Distinct navigation behaviors in Aedes, Anopheles and Culex mosquito larvae

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ABSTRACT

Mosquitoes spread deadly diseases that impact millions of people every year. Understanding mosquito physiology and behavior is vital for public health and disease prevention. However, many important questions remain unanswered in the field of mosquito neuroethology, particularly in our understanding of the larval stage. In this study, we investigate the innate exploration behavior of six different species of disease vector mosquito larvae. We show that these species exhibit strikingly different movement paths, corresponding to a wide range of exploration behaviors. We also investigated the response of each species to an appetitive food cue, aversive cue or neutral control. In contrast to the large differences in exploration behavior, all species appeared to gather near preferred cues through random aggregation rather than directed navigation, and exhibited slower speeds once encountering food patches. Our results identify key behavioral differences among important disease vector species, and suggest that navigation and exploration among even closely related mosquito species may be much more distinct than previously thought.

KEY WORDS: Chemosensory, Disease vector, Chemotaxis, Olfaction, Gustation, Foraging

INTRODUCTION

Mosquitoes are global disease vectors that transmit diseases such as malaria, Chikungunya and dengue fever. To limit the spread of these disease vector mosquitoes, researchers have identified larval mosquito control as a highly effective public health tool (Weeks et al., 2019). In particular, naturally occurring larvicides such as methionine and Bacillus sp. bacteria have recently increased in popularity as an environmentally safe alternative to synthetic insecticides such as DDT (Weeks et al., 2019; Regis et al., 2000). These larvicides must be ingested by larvae to be effective, and many factors affect larval feeding rate, including foraging strategy, chemosensory preference and competition with conspecifics or individuals from other species (Hartman, 2016; Ramoska and Hopkins, 1981; Merritt et al., 1992). In addition, larval behaviors and development rate also play an important role in adult population levels. For instance, direct competition for limited food resources at the larval stage is thought to be a major factor in the presence of certain disease vectors (Bevins, 2008; Alto et al., 2005). Qualitative studies of mosquito larvae have shown different patterns of feeding and swimming behaviors (Ramoska and Hopkins, 1981; Merritt et al., 1992; Yee et al., 2004), although these inter-specific differences appear to be flexible and dependent upon the environment (Yee et al., 2004). Despite growing interest (Skiff and Yee, 2014; Reiskind and Shawn Janairo, 2018; Zahouli et al., 2017), the strategies larvae use to locate sources of food, or respond to environmental stimuli, including sources of nutrients or toxic chemicals, remain poorly understood across many disease vector species. A better understanding of larval navigation and foraging behavior across mosquito species may help inform vector control techniques by suggesting where, when and how much ingestible larvicide to apply to maximize mosquito control while minimizing cost and environmental impact.

Comparative approaches can provide important insights into the sensory and evolutionary bases of behavior. Despite the limited data on chemosensory behaviors by mosquito larvae, previous work has demonstrated differences across species in locomotion and responses to food-related stimuli (Skiff and Yee, 2014), with these differences attributed to the adaptive use of nutrient resources and impacts of competition. For instance, larvae of multiple species of Culex or Aedes may inhabit spatially restricted containers where individuals experience interspecific competition for limited resources (Bevins, 2008; Yee et al., 2004; Juliano, 2009; Workman and Walton, 2003). Although these species may exhibit differences in locomotory and feeding responses, such as suspension feeding or browsing on surfaces, they can exhibit similar behaviors when experiencing similar environmental resources, such as browsing on the surface of a leaf (Yee et al., 2004). Larval resource use and competition have important effects on adult body size, and in the case of Aedes albopictus, can increase arboviral infection. Characterizing larval behaviors under different chemosensory conditions may allow us to determine how different species respond to different microhabitats or competitive environments.

