

# Distinct navigation behaviors in six species of disease vector mosquito larvae

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IN PREPARATION FOR: THE JOURNAL OF EXPERIMENTAL BIOLOGY. 2020

## 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 investigate the response of each species to an appetitive food cue, aversive cue or neutral control. By contrast to the large differences in exploration behavior, all species appeared to gather near preferred cues through random aggregation rather than directed navigation. Our results identify key behavioral differences among important disease vector species, and suggests that navigation and exploration among even closely related mosquito species may be much more distinct than previously thought.

## 1 Introduction

2 Mosquitoes are global disease vectors that transmit dis-  
3 eases such as malaria, Chikungunya, and dengue fever.  
4 To limit the spread of these disease vector mosquitoes,  
5 researchers have identified larval mosquito control as  
6 a highly effective public health tool [1]. One particu-  
7 lar mechanism for control involves targeting mosquito  
8 larvae during growth and development. In particular,  
9 naturally occurring larvicides such as methionine and  
10 *Bacillus spp.* bacteria have recently increased in pop-  
11 ularity as an environmentally safe alternative to syn-  
12 thetic insecticides like DDT [1,2]. These larvicides must  
13 be ingested by larvae to be effective, and many factors  
14 affect larval feeding rate, including foraging strategy,  
15 chemosensory preference, and competition with con-  
16 specifics or individuals from other species [3–5]. In  
17 addition, larval behaviors and development rate also  
18 play an important role in adult population levels. For  
19 instance, direct competition for limited food resources  
20 at the larval stage is thought to be a major factor in  
21 the presence of certain disease vectors [6, 7]. Qualita-  
22 tive studies of mosquito larvae have also shown different  
23 patterns of feeding and swimming behaviors [4,5,8], al-  
24 though these inter-species differences are poorly charac-  
25 terized. But despite growing interest [9–11], the strate-  
26 gies larvae use to locate sources of food, and their asso-  
27 ciated chemosensory behaviors, remain poorly under-  
28 stood across many disease vector species. A better un-  
29 derstanding of larval navigation and foraging behavior  
30 across mosquito species may help inform vector control  
31 techniques by suggesting where, when, and how much  
32 ingestible larvicide to apply to maximize mosquito con-  
33 trol while minimizing cost and environmental impact.

34 Mosquito larvae may also provide important insight

35 into the algorithms associated with search behaviors  
36 by aquatic insects. A previous study has shown that  
37 *Ae. aegypti* larvae find food randomly, rather than  
38 demonstrating directed motion toward preferred cues.  
39 Once a food-rich area is located, larvae decrease  
40 their swimming speed to remain in the favorable  
41 environment [12]. Previous studies in *Anopheles albi-*  
42 *manus* [13] and *Aedes vexans* [14] larvae showed that  
43 these species also discover food at random, although  
44 the methods used in these studies prevented deeper  
45 analysis into the mechanism of aggregation. This is  
46 an unusually simple foraging strategy rarely found in  
47 other insects, or even in adult mosquitoes [15]. Do  
48 other disease vector mosquito species also forage by  
49 randomly encountering food cues?

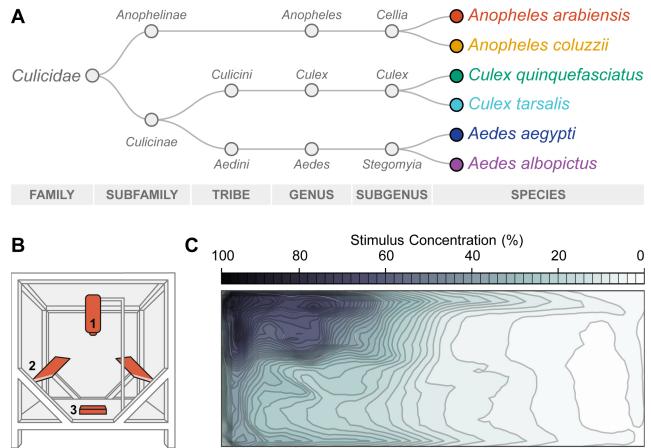
50 In this study we investigated the foraging and  
51 navigation behavior of six species of mosquito larvae,  
52 drawn from the three major disease vector genera  
53 *Aedes*, *Anopheles*, and *Culex* [16] (Fig 1A). These  
54 species were selected for their importance to public  
55 health and for their diversity in ecological special-  
56 ization and habitat choice. In the *Aedes* genus we  
57 investigated *Aedes aegypti* and *Aedes albopictus* —  
58 the most important vectors of dengue fever and  
59 yellow fever. Although these two species are closely  
60 related, *Ae. aegypti* preferentially breeds in manmade  
61 containers [17] while *Ae. albopictus* is a generalist that  
62 inhabits rural and forested areas [18]. In the *Anopheles*  
63 genus we examined the malaria vectors *Anopheles*  
64 *arabiensis* and *Anopheles coluzzii*. Interestingly,  
65 although *An. arabiensis* and *An. coluzzii* inhabitat  
66 similar human-associated larval habitats [19, 20],  
67 *An. arabiensis* drastically outcompetes *An. coluzzii*  
68 in mixed-species larval competition [3]. This sug-

gests 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 correspond 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*) [21]. Our exploratory study reveals striking differences in exploration behavior between all six species.

