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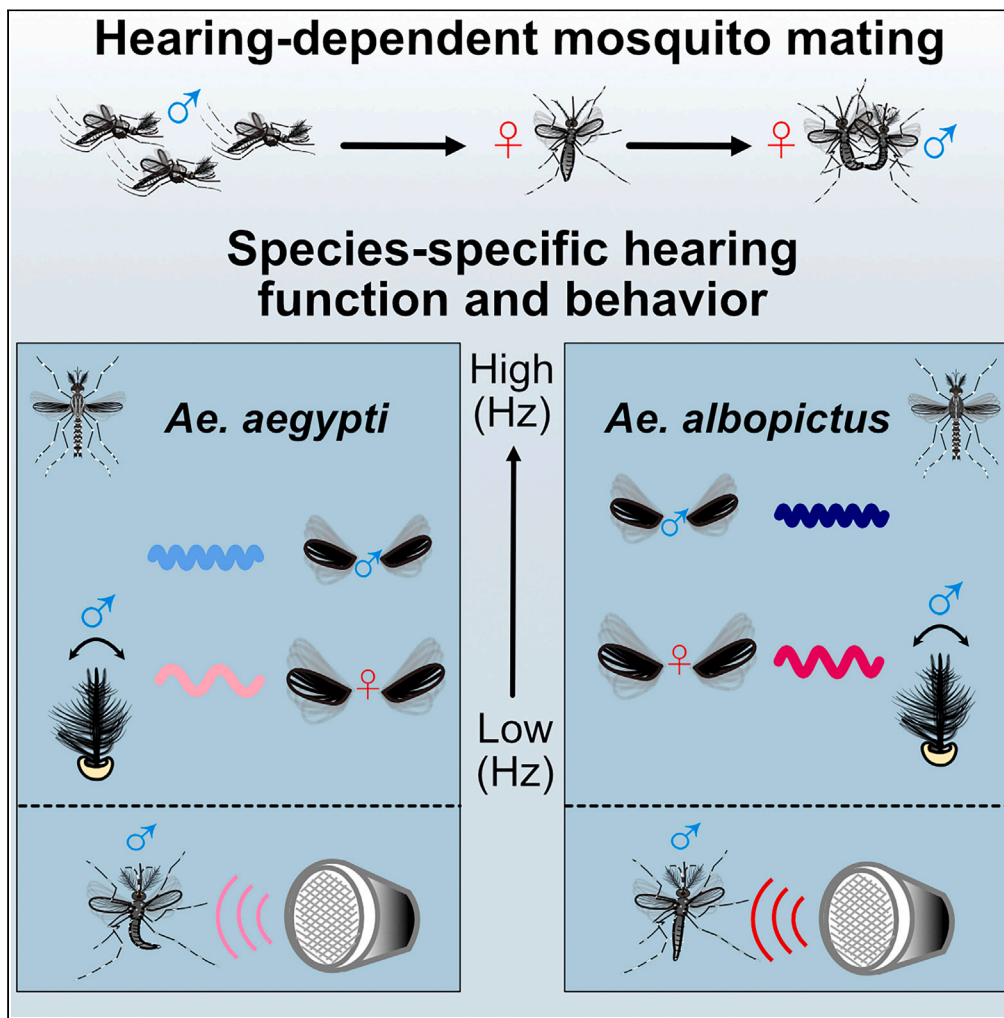
Differences in male *Aedes aegypti* and *Aedes albopictus* hearing systems facilitate recognition of conspecific female flight tones

Hearing-dependent mosquito mating

Species-specific hearing function and behavior

Ae. aegypti

Ae. albopictus

High (Hz)

Low (Hz)

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Highlights

Two sister *Aedes* mosquito species show differences in multiple auditory components

Male ears of each species are tuned to different frequencies of sound

Males from each species show distinct responses to female sounds

Male electrical, but not mechanical, tuning frequency is conserved across species

