to octenol as previously described. We first used bulbocapnine (10^{-6} M) and then tested a combination of three drugs (bulbocapnine, SCH-23390 and fluphenazine) at either 10^{-6} M or 10^{-3} M. Ten microliters of the drug solution was diluted in 10 mL sucrose to which we added a few droplets of green food colorant in order to check for sucrose intake by female mosquitoes through their abdomen by transparency. One microliter of 1 mM DMSO (Sigma, Saint Louis, MO, USA) was added to the solution to help preventing any chemical degradation. Cotton balls imbibed with this solution were placed above containers with female mosquitoes and were given 24 hr to feed before training. Mosquitoes fed with bulbocapnine alone were still able to learn (PI = −0.46; $p < 0.05$, binomial test), but those fed with the drug combination did not show learning (PI = −0.14 and 0, at 10^{-6} and 10^{-3} M respectively; $p > 0.05$, binomial test; for both concentrations).

Knockdown of Dop1 using RNA interference: Double-stranded RNA (dsRNA) of *Dop1* and *Drosophila* nautilus (non-targeting control, #M68897) genes were synthesized by *in vitro* transcription using the MEGAscript RNAi kit (ThermoFisher Scientific, Waltham, MA, USA - AM1626) following the manufacturer's recommendations. The integrity of the products was assessed by agarose gel electrophoresis (0.8%) to ensure that the fragments were of the proper size and not degraded. After synthesis, the dsRNA was precipitated using sodium acetate and ethanol and resuspended in nuclease free water (ThermoFisher Scientific, Waltham, MA, USA). The concentration and integrity of the dsRNA were determined by spectrometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA) and electrophoresis. The dsRNA was then kept at −80°C until the injections were performed. Before the injection, the dsRNA was thawed and diluted in water to the desired concentration. Injections were performed using a pulled borosilicate pipette [*sensu* 26]. The pupae were briefly anesthetized on ice before injection and maintained on a cold aluminum block during the whole injection process. Each pupa received a microinjection of 66 nL dsRNA diluted in water which represents a concentration of 100 ng of dsRNA. The injected pupae were then placed in a plastic container of water (BioQuip, Rancho Dominguez, CA, USA - 1425DG) to recover until emergence. The injection of 100 ng of *Dop1* dsRNA led to a survival of 50% of the pupae while 95% of the pupae emerged after being injected with the non-targeting control dsRNA (Figure S4). The level of knockdown was assessed with RT-qPCR and western blots. We observed a decrease in the mRNA for *Dop1* in 60% of the injected mosquitoes and the knockdown was of about 30% (Figure S4), similar to what was found in other studies [52].

After the behavioral experiments, the mosquitoes' heads were excised on a cold aluminum block and flash frozen using liquid nitrogen to preserve the RNA integrity. The total RNA of the mosquitoes' heads was extracted using the RNAqueous Micro Kit (ThermoFisher Scientific, Waltham, MA, USA - AM1931) following the manufacturer's instructions. The total amount of RNA per sample was measured by spectrometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA) and a 1% agarose gel was ran to assess for RNA quality and integrity as well as for possible gDNA contamination. 80 ng of RNA were then used for the reverse transcription reaction. We used the SuperScript III First-Strand Synthesis Supermix for qRT-PCR kit (ThermoFisher - Cat No. 11752–250) to produce cDNA following the manufacturer's instructions. The qPCRs were prepared using the SYBR Select Master Mix (ThermoFisher Scientific, Waltham, MA, USA - Cat. No. 4472903) and were performed on an ABI StepOnePlus Real Time PCR machine (ThermoFisher Scientific, Waltham, MA, USA) using specific primers (*Dop1-F*: 5′-AACGATTTACTGGG CTACTGG-3′; *Dop1-R*: 5′-CCTTGATGT GGATGTACCGG-3′; *RPS7-F*: 5′-TCAGTGTACAA GAAGCTGACCGGA-3′ ; *RPS7-R*: 5′-TTCCGCGCGCGCTCACTTATTAGATT-3′ - Integrated DNA Technologies, Coralville, IA, USA). *RPS7* was used as a housekeeping gene. Triplicates of each reaction were done for a single PCR plate (TempPlate Semi-Skirt 0.1mL PCR plates, USA Scientific, Inc., Ocala, FL, USA - Cat. No. 1402–9100; MicroAmp Clear Adhesive Films, ThermoFisher Scientific, Waltham, MA, USA - Cat. No. 4306311) and the reactions were performed twice. The expression of *Dop1* relative to the endogenous control was determined using the quantitative ΔΔCt method from the StepOne Software v2.3.

CRISPR/Cas9: The short guide RNAs (sgRNAs) used for CRISPR/Cas9 were designed to target the first exon of the conserved *Dop1* (AAEL003920)(Figure S4; Table S1). To define the sgRNA genomic target sites several factors were considered. First, *Ae. aegypti* transcriptional databases were utilized to confirm RNA expression of putative target regions [53]. We then performed blast searches to hunt for conservation and discovered an important conserved receptor domain termed 7tm-4 superfamily domain (pfam13853) that we decided to target [54]. To minimize potential off-target effects, we confirmed specificity of our sgRNAs using publicly available bioinformatic tools [55] and selected the most specific sgRNAs within our target region.

Immunohistochemistry—The polyclonal antiserum against tyrosine hydroxylase (ImmunoStar, Hudson, WI, USA - Cat. no. 22941) was used at a concentration of 1:50 and monoclonal antisera against synapsin I (Sigma-Aldrich, St. Louis, MO, USA - Cat. No. WH0006853M7) were used at a concentration of 1:100 for immunohistochemistry. The antibody against the D1-like dopamine receptor, Dop1 was custom made by 21st Century Biochemicals against a synthetic peptide corresponding to amino acids 138–154 of the *Ae. aegypti* protein, affinity purified, and used at a concentration of 1:100 for immunohistochemistry. This antibody was also used at a concentration of 1:1000 for western blot assays and recognizes a band with a mass of ~72 kDa. Deglycosylation of protein samples with glycerol-free PNGase F (New England BioLabs, Ipswich, MA, USA - Cat. No. P0705) resulted in detection of a band at the expected molecular weight of ~41 kDa. To further test specificity of this antibody, sections of *Ae. aegypti* brain tissue were divided into

two wells and incubated with either antibody preadsorbed with 100 μM of the Dop1 peptide (used to produce the antibody in rabbit) or with antibody alone and then processed for immunohistochemistry, as described below. Both wells were additionally incubated with antisera against synapsin I as a positive control for staining. Preadsorption with peptide from Dop1 abolished *Dop1*-like immunoreactivity, while synapsin-like immunoreactivity remained (Figures 5).