## 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 [12] (Fig 1B). We first investigated behavior in clean water during the 15 minute 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). 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, while the two *Anopheles* species interspersed long rests with fast, straight sprints. The two *Aedes* species spent the majority of the time moving, albeit at a much lower mean speed than other species. To further investigate these observations, we quantified ten 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 [22], 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 minute 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



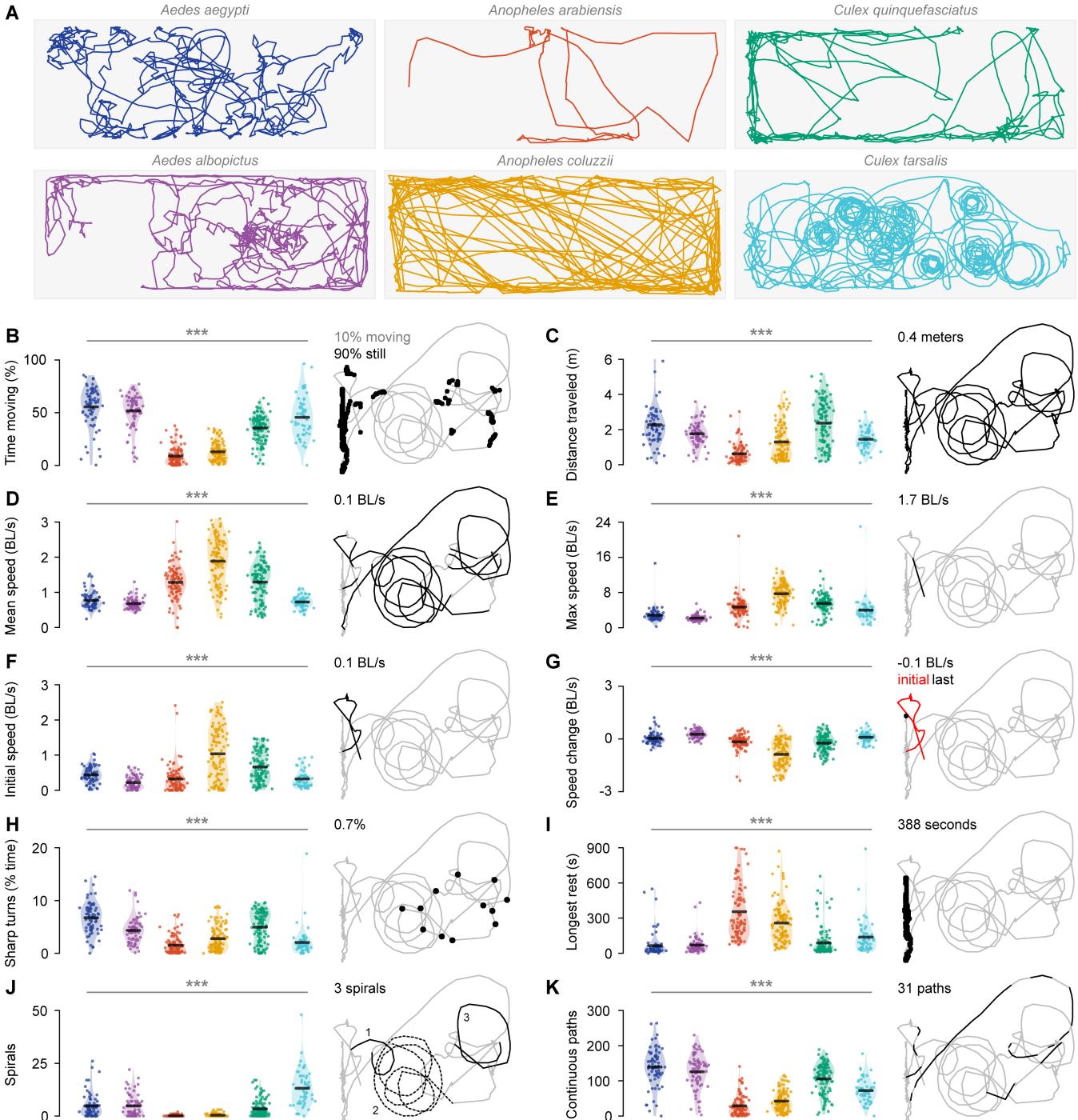
**Figure 1: Summary of experimental design and methods.** **A:** A simplified phylogeny of the six mosquito species investigated in this manuscript, adapted from [16] (branch lengths are not representative of phylogenetic distance). **B:** Diagram of the experimental rig, adapted from [12], including a Basler Scout Machine Vision GigE camera (1), infrared lighting (2) and a behavior arena filled with water (3). **C:** A map of stimulus distribution within the arena at 15 minutes post-stimulus addition, adapted from [12].

all speed measurements to each individual's body length (body lengths·s<sup>-1</sup>). Finally, some metrics were intended to measure the physiological capacity of the starved larvae, such as the longest rest period and maximum observed speed.

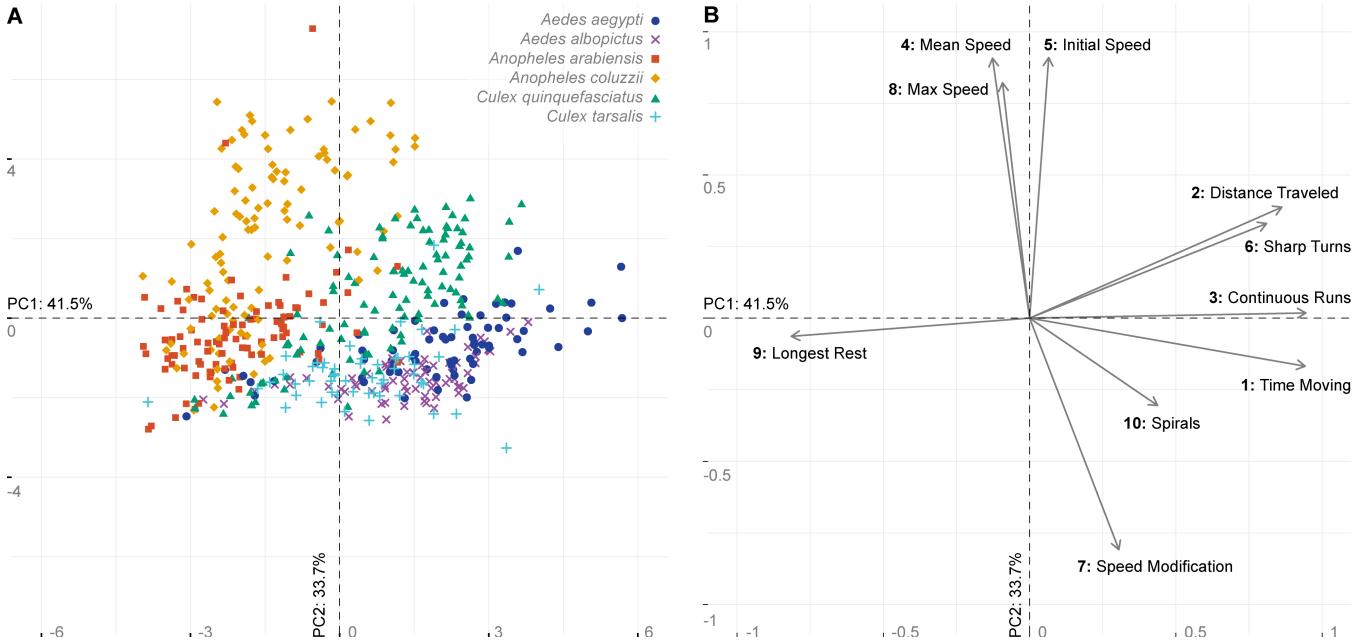
### Exploration differences among species

