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Neuromodulation and differential learning across mosquito species

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Mosquitoes can change their feeding behaviours based on past experiences, such as shifting from biting animals to biting humans or avoiding defensive hosts (Wolff & Riffell 2018 *J. Exp. Biol.* **221**, jeb157131. (doi:10.1242/jeb.157131)). Dopamine is a critical neuromodulator for insects, allowing flexibility in their feeding preferences, but its role in the primary olfactory centre, the antennal lobe (AL), remains unclear (Vinauger *et al*. 2018 *Curr. Biol.* **28**, 333–344.e8. (doi:10.1016/j.cub.2017.12.015)). It is also unknown whether mosquitoes can learn some odours and not others, or whether different species learn the same odour cues. We assayed aversive olfactory learning in four mosquito species with different host preferences, and found that they differentially learn odours salient to their preferred host. Mosquitoes that prefer humans learned odours found in mammalian skin, but not a flower odour, and a nectar-feeding species only learned a floral odour. Comparing the brains of these four species revealed significantly different innervation patterns in the AL by dopaminergic neurons. Calcium imaging in the *Aedes aegypti* AL and three-dimensional image analyses of dopaminergic innervation show that glomeruli tuned to learnable odours have significantly higher dopaminergic innervation. Changes in dopamine expression in the insect AL may be an evolutionary mechanism to adapt olfactory learning circuitry without changing brain structure and confer to mosquitoes an ability to adapt to new hosts.

1. Introduction

Over 3500 species of mosquitoes inhabit every continent except Antarctica and exhibit diverse food preferences, including feeding from flowers and a variety of blood hosts, such as humans, frogs and birds [1,2]. Although each mosquito species may possess a suite of genes associated with the sensory detection of their preferred blood hosts, there is nonetheless a high degree of behavioural flexibility even for those mosquitoes that specialize on a specific host. For example, mosquitoes may prefer certain subpopulations within a host species or may adapt to biting new hosts when their preferred is not available [3,4]. How mosquitoes choose their hosts and adapt their preferences remain open questions.

There are many factors contributing to host preference in mosquitoes, but one understudied factor is olfactory learning [5]. Learning from past experiences can hone preferences which may explain why mosquitoes target only a subset of the host population. Indeed, in disease vectors such as mosquitoes, 20% of the host population may be responsible for 80% of disease transmission [6]. Recent advances in *Aedes aegypti*, the yellow fever mosquito, revealed that this species selectively formed associative olfactory memories with odours associated with their hosts, but not other odours that lacked a strong connection to *Ae. aegypti's* natural history [7,8]. It remains unknown whether the same subset of odours learned by one species can also be learned by others or what might mediate differences in learning abilities. One possibility is dopamine, an important neuromodulator in the insect nervous system, necessary for encoding long-term olfactory memories [5,7]. In the *Ae. aegypti* brain, dopaminergic neurons selectively innervate specific subunits called glomeruli in the

mosquito antennal lobes (electronic supplementary material, figure S1A), the primary processing centres of olfactory information, possibly allowing enhanced neuromodulation and drive of glomeruli that encode specific learnable odourants. Nonetheless, it remains an open question whether other mosquito species show a similar aptitude for learning, or what is the relationship between the saliency of the learned odours and differential dopaminergic innervation. While higher-order centres called mushroom bodies are known to mediate olfactory learning, we hypothesize that this differential dopaminergic innervation across glomeruli plays a role in differential learning across odours and species-specific differences.

To address these knowledge gaps, in this article we test the hypothesis that mosquitoes learn odours that are salient in the context of their preferred hosts, and that differences in the dopaminergic innervation of olfactory loci in their brain reflect these differences. We compare the ability to learn host and plant odours across four species of mosquitoes with varying host preferences and examine their electrophysiological responses to different host odours. We also quantify the pattern of dopaminergic innervation in their brain. Using *Ae. aegypti* as a model, we then test the hypothesis that learned odours are encoded in subregions of the mosquito olfactory lobe with high levels of dopamine expression while unlearned odours are encoded in subregions with lower dopamine expression levels. Using this integrative approach, our results demonstrate that mosquitoes have species-specific learning abilities that may be evolutionarily tied to neuromodulatory circuits in their olfactory system.

2. Results

(a) Differential behaviours to hosts and host odourants

Mosquitoes share conserved brain architecture with other diptera, but they engage in a wide variety of host-feeding behaviours, from blood-feeding on animal or human hosts to consuming ant regurgitation or solely feeding on plant fluids [1,2,9–11]. We chose representative taxa from across the Culicidae: *Ae. aegypti*, *Anopheles stephensi*, *Toxorhynchites amboinensis and Culex quinquefasciatus* (figure 1*a*). As adults, females of these species prefer to feed on humans (*Ae. aegypti*, *An. stephensi*), birds (*Cx. quinquefasciatus*) and flowers (*Tx. amboinensis*) [2,10]. We first examined behaviours in these four species to assess their responses to host odours. Experiments were conducted in a Y-olfactometer that allowed testing of the mosquito preferences to different host odours (human, chicken and flower scents) compared to a mineral oil (no odour) control. Although each species responded in a distinct manner to the host scents, both *Ae. aegypti* and *An. stephensi* exhibited the strongest attraction to the human scent (binomial exact test, $p = 0.01$ and $p = 0.004$ for *Ae. aegypti* and *An. stephensi*, respectively). By contrast, *Cx. quinquefasciatus* exhibited the strongest attraction to the chicken scent (binomial exact test, $p = 0.04$), closely followed by an attraction to the human scent (binomial exact test, $p = 0.15$) (figure 1*a*), whereas *Tx. amboinensis* only showed attraction to the flower scent (binomial exact test, $p = 0.02$).

Hosts and flowers emit distinct odourants in their scents [3,5,7,12–14] (figure 1*b*). We therefore next examined antennal responses in these four species to assess whether or not they can detect representative host odours. Antennal responses to three odourants—1-octen-3-ol and hexanoic acid (both associated with vertebrate hosts), and linalool, a terpene alcohol produced by plants and a component of many floral scents [12–14] (figure 1*b*)—were recorded via electroantennogram (EAG), which is thought to measure the bulk sum of olfactory sensory neuron response on the antennae [15]. Although each species exhibited a significantly different pattern of responses to the odourants (figure 1*c*; ANOVA: $p < 0.05$), the three odourants consistently elicited robust responses across the different species. Together, these odourants allowed us to examine whether differential learning of odours is related to their host/feeding preferences.

(b) Differential learning behaviours across species

Could mosquitoes differentially learn the odourants that are distinctive features in the scents of their cognate host or plant odour? To test this, female mosquitoes from each species were trained in an aversive conditioning paradigm (electronic supplementary material, figure S1B, [7]) using mechanical shock as the unconditioned stimulus (US) and one of the three odourants used in the EAG experiments as the conditioned stimulus (CS). Although appetitive conditioning experiments conducted by our group also showed odourant-specific learning, in this study we used an aversive conditioning paradigm which allows use of the same unconditioned stimulus (US; a mechanical shock that mimics 'swatting') across species. Mosquitoes were divided into three treatment groups: 'naive' mosquitoes had no prior exposure to any test odours, 'trained' mosquitoes were exposed to 10 pairings of the US and CS, and 'unpaired' mosquitoes were handled in the same manner as trained mosquitoes, but odour and mechanical shock were presented randomly without temporal contingency. The unpaired treatment group was used as an additional control when mosquitoes exhibited learned responses to the US + CS pairing. After 24 h, mosquitoes were individually tested in a two-choice Y-maze olfactometer, and a preference index was calculated (electronic supplementary material, figure S1B). Similar to previous studies, if the association was learned, we expected the preference index of trained mosquitoes to be significantly lower than the naive and unpaired groups [7,16].