Animals were immobilized by refrigeration at 4°C and heads were removed into cold (4°C) fixative containing 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS, Sigma-Aldrich, St. Louis, MO, USA -Cat. No. P4417). Heads were fixed for 1 h and then brains were dissected free in PBS containing 4% Triton X-100 (PBS-TX; Sigma-Aldrich, St. Louis, MO, USA - Cat. No. X100). Brains were incubated overnight at 4°C in 4% PBS-TX. The next day, brains were washed three times over 10 minutes each in 0.5% PBS-TX and then embedded in agarose. The embedded tissue was cut into 60 μm serial sections using a Vibratome. Sections were placed in a well plate for further processing. Sections were washed in PBS containing 0.5% PBS-TX six times over 20 minutes. Then 50 μL normal serum was added to each well containing 1,000 μL PBS-TX. After 1 hour, primary antibody was added to each well and the well plate was left on a shaker overnight at room temperature. The next day, sections were washed six times over 3 h in PBS-TX. Then 1,000 μL aliquots of PBS-TX were placed in tubes with 2.5 μL of secondary Alexa Fluor 488 or Alexa Fluor 546-conjugated IgGs (ThermoFisher Scientific, Waltham, MA, USA) and centrifuged at 13,000 rpm for 15 minutes. A 900 μL aliquot of this solution was added to each well. The well plate was left on a shaker to gently agitate the sections overnight at room temperature. Tissue sections were then washed in PBS six times over 3 h, embedded on glass slides in Vectashield (Vector Laboratories, Burlingame, CA, USA - Cat. No. H-1000). Brain tissue was imaged using a Leica SP5 laser scanning confocal microscope. Maximum intensity projections were processed using ImageJ (National Institutes of Health) and selected images were assembled using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA).

For western blots, brain tissue for *Ae. aegypti* was homogenized in lithium dodecyl sulfate (LDS) sample buffer with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The Novex electrophoresis system was used for protein separation [56].

Electrophysiology mosquito preparation—A total of 74 units recorded from 22 individuals, were exposed to a total of 418 odor stimulations in the electrophysiology experiments. Mosquitoes were immobilized on ice and mounted on a custom-designed holder (Figure 4B) using UV-cured glue (Bondic, Non Toxic Liquid Plastic Welder, BondicUSA, Fairfield NJ, USA). Each mosquito was tethered to the holder by the head capsule and the anterior-dorsal tip of the thorax, allowing steady electrophysiological recordings while the mosquito beats its wings in a fictive form of flight (Figure 4). All six legs were removed to prolong the flight bouts. A hole was cut in the cuticle of the head capsule to expose the antennal lobes, and then trachea and muscles 8 and 11 were removed. The brain was superfused continuously with temperature-controlled physiological saline solution (20oC) using a bipolar temperature controller and an in-line heater/cooler (CL-100 and SC-20, Warner Instruments).

The extracellular saline was made based on the Beyenbach and Masia recipe [57] and contained 150.0 mM NaCl, 25.0 mM N-2-hydroxyethyl-piperazine-N'−2-ethanesulfonic acid (HEPES), 5.0 mM sucrose, 3.4 mM KCl, 1.8 mM $NaHCO_3$, 1.7 mM $CaCl_2$, and 1.0 mM $MgCl_2$. The pH was adjusted to 7 with 1 M NaOH. Immediately before the experiment, dopamine was dissolved to 1 μM in extracellular saline (this is equivalent to concentrations used in other studies of olfactory learning [58]. A drip system comprising two 100 mL reservoirs – one containing dopamine, and the other saline – converged on the two-channel temperature controller to facilitate rapid switching from normal physiological saline solution to dopamine and back. Dopamine was superfused directly into the holder near to the opening of the head capsule and recorded neuropil. The odor-evoked responses were first recorded under normal physiological saline solution and then repeated under dopamine diluted in normal saline solution, and finally the normal saline wash.

Coupled extracellular and behavioral recordings, spike sorting, and analysis —The tethered mosquito was placed on a Nikon FN-1 microscope (Eclipse FN1, Nikon Instruments Inc., Melville, NY, USA) under 20X objective (UMPlanFI, Olympus, Japan) to allow precise positioning of the recording electrode in one of the antennal lobes. Electrodes were pulled from quartz glass capillaries using a Sutter P-2000 laser puller and filled with 0.1 M LiCl. The electrode was positioned under visual control using the FN1 microscope and advanced slowly through the antennal lobe using a micromanipulator (PM10 - World Precision Instruments) until spikes were apparent in the recording channel. To determine the position of the recordings, the tip of each electrode was dipped into a solution of 2% Texas Red (ThermoFisher Scientific, Waltham, MA, USA) dissolved in 0.5 M potassium chloride solution before placement in the brain. After recording experiments, brains were imaged and z stacks were taken at 1 μm steps using a two-photon microscope (Prairie Technologies Inc.).

Electrophysiological signals were amplified 10,000X and filtered (typically 0.1–5 kHz) (A-M Systems Model 1800, Sequim, WA, USA), recorded and digitized at 10 kHz using WinEDR software (Strathclyde Electrophysiology Software, Glasgow, UK) and a BNC-2090A analog-to-digital board (National Instruments, Austin, TX, USA) on a personal computer. Spike data were extracted from the recorded signal and sorted using a clustering algorithm based on the method of principal components (PCs) (Offline Sorter; Plexon, Dallas, TX, USA). Only those clusters that were separated in three-dimensional space (PC1–PC3) after statistical verification (multivariate ANOVA: $p < 0.1$) were used for further analysis (2–6 units were isolated per preparation; $n = 22$ preparations from as many mosquitoes; Figure S5A). Each spike in each cluster was time-stamped, and these data were used to create raster plots and to calculate peri-stimulus time histograms (PSTHs), interspike interval histograms, and rate histograms.