Mosquito larvae may also provide important insight into the algorithms associated with search behaviors by aquatic insects. A previous study has shown that Aedes aegypti larvae find food randomly, rather than demonstrating directed motion toward preferred cues. Once a food-rich area is located, larvae decrease their swimming speed to remain in the favorable environment (Lutz et al., 2019). Previous studies in Anopheles albimanus (Aly and Mulla, 1986) and Aedes vexans (Aly, 1985) larvae showed that these species also discover food at random, although the methods used in these studies prevented deeper analysis into the mechanism of navigation. This is an unusually simple foraging strategy rarely found in other insects, or even in adult mosquitoes (Takken and Knols, 2010). Do other disease vector mosquito species also forage by randomly encountering food cues?

In the present study, we investigated the foraging and navigation behavior of six species of mosquito larvae, drawn from the three major disease vector genera Aedes, Anopheles and Culex (Ruzzante et al., 2019) (Fig. 1A). These species were selected for their importance to public health and for their diversity in ecological specialization and habitat choice. In the Aedes genus, we
investigated *Ae. aegypti* and *Ae. albopictus* – the most important vectors of dengue fever and yellow fever. Although these two species are closely related, *Ae. aegypti* preferentially breeds in manmade containers (Christophers, 1960), while *Ae. albopictus* is a generalist that may also inhabit rural and forested areas (Mousson et al., 2005). In the *Anopheles* genus, we examined the malaria vectors *An. arabiensis* and *An. coluzzii*. Interestingly, although *An. arabiensis* and *An. coluzzii* inhabit similar human-associated larval habitats (Minakawa et al., 1999; Etang et al., 2016), *An. arabiensis* drastically outcompetes *An. coluzzii* in mixed-species larval competition (Hartman, 2016). This suggests that these closely related species may rely on different foraging strategies, and that larval habitat specialization may not solely predict foraging behavior. Finally, we investigated *Culex quinquefasciatus*, a container-breeding mosquito, and *Culex tarsalis*, which breed in large vegetative areas such as rice fields. These two *Culex* species exhibit oviposition behavior that corresponds to predation risk in their natural larval habitat (high risk and high predator avoidance for *C. tarsalis*, low risk and low predator avoidance for container-dwelling *C. quinquefasciatus*) (Van Dam and Walton, 2008). Our exploratory study reveals striking differences in exploration behavior between all six species.

**MATERIALS AND METHODS**

Mosquitoes

Six species of wild-type mosquitoes were obtained from BEI Resources (National Institute of Allergy and Infectious Diseases, National Institutes of Health): *Ae. aegypti* (strain COSTA RICA, MRA-726, contributed by William G. Brogdon), *Ae. albopictus* (strain ATM-NJ95, Centers for Disease Control and Prevention for distribution by BEI, NR-48979), *An. arabiensis* (strain COSTA RICA, MRA-726, contributed by William G. Brogdon), *An. coluzzii* (strain Ngousso, MRA-1279, contributed by Frédéric Simard), *C. quinquefasciatus* (strain JHB, NR-43025) and *C. tarsalis* (strain YOLO, NR-43026). All species were reared in Milli-Q water in a shallow tray (26×35×4 cm) and fed with fish food (Petco; Hikari Tropic First Bites). Larvae were reared using the circadian cycle recommended by species-specific BEI rearing guidelines (16 h:8 h light:dark for *C. tarsalis*; 12 h:12 h for all other species). One day before the experiment, L3-stage larvae were isolated in Falcon™ 50 ml conical centrifuge tubes (Thermo Fisher Scientific, Waltham, MA, USA) containing ~15 ml Milli-Q water. Larvae were denied food for at least 24 h before the experiment. Animals that died before eclosion or pupated during the experiment were omitted, and all animals were tested during the light phase of their circadian cycle.