Training and testing the different species to the odourants and mechanical shock showed that trained *Ae. aegypti* significantly avoided both hexanoic acid and 1-octen-3-ol compared to naive or unpaired groups (figure 2*a*, electronic supplementary material, figure S2A, binomial exact test, $p = 0.007$ trained versus naive and $p = 0.011$ trained versus unpaired for 1-octen-3-ol and $p = 0.0005$ trained versus naive and $p = 0.014$ trained versus unpaired for hexanoic acid). However, naive and trained *Ae. aegypti* did not significantly differ in their preference for linalool (binomial exact test, $p = 0.832$ trained versus naive). These results suggest that *Ae. aegypti* learned to avoid the odours associated with their preferred mammalian hosts, but not a common flower odourant. Like *Ae. aegypti*, *An. stephensi* also learned to avoid 1-octen-3-ol (figure 2*b*, electronic supplementary material, figure S2A, binomial exact test, $p = 0.0006$ trained versus naive, $p < 0.0001$ trained versus unpaired). However, when hexanoic acid or linalool was used as the conditioned stimulus, trained *An. stephensi* exhibited lower preference indices compared to naive mosquitoes, but values were not significantly different (binomial exact test, $p = 0.241$ trained versus naive for hexanoic acid and $p = 0.223$ trained versus naive for linalool).

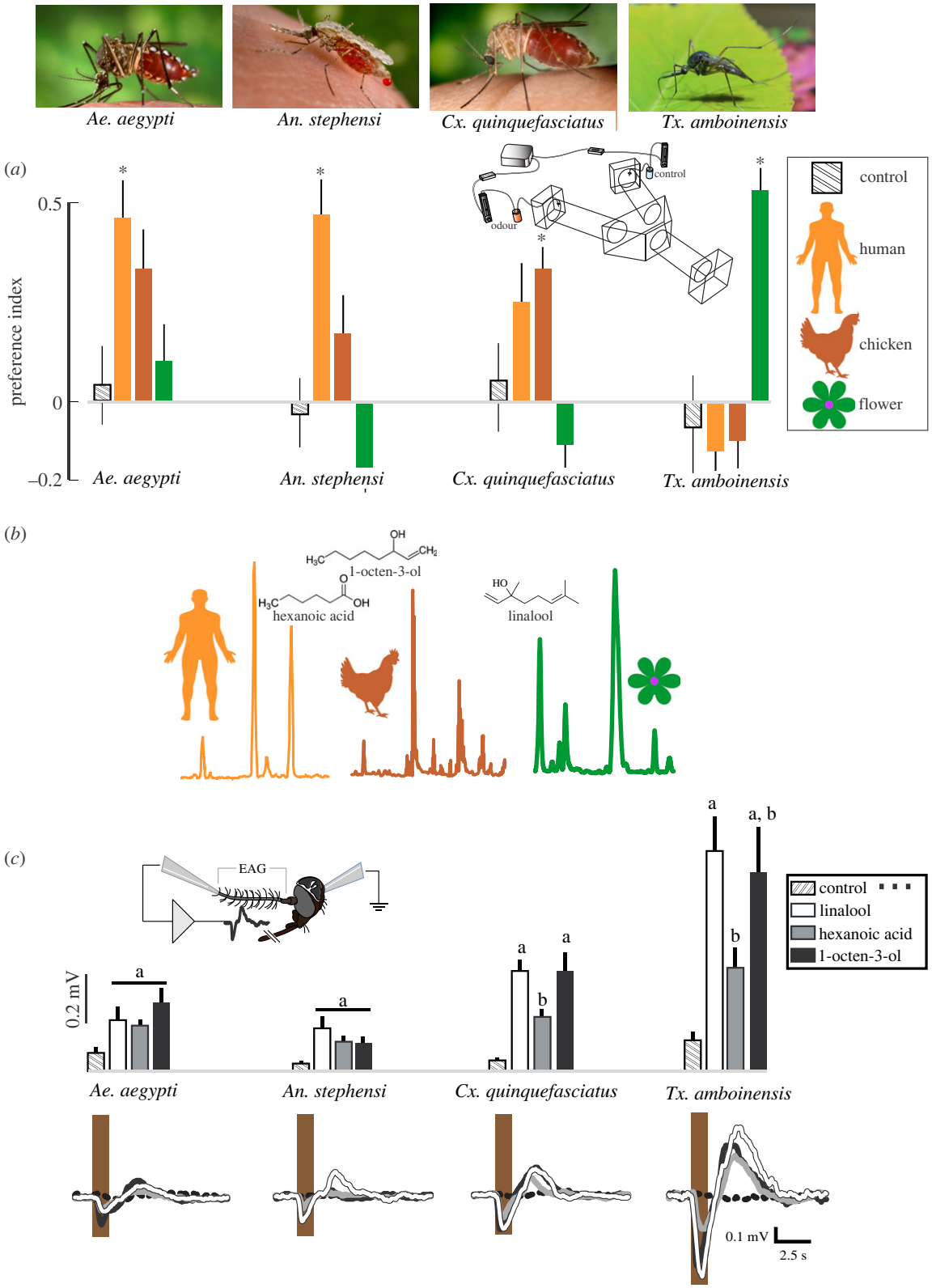

Figure 1. Behavioural and antennal responses to host and plant odours vary across species. (*a*) Four species were tested in the Y-olfactometer to host odours: *Ae. aegypti*, *An. stephensi*, *Cx. quinquefasciatus* and *Tx. amboinensis*. Histograms are the preference indices of the mosquito responses to different host odours (human, chicken, flower and the mineral oil (no odour) control). Asterisks denote a statistically significant preference for the host odour from zero ($p < 0.05$). (*b*) GCMS ion chromatograms of the headspace of humans, chickens and flower. Odourants used in subsequent EAG and olfactory conditioning assays included those from human hosts (1-octen-3-ol and hexanoic acid) and flowers (linalool). (*c*) Histograms are the mean (±SE) EAG responses of the mosquitoes to the different odourants (1-octen-3-ol, hexanoic acid and linalool). For the strongly anthropophilic species, *Ae. aegypti* and *An. stephensi*, responses to all three odours were not statistically significant ($p > 0.05$). By contrast, hexanoic acid elicited significantly lower responses compared to the other odourants in *Cx. quinquefasciatus* and linalool for *Tx. amboinensis* (*t*-test: $p < 0.05$). Letters denote significant differences between odour stimuli (*t*-test: $p < 0.05$). (*Bottom*) Average traces from five individuals of each species to the three odourants (1-octen-3-ol; linalool; hexanoic acid and solvent (mineral oil) control).