We next investigated whether or not 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 ten navigation variables (Fig S1). We found significant differences among the six mosquito species (Fig 3, perMANOVA p<0.001, pseudo F=70.7). 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: very fast animals (upper left); animals that traveled a long distance and conducted many sharp turns (upper right); animals that rested for long periods of time (lower left); and animals that traveled in spiral patterns and changed their behavior drastically during the 15 minute period (lower right).

We next asked if sister mosquito species display navigation behavior that is more similar to each other than



**Figure 2: Larvae exhibit species-specific behavioral differences in the absence of chemosensory stimuli.** **A:** Example trajectories visualizing one individual from each species, navigating in clean water. **B-K:** Species-specific distributions for each of ten navigation variables quantified from larval behavior in clean water. For each variable a violin plot visualizes the distribution for each species, with scatter points showing values for individual animals. A black bar marks the mean value for each species. Asterisks above each plot represents the significance of differences across species (Kruskal-Wallis test; \*\*\*:  $p < 0.001$ ). For all plots, a sample trajectory from a *Cx. quinquefasciatus* individual is shown on the right in gray, with red and black lines highlighting sections of the trajectory included in that variable. From left to right in all graphs: *Ae. aegypti* (navy); *Ae. albopictus* (purple); *An. arabiensis* (red); *An. coluzzii* (yellow); *Cx. quinquefasciatus* (green); *Cx. tarsalis* (aqua). **B:** Time spent moving (%) **C:** Total distance traveled (meters) **D:** Mean speed when moving (body lengths·s<sup>-1</sup>) **E:** Maximum speed (body lengths·s<sup>-1</sup>) **F:** Initial speed, or mean speed in first minute (body lengths·s<sup>-1</sup>) **G:** Speed modification, or difference in mean speed between first and last minutes (body lengths·s<sup>-1</sup>) **H:** Sharp turns (% total time spent turning  $>45^\circ$ ) **I:** Longest continuous rest period (s) **J:** Number of spirals **K:** Number of continuous paths that are not spirals.



**Figure 3: Larvae exhibit species-specific behavioral differences in the absence of chemosensory stimuli.** **A:** PCA biplot of ten individual variables describing larval trajectories based on the Euclidean distance matrix visualized in Fig S1. PC1 and PC2 explain a significant proportion of variance in the original data (Monte Carlo test, PC1: 41.5%,  $p<0.001$ ; PC2: 33.7%,  $p<0.001$ ), while all other PC axes did not ( $p>0.05$ ). Scatter points depict individual larvae in the ordination space, colored by species: *Ae. aegypti* (navy circles,  $n=67$ ), *Ae. albopictus* (purple x markers,  $n=70$ ), *An. arabiensis* (red squares,  $n=93$ ), *An. coluzzii* (yellow diamonds,  $n=108$ ), *Cx. quinquefasciatus* (green triangles,  $n=110$ ), and *Cx. tarsalis* (aqua + markers,  $n=53$ ). **B:** Vector arrows (1-10) indicate the direction of variable gradients in the ordination space. Ordered from highest to lowest contribution to PC1 and PC2, these variables include: **1:** Time spent moving (variable contribution to PC1: 21.4%; PC2: 0.8%); **2:** Distance traveled (PC1: 17.9%; PC2: 4.5%); **3:** Continuous paths (PC1: 21.5%; PC2: 0%); **4:** Mean speed (PC1: 0.4%; PC2: 24.5%); **5:** Initial speed in first minute (PC1: 0.1%; PC2: 24.6%); **6:** Sharp turns (PC1: 15.8%; PC2: 3.2%); **7:** Speed modification, or the difference in mean speed between first and last minutes (PC1: 2.2%; PC2: 19.4%); **8:** Maximum speed (PC1: 0.2%; PC2: 20.1%); **9:** Longest rest period (PC1: 16%; PC2: 0.1%); and **10:** Spirals (PC1: 4.6%; PC2: 2.8%)

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). Interestingly, we observed that both *Aedes* species were more similar to each other than to any other species (comparison of pseudo-F statistics across species-species pairs). 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* 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.

#### 174 **Larval response to attractive and aversive cues**

175 Next, we examined the change in larval behavior after  
 176 introduction of 0.5% food extract, 10mM quinine,  
 177 or distilled water. These stimuli were chosen to  
 178 investigate larval cue-finding behavior, because previous  
 179 studies have shown that these cues elicit robust  
 180 preferences in *Ae. aegypti* mosquito larvae [12, 23].  
 181 Corroborating a previous study [12], we found that  
 182 *Ae. aegypti* significantly preferred 0.5% food extract.  
 183 To quantify preference, we normalized the median  
 184 concentration preferred by each larva during the  
 185 experiment phase, to the corresponding larval behavior  
 186 during the acclimation phase. *Ae. aegypti* preferred a  
 187 median food concentration of 20% more than would  
 188 be expected from their pre-experiment behavior  
 189 ( $p=0.0002$ , pairwise t-test). We observed similar  
 190 attraction for all other species (Fig 4A): *Ae. albopictus*  
 191 (+32%,  $p<0.001$ ), *An. arabiensis* (+13%,  $p=0.005$ ),  
 192 *An. coluzzii* (+7%,  $p=0.04$ ), *C. quinquefasciatus*  
 193 (+14%,  $p=0.006$ ), and *C. tarsalis* (+16%,  $p=0.001$ ).  
 194 Further, we investigated changes in larval behavior  
 195 after introduction of 10mM quinine, an aversive  
 196 tastant. Similar to our previous study [12], *Ae. aegypti*  
 197 significantly avoided quinine, preferring a median