To couple electrophysiological and behavioral responses, we used a set-up [59, 60] where an infrared camera (PointGrey Firefly MV FMVU-03MTC) was placed below the preparation. This set-up allowed an easy positioning of the recording electrodes, visualization of the flight responses, and stimulation of the preparation with olfactory stimuli. IR LEDs were used to illuminate the wings, abdomen and proboscis, and images were recorded at 60 frames/s. A Python-based open source software (Kinefly [61]) calculated the wingbeat

stroke amplitudes for each wing per frame. Because mosquito wing-beat frequencies are well above 400 Hz (and above the frame rate of the camera), and since the product of frequency and amplitude rises linearly with increasing flight force [49], wing stroke amplitude was used as a proxy for surge behavior in free flying insects. Wingstroke amplitude was timestamped and acquired simultaneously with electrophysiological recordings. Amplitude data were averaged across preparations and depicted as the absolute change in amplitude so that comparisons were possible across odors, including attractants, but also neutral and aversive compounds.

QUANTIFICATION AND STATISTICAL ANALYSES

Olfactometer behavioral data analysis—Binary data collected in the olfactometer were analyzed and all statistical tests were computed using R software (R Development Core Team [62]). Comparisons were performed by means of the Exact Binomial test ($\alpha = 0.05$). For each treatment, the choice of the mosquitoes in the olfactometer was either compared to a random distribution of 50% on each arm of the maze or to the distribution of the corresponding control when appropriate. For binary data, the standard errors (SE) were calculated as in [63]:

$$SEM = \left(\frac{p(1-p)}{n}\right)^{\frac{1}{2}}$$

For each experimental group, a preference index (PI) was computed in the following way: PI = [(number of females in the test arm – number of females in the control arm) / (number of females in the control arm + number of females in the test arm)]. A PI of +1 indicates that all the motivated mosquitoes chose the test arm, a PI of 0 indicates that 50% of insects chose the test arm and 50% the control arm, and a PI of −1 indicates that all insects chose the control arm of the olfactometer (adapted from [18]). Means of instantaneous flight speeds were analyzed in Excel and flight speed comparisons were made in R, by means of Student's t test $\alpha = 0.05$).

Flight arena behavioral data analysis—Flight arena data was analyzed by the mean response for each individual that was calculated from the saved trials and used as a replicate to calculate the mean response for each treatment group. This latter was calculated using the difference in frequency before (200 ms before the odor stimulation) and after the odor delivery (starting at 1 s and ending 3 s after the pulse). One-tailed Student's t tests for paired samples were used to test for differences from baseline and t tests for independent samples were used to test for differences between groups. All analyses were performed in R.

Electrophysiological data analysis—Analyses of electrophysiological data were performed with R (R Core Team [62]) and Neuroexplorer (Nex Technologies, Winston-Salem, NC, USA) using a bin width of 20 ms, unless noted otherwise. We quantified the control corrected response for every unit by calculating a response index (RI). RI values reflect the deviation from the mean response of all units across all odors in one ensemble, as $RI = (R_{odor} - R_m)/SD$, where R_{odor} is the number of spikes evoked by the test odor minus

the number evoked by the control stimulus, R_m is the mean response, and SD is the standard deviation across the data matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Aversive learning by mosquitoes suppresses responses to human hosts
- Mosquitoes can learn the association between mechanical shock and certain odorants
- CRISPR/Cas9 modification of the dopamine-1 receptor prevents learning
- Dopamine causes heterogeneous modulation of antennal lobe neurons

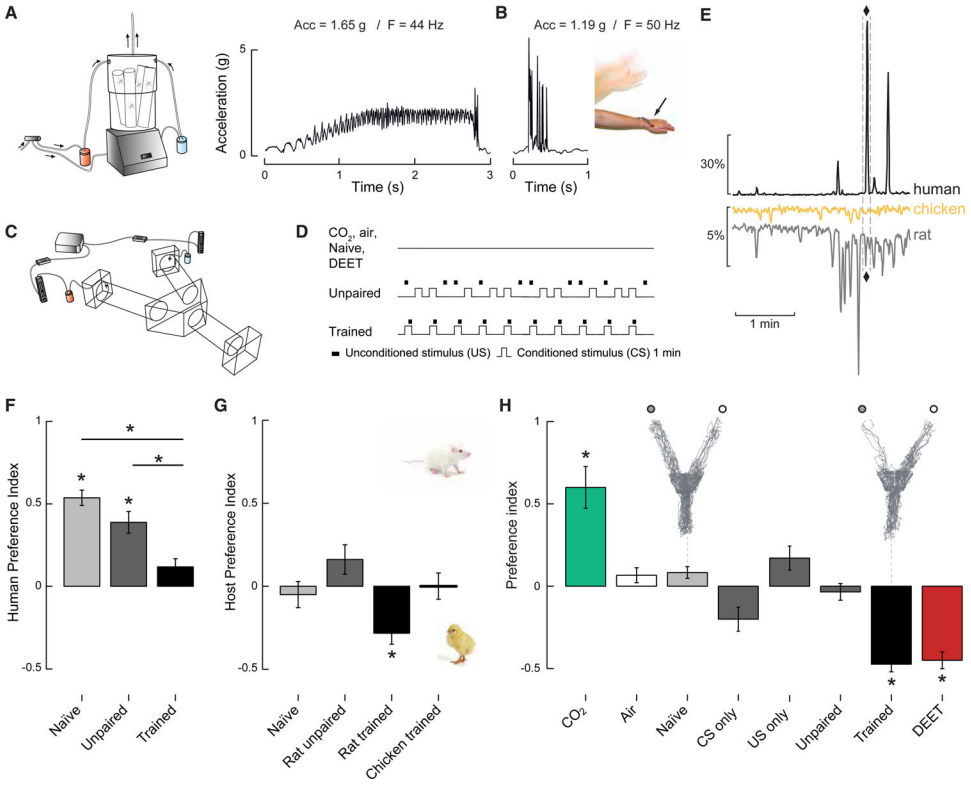

Figure 1. Mosquitoes Aversively Learn Host Odors and Single Odorants

(A and B) Left: Aversive training device: mosquitoes are enclosed in individual tubes and stimulated with a mechanical shock from the vortexer and odor (or solvent control) delivered from a scintillation vial. Right: Accelerometer recordings from inside the training device and (B) from an individual's arm allowed us to scale the appropriate forces experienced by a mosquito exposed to host defensive behavior.