By contrast, *Cx. quinquefasciatus*, which rarely feed on mammalian hosts and prefer avian hosts when they are available, did not show an ability to learn these same odours (figure 2*c*). Previous work has shown that *Cx. quinquefasciatus*, unlike *Ae. aegypti* and *An. stephensi*, are repelled by 1-octen-3-ol, which is found in mammalian, but

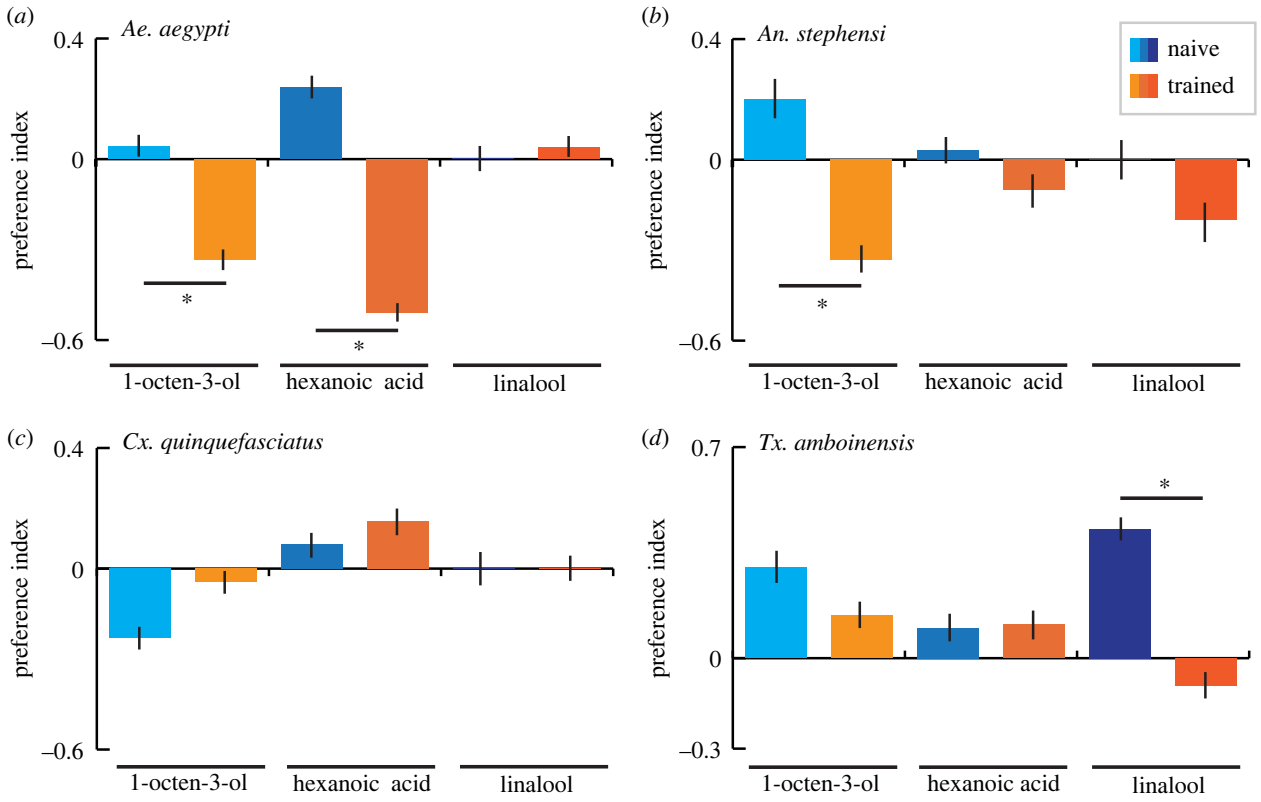

Figure 2. Mosquitoes learn odours associated with their preferred host. Mosquito preference index (PI) of (*a*) *Ae. aegypti*, (*b*) *An. stephensi*, (*c*) *Cx. quinquefasciatus* and (*d*) *Tx. amboinensis* females to 1-octen-3-ol, hexanoic acid and linalool in naive and trained groups. Asterisks in (*a–c*) represent statistical significance from zero while asterisk in (*d*) represents statistical significance from naive PI (binomial exact test: $p < 0.05$). Bars are the mean ± SEM.

not avian skin volatiles [17,18]. However, we did not observe significant differences in preference between naive and trained groups for either 1-octen-3-ol, hexanoic acid or linalool (figure 2*c*, binomial exact test, $p = 0.928$ trained versus naive for 1-octen-3-ol, $p = 0.829$ trained versus naive for hexanoic acid, $p = 0.566$ trained versus naive for linalool) although activity levels were high, with 93–100% of *Cx. quinquefasciatus* making a choice in the olfactometer (electronic supplementary material, table S1).

We next tested a nectar-feeding (non-haematophagous) mosquito, *Tx. amboinensis* to assess whether this species could learn associations with either vertebrate host or plant odours. When tested against 1-octen-3-ol or hexanoic acid, the trained mosquitoes did not differ significantly in preferences from the naive groups (figure 2*d*) (binomial exact test, $p = 0.204$ for 1-octen-3-ol and $p = 0.755$ for hexanoic acid). However, *Tx. amboinensis* was the only species in this study to learn the association between linalool and the mechanical shock (electronic supplementary material, figure S2A, binomial exact test, $p = 0.002$ trained versus naive and $p = 0.009$ trained versus unpaired), suggesting that like *Ae. aegypti* and *An. stephensi*, *Tx. amboinensis* learned the odour associated with their preferred food resource.

(c) Differential localization of tyrosine hydroxylase-like immunoreactivity across species

The relationship between learning and dopaminergic innervation in the antennal lobe of mosquitoes remains unknown. In *Ae. aegypti*, there is strong, differential innervation in specific glomeruli and, in parallel, these mosquitoes can learn only specific odourants [7,8]. As a first step to examine whether differential learning across mosquitoes is associated with variations in dopamine innervation, we used antisera against tyrosine hydroxylase (TH), which specifically stain dopaminergic neurons, to localize this neuromodulator in the brain of each species [19,20]. In particular, we focused on olfactory circuitry, including the antennal lobes (ALs), which are the primary olfactory brain structures in insects, and the mushroom bodies (MBs), which are higher-order centres implicated in learning and memory (electronic supplementary material, figure S1A).

Although TH-like immunoreactivity was localized in the ALs and MBs of all four species, the degree of dopaminergic innervation varied dramatically. The most striking difference in TH-like immunoreactivity across mosquito species was differential innervation of the ALs and their substructures (figure 3*a–d*). Each AL is subdivided into spherical-shaped compartments of dense neuropil called olfactory glomeruli. Olfactory receptor neurons (ORNs) expressing the same ligand-gated receptors terminate convergently, generally in one glomerulus where they synapse onto local interneurons (LNs) and projection neurons (PNs) that relay information to higher-order centres such as the MBs and lateral horns [22,23]. This organization compartmentalizes the encoding of single odour chemicals into one or very few glomeruli, and the pattern of activity across glomeruli forms a combinatorial code designating an identity to complex odours [24]. In *Ae. aegypti*, the ALs are extensively innervated with dopaminergic neurons, but TH-like immunoreactivity was heterogeneous across glomeruli (figure 3*a*,*a*′). Higher concentrations of TH were observed in the more anteromedial glomeruli, such as the AM1, AD2, MD1, MD2 and MD3 glomeruli [25]. Similar extensive but heterogeneous dopaminergic innervation of the ALs was observed in *Tx. amboinensis*. However, the pattern of high- and low-concentration glomeruli was different in this