Article

Differences in male *Aedes aegypti* and *Aedes albopictus* hearing systems facilitate recognition of conspecific female flight tones

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SUMMARY

When *Aedes albopictus* mosquitoes invade regions predominated by *Aedes aegypti*, either the latter can be displaced or the species can coexist, with potential consequences on disease transmission. Males from both species identify females by listening for her flight sounds. Comparing male hearing systems may provide insight into how hearing could prevent interspecific mating. Here, we show that species-specific differences in female wing beat frequencies are reflected in differences in male ear mechanical tuning frequencies and sound response profiles. Though *Aedes albopictus* males are attracted to sound, they do not readily display abdominal bending, unlike *Aedes aegypti*. We observed interspecific differences in male ear mechanical, but not electrical, tuning, suggesting a conserved primary auditory processing pathway. Our work suggests a potential role for hearing in the premating isolation of *Aedes aegypti* and *Aedes albopictus*, with implications for predicting future dynamics in their sympatric relationships and our understanding of mosquito acoustic communication.

INTRODUCTION

Aedes aegypti (*Ae. aegypti*) and *Aedes albopictus* (*Ae. albopictus*) act as the primary and secondary vectors for several major mosquito-borne diseases, including dengue and Zika. The global distributions of both species have expanded rapidly over the past few decades, driven in part by factors such as increased levels of urbanization and climate change.[1] *Ae. albopictus*, though predominantly found in temperate regions, can thrive in a plethora of environmental settings.[1,2] The invasion of *Ae. albopictus* into (tropical and sub-tropical) regions previously dominated by *Ae. aegypti* led to the rapid displacement of *Ae. aegypti* (~1–3 years) in the 1980s throughout much of the southeastern USA, as well as more recently in Bermuda.[3,4] Several lab- and field-based studies suggest that asymmetric reproductive interference favoring *Ae. albopictus* over *Ae. aegypti* could be driving this displacement.[5–9]

Competitive displacement of *Ae. aegypti* may not always be the outcome when the two species meet, with *Ae. albopictus* failing to colonize certain southern cities of USA where *Ae. aegypti* predominates.[8] *Ae. aegypti* and *Ae. albopictus* have also been reported to be in sympatry for more than a few decades, possibly with current population distributions stabilized since the initial invasion of *Ae. albopictus*.[10–12] The driving forces shaping these population dynamics remain unknown, though it has been proposed that avoidance of interspecific mating may have rapidly evolved under the strong selection pressure of reproductive costs associated with heterospecific mating.[8,9,13–15] One way to better understand interspecific mating between *Ae. aegypti* and *Ae. albopictus* is to study their shared precopulatory courtship behaviors.[16]

One such shared courtship behavior is the male attraction to conspecific female flight sounds (male phonotaxis).[17–21] In both species, male and female wing beat frequencies (WBFs) are sexually dimorphic, enabling males to discriminate female flight sounds from their own.[22] Interspecific differences in WBFs have previously been identified in *Ae. aegypti* and *Ae. albopictus*, with the latter species displaying higher WBFs for both sexes.[23–28] Interestingly, however, similar studies comparing two *Anopheles* species, *Anopheles gambiae* (*An. gambiae*) and *Anopheles coluzzii* (*An. coluzzii*), did not identify such differences between species.[22] Despite apparent interspecific differences in female *Aedes* WBFs, it remains unclear if corresponding differences in the male hearing systems exist to enable discrimination of combinations of conspecific and heterospecific female flight sounds.