**Preparation of odor stimuli**

Stimuli were used that elicited robust behavioral responses across species. Two stimuli were used: an attractive food solution and quinine, a compound that elicits aversion in *Ae. aegypti* larvae (Lutz et al., 2019). The food extract solution was made fresh daily by dissolving 0.5% food (Petco; Hikari Tropic First Bites) in Milli-Q water for 1 h, then passing the mixture through a 0.2 μm filter (VWR International, 28145-477) to remove solid particulates. Quinine hydrochloride was prepared at 10 mmol l−1 in Milli-Q water (Sigma-Aldrich, Q1125). For all species, we saw no difference in mortality between the three treatments (*P*=1 for all species; Fig. S2), suggesting that exposure to quinine or food extract did not significantly harm larvae physiologically.

**Behavior arena and imaging**

We computed the trajectories of individual larvae in a custom behavior arena as previously described (Lutz et al., 2019; Bui et al., 2019). Briefly, individual larvae were introduced to an 8×3 cm rectangular behavior arena containing 20 ml of distilled Milli-Q water. Larvae were allowed to acclimate within the dark arena for 15 min, while being recorded by a Basler Scout Machine Vision GigE camera under infrared light. Subsequently, 100 μl of one stimulus was pipetted gently into the upper left corner of the arena (Fig. 1C), and larval behavior was recorded for an additional 15 min. In a separate experiment without larvae, we pipetted 100 μl of fluorescein dye into an identically shaped arena, in order to map stimulus concentration within the arena throughout the 15 min experiment (Lutz et al., 2019) (Fig. 1C). Trajectory paths were extracted from each video using Multitracker software by van Breugel et al. (2018) and additional code.
developed previously (Lutz et al., 2019). We visually inspected each trajectory path and manually corrected errors and omissions introduced by the tracking software.

**Trajectory quantification**

During foraging and swimming behaviors, mosquito larva can exhibit species-specific differences in their swimming kinematics and behaviors, including changing the duration of activity (Silh, 1986), increasing or decreasing their swim speeds, or exhibiting complex changes in locomotion (Merritt et al., 1992). We thus quantified 10 aspects of larval navigation in clean water to represent many of these ecologically relevant behaviors. Time spent moving was quantified as a percentage (0–100%), with movement defined as >1 mm s⁻¹. Total distance traveled was measured in meters. To normalize for any size-specific differences across individuals or species (Fig. S2), we converted larval speed measurements into body lengths per second (BL s⁻¹). Experimenters were blind to larval species or sex when measuring body lengths. Thus, the units BL s⁻¹ were used for quantifying maximum speed, mean speed when moving, mean speed in the first minute, and the difference in mean speed between first and last minutes. Spirals were defined as a distinct time period in which larvae engaged in >4 s of continuous spiraling movement. Sharp turns were defined as turns of >45 deg conducted at a speed of >4 mm s⁻¹. Continuous paths were defined as sustained movement at the same Δ angle, not including spirals. Rests were defined as periods of time >10 s of no movement.

**Statistical analyses**

Statistical analyses were performed in R (https://www.r-project.org/) and in Python (https://www.python.org/). We used a non-parametric Kruskal–Wallis test with Bonferroni correction to compare navigation characteristics across species for each of the 10 aspects of larval navigation (Fig. 2), because we found that not all variables followed a normal distribution (Shapiro–Wilk test, P>0.05). To create the Euclidean distance matrix for larval similarity analysis (Fig. S1), we first standardized all variables to zero mean and unit variance. To compare larval trajectories across species, both as a group of six and in species–species pairs, we used a perMANOVA and test of multivariate dispersion ANOVA with a Bonferroni correction (Table 1). We used a Monte Carlo permutation test to select significant eigenvectors for visualization in our PCA ordination (Fig. 3). We used a pairwise t-test to compare larval preference for different stimuli for each species (Fig. 4A). Preference was defined as the median concentration preferred by the larvae during the 15 min experiment, normalized to the areas chosen by the same larva during the preceding 15 min acclimation phase. This normalization was necessary to control for innate larval preference for corners or walls reported in some species (Lutz et al., 2019 preprint). Discovery time across different stimuli were compared for each species using a non-parametric Kruskal–Wallis test (Fig. 4B). A non-parametric test was used because we found that discovery time data did not follow a normal distribution (Shapiro–Wilk test, P>0.05). Discovery time was defined as the time taken (in seconds) to first encounter a section of the behavioral arena ≥50% concentration, normalized to the time taken to first encounter the same area during the clean water acclimation period. We used a Fisher’s exact test with Bonferroni correction to assess mortality differences among larval species, as well as among experimental treatments in larvae of the same species (Fig. S2). We used a non-parametric Kruskal–Wallis test to compare body length between different species (Fig. S2), because we found that body length data did not follow a normal distribution for all species (Shapiro–Wilk test, P>0.05).