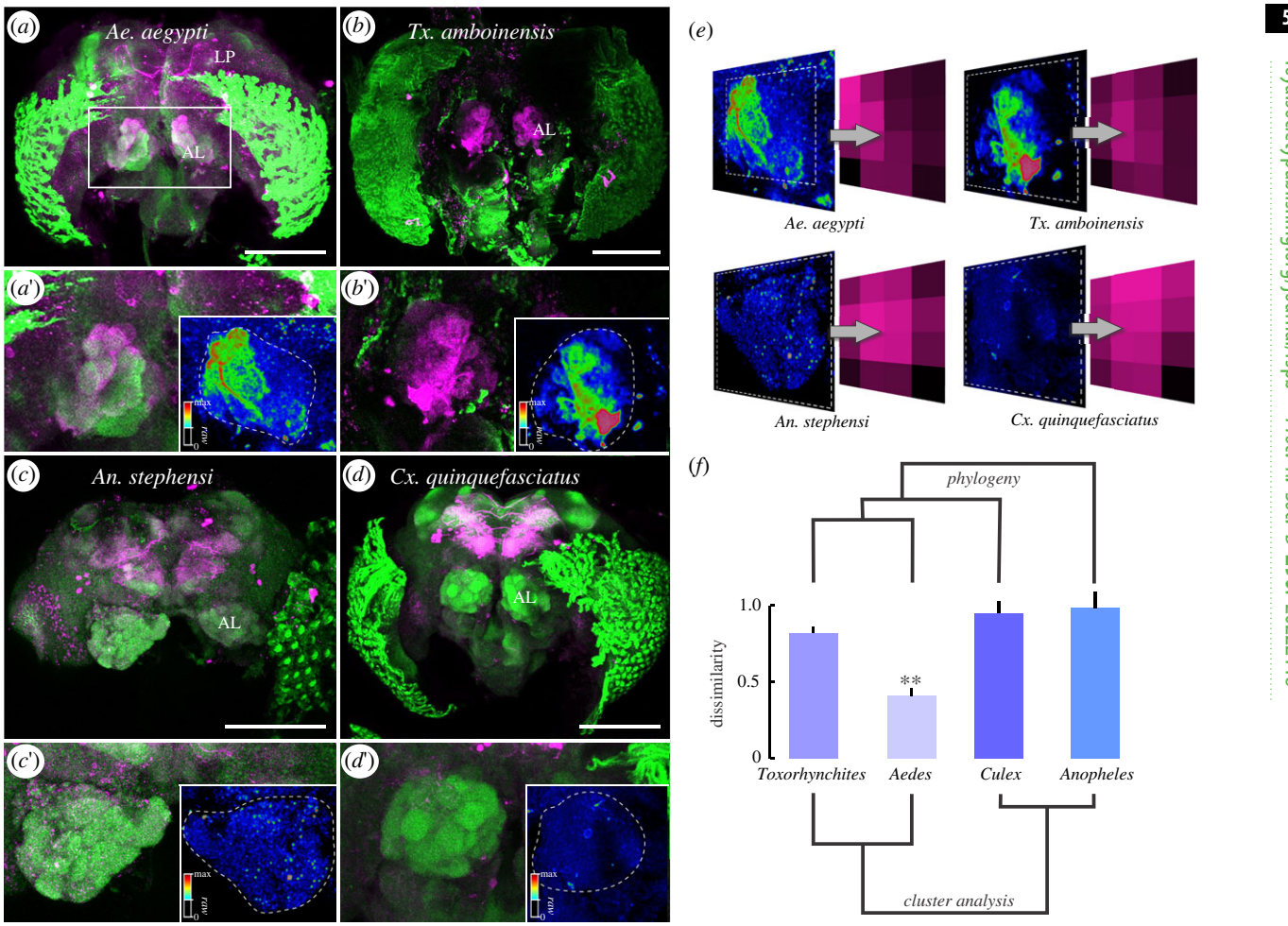

Figure 3. Differential dopaminergic innervation of antennal lobes across mosquito species and necessity for learning. Maximum projection views of confocal image stacks from (*a*) *Ae. aegypti*; (*b*) *Tx. amboinensis*; (*c*) *An. stephensi*; and (*d*) *Cx. quinquefasciatus* female whole brains labelled with antisera against tyrosine hydroxylase (TH) to stain dopaminergic neurons (magenta) and phosphorylated CaMKII (pCaMKII, green) for background staining. The box in (*a*) represents the areas magnified in (*a*'–*d*'). Insets: Tyrosine hydroxylase intensity pseudocoloured using the Rainbow RGB look-up table in ImageJ. Dashed outlines delimitate AL boundaries. Scale bars = 100 µm in (*a,c,d*) and 200 µm in (*b*). AL = antennal lobe, LP = lateral protocerebrum. (*e*) Normalized heat maps of average pixel intensity in 16 quadrants of the antennal lobes of *Ae. aegypti*, *Tx. amboinensis*, *An. stephensi*; and *Cx. quinquefasciatus* females. (*f*) Dissimilarity index of average pixel intensity matrices from (*e*) overlaid with relative phylogenetic positions [21] and cluster analysis.

species, with higher concentrations localized in posterolateral glomeruli (figure 3*b*,*b*').

TH-like immunoreactivity signals were lower in the ALs of *An. stephensi*, but dopaminergic innervation was observed surrounding glomeruli, especially in the lateral region of the antennal lobes (figure 3*c*'). By contrast, in *Cx. quinquefasciatus* the TH signal was not detectable above background in the ALs, although extensive TH-like immunoreactivity was observed in the lateral protocerebrum, surrounding the MBs in this species (figure 3*d*'). We used two different antisera, one raised against TH from rat (AB_572268) and one from *Manduca sexta* [26], and both revealed the same pattern of immunoreactivity.

To quantify differences in localization patterns across species, we calculated signal to noise (average pixel intensity divided by background) in a 4 × 4 grid overlaid on confocal images of TH-like immunoreactivity in the antennal lobes (figure 3*e*). Comparing the pattern of TH innervation in the AL across species showed that each had a statistically different innervation pattern (figure 3*f*; electronic supplementary material, table S1; Procrustes analysis: $p < 0.05$). This was further verified by specifically examining TH intensities in the more anteromedial glomeruli (for *Ae. aegypti*, where MD glomeruli are located), which showed significant differences between species (Kruskal Wallis test: $\chi^2 = 21.65$, $p < 0.001$).

(d) Dopamine is necessary for aversive olfactory learning in *Ae. aegypti* and *An. stephensi*

The differences in dopaminergic innervation of ALs across mosquito species raise the question of the importance of dopamine for aversive learning in other mosquito species. In *Ae. aegypti*, pharmacological knock-down of dopamine receptors abolished learning [7]. We asked whether dopamine is necessary for learning in distantly related species. In our study, *An. stephensi* is most distantly related to *Ae. aegypti*; their lineages having diverged over 200 million years ago [21]. To answer our question, we aversively trained two more groups of *An. stephensi* using 1-octen-3-ol as the conditioned stimulus: one group was injected in the thorax with a dopamine receptor antagonist (SCH-23390), and the other group was injected with physiological saline. In *Ae. aegypti*, SCH-23390 abolishes the associative learning of 1-octen-3-ol, replicating the result of dsRNA or CRISPR knock-down of dopamine receptor DOP1 [7]. As observed in *Ae. aegypti*, injection with the dopamine antagonist abolished aversive learning of 1-octen-3-ol in *An.*

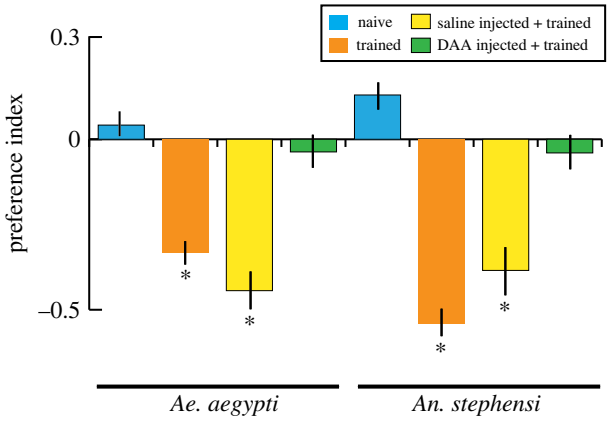

Figure 4. Necessity for dopamine in mosquito olfactory learning. Preference index of female *Ae. aegypti* and *An. stephensi* mosquitoes for 1-octen-3-ol in naive (blue), trained (orange), saline-injected + trained (yellow), and D1 dopamine receptor antagonist SCH 23390-injected (DAA) (green) treatment groups. Asterisks represent statistical significance from zero (binomial exact test: $p < 0.05$). Bars are the mean ± SEM.

stephensi while saline-injected mosquitoes learned to avoid this odour (figure 4, [7]). In both treatment groups, the injection procedure did not cause any motor defects. Despite differential dopaminergic innervation of ALs, both species required dopamine in the brain to form aversive associative memory of the host odour although we could not distinguish between effects at the level of the mushroom bodies versus the antennal lobes.