	<i>Aedes aegypti</i>	<i>Aedes albopictus</i>	<i>Anopheles arabiensis</i>	<i>Anopheles coluzzii</i>	<i>Culex quinquefasciatus</i>	<i>Culex tarsalis</i>
<i>Aedes aegypti</i>		11.12 ***	89.92 ***	115.66 ***	25.66 ***	20.13 ***
<i>Aedes albopictus</i>	5.54		99.66 ***	118.58 ***	38.73 ***	16.96 ***
<i>Anopheles arabiensis</i>	0.76	0.03		33.10 ***	71.41 ***	48.50 ***
<i>Anopheles coluzzii</i>	1.44	6.79	18.19 ***		69.04 ***	63.56 ***
<i>Culex quinquefasciatus</i>	4.90	18.77 ***	21.33 ***	0.06		29.69 ***
<i>Culex tarsalis</i>	0.02	4.84	0.22	3.15	3.21	

**Table 1: Comparisons of navigation patterns among species.** Values in upper right half of the matrix represent pseudo-F statistics from pairwise perMANOVA tests. Asterisks after each value indicate the significance of the corresponding p-value after Bonferroni correction: \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ ; no asterisk: not statistically significant. 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 differences in behavioral diversity among individuals, rather than in differences in raw behavioral metrics.

concentration of 9% less than 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 ( $p>0.05$ ).

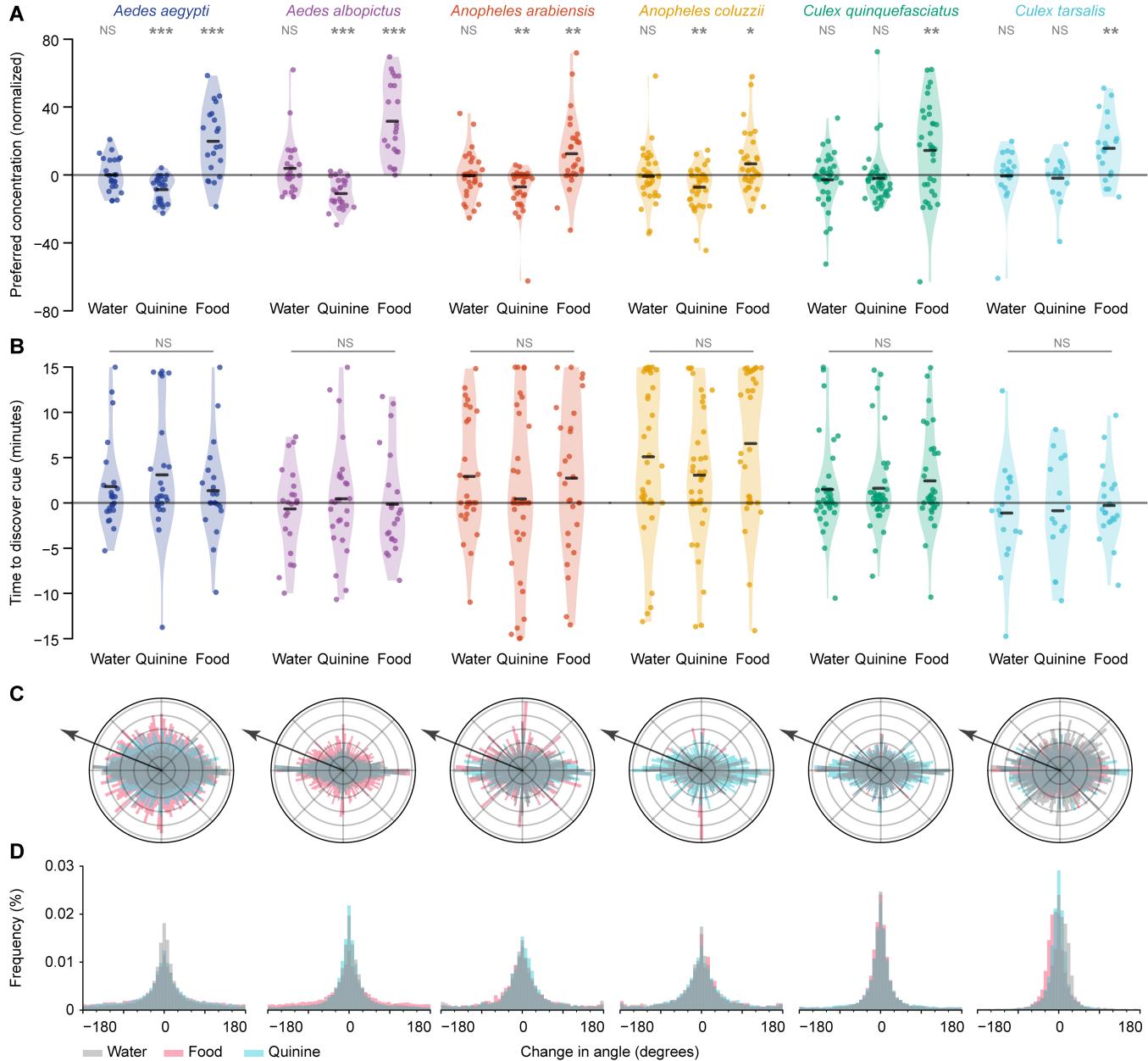
We next explored the method of larval aggregation near food sources. In our previous work, we found that *Ae. aegypti* explore their environment using a non-directional search strategy that results in random discovery of preferred cues [12]. In all species in this study, we found that larval preference 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 preferred direction (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 [12] and researcher observations [13, 14].