**RESULTS**

**Larval exploration behavior in clean water**

To study the navigation behavior of each mosquito species, we used a semi-automated video analysis method previously reported in Lutz et al. (2019) (Fig. 1B). We first investigated behavior in clean water during the 15 min acclimation period (Ae. aegypti n=67; Ae. albopictus n=70; An. arabiensis n=93; An. coluzzii n=108; C. quinquefasciatus n=110; C. tarsalis n=53). The arena size used in these experiments (3×8 cm) was chosen based on previous field research showing that 95% of Ae. aegypti and Ae. albopictus oviposition sites in the field were man-made containers, and that more than a quarter of these observed oviposition sites were under 5 cm in radius (Chan et al., 1971), comparable to our experimental arena. Similarly, An. gambiae larvae were most commonly found in very small pools such as animal hoofprints in a different field study (Minakawa et al., 2004).

We observed striking behavioral differences across mosquito species in many aspects of exploration behavior (Fig. 2A). For example, C. tarsalis explored the environment slowly using distinctive sweeping circles (mean number of spirals=13.1), while the two Anopheles species interspersed long rests with fast, straight sprints (An. arabiensis mean number of spirals=0.2; An. coluzzii 0.4). The two Aedes species, as well as C. tarsalis, spent the majority of the time moving (Ae. aegypti 56%; Ae. albopictus 52%; C. tarsalis 46%), albeit at a much lower mean speed (Ae. aegypti 0.8 BL s⁻¹; Ae. albopictus 0.7 BL s⁻¹; C. tarsalis 0.7 BL s⁻¹) compared with other species (mean speed 1.3–1.9 BL s⁻¹; time spent moving 9–35%). To further investigate these observations, we quantified 10 different aspects of each larval trajectory, based on metrics we believed to be relevant to foraging and exploration behavior (Fig. 2B–K). We found significant differences across species in all quantified measures (Fig. 2B–K; P<0.001, Kruskal–Wallis test with Holm–Bonferroni correction).

For example, some metrics measure the frequency of exploration behavior in starved larvae, such as time spent moving and total distance traveled. Other metrics quantify known search behavior patterns in insects, such as the looping spirals observed in local search behavior (Bell, 1990), the frequency of sharp turns and the number of continuous straight-line paths. Some metrics were added to assess larval response to disturbance. For example, introducing animals to the arena during the acclimation phase is likely to elicit disturbance response to mechanical movement. Thus, we measured the mean speed of animals during the initial minute following introduction to the arena, as well as throughout the entire 15 min acclimation period. We also calculated a metric subtracting the speed during the initial minute from the last (15th) minute, to quantify the change in larval behavior post-disturbance. Because we found significant differences in larval size across species (Fig. S2), we normalized all speed measurements to each individual’s body length (BL s⁻¹). Finally, some metrics were intended to measure the physiological capacity of the starved larvae, such as the longest rest period and maximum observed speed.