(C) Y-maze olfactometer used in behavioral experiments. Mosquitoes are released in the starting chamber, fly upwind, and then have the choice between two arms, each delivering a different odor stimulus.

(D) Sequences of event delivery (i.e., shock [unconditioned stimulus, US], odor [conditioned stimulus, CS], and inter-trial interval, ITI) during the experiments.

(E) Representative GCMS chromatograms of the different host species: human (black, top), chicken (middle, yellow), and rat (gray, bottom). The octenol peak is indicated by the diamond sign.

(F) Mosquito human host preference represented as a preference index computed from the distribution of insects in the olfactometer.

(G) Mosquito host preference between the rat and the chicken scents, represented as a preference index.

(H) Mosquito preference for a CO2-positive control (green bar), a DEET-negative control (red bar), and octenol (all other bars). Above the naive and trained groups, flight trajectories of individual mosquitoes in response to octenol (gray circle) and a control (white circle).

(F–H) Each bar is the mean ± SE from 15–71 mosquitoes; asterisks denote responses that are significantly different from random or from their respective control (binomial test: $p < 0.05$). See also Figures S1 and S2.

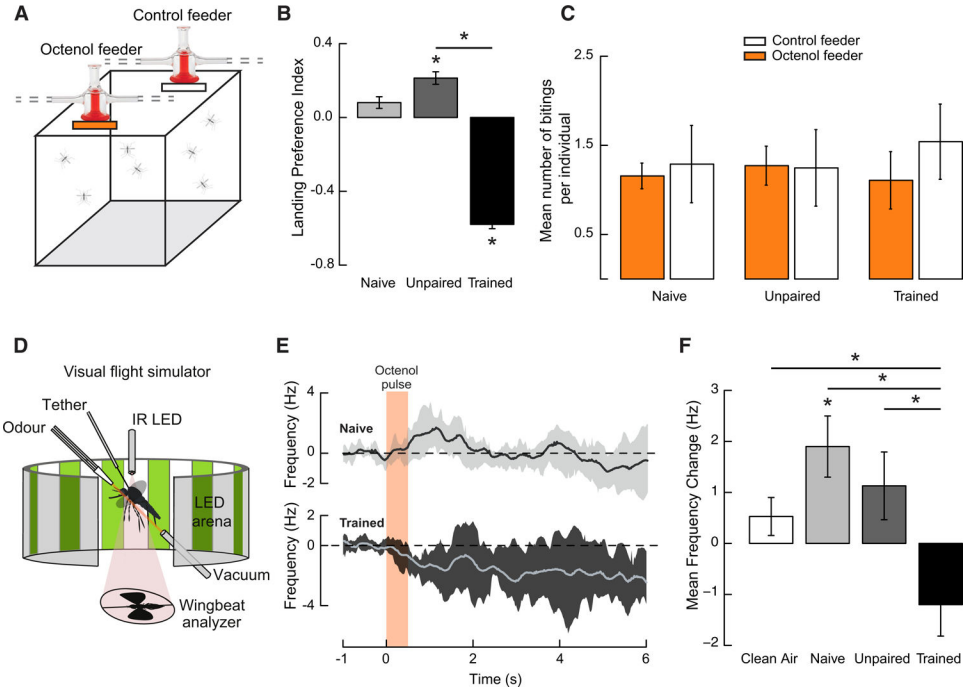

Figure 2. Aversive Learning Modifies Odor-Guided Feeding Preferences and Flight Responses

(A) Experimental setup for testing mosquitoes' odor-guided feeding and biting behavior. Each feeder provided heparinized bovine blood and were scented with either octenol or water.

(B) Mosquito landing preference index for either one of the two artificial feeders, for the naive, unpaired, and trained groups. Bars are the mean ± SE, with each bar representing 9–10 groups of 17 responsive female mosquitoes; asterisks denote distributions that are significantly different from random (binomial test: $p < 0.05$).

(C) Average number of biting per individual on each of the two feeders for the naive, unpaired, and trained groups.

(D) Visual flight simulator [30, 31] used to record wing kinematics from a tethered mosquito.

(E) Stimulus-trigger-averaged changes in wingbeat frequency (solid line) in response to a pulse of octenol (light orange bar) for the naive and the trained groups. Solid lines are the mean (16–23 mosquitoes) for the different treatment groups; shaded areas represent the mean ± the first quartiles.

(F) Frequency response to a pulse of air (white bar) or octenol for the naive (light gray bar), unpaired (dark gray bar), and trained (black bar) groups. Each bar is the mean ± SE of 16–23 responsive female mosquitoes; asterisks denote significant responses compared to zero when located above bars or between groups when located above horizontal lines ($p < 0.05$, Student's t test, $t > 1.57$). See also Figure S3.