In our previous study, we were able to conduct deep analyses into the mechanism of *Ae. aegypti* navigation, using a dataset of over 500 individual animals observed independently. In this current study, we did not have the reagent resources or manpower to conduct the 3,000 experiments necessary for similar analyses across species. Nevertheless, we visualized some of the same behavioral changes as a reference for future experiments by other researchers (Fig S3, Fig S4, Fig S5). We found several interesting patterns in this visualization. For example, in the vast majority of

cases, animals did not appear to change their kinematic 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 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). Although further experiments are necessary to understand these results, it is likely that the *Anopheles* and *Culex* species in our study use different navigation mechanisms to aggregate near cues, such as adjusting turning frequency or movement direction.

## 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, it is possible that larvae may exhibit different



**Figure 4: Larval gathering near preferred cues is more consistent with passive aggregation than active navigation.** **A:** All larval species were significantly attracted to food extract. With the exception of the two *Culex* species, larvae were also significantly repelled by the aversive tastant quinine. **B:** However, none of the six larval species were able to change their navigation behavior to find food faster, or delay occupying high-concentration areas of quinine: *Ae. aegypti* ( $p=0.60$ , Kruskal-Wallis test); *Ae. albopictus* ( $p=0.71$ ); *An. arabiensis* ( $p=0.52$ ); *An. coluzzii* ( $p=0.22$ ); *Cx. quinquefasciatus* ( $p=0.64$ ); *Cx. tarsalis* ( $p=0.93$ ). **C:** For all species, animals did not appear to change their behavior to move towards or away from any stimulus. Polar plots showing aggregated movement direction for all animals throughout the 15 minute experiment for water (grey), food (pink) or quinine (light blue). Arrows mark the approximate direction of each stimulus. Each bar in the polar plot represents the proportion of time that animals moved in that specific direction. For visualization purposes, all polar plots are normalized to the maximum frequency for each stimulus. **D:** Turning frequency for each species did not appear to change depending on the added stimulus. Frequency histograms for aggregated larval turning frequency in response to the addition of water (grey), food (pink) or quinine (light blue). Y axis ranges are identical for all six plots. C-D: To reduce noise from passively drifting animals, plots only include data where larvae were moving at  $1\text{mm}\cdot\text{s}^{-1}$ .

navigation strategies in larger environments. 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 [3, 24, 25]. It is possible that this competitive environment drives larvae to exploit

284 different foraging niches through different navigation  
285 strategies. Alternatively, it is possible that the different  
286 environmental conditions preferred by each species,  
287 such as lakes, streams, and containers, result in different  
288 navigation strategies consistent across habitats.  
289 Although our study did not examine enough species to  
290 quantitatively answer this question, it is interesting to  
291 note that *Aedes* and *Anopheles* mosquito larvae exhibited  
292 greater similarity to sister species even when the  
293 sister species inhabited vastly different natural larval  
294 habitats. By contrast, the two *Culex* species exhibited  
295 the greatest similarity toward non-sister species that  
296 inhabited similar ecological environments.

297 It is important to note that our investigative  
298 study may not address important characteristics of  
299 mosquitoes found in the wild. For example, although  
300 all larvae analyzed in this study successfully completed  
301 development under the same laboratory rearing conditions,  
302 it is likely that environmental variables including  
303 temperature, humidity, and water depth were more  
304 optimal for some species than others. Indeed, species  
305 exhibited significantly different mortality rates post-  
306 experiment ( $p=0.003$ , Fisher's exact test), suggesting  
307 that the 24-hour starvation period may have been  
308 more stressful for some species (Fig S2). In addition,  
309 our experimental trials only observed larvae for a total  
310 of 15 minutes after stimulus addition, and it is possible  
311 that larvae may exhibit different behaviors at longer  
312 time scales.

313 Nevertheless, we believe that this study reveals an  
314 important area of future research. To our knowledge,  
315 this is the first study to quantitatively compare ex-  
316 ploration behavior among mosquito larvae using ma-  
317 chine vision rather than researcher observations. Even  
318 among the small subset of species examined in this  
319 study, we saw immediate and clear differences in explo-  
320 ration, stimulus preference, and chemosensory naviga-  
321 tion. Future studies incorporating additional mosquito  
322 species — especially outgroups that are not disease vec-  
323 tors — would add fascinating comparisons that may  
324 help clarify the evolutionary basis of exploration be-  
325 havior in mosquitoes. Because laboratory arena sizes  
326 and rearing conditions are already within the natural  
327 range of many mosquito species, mosquito larvae may  
328 be a promising field for phylogenetic behavior questions  
329 from a practical perspective. Finally, our results un-  
330 derscore the importance of understanding disease vec-  
331 tor behavior at all life history stages. We suggest that  
332 species-specific vector control research may be partic-  
333 ularly important to improving disease prevention meth-  
334 ods.

## 335 Materials and Methods

### 336 Insects

337 Six species of wild-type mosquitoes were obtained

338 from BEI Resources (NIAID, NIH): *Ae. aegypti* (Strain  
339 COSTA RICA, MRA-726, contributed by William G.  
340 Brogdon), *Ae. albopictus* (Strain ATM-NJ95, Centers  
341 for Disease Control and Prevention for distribution  
342 by BEI, NR-48979), *An. arabiensis* (Strain DON-  
343 GOLA, MRA-856, contributed by Mark Q. Benedict),  
344 *An. coluzzii* (Strain Ngousso, MRA-1279, contributed  
345 by Frédéric Simard), *C. quinquefasciatus* (Strain JHB,  
346 NR-43025), and *C. tarsalis* (Strain YOLO, NR-43026).  
347 All species were reared in milliQ water in a shallow tray  
348 (26×35×4cm) and fed with fish food (Petco; Hikari  
349 Tropic First Bites). Larvae were reared using the  
350 circadian cycle recommended by species-specific BEI  
351 rearing guidelines (16:8 light:dark for *C. tarsalis*; 12:12  
352 for all other species). One day before the experiment,  
353 L3-stage larvae were isolated in Falcon™ 50mL conical  
354 centrifuge tubes (Thermo Fischer Scientific, Waltham,  
355 MA, USA) containing ~15mL milliQ water. Starved  
356 larvae were denied food for at least 24 hours before  
357 the experiment. Animals that died before eclosion or  
358 pupated during the experiment were omitted, and all  
359 animals were tested during the light phase of their  
360 circadian cycle.