**Exploration differences among species**

We next investigated whether these observed differences were consistent with known phylogenetic relationships. In particular, do different species exhibit different trajectory patterns when all navigation variables are considered? To answer this question, we created a Euclidean distance matrix of larval trajectories by incorporating all 10 navigation variables (Fig. S1). We found significant differences among the six mosquito species (perMANOVA P<0.001, pseudo-F=70.7; Fig. 3). To visualize
these results, we reduced the dimensionality of this data using principal component analysis (PCA) based on the same Euclidean distance matrix (Fig. 3A). PC1 and PC2 explained a significant proportion of variation in the data, but not subsequent PC axes (Monte Carlo permutation test, PC1, \( P < 0.001 \); PC2, \( P < 0.001 \); PC3–10, \( P > 0.05 \)). In brief, we found that larvae appeared to cluster in ordination space into four distinct categories: (1) very fast animals (upper left); (2) animals that traveled a long distance and conducted many sharp turns (upper right); (3) animals that rested for long periods of time (lower left); and (4) animals that traveled in spiral...
patterns and changed their behavior drastically during the 15 min period (lower right).

We next asked whether sister mosquito species display exploratory behavior that is more similar to each other than to other species. A post hoc pairwise perMANOVA for each species–species pair showed that all species differed significantly in navigation from each other, including sister species (Table 1). We observed that both An. arabiensis species were more similar to each other than to any other species (comparison of pseudo-F statistics across species–species pairs), and both Anopheles species were also closest to each other than to non-sister species. However, C. quinquefasciatus was most similar to Ae. aegypti, while C. tarsalis was most similar to Ae. albopictus. It is interesting to note that C. quinquefasciatus and Ae. aegypti both inhabit man-made containers, while C. tarsalis and Ae. albopictus can inhabit large vegetated areas such as rice fields and lakes. Although our study only compares six species and is not intended to draw phylogenetic conclusions, our limited panel of results suggest that both evolutionary history and ecological specialization may correlate with similar navigation behaviors in different species.

Larval response to attractive and aversive cues

Next, we examined the change in larval behavior after the introduction of 0.5% food extract, 10 mmol l⁻¹ quinine or distilled water. These stimuli were chosen to investigate larval cue-finding behavior, because previous studies have shown that these cues elicit robust preferences in Ae. aegypti mosquito larvae (Lutz et al., 2019; Bui et al., 2019). Corroborating a previous study (Lutz et al., 2019), we found that Ae. aegypti significantly preferred 0.5% food extract. To quantify preference, we normalized the median concentration preferred by each larva during the experiment phase, to the corresponding larval behavior during the acclimation phase. Ae. aegypti preferred a median food concentration of 20% more than would be expected from their pre-experiment behavior (P=0.0002, pairwise t-test). We observed similar attraction for all other species (Fig. 4A): Ae. albopictus (+32%, P<0.001), An. arabiensis (+13%, P=0.005), An. coluzzii (+7%, P=0.04), C. quinquefasciatus (+14%, P=0.006) and C. tarsalis (+16%, P=0.001). Further, we investigated changes in larval behavior after the introduction of 10 mmol l⁻¹ quinine, an aversive tantant. Similar to our previous study (Lutz et al., 2019), Ae. aegypti significantly avoided quinine, preferring a median concentration of 9% less than what would be expected from their pre-experiment behavior (P<0.001). We observed similar aversion in Ae. albopictus (–11%, P<0.001), An. arabiensis (–7%, P=0.002) and An. coluzzii (–7%, P=0.001) (Fig. 4A). Interestingly, neither C. quinquefasciatus nor C. tarsalis exhibited aversion to quinine (C. quinquefasciatus P=0.42; C. tarsalis P=0.60). In response to the addition of distilled water – a negative control for mechanical disturbance – all species exhibited no change in preference (P>0.05).

We next explored the mechanism of larval navigation to food sources. In our previous work, we found that Ae. aegypti explore their environment using a non-directional search strategy that results in random discovery of preferred cues (Lutz et al., 2019). In all species in this study, we found that larval preference also appeared to be consistent with random cue discovery. Discovery time did not significantly differ between water, food and quinine for any species (P>0.05, Kruskal–Wallis test; Fig. 4B), suggesting that larvae encounter environmental cues by random chance. In addition, we did not observe strong differences in orientation (Fig. 4C) or turn frequency (Fig. 4D) between different stimuli for any of the six species. Although surprising, these results are consistent with earlier literature using both video-tracking methods (Lutz et al., 2019) and researcher observations (Aly and Mulla, 1986; Aly, 1985).