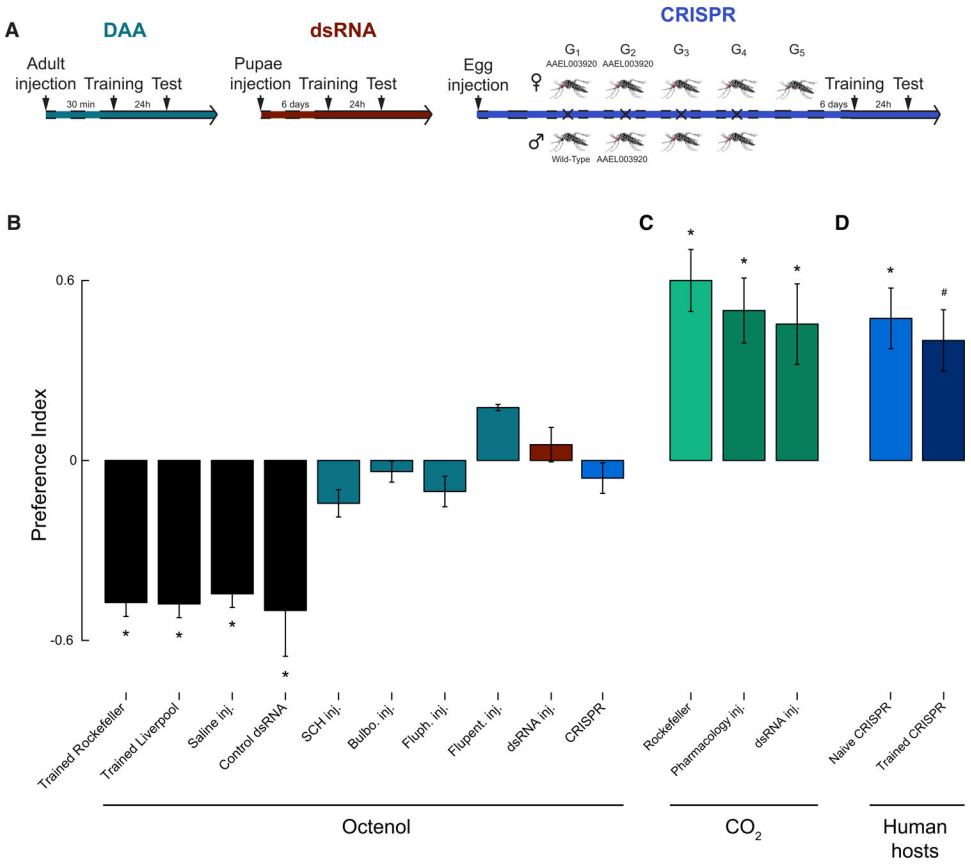

Figure 3. Dopamine Is Involved in Mosquito Learning

(A) Left: Dopamine receptor antagonists (DAA) (SCH-23390, bulbocapnine, flupentixol, and fluphenazine) were injected in the thorax of 6-day-old female mosquitoes that were trained 30 min post injection and tested 24 hr later. Center: *Dop1* and control dsRNA were injected in 1-day-old pupae, and after 6 days post emergence, mosquitoes were trained and tested. Right: CRISPR/Cas9 constructs were injected in embryos. Mutants were backcrossed, screened and selected by sequencing for five to eight generations before being trained at 6 days old.

(B–D) Mosquito choice in the olfactometer represented as a preference index. Trained mosquitoes from the Rockefeller, Liverpool strain, saline-injected, and dsRNA-injected Rockefeller lines were not significantly different in their learning performances ($p > 0.05$, binomial test; black bars). By contrast, mosquitoes injected with dopamine receptor antagonists (blue-green bars), dsRNA-injected (red bar), and CRISPR mosquitoes (blue bar) showed no learning. Mosquitoes injected with dopamine receptor antagonists (SCH-23398, 10^{-6} M) or dsRNA, as well as CRISPR mosquitoes, were still responding to positive controls such as CO_2 (C) or host odors (D). When human scents were used during training, CRISPR mosquitoes showed no learning ($p = 0.79$, binomial test). Each bar (mean ± SE) representing 11–29 responsive female mosquitoes; asterisks indicate distributions that are significantly different from random ($p < 0.05$, binomial test); # indicates $p < 0.06$ when the response of the trained CRISPR was compared to chance. See also Figures S2 and S4.