### 361 Preparation of Odor Stimuli

362 Stimuli were used that elicited robust behavioral re-  
363 sponses across species. Two stimuli were used: An at-  
364 tractive food solution and quinine, a compound that  
365 elicits aversion in *Ae. aegypti* larvae [12]. The food ex-  
366 tract solution was made fresh daily by dissolving 0.5%  
367 food (Petco; Hikari Tropic First Bites) in milliQ wa-  
368 ter for one hour, then passing the mixture through a  
369 0.2μm filter (VWR International #28145-477) to re-  
370 move solid particulates. Quinine hydrochloride was  
371 prepared at 10mM in milliQ water (Aldrich #Q1125).  
372 For all species, we saw no difference in mortality be-  
373 tween the three treatments ( $p=1$  for all species, Fig S2),  
374 suggesting that exposure to quinine or food extract did  
375 not significantly harm larvae physiologically.

### 376 Behavior Arena and Imaging

377 We computed the trajectories of individual larvae in a  
378 custom behavior arena as previously described [12, 23].  
379 Briefly, individual larvae were introduced to a 8×3cm  
380 rectangular behavior arena containing 20mL of distilled  
381 milliQ water. Larvae were allowed to acclimate within  
382 the dark arena for 15 minutes, while being recorded by  
383 a Basler Scout Machine Vision GigE camera under in-  
384 frared light. Subsequently, 100μL of one stimulus was  
385 pipetted gently into the upper left corner of the arena  
386 (Fig 1C), and larval behavior was recorded for an ad-  
387 dditional 15 minutes. In a separate experiment without  
388 larvae, we pipetted 100μL of fluorescein dye into an  
389 identically shaped arena, in order to map stimulus con-  
390 centration within the arena throughout the 15 minute  
391 experiment [12] (Fig 1C). Trajectory paths were ex-

392 tracted from each video using Multitracker software by  
393 Floris van Breugel [26] and additional code developed  
394 previously [12]. We visually inspected each trajectory  
395 path and manually corrected errors and omissions in-  
396 troduced by the tracking software.

### 397 Trajectory Quantification

398 During foraging and swimming behaviors, mosquito  
399 larva can exhibit species-specific differences in their  
400 swimming kinematics and behaviors, including  
401 changing the duration of activity [27], increasing or  
402 decreasing their swim speeds, or exhibiting complex  
403 changes in locomotion [5]. We thus quantified ten  
404 aspects of larval navigation in clean water to represent  
405 many of these ecologically relevant behaviors. Time  
406 spent moving was quantified as a proportion (0-100%),  
407 with movement defined as  $>1\text{mm}\cdot\text{s}^{-1}$ . Total distance  
408 traveled was measured in meters. To normalize for any  
409 size-specific differences across individuals or species  
410 (Fig S2), we converted larval speed measurements into  
411 body lengths per second. Experimenters were blind  
412 to larval species or sex when measuring body lengths.  
413 Thus, body lengths·s $^{-1}$  were used for quantifying  
414 maximum speed, mean speed when moving, mean  
415 speed in first minute, and the difference in mean speed  
416 between first and last minutes. Spirals were defined  
417 as a distinct time period in which larvae engaged in  
418  $>4$  seconds of continuous spiraling movement. Sharp  
419 turns were defined as turns of  $>45^\circ$  conducted at a  
420 speed of  $>4\text{mm}\cdot\text{s}^{-1}$ . Continuous paths were defined  
421 as sustained movement at the same  $\Delta$  angle, not  
422 including spirals. Rests were defined as periods of time  
423  $>10$  seconds of no movement.

### 424 Statistical Analyses

425 Statistical analyses were performed in R [28] and  
426 in Python [29]. We used a non-parametric Kruskal-  
427 Wallis test with Bonferroni correction to compare nav-  
428 igation characteristics across species for each of the ten  
429 aspects of larval navigation (Fig 2), because we found  
430 that not all variables followed a normal distribution  
431 (Shapiro-Wilk test,  $p>0.05$ ). To create the Euclidean  
432 distance matrix for larval similarity analysis (Fig S1),  
433 we first standardized all variables to zero mean and  
434 unit variance. To compare larval trajectories across  
435 species, both as a group of six and in species-species  
436 pairs, we used a perMANOVA and test of multivariate  
437 dispersion ANOVA with a Bonferroni correction (Ta-  
438 ble 1). We used a Monte Carlo permutation test to  
439 select significant eigenvectors for visualization in our  
440 PCA ordination (Fig 3). We used a pairwise t-test to  
441 compare larval preference for different stimuli for each  
442 species (Fig 4A). Preference was defined as the me-  
443 dian concentration preferred by the larvae during the  
444 15 minute experiment, normalized to the areas chosen  
445 by the same larva during the preceding 15 minute ac-

446 climation phase. This normalization was necessary to  
447 control for innate larval preference for corners or walls  
448 reported in some species [12]. Discovery time across  
449 different stimuli were compared for each species using  
450 a non-parametric Kruskal-Wallis test (Fig 4B). A non-  
451 parametric test was used because we found that dis-  
452 covery time data did not follow a normal distribution  
453 (Shapiro-Wilk test,  $p>0.05$ ). Discovery time was de-  
454 fined as the time taken (in seconds) to first encounter a  
455 section of the behavioral arena  $\geq 50\%$  concentration,  
456 normalized to the time taken to first encounter the  
457 same area during the clean water acclimation period.  
458 We used a Fisher's Exact Test with Bonferroni correc-  
459 tion to assess mortality differences among larval species,  
460 as well as among experimental treatments in larvae of  
461 the same species (Fig S2). We used a non-parametric  
462 Kruskal-Wallis test to compare body length between  
463 different species (Fig S2), because we found that body  
464 length data did not follow a normal distribution for all  
465 species (Shapiro-Wilk test,  $p>0.05$ ).