In our previous study, we were able to conduct deep analyses and simulations into the mechanism of Ae. aegypti navigation, using a dataset of over 500 individual animals observed independently, with approximately 2 million total data points. In the present study, we did not have the necessary data to conduct the simulations or 3000 experiments necessary for similar analyses across species. Nevertheless, we visualized some of the same behavioral changes as a reference for future experiments (Figs S3, S4 and S5). We found several interesting patterns in these datasets. For example, in the vast majority of cases, animals did not appear to change their behavior – such as the number of looped searches or sharp turns – after addition of the stimulus. In cases where animals did change their behavior – such as An. coluzzii, which decreased initial speed in the post-stimulus period – animals seemed to exhibit the same behavioral changes for all experiments, independently of the stimulus added. Corroborating our previous results, we found that

Table 1. Comparisons of swimming patterns among species

<table>
<thead>
<tr>
<th>Species Pair</th>
<th>Ae. aegypti</th>
<th>Ae. albopictus</th>
<th>An. arabiensis</th>
<th>An. coluzzii</th>
<th>C. quinquefasciatus</th>
<th>C. tarsalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ae. aegypti</td>
<td>–</td>
<td>11.12***</td>
<td>89.92***</td>
<td>115.66***</td>
<td>25.66***</td>
<td>20.13***</td>
</tr>
<tr>
<td>Ae. albopictus</td>
<td>5.54</td>
<td>–</td>
<td>99.66***</td>
<td>118.58***</td>
<td>38.73***</td>
<td>16.96***</td>
</tr>
<tr>
<td>An. arabiensis</td>
<td>0.76</td>
<td>0.03</td>
<td>–</td>
<td>33.10***</td>
<td>71.41***</td>
<td>48.50***</td>
</tr>
<tr>
<td>An. coluzzii</td>
<td>1.44</td>
<td>6.79</td>
<td>18.19***</td>
<td>–</td>
<td>69.04***</td>
<td>63.56***</td>
</tr>
<tr>
<td>C. quinquefasciatus</td>
<td>4.90</td>
<td>18.77***</td>
<td>21.33***</td>
<td>0.06</td>
<td>–</td>
<td>29.69***</td>
</tr>
<tr>
<td>C. tarsalis</td>
<td>0.02</td>
<td>4.84</td>
<td>0.22</td>
<td>3.15</td>
<td>3.21</td>
<td>–</td>
</tr>
</tbody>
</table>

Values in the upper right half of the matrix represent pseudo-F-statistics from pairwise perMANOVA tests. Asterisks after each value indicate the significance of the corresponding F-value after Bonferroni correction: **P<0.01; ***P<0.001. In the upper right, significant values with a high pseudo-F-statistic represent species–species pairs that exhibit statistically significant differences in overall navigation behavior. Values in the lower left half of the matrix represent F-statistics for a pairwise test of multivariate dispersion (ANOVA with Bonferroni correction). Significant values with a high F-statistic in the bottom half of the matrix represent species–species pairs with statistically significant differences in the intra-species variability of navigation behavior. These results suggest that some, but not all, of the observed differences between species–species pairs may be due to variance among individuals, rather than to differences in raw behavioral metrics.
in our current work, *Ae. aegypti* also appear to aggregate near preferred cues by decreasing their movement speed near preferred areas (Fig. S4). Interestingly, we observed the same pattern for *Ae. albopictus* but not for any other species (Fig. S4). Similarities between closely related species were reflected in the PCA analyses, whereas the exploration responses were often distinct between species (Fig. 3A, Fig. S4K). However, once food was added, all the species significantly slowed their swimming speeds, causing them to cluster in the PCA space (Fig. S4N). Although further experiments are necessary to understand these results, it is likely that the *Anopheles* and *Culex* species in our study use different kinematic changes to navigate with respect to chemosensory cues, such as adjusting their turning frequency.