(e) Relationship between odour-evoked responses in the *Ae. aegypti* AL and dopaminergic innervation

Learning abilities of different mosquito species and the importance of dopamine in this behaviour raised the question of what is the functional relationship between the odour-evoked glomerular response and dopaminergic innervation? To begin to understand this relationship in *Ae. aegypti*, we used our GCaMP6s line to record the MD3 glomerular responses to octen-3-ol—it's cognate odourant via the AeaegOR8 receptor expressed on the maxillary palps [27]—when dopamine (10^{-7} *M*) was focally applied by iontophoresis onto the glomerulus (figure 5*a*). Comparing the MD3 glomerular responses to different odourants, when stimulated with 1-octen-3-ol during the pre-dopamine period (saline only), the MD3 glomerulus showed a strong calcium response that was significantly greater than the mineral oil (no odour) control (Kruskal-Wallis test: $p < 0.0001$; and with multiple comparisons: $p < 0.0001$) (figure 5*b*,*c*). However, dopamine application significantly increased the MD3 responses (Kruskal-Wallis test: $p = 0.014$), with the MD3 response to 1-octen-3-ol showing a 5% to 102% increase. The effects of dopamine application could be washed out, which returned the MD3 response to the pre-application condition (multiple comparisons between pre and wash phases: $p = 0.96$) (figure 5*b*,*c*). Importantly, odour-evoked responses by other AL glomeruli greater than 25 µm away from the MD3 glomerulus showed no modulatory changes during dopamine application (Kruskal–Wallis test for glomerular dopamine modulation: $p = 0.20$), indicating that dopamine was local and specific in its neuromodulatory effect.

To better understand the relationship between the glomerular map of odour-evoked responses and compare relative concentrations of TH expression between the glomeruli that encode these odours, we examined glomerular responses to 1-octen-3-ol, hexanoic acid, linalool and the mineral oil control throughout the AL (figure 5*d–j*; electronic supplementary material, figure S2B,C; figure S3). Serial imaging of depths across the AL—composed of more than 53 glomeruli [25]—enabled us to analyse 18 glomeruli that were reliably identifiable across preparations based on their position. Some of these glomeruli are responsive to odourants like nonanal, lilac aldehyde and other compounds [28,29], some of which are not learnable by *Ae. aegypti*. Stimulation with 1-octen-3-ol elicited the largest response in the MD3 glomerulus (Kruskal-Wallis test with multiple comparisons: $p = 0.0019$; figure 5*e*,*h*), whereas other glomeruli were not significantly different from one another in their responses to 1-octen-3-ol ($p > 0.05$), although their responses (e.g. AM4, PC1) were still significantly greater than mineral oil (no odour) control ($p < 0.05$). Hexanoic acid only elicited significant responses in the MD1 glomerulus ($p < 0.01$) compared to other glomeruli, although other glomeruli were also mildly activated (e.g. V3, PD1, PC1 and PL6; figure 5*f*,*i*). One glomerulus, AL3 responded significantly to hexanoic acid but also responded robustly to 1-octen-3-ol, linalool and every other odour we tested and may be very broadly tuned (electronic supplementary material, figure S3B,C). Similar to hexanoic acid, linalool elicited broad responses across glomeruli, although only the responses by the AM4 glomerulus was significantly higher ($p = 0.041$; figure 5*g*,*j*).

We next examined the relationship between dopaminergic innervation and glomerular responses to odourants. Dopaminergic innervation was mapped to each glomerulus by staining for TH and calculating the signal-to-noise (average pixel intensity within the glomerulus divided by average background pixel intensity) correlated with odourant-evoked responses across the glomerular ensemble (figure 5*k*; electronic supplementary material, figure S1C). When we compared the relationship between 1-octen-3-ol responses and TH levels across glomeruli, we found a significant relationship between the glomerular responses and dopamine levels (linear model: $r = 0.52$; $F_{1,15} = 5.68$; $p = 0.03$), with MD1 responses also reflected in the strong dopaminergic innervation ($p < 0.05$). Similarly, the strong odour-evoked responses by MD3 to hexanoic acid were also reflected in the significant dopaminergic innervation to this glomerulus ($p < 0.01$), with 1.7 to 7.3-fold higher innervation than other glomeruli. By contrast, for linalool, there was no relationship between the glomerular responses and dopamine levels ($p = 0.25$). Together, these results indicate the glomeruli encoding odours learned by *Ae. aegypti* had high levels of dopaminergic innervation relative to other glomeruli (figure 5*l*).

3. Discussion

Olfactory learning has long been implicated in shaping insect behaviours when preferred resources are scarce, however, the degree of learning by different mosquito species and its relationship to their host preferences has remained an open question. Here, we show that learning preferred-host odours may be a common phenomenon across mosquitoes, and that dopamine plays an important role in mediating this process. Furthermore, using *Ae. aegypti*, our results suggest a