### 466 Acknowledgements

467 We thank Floris van Breugel for assistance in developing methods  
468 for video data analysis, and Julian Olden for advice on statistical  
469 methods. We also thank Binh Nguyen and Kara Kiyokawa for  
470 maintaining the Riffell lab mosquito colony, and Dustin Miller  
471 for advice on rearing mosquitoes procured from the CDC.

### 472 Author Contributions

473 Conceptualization: E.K.L. and J.A.R.; Methodology: E.K.L.  
474 and J.A.R.; Software: E.K.L.; Investigation: K.T.H. and E.K.L.;  
475 Resources: E.K.L. and J.A.R.; Data Curation: E.K.L. and  
476 K.T.H.; Writing — Original Draft: E.K.L; Writing — Review  
477 and Editing: E.K.L, J.A.R., and K.T.H.; Visualization: E.K.L.  
478 and K.T.H.; Supervision: J.A.R.; Project Administration:  
479 J.A.R.; Funding acquisition: J.A.R., E.K.L., and K.T.H.

### 480 Declaration of Interests

481 The authors declare no competing financial interests.

### 482 Funding

483 This work was supported in part by the National Institute of  
484 Health grant 1RO1DCO13693-04 to J.A.R.; National Science  
485 Foundation grants IOS-1354159 to J.A.R. and DGE-1256082  
486 to E.K.L.; Air Force Office of Sponsored Research under grant  
487 FA9550-16-1-0167 to J.A.R.; CoMotion Innovations Scholarship  
488 to K.T.H.; Robin Mariko Harris Award to E.K.L.; and the  
489 Margo and Tom Wyckoff Award to E.K.L.