DISCUSSION

Our results raise several interesting questions for future research. In our experiments investigating larval cue-finding, we predicted that larvae may exhibit navigation strategies adapted to their environment, with species living in small containers displaying different strategies than those that breed in larger lakes or streams. However, our results showed that the six different species of mosquito larvae were strikingly homogeneous in their chemosensory responses to food: none of the species were able to change their behavior to find food cues faster in our experimental paradigm. Are there intrinsic physical properties to chemical diffusion in small, stagnant aquatic environments that makes more directed navigation particularly difficult? Although many of the species examined are naturally adapted to habitats of similar size to the experimental arena (Skiff and Yee, 2014; Christophers, 1960), it is possible that larvae may exhibit different navigation strategies in larger environments where the chemical gradients may be shallower or influenced by turbulent kinetic motion. Additionally, are there physiological limitations to larval chemosensation, such as the sensitivity of receptors or complexity of neural processing circuits, that prevent larvae from utilizing more complex navigation processes?

Second, is there an evolutionary benefit to different navigation behaviors exhibited by each species in clean water? Interspecific larval competition significantly affects distribution of mosquito species in the wild and in laboratory experiments (Hartman, 2016; Juliano, 1998; Braks et al., 2004). It is possible that this competitive environment drives larvae to exploit different foraging niches through different navigation strategies. Alternatively, it is possible that the different environmental conditions preferred by each species, such as lakes, streams and containers, result in different navigation strategies consistent across habitats. Although our study did not examine enough species to quantitatively answer this question, it is interesting to note that *Aedes* and *Anopheles* mosquito larvae exhibited greater similarity to sister species even when the sister species inhabited vastly different natural larval habitats. By contrast, the two *Culex* species exhibited the greatest similarity toward non-sister species that inhabited similar ecological environments.

It is important to note that our investigative study may not address important characteristics of mosquitoes found in the wild. For example, although all larvae analyzed in this study successfully completed development under the same laboratory rearing conditions, it is likely that environmental variables including temperature, concentration of dissolved organic matter, and water depth were more optimal for some species than for others. Indeed, species exhibited significantly different
mortality rates post-experiment, suggesting that the 24 h starvation period may have been more stressful for some species (Fig. S2). In addition, our experimental trials only observed larvae for a total of 15 min after stimulus addition, and it is possible that larvae may exhibit different behaviors over longer time scales.

Nevertheless, we believe that this study reveals an important area of future research. To our knowledge, this is the first study to quantitatively compare exploration behavior among mosquito larvae using machine vision rather than researcher observations. Even among the small subset of species examined in this study, we saw immediate and clear differences in exploration, stimulus preference and chemosensory navigation. Future studies incorporating additional mosquito species – especially outgroups that are not disease vectors – would add fascinating comparisons that may help clarify the


Supplementary Figures

**Fig S1: Euclidean distance matrix of individual differences between larvae.**
Euclidean distance matrix incorporating all ten navigation behavior variables in clean water. Each row or column visualizes one individual larva, arranged in species groups and then in order of experiment date. White lines mark boundaries between different species. Higher Euclidean distances (arbitrary units) represent lower similarity between individuals. The diagonal of black cells (upper left corner to the lower right corner) indicate self-self comparisons (distance = 0).
Fig S2: Larval size and mortality are significantly different across species.

A: Size of starved L3 larvae differed significantly across species (p<0.001, Kruskal-Wallis test with Bonferroni correction). Scatter points depict individual larvae, colored by species from left to right: *Ae. aegypti* (navy, n=67, mean=5.33mm), *Ae. albopictus* (purple, n=70, mean=5.03mm), *An. arabiensis* (red, n=93, mean=4.53mm), *An. coluzzii* (yellow, n=108, mean=4.56mm), *C. quinquefasciatus* (green, n=110, mean=4.95mm), and *C. tarsalis* (aqua, n=53, mean=4.54mm). Horizontal black bars visualize the mean of each group. Due to these size differences across species, speed measurements were standardized to larval body size for each individual.