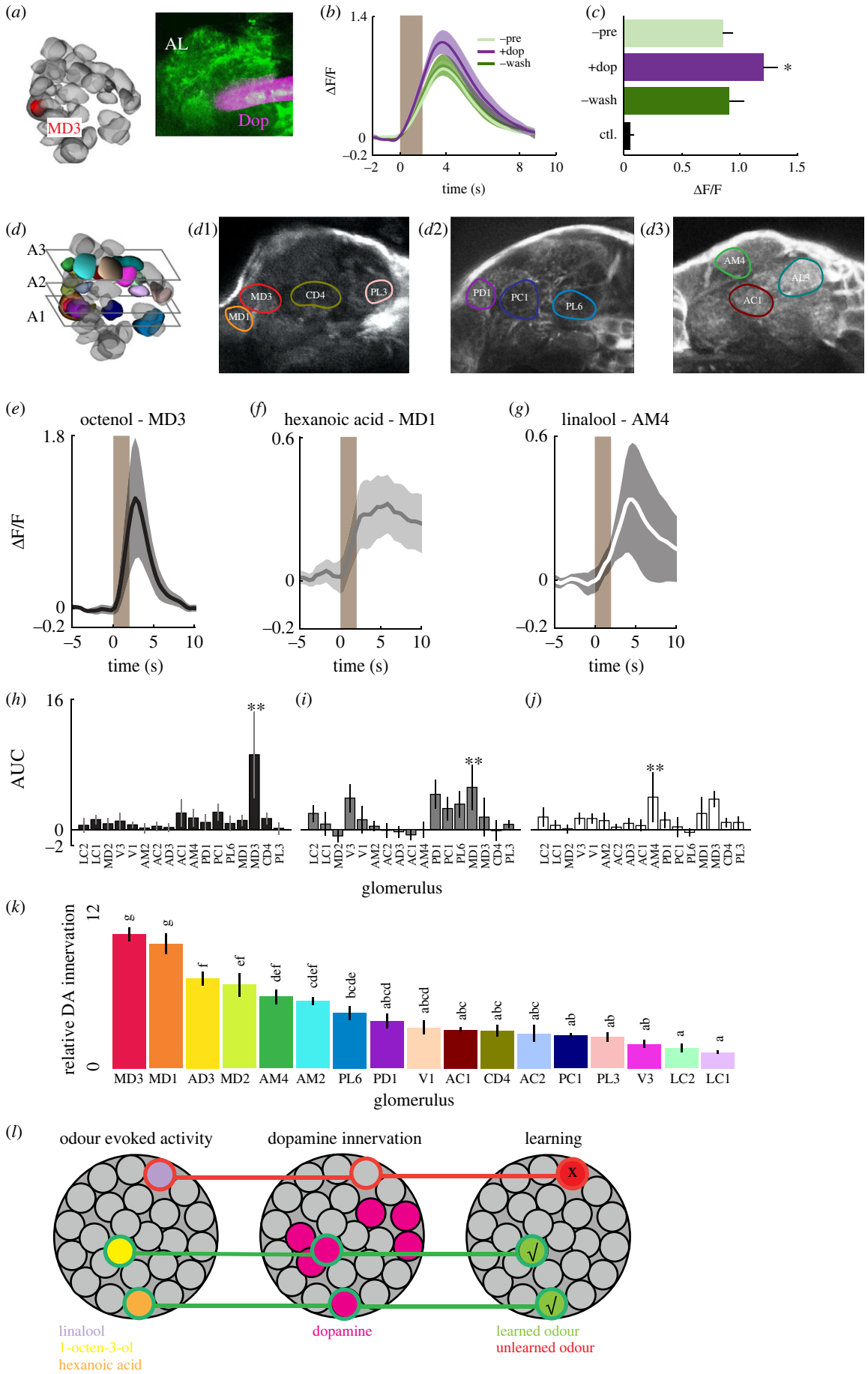

Figure 5. (*Caption overleaf.*)

relationship between dopaminergic innervation to specific antennal lobe glomeruli and a mosquito's ability to learn the odour encoded therein (figure 5*l*).

Dopaminergic modulation in olfactory learning has primarily been studied in the mushroom bodies [30–32], which receive afferents from the ALs and integrate signals

Figure 5. (*overleaf*.) Odour-evoked activity and relative dopaminergic innervation of olfactory glomeruli. (*a*, *left*) three-dimensional schematic of the *Ae. aegypti* female antennal lobe. The MD3 glomerulus (responsive to 1-octen-3-ol) is highlighted in red. (*a*, *right*) Image showing the AL (green) and microelectrode filled with dopamine (magenta). (*b*) Temporal response of the MD3 glomerulus when stimulated with 1-octen-3-ol during the pre-, dopamine-application, and wash phases of the experiment. Lines are the mean, area is the ±SEM. (*c*) Integrated response to 1-octen-3-ol over the peak 2-s period for the MD3 glomerulus during the different phases of dopamine application. Ctl is the mineral oil (no odour) control. Bars are the mean ± SEM; asterisk denotes significant difference between the experimental phases (*$p < 0.05$). (*d*) Three-dimensional schematic of the *Ae. aegypti* female antennal lobe. Glomeruli analysed in this study are highlighted and colours correspond to their labels in K. Representative frames from calcium imaging videos with outlined regions of interest are shown at depths (distances along anterior-posterior axis measured from anterior surface of the antennal lobe) 75–80 µm (*d*1), 50–60 µm (*d*2) and 20–30 µm (*d*3). Normalized averages of calcium responses (ΔF/F) to (*e*) 1-octen-3-ol in glomerulus MD3, (*f*) hexanoic acid in glomerulus MD1 and (*g*) linalool in glomerulus AM4. (*h–j*) Average area under the curve (AUC) of each odour response for 10 s beginning at the time of stimulus onset when mosquitoes were presented with (*h*) 1-octen-3-ol, (*i*) hexanoic acid and (*j*), linalool. Asterisks represent the level of significance in a linear regression model. **$p < 0.01$. (*k*) Relative dopamine (DA) innervation calculated as normalized average signal to noise from TH-like immunoreactivity (average pixel intensity of glomerulus divided by average pixel intensity of background). Letters represent significance groups (one-factor ANOVA and Tukey *post hoc* tests, $p < 0.05$). (l) Model of putative relationship between dopaminergic innervation of olfactory glomeruli and learning behaviour. Odours that are learned by *Ae. aegypti* are encoded in glomeruli with higher levels of dopaminergic innervation.

from different senses as well as reward and punishment. However, neuromodulation at the level of the AL can induce neuroplasticity, mediate gain control of olfactory signals and change the strength of output to higher-order centres like the mushroom bodies [33]. In honeybees, over 40% of observed AL neurons were sensitive to dopamine, which attenuates Ca^{2+}-activated K^+ currents [34], and this modulation likely impacts population coding activity in projection neurons and therefore learning. Indeed, in honeybees, associative learning elicits changes in the volume of specific glomeruli in the AL [35], as well as odour-evoked responses in glomerular activity and increases in odour discriminability [36]. Antennal lobes in the hawkmoth, *Manduca sexta* are innervated by extrinsic dopaminergic neurons with differential modulatory effects on subsets of AL neurons, potentially due to the expression of three different dopamine receptors [37]. As observed in this study, most hawkmoth AL neurons increased odour-evoked responses during dopamine application and this modulation was necessary for aversive learning of floral scents from host plants. In *Ae. aegypti*, dopamine also has differential modulatory effects on glomeruli encoding different odours. For example, representation of 1-octen-3-ol in the AL was significantly changed upon application of dopamine, increasing its discriminability with respect to other stimuli [7]. Here we show that glomeruli tuned to odours learned by mosquitoes, such as 1-octen-3-ol, are highly innervated with dopaminergic fibres compared to other glomeruli. The specific identities of modulatory neurons innervating glomeruli are yet to be determined, although another dipteran, *Drosophila melanogaster* possesses a subset of dopaminergic local interneurons, which may be conserved in mosquitoes [38]. Improved genetic tools may allow us to functionally image and interrogate their role in AL modulation and downstream effects on MB Kenyon cells.

In previous studies with *Ae. aegypti*, dopamine increased the odour-evoked excitability of AL neurons [7], and this was analogous to our calcium imaging results in the MD3 glomerulus (figure 5*b*,*c*). To further examine how dopaminergic innervation might modulate or amplify the input to downstream circuits in the MB, we conducted an analysis by convolving the odour-evoked response and the TH levels to model the putative excitatory drive from each glomerulus (electronic supplementary material, figure S4). Results showed that for both 1-octen-3-ol and hexanoic acid, activity in MD1 and MD3 glomeruli could potentially be amplified by 110- and 63-fold higher levels, respectively, over other glomeruli. For linalool, a similar pattern emerged, with the AM4 glomerulus significantly elevated over non-innervated glomeruli (Kruskal-Wallis test with multiple comparisons: $p < 0.05$; figure 5*h*), although the putative drive from the MD1 and MD3 glomeruli was also elevated. Our experiments testing the focal release of dopamine indicate that this selective increase in glomerular response could be an important feature of the *Ae. aegypti* AL. Although these results need to be further verified experimentally, they indicate that dopamine could significantly modify the strength and specificity of glomerular drive to the MB, and hence impact learning.

This study also highlights the importance of a comparative approach to understanding mosquito learning and food preferences. Our results suggest those species that prefer to feed on human blood aversively learned odours associated with mammalian skin volatiles but not a flower odour and the opposite was true of mosquitoes that feed solely on plant carbohydrates. Although the number of tested odourants in our behavioural and electrophysiological assays was relatively small, the odourants evoked similar levels of afferent drive to the antennal lobe (figure 1) and reflected the odourants from different nutrient resources the mosquitoes might use [13,27].