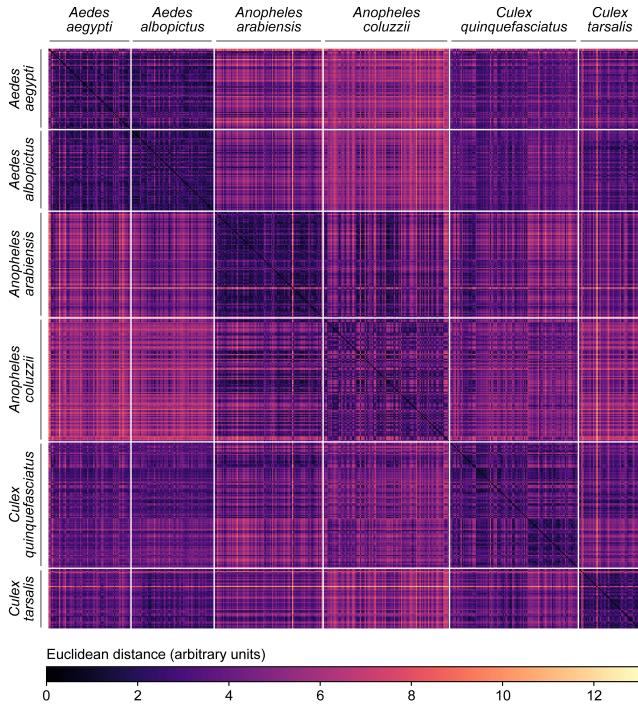
### 490 Additional Files

491 Code associated with this manuscript can be found at:  
492 <https://github.com/eleanorlutz/aedes-aegypti-2020>.

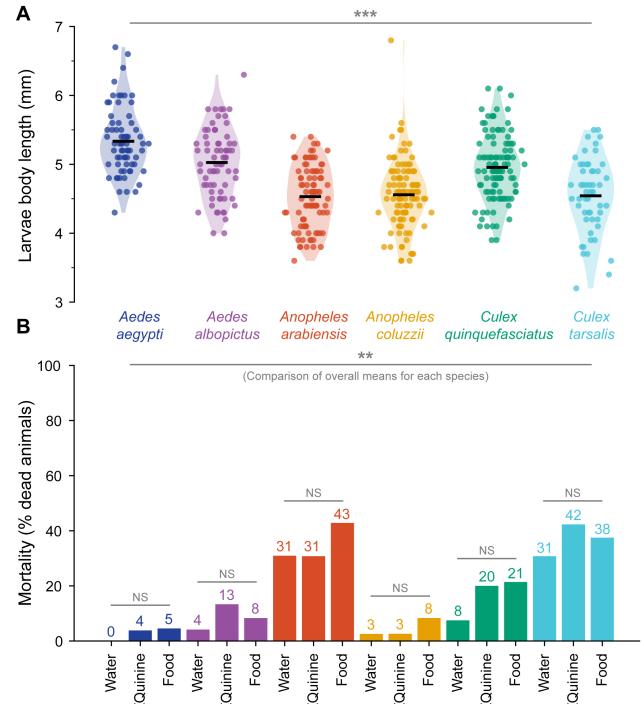
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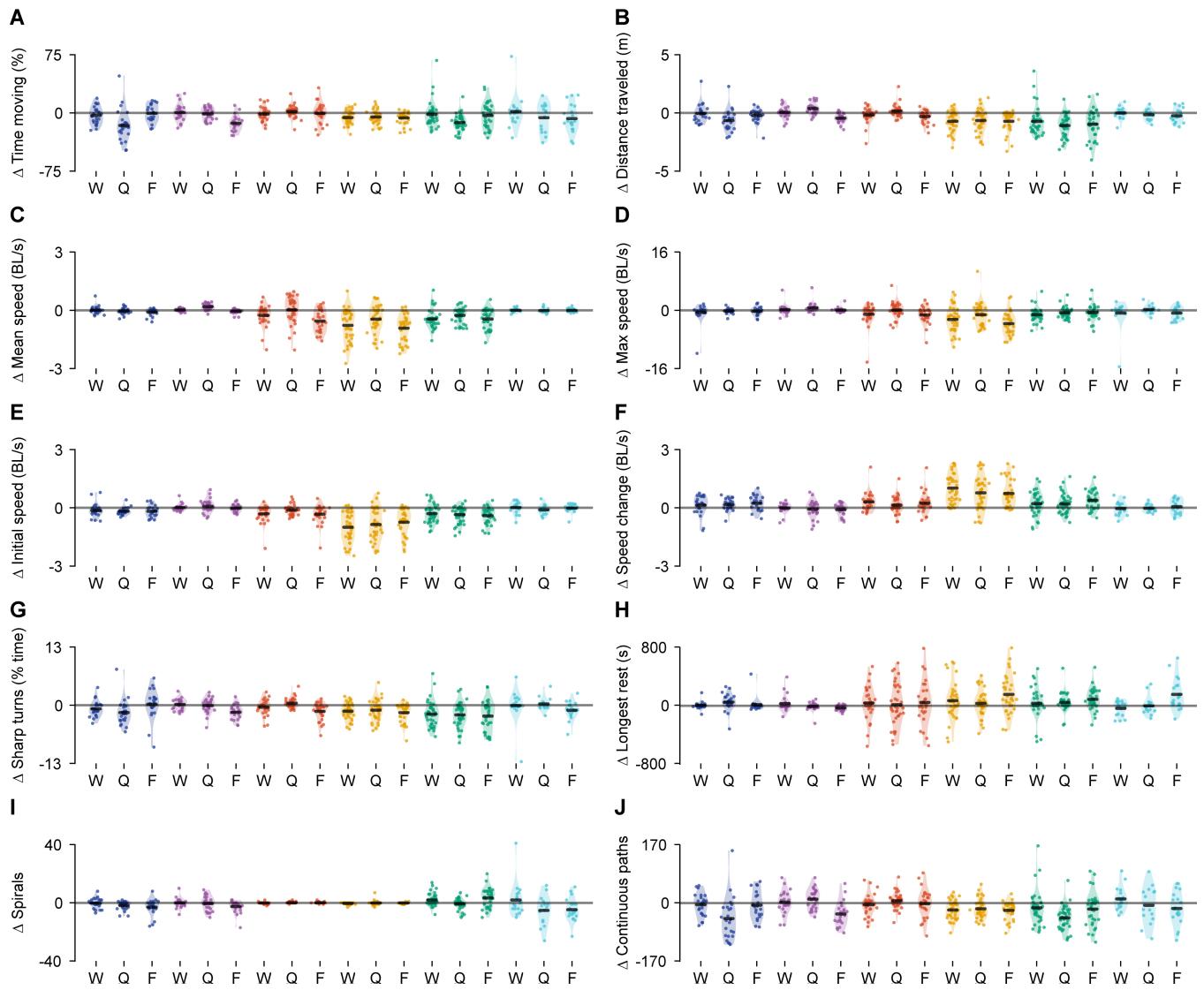
## Supplementary Figures



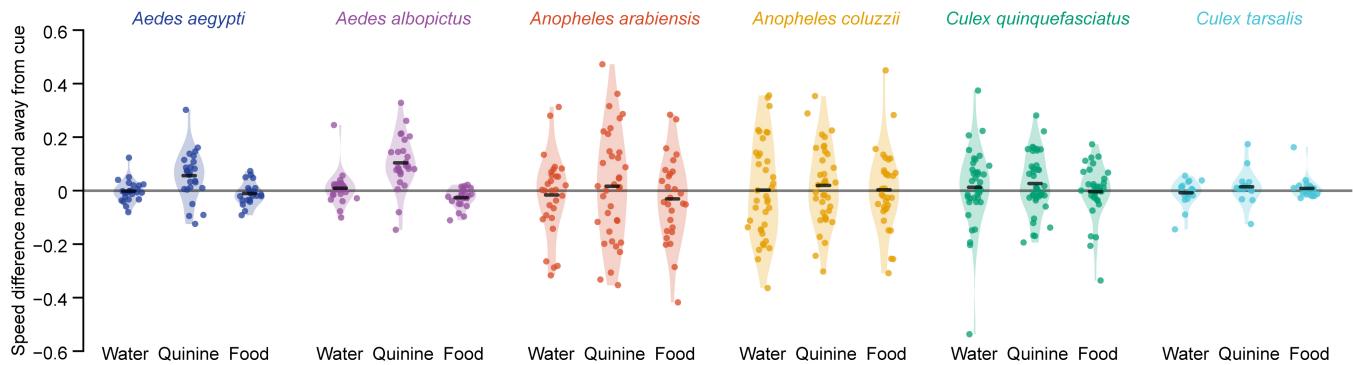
**Figure S1: Euclidean distance matrix of individual differences between larvae.** Euclidean distance matrix incorporating all ten navigation behavior variables in clean water. Each row or column visualizes one individual larvae, arranged in species groups and then in order of experiment date. White 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 lower right corner) indicate self-self comparisons (distance = 0).



**Figure 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), *Cx. quinquefasciatus* (green,  $n=110$ , mean=4.95mm), and *Cx. 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.



**Figure S3: Changes in kinematic navigation behavior 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); *Cx. quinquefasciatus* (green); and *Cx. 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·s<sup>-1</sup>) **D:** Maximum speed (body lengths·s<sup>-1</sup>) **E:** Initial speed, or mean speed in first minute (body lengths·s<sup>-1</sup>) **F:** Speed modification, or difference in mean speed between first and last minutes (body lengths·s<sup>-1</sup>) **G:** Sharp turns (% total time spent turning >45°) **H:** Longest continuous rest period (s) **I:** Number of spirals **J:** Number of continuous paths that are not spirals.



**Figure 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* aggregate near 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 a similar 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.

**Figure S5 (following page): 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**), *Cx. quinquefasciatus* (**E**), and *Cx. 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.