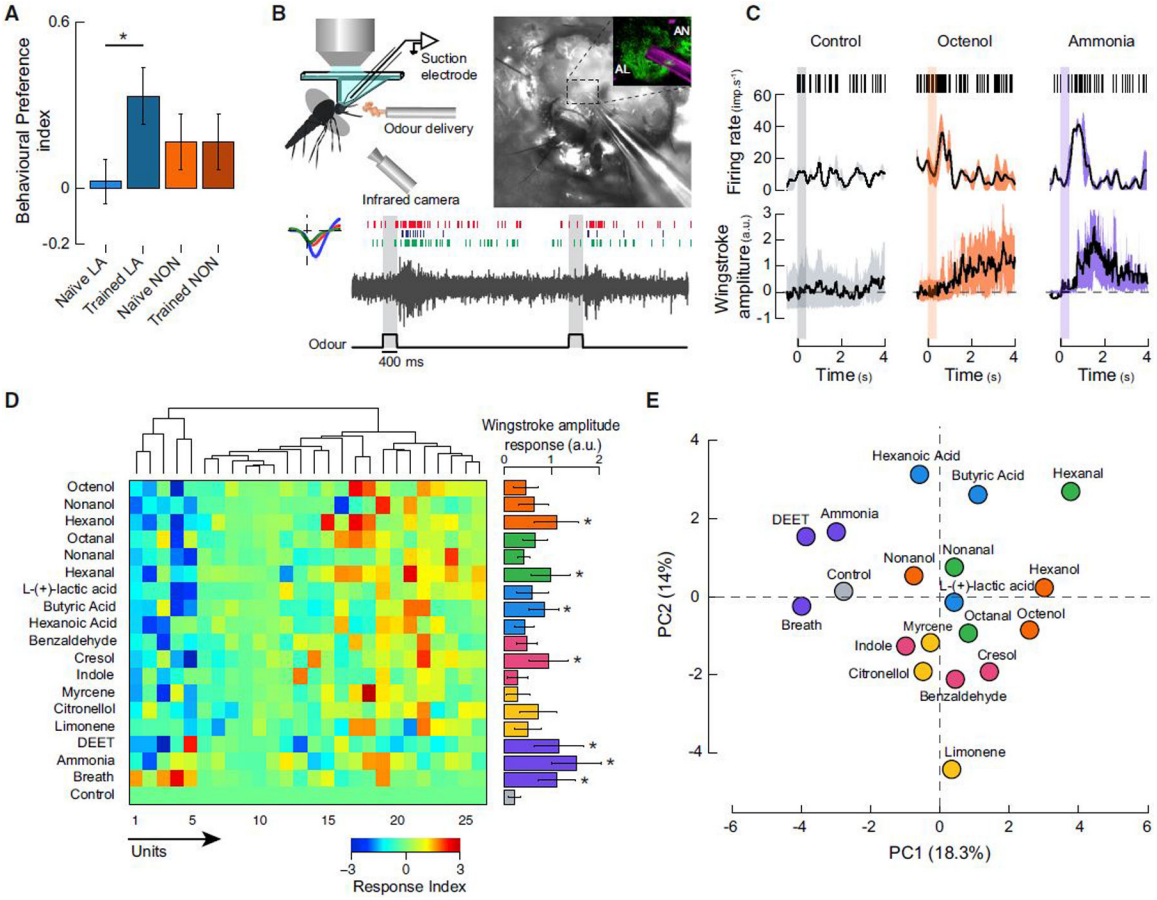

Figure 4. Odor Stimuli Are Learned and Represented Differentially in the Mosquito Brain

(A) Mosquito preference index (PI) for L-(+)-lactic acid (LA, blue bars) and 1-nonanol (NON, orange bars), tested in the olfactometer. Each bar is the mean ± SE from 21–39 responsive female mosquitoes; asterisks denote $p < 0.05$ (binomial test).

(B) Top left: Electrophysiological preparation for simultaneous flight behavior and suction electrode recording from the mosquito antennal lobe (AL), which receives olfactory input from the antenna and maxillary palps. Top right: Picture of the suction electrode inserted in the right AL of a mosquito. Inset: Representative electrode position (5 mm tip diameter, purple) relative to the AL (green) and antennal nerve (AN). Bottom: Representative raw recording and raster plot showing the responses of three units after the delivery of 400 ms pulses of octenol (gray bar).

(C) Top: Raster plots and peri-event histograms of the mean (± variance) responses of an isolated unit from the suction electrode recordings. Bottom: Stimulus trigger-averaged responses in wingstroke amplitude (± first quartiles) to olfactory stimulation. Vertical shaded bars represent the odor stimulus: clean air, gray; octenol, orange; ammonia, purple.

(D) Left: Neural ensemble response to the odor panel (rows 1–19), plotted as a color-coded response matrix across neural units (columns) (n = 8 preparations). Right: Normalized absolute change in mean wingstroke amplitude (a.u. ± SE) in response to each odor of the panel and color coded according to the chemical class of the odorant (see [E]). Asterisks

denote responses that are significantly different from the control (Student's t test: n = 10–16; $t > 2.38$; $p < 0.05$).

(E) Principal components analysis of the ensemble responses. a–e: color fills are indicative of the chemical class of the odorant (orange, alcohols; green, aldehydes; blue, carboxylic acids; pink, aromatic and phenolic compounds; yellow, monoterpenes; purple, other compounds; gray, mineral oil control). See also Figure S5.