The evolution of neuromodulatory inputs to sensory and motor circuits played a role in mediating species-specific differences in behaviour across animal phyla [39]. Our study suggests that evolution of species-specific host preferences in mosquitoes may have co-evolved with differences in dopaminergic innervation to the antennal lobe. For all four species, patterns of dopaminergic innervation varied, although the basic morphology and circuit organization is conserved. Indeed, mosquitoes share ground pattern organization of olfactory circuitry with all insects and glomerular structure is conserved across invertebrate olfactory lobes and vertebrate olfactory bulbs [40]. An evolved increase in dopaminergic input to certain glomeruli may prime those circuits to relay odour-specific information to higher-order centres of learning and memory. When host availability changes on a short evolutionary timescale, mosquitoes may adapt to learning new olfactory signals by modifying dopaminergic inputs to prime the most salient odour circuits. Dopaminergic systems are involved in arousal, developmental processes and results from this study suggest flexibility in host preferences [41,42]. Moreover, olfactory behaviours, including learning, are critical for mosquito preferences and biting of human hosts, and thereby the spread of diseases

that afflict over a billion people annually. Therefore, unraveling the neural basis of learning in mosquitoes provides motivation for the development of new strategies for their control.

4. Material and methods

Methods for the antibodies, immunohistochemistry, image analysis, electroantennogram, and behavioural responses to host odours can be found in the electronic supplementary material.

(a) Experimental model and subject details

Aedes aegypti (wild-type: MRA-734, ATCC, Manassas, VA, USA; and our *PUb-GCaMP6s* line), *An. stephensi* (MRA-128, Strain STE2, CDC, Atlanta, GA, USA) and *Cx. quinquefasciatus* (NR-43025, Strain JHB, CDC, Atlanta, GA, USA) were maintained in breeding colonies at the University of Washington. *Toxorhychites amboinensis* eggs were a generous donation from Jason Pitts (Baylor University, Waco, TX, USA) and were also used to establish a new breeding colony.

Mosquitoes were reared at 25 ± 1°C, 60 ± 10% relative humidity (RH) in climatic chambers that maintained a 12 h light cycle [12 : 12]. *Aedes aegypti*, *An. stephensi* and *Cx. quinquefasciatus* mosquitoes were fed heparinized bovine blood (Lampire Biological Laboratories, Pipersville, PA, USA) through a parafilm membrane attached to a double-walled glass feeder (D.E. Lillie Glassblowers, Atlanta, GA, USA; 2.5 cm internal diameter) circulating water at 37° C. All species were provided with 10% sucrose. Eggs were hatched in trays of deionized water and larvae were provided with powdered fish food (Hikari Tropic 382 First Bites - Petco, San Diego, CA, USA). *Toxorhynchites amboinensis* eggs were hatched in cups of deionized water and larvae were placed in separate containers of deionized water (to prevent cannibalism) and fed *Ae. aegypti* larvae. Pupae of all species were collected and placed in mosquito breeding jars and, once eclosed, they were maintained on 10% sucrose (BioQuip, Rancho Dominguez, CA, USA). Mosquitoes used in all experiments were given a chance to mate, but were non-blood fed and isolated at 6 days post-emergence.

(b) Method details

(i) Two-photon calcium imaging

Odour-evoked responses were imaged in the AL of female *Ae. aegypti* expressing genetically encoded polyubiquitin-GCaMP6s during tethered flight [43]. Calcium responses were visualized on a Prairie Ultima IV multiphoton microscope (Prairie Technologies) and Ti-sapphire laser (Chameleon Ultra, Coherent). Laser power was adjusted to 20 mW at the rear aperture of the objective lens (Nikon NIR Apo, 40× water immersion lens, 0.8 NA), GCaMP fluorescence was bandpass filtered with a HQ 525/50 m-2p emission filter (Chroma Technologies) and photons collected using a multialkali photomultiplier tube.

Individual mosquitoes were cold-anaesthetized on ice and transferred to a Peltier-cooled holder while the head was glued to a three-dimensional printed stage. This custom stage is shaped like an inverted pyramid with a window at the vertex to permit superfusion of saline to the head capsule and room for the mosquito to move its abdomen and beat its wings. After gluing the head to the stage, a window was cut in the cuticle to expose the brain and a cold, oxygenated saline drip was placed in the well. A continuous stream of filtered air was directed towards the mosquito's antennae and odours were injected through an odour cartridge into this stream via solenoid valve for 2 s per stimulation. Odour cartridges consisted of glass syringes containing filter paper and 2 µl of either mineral oil or odour chemical (1-octen-3-ol, hexanoic acid or linalool) diluted in mineral oil at 1 : 100. For each odour stimulation, images of the antennal lobe were collected at 2 Hz over 30 s, beginning 10 s before odour stimulus onset. These images—500 µm by 600 µm window, sampled at 2 Hz—were time-stamped and synchronized with the time course of the odour-stimulus. Calcium responses were collected while imaging at focus planes 20–30 µm, 50–60 µm or 70–80 µm from the top of the antennal lobe in the anterior–posterior neuraxis.

Images were examined in Fiji and if two-dimensional movement occurred, the images were exported to Matlab (v2018; Mathworks, Natick, MA, USA) for Gaussian filtering (2 × 2 pixel; $\sigma = 1.5$–3) and alignment using a single frame as the reference at a given imaging depth and subsequently registered to every frame to within 0.25 pixel. Glomerular ROIs were determined based on the clear boundaries around glomeruli, especially during odour stimulation. We also note that the GCaMP6s is expressed in multiple cell types in the *Ae. aegypti* brain and AL, including glia, projection neurons, local interneurons and olfactory sensory neurons. However, immunohistochemical assays using antibodies against glutamine synthetase (glia) and GFP (GCaMP6 expression) revealed little overlap although neuronal cell bodies were GFP-positive (electronic supplementary material, figure S2B,C). Glia appeared in astroglial-like projections, mainly concentrated around the exterior of the glomeruli, whereas during odour stimulation dendrites and axons from projection neurons and local interneurons within the interior of the glomeruli often become apparent. Odour evoked calcium responses were calculated as the change in fluorescence for each glomerulus in each frame t of a time series using the formula: $\Delta F/F_0 = (F_t - F_0)/F_0$, where F_0 is the average intensity during the baseline period prior to the presentation of the odour stimulus. For each odour, the area under the curve (AUC)—reflecting the total response of the glomerulus and calculated by summing the $\Delta F/F$ for each frame from the stimulus onset to the signal return to baseline—was calculated for each glomerulus. We used the glomerular AUC for most statistical tests as this may reflect the total input to the MB. Finally, optical sections (1 µm) through the AL were collected with the same multiphoton microscope and olfactory glomeruli were reconstructed in three-dimensional using ImageJ and the Segmentation Editor plugin ($n = 12$ mosquitoes).

For dopamine application experiments, dopamine (10^{-7} M) was dissolved in saline and administered to the preparation through a microelectrode placed at the surface of the MD3 glomerulus in the *Ae. aegypti* AL (GCaMP6s line). Positive current (50 ms pulses) was passed through the microelectrode via an iontophoresis system (World Precision Instruments) to administer the dopamine onto the glomerulus. A negative retaining current was used during the pre and wash phases (controls) of the experiment ($n = 6$ mosquitoes).