B: Post-experiment mortality for each species and treatment. Numbers shown above each bar mark the percentage value for each treatment. Differences were statistically significant across species (p=0.003, Fisher’s Exact Test with Holm-Bonferroni correction), but not across the three treatments for the same species (p=1 for all species). Asterisks indicate the significance of the corresponding p-value: *: p<0.05; **: p<0.01; ***: p<0.001; NS: not statistically significant. All animals that died before eclosion (shown here) were omitted from analyses.
Fig S3: Changes in kinematic swimming responses following stimulus addition.

A-J: Changes in each of the ten navigational behavior measurements following the addition of water (W), quinine (Q), or food extract (F). From left to right in all plots: *Ae. aegypti* (navy); *Ae. albopictus* (purple); *An. arabiensis* (red); *An. coluzzii* (yellow); *C. quinquefasciatus* (green); and *C. tarsalis* (aqua). Each scatter point represents one individual, and the y axis shows the change in the navigational variable.
(experiment period - acclimation period). Thus, a point lying at 0 represents no change in behavior following addition of the stimulus. **A:** Time spent moving (%) **B:** Total distance traveled (meters) **C:** Mean speed when moving (body lengths \( \cdot \) s\(^{-1}\) [BL/s]) **D:** Maximum speed (body lengths \( \cdot \) s\(^{-1}\) [BL/s]) **E:** Initial speed, or mean speed in first minute (body lengths \( \cdot \) s\(^{-1}\) [BL/s]) **F:** Speed modification, or difference in mean speed between first and last minutes (body lengths \( \cdot \) s\(^{-1}\) [BL/s]) **G:** Sharp turns (% total time spent turning >45°) **H:** Longest continuous rest period **I:** Number of spirals **J:** Number of continuous paths that are not spirals. **K-N:** Representation of the same ten variables as in **A-J,** in PCA space. **K:** Reproduction of acclimation data for all animals visualized in Fig 3A. **L:** Experiment data from all animals responding to quinine, reprojected into the same PC space as in **K.** **M:** Experiment data from all animals responding to water, reprojected into the same PC space as in **K.** **N:** Experiment data from all animals responding to food, reprojected into the same PC space as in **K.
Fig S4: Species exhibit potential differences in navigation strategy toward preferred areas.

In a previous study, we conducted deep analyses into the mechanism of *Ae. aegypti* navigation, using a dataset of over 500 individual animals observed independently. This study revealed that *Ae. aegypti* locate preferred cues by decreasing their movement speed near preferred areas. In this current study, we did not have the reagent resources or manpower to conduct the 3,000 experiments necessary for an equally robust analysis. Nevertheless, we visualized the speed near and far from cues for each species and each stimulus. In this graph, each scatter point represents one individual, and the y axis shows the change in speed near the experimental cue (speed in areas >50% concentration - speed in areas <50% concentration), normalized to larval behavior in corresponding sections of the arena in the 15 minute acclimation period.
Fig S5: Larval distribution and trajectory maps for all species and experimental conditions. A-F: Distribution histograms across the x axis (above) and trajectory maps (below) of all starved animals during the experiment phase for water (left column), quinine (center column), and food (right column); Ae. aegypti (A), Ae. albopictus (B), An. arabiensis (C), An. coluzzii (D), C. quinquefasciatus (E), and C. tarsalis (F). Although trajectories are shown aggregated into one image for each panel, all animals were tested individually. Scatter points show the position of each animal at the end of the experiment. It is important to note that these histograms show the aggregated position data from all animals throughout the entire 15 minute experiment. Thus, a single animal exhibiting strong attraction or aversion may disproportionately influence this data visualization. For statistical tests reported in this paper, a single preference value was calculated for each animal (Fig 4A) to avoid such effects. Note that the distribution histograms for An. arabiensis and An. coluzzii appear particularly sparse, because these two Anopheles species spent the majority of the experiment at rest.