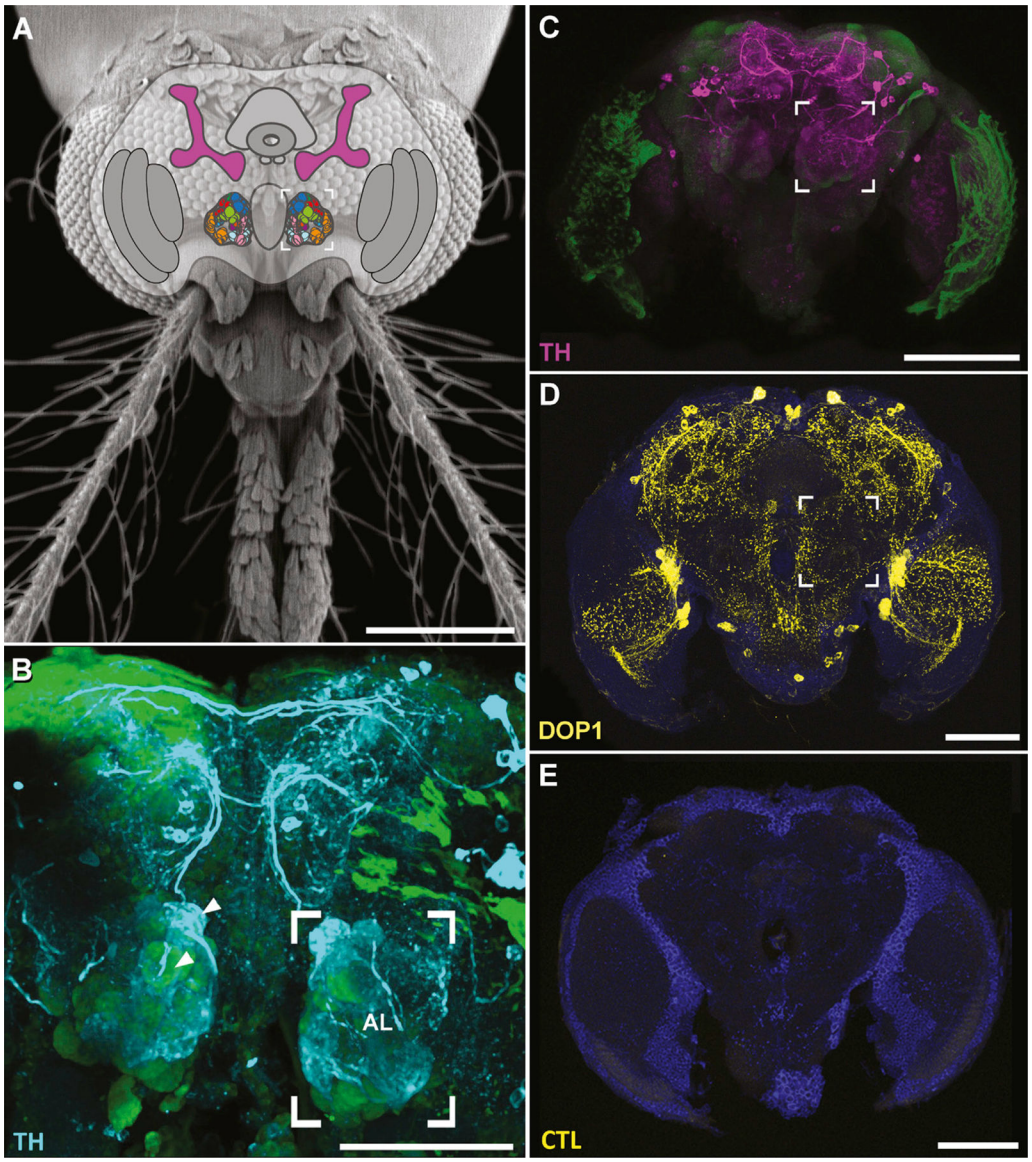

Figure 5. Tyrosine Hydroxylase and Dopamine Receptor Immunoreactivity

(A) Schematic of the *Ae. aegypti* brain superimposed on a scanned electron microscope image [34]. Highlighted regions include the AL (multicolored to represent individual glomeruli that receive input from olfactory receptor neurons) and the mushroom bodies (MB), implicated in learning and memory. The open box around the AL is used to indicate the corresponding location in panels (B)–(D). CX, central complex; OL, optic lobes. Scale bar, 500 μm.

(B) Confocal micrograph of a whole *Ae. aegypti* brain stained with antibodies against tyrosine hydroxylase (magenta) shows immunoreactivity concentrated in the lateral protocerebrum and AL. Background fluorescence in green. Scale bar, 100 μm.

(C) Confocal micrograph of a *Ae. aegypti* brain stained with antibodies against tyrosine hydroxylase (cyan) shows heterogeneous innervation of dopaminergic neurons across

antennal lobe glomeruli. Open box bounds the right AL. Background fluorescence in green. Scale bar, 60 μm.

(D) A 60 μm section of *Ae. aegypti* brain stained with antibodies against the mosquito dopamine-1 receptor-1 Dop1 (yellow) shows these receptors enriched in the lateral protocerebrum around the MB as well as localized around the AL. Background fluorescence in blue (synapsin). Scale bar, 100 μm.

(E) A representative section from the same brain that was preadsorbed with a synthetic peptide corresponding to amino acids 138–154 of Dop1 shows synapsin immunoreactivity (blue), but Dop1 immunoreactivity (yellow) is abolished. Scale bar, 100 mm. See also Figures S4 and S6.