(ii) Mosquito training protocol and control groups

A total of 1944 individual female mosquitoes were used in the behavioural conditioning experiments (electronic supplementary material, table S2). Aversive conditioning was performed as in Vinauger *et al.*, ([7], electronic supplementary material, figure S1B)—using a forward-paired paradigm and a computer-controlled system that allowed precise timing of the unconditioned and conditioned stimuli. Briefly, female mosquitoes were individually separated into plastic containers with mesh openings and allowed to acclimate for 120 s in a training chamber with a tube delivering clean (medical grade) air (30 cm s^{-1}, 23°C, 50% RH). Then, an odour was delivered via solenoid valve into a separate air stream for 60 s. During the last 30 s of odour presentation, the odour was paired with a mechanical shock delivered by a vortexer (1.65 g at 44 Hz). Odour and mechanical shock pairings were presented 10 times with a 2-min inter-trial interval (ITI). During the ITI, odour was vacuumed out of the training chamber while the clean airline

continued to flow. After the training session was completed, mosquitoes were placed in a climatic chamber (25° C; 60% RH; 12–12 h L : D) for 24 h before testing in a Y-maze olfactometer. Both training and testing occurred at 10 h into the light phase of the 12 : 12 light cycle for *Ae. aegypti* and *An. stephensi*, and at 8 h for *Tx. amboinensis*. *Cx. quinquefasciatus* were trained and tested at the onset of the dark phase in the 12 : 12 light cycle. These time periods reflect the peak activity time of the mosquitoes.

Two control groups were compared to the trained group to test the effects of aversive conditioning. A naive group was tested for each species. However, when the preference of trained mosquitoes for an odour differed significantly from naive (i.e. the mosquitoes learned), an unpaired group was tested as well to control for the timing effects of the unconditioned and conditioned stimulus inputs. Unpaired mosquitoes were placed in the training chamber and handled in the same manner as trained mosquitoes except that odour and mechanical shock were delivered semi-randomly, 60 s apart and without overlap. Each group was placed in the climatic chamber for 24 h before testing.

In addition, conditioned-stimulus-only, unconditioned-stimulus-only, and backward-paired training groups were analysed and tested in *Ae. aegypti* mosquitoes (e.g. Vinauger *et al.*, [7]). These groups were examined to determine if mosquitoes exhibited similar responses as other insects in these conditioning paradigms. *Ae. aegypti* mosquitoes in these groups did not exhibit learned responses (binomial test: $p > 0.32$) and were not significantly different from naive groups [7].

Training and testing trials were conducted in blocks of 12 or 24 mosquitoes. Certain groups were tested throughout the study period to validate results and therefore have higher sample sizes. These include 'unpaired' *Ae. aegypti* tested against 1-octen-3-ol, naive *Ae. aegypti* tested against linalool, and trained *Cx. quinquefasciatus* tested against hexanoic acid.

(iii) Behavioural testing in the olfactometer

Odour preference was tested using a custom-built, Plexiglas Y-maze olfactometer as described by Vinauger *et al.*, [7]. Mosquitoes were placed in a starting chamber attached to a cylindrical entry tunnel (30 cm long, 10 cm diameter), leading to a central chamber connected to two symmetrical 'choice' tunnels (both 39 cm long, and 10 cm diameter). Fans drew air into each 'choice' arm through a charcoal filter and a honeycomb filter to create uniform laminar flow at 20 cm s^{-1}. Odour and control stimuli were delivered by pumping air into the 'choice' tunnels via Teflon tubing connected to one of two 20 ml scintillation vials containing 10 ml of either mineral oil (control) or tested odour (1-octen-3-ol 1 : 100, hexanoic acid 1 : 100, linalool 1 : 10 000 diluted in mineral oil). Odours were tested at these concentrations because they did not elicit strong aversive responses but still elicited behavioural responses. Moreover, odourants and concentrations are typical of other studies, and mosquitoes are sensitive to these odourants at even lower concentrations [44–46]. Control and tested odour presentation were randomized daily between the left and right 'choice' arms and the maze was cleaned with 70% and 100% ethanol after each experiment. Behavioural testing was performed in a climatic chamber at 27°C and 70% RH.

Each test consisted of placing a single female mosquito in the starting chamber of the olfactometer, then the experimenter exited the climatic chamber and simultaneously monitored and filmed the maze via webcam. The first choice of the mosquito was recorded when she crossed the entry of a 'choice' arm. Mosquitoes that did not make a choice within 5 min were marked as active and non-responsive if they flew as far as the central chamber and they were marked as inactive and non-responsive if they did not leave the starting chamber. These two non-responsive groups were discarded from the preference analysis. Activity and per cent choice for each species and group is reported in electronic supplementary material, table S2.

(c) Pharmacology

To test the necessity of dopamine for aversive olfactory learning, female mosquitoes were injected with either saline or the Dop1 receptor antagonist SCH-23390 (Sigma Aldrich, St. Louis, MO, USA) dissolved in saline at a concentration of 10^{-6} M. This concentration was chosen based on our previous work [7], and subsequent experiments testing a two fold lower concentration (5×10^{-7} M) still suppressed learned responses, whereas a 200-fold lower concentration did not suppress learned responses. Injections were performed as in Vinauger *et al.* [7] and others [45,46]. Using the same Dop1 receptor antagonist allowed us to compare between species, and we note that creating Dop1 knock-outs through CRISPR is still a work in progress in these other mosquito species. In *Ae. aegypti*, dsRNA knock-down and CRISPR-mediated knock-out of the Dop1 receptor produced a similar lack of learned responses as using SCH-23390, but all these manipulations did not cause any locomotory defects or influence the innate response to CO_2 [7]. Briefly, 6-day old females that were mated but not blood-fed were isolated and cold-immobilized. Borosilicate micropipettes were filled with either saline or SCH-23390 and attached to a Picospritzer (Picospritzer III, Parker Hannifin, NJ, USA). Two drops of 65 nl each were injected into the thorax and then the mosquitoes rested for 30 min before training. These injection procedures are similar to other studies using pharmacological interventions [7,47,48]. Training and testing of the saline control and dopamine antagonist groups were performed as described above.

(d) Quantification and statistical analysis

Analyses were performed in R. For the binary data collected in the olfactometer, comparisons were performed by means of the binomial exact 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:

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

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.

For the calcium imaging, EAG and IHC data, whenever the data did not meet the normality assumption of the ANOVA, a Kruskal–Wallis test (with *post hoc* Tukey test for multiple comparisons) was performed. A linear regression model was used to examine the relationship between glomerular responses and TH-innervation. To examine the similarity in the pattern of dopamine labelling between species (figure 3*f*), for each individual, the pixel intensity in 16 quadrants of the antennal lobes of *Ae. aegypti*, *Tx. amboinensis*, *An. stephensi* and *Cx. quinquefasciatus* females were quantified. The resulting intensity values were transformed into a vector and analysed by cluster analysis to determine the dissimilarity indices (normalized Euclidean distances) for each species, which could be compared using a Kruskal-Wallis test. To further examine the different patterns of innervation, a Procrustes analysis (PA) was used to compare dopamine innervation in the mosquito ALs. We used a PA code from David L. Jones (http://www.rsmas.

miami.edu/personal/djones/). The PA has been widely used in the field of sensory biology and in comparison to other multivariate analyses (e.g. PCA) does not provide dimensional reduction of large data sets through orthogonal linear transformation. Instead, the PA is a procedure that minimizes the sum-of-squared differences between two configurations (i.e. data matrices) in a multivariate Euclidean space. The PA attempts to match one vector to another vector through matrix translation, scaling and rotation, and allows the determination of the similarity between two vectors based on landmarks and the specific (i,j) nodes in which that similarity lies. Therefore, this analysis provides a determination of the regions of the AL that produce similar values.

Data accessibility. Code is available from the J.A.R.'s GitHub account (https://github.com/riffelllab). Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.66t1g1k5k [49] and are provided in the electronic supplementary material [50].

Authors' contributions. G.H.W.: conceptualization, formal analysis, visualization, writing—original draft; C.V.: investigation, methodology; C.L.: investigation, methodology; E.R.: investigation; J.A.R.: conceptualization, funding acquisition.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare no competing interests.

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