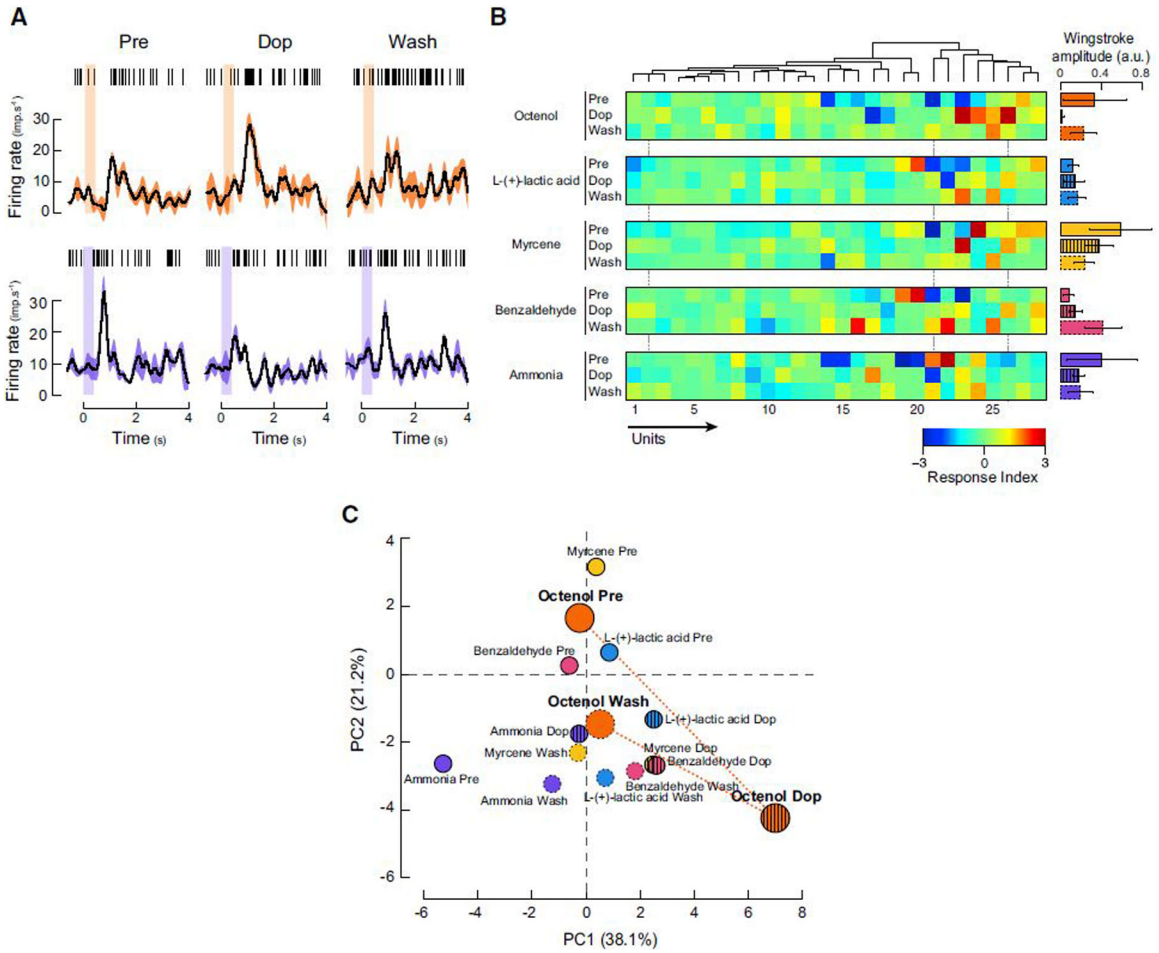

Figure 6. Dopamine Selectively Modulates Antennal Lobe Neurons

(A) Top: Raster plots and peri-event histograms of the mean (± variance) responses of an isolated unit from the electrode recordings. Bottom: Stimulus trigger-averaged responses in wingstroke amplitude (± first quartiles) to olfactory stimulation. Vertical shaded bars represent the odor stimulus: octenol, orange; ammonia, purple. Each column corresponds to the responses before (Pre), during (Dop), and after (Wash) dopamine application.

(B) Left: Neural ensemble response to a subset of five odorants (octenol, lactic acid, myrcene, benzaldehyde, ammonia) before (Pre), during (Dop), and after (Wash) dopamine application. Responses are plotted as a color-coded response matrix across the neural units (columns). Vertical dashed lines indicate examples of units for which the response either does not change (unit 2), decreases (unit 21 for ammonia), or increases (unit 26 for all odors except ammonia) during dopamine application. Right: Normalized mean wingstroke amplitude change (a.u.) in response to each odor of the panel, before (open bars), during (hatched bars), and after (dashed bars) dopamine application. Bars are the mean ± SE.

(C) Principal components analysis of the ensemble responses. Borders and color fills are indicative of the odorant (orange, octenol; blue, lactic acids; pink, benzaldehyde; yellow, myrcene; purple: ammonia) and of the treatment (solid line, Pre; hatchings, Dop; dashed lines, Wash). See also Figures S5 and S6.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-tyrosine hydroxylase	ImmunoStar	Cat#22941; RRID: AB_572268
Mouse monoclonal anti-synapsin I	Sigma-Aldrich	Cat#WH0006853M7; RRID: AB_1843825
Rabbit polyclonal anti-Dop1	This paper	Cat# Riffell_DOP1, RRID:AB_2716715
Biological Samples		
Heparinized bovine blood	Lampire Biological Laboratories	Bovine blood
Chemicals, Peptides, and Recombinant Proteins		
Bulbocapnine	Sigma-Aldrich	D046–25MG
SCH-23390	Sigma-Aldrich	D054–5MG
Fluphenazine	Sigma-Aldrich	F4765–1G
Flupentixol	Sigma-Aldrich	Y0000054
All odorants from electrophysiological experiments	Sigma-Aldrich	N/A
Critical Commercial Assays		
MEGAscript RNAi Kit	Thermofisher Scientific	AM1626
DNeasy Blood and Tissue Kit	QIAGEN	69504
Qiaquick PCR Purification Kit	QIAGEN	28104
RNAqueous Micro Kit	Thermofisher Scientific	AM1931
SYBR Select Master Mix	Thermofisher Scientific	4472903
SuperScript III First-Strand Synthesis Supermix for qRT-PCR kit	Thermofisher Scientific	11752–250
PNGase F	New England BioLabs	P0705
Experimental Models: Organisms/Strains		
Aedes aegypti Rockefeller strain	BEI	ROCK
Aedes aegypti Liverpool strain	BEI	LVP- IB12
Aedes aegypti CRISPR mutant Liverpool strain	This study	Dop1 mutant
Rattus norvegicus Sprague Dawley strain	Taconic Bioscience Inc.	SD

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Gallus gallus White Leghorn strain	Featherland Farms, Coburg, OR	WL
Homo sapiens	Seattle, WA, USA	N/A
Oligonucleotides		
P1 = TGCAGGTGTTTTTCTATCGATTGTGAT	This study	N/A
P2 = ACATGACATCGAACGCCACCC	This study	N/A
P3 = GAAATTAATACGACTCACTATAGGACACCGAGCGGAGTCTGCGGGTTTTAGAGCTAGAAATAGC	This study	N/A
P4 = GAAATTAATACGACTCACTATAGGTGCCATCGCCGATCTGTTCGGTTTTAGAGCTAGAAATAGC	This study	N/A
P5 = AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	This study	N/A
Dop1-R: 5′-TAATACGACTCACTATAGGGATCAGATGGCGGATACCT GTT-3′	Integrated DNA Technologies	N/A
Dop1-F: 5′-TAATACGACTCACTATAGGGAACTCTACTGCTACGCCCAGA-3′	Integrated DNA Technologies	N/A
Dop1-F: 5′-AACGATTTACTGGG CTACTGG-3′	Integrated DNA Technologies	N/A
Dop1-R: 5′-CCTTGATGTGGATGTACCGG-3′	Integrated DNA Technologies	N/A
RPS7-F: 5′-TCAGTGTACAA GAAGCTGACCGGA-3′	Integrated DNA Technologies	N/A
RPS7-F: 5′-TCAGTGTACAA GAAGCTGACCGGA-3′	Integrated DNA Technologies	N/A
Recombinant DNA		
Cas9 protein	PNA-bio	CP01
Software and Algorithms		
Kinefly	Custom	https://github.com/ssafarik/Kinefly
R	R Development Core Team	N/A
MATLAB	The MathWorks, Inc.,	MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States
ImageJ	NIH	https://imagej.nih.gov/ij/
WinEDR	Strathclyde Electrophysiology Software	http://spider.science.strath.ac.uk/sipbs/software_ses.htm
StepOne Software v2.3	Thermofisher Scientific	N/A
ChemStation software	Agilent Technologies	N/A
Neuroexplorer	Nex Technologies	http://www.neuroexplorer.com/
Adobe Photoshop CS4	Adobe Systems	N/A
Other		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Wingbeat Analyzer	JFI Electronics / University of Chicago	N/A
Mosquito Electrophysiology Holder	This study	J. Riffell