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Mosquito ears comprise a flagellum that deflects in response to airborne vibrations and a Johnston's organ (JO), the site of mechanotransduction.[29] Mosquito hearing systems also contain an auditory efferent system unique among insects.[29] Male ears are highly sensitive, with the flagellum being actively tuned to female WBFs.[30] Spectral matching between the sender's signal (female WBFs) and the receiver's signal recognition properties (male ear mechanical tuning) has been proposed to underlie male phonotaxis, a process that relies heavily on the active hearing process of male ears.[31] Frequency matching between sender and receiver in an acoustic-based communication system has previously been elegantly demonstrated in *Drosophila* (for mechanical tuning), as well as grasshoppers and amphibians.[32–34] Frequency mismatch in such an acoustical coupling could thus disrupt male mosquito phonotaxis.

For mosquitoes that rely on hearing for mating, the male JO neural response (electrical tuning) is not particularly sensitive to frequencies similar to the female WBF; instead, the electrical tuning of the male JO appears most sensitively tuned to the difference between male and female WBFs (referred to as the difference tone).[35–38] Therefore, for *Anopheles*, *Culex*, and *Aedes* males, while overlap exists between male ear mechanical and electrical tuning ranges, male ears are mechanically and electrically most sensitive to distinct frequencies, with unclear underlying molecular mechanisms.[22,35–37,39] Crucially and interestingly, similar estimates of male ear electrical tuning have been found across species.[35] Mismatch in the most sensitive frequencies of male ear mechanical and electrical tuning have prompted researchers to suggest that male ears may instead be detecting the additional tones (distortion products) generated from the non-linear mixing of their own WBFs and conspecific female WBFs to acoustically detect flying females, a phenomenon arising from the non-linearities of the flagellum receiver and referred to as a distortion product (DP) based communication system.[36,37,40,41]

Given the indispensable role of male hearing in mosquito courtship, this sensory system could be involved as part of a premating barrier that prevents interspecific mating between *Ae. aegypti* and *Ae. albopictus*, facilitating the co-existence of both species in specific locations.[42] In this study, we aimed to investigate potential differences in the male hearing systems between *Ae. aegypti* and *Ae. albopictus* to understand how these differences may contribute toward species-specific recognition of female WBFs.

Here, we linked female WBFs to male ear mechanical tuning and male phonotaxis in both species. We found species-specific differences in WBFs, mechanical tuning and phonotaxis, with the relative increase in female *Ae. albopictus* WBFs driving corresponding differences in the latter two components in males. Although male mechanical tuning differed between the two species, electrical tuning was found to be not significantly different when using both large sweep stimulation and while measuring threshold responses. This could imply a conserved primary auditory processing pathway across species. We further found that *Ae. albopictus* males were significantly less likely to bend their abdomen in response to female-like tone stimulation, suggestive of finer interspecific differences in hearing-related behaviors.

RESULTS

Interspecific differences in female WBFs are reflected in male ear mechanical frequencies

First, we reviewed previous reports in which both species were tested and found consistently higher WBFs reported for male and female *Ae. albopictus* as compared to *Ae. aegypti* (Figure S1A; Table S1).[23–28] We calculated average values of 479 Hz for *Ae. aegypti* females compared to 512 Hz for *Ae. albopictus*, and 721 Hz for *Ae. aegypti* males compared to 769 Hz for *Ae. albopictus* males. However, there were significant differences in experimental conditions between these reports, which have been reported to influence mosquito WBFs, hearing function and hearing behaviors.[35,43] Therefore, we measured WBFs in our environmental conditions to enable direct comparisons between female flight sounds, male hearing function, and male phonotaxis responses.

To enable more accurate comparisons between functional and behavioral experiments, we recorded male and female WBFs and ear mechanical tuning of both species in our lab-based settings (Figures 1A and 1B). Female *Ae. albopictus* WBFs were significantly higher than female *Ae. aegypti* WBFs (Welch's t-test; $p = 9.381 \times 10^{-9}$; Table 1; Figure 1C), with means of 566 Hz and 532 Hz. Similarly, male *Ae. albopictus* WBFs were higher than male *Ae. aegypti* WBFs (Welch's t-test; $p = 0.0007857$; Figure S1B; Table 1), with means of 921 Hz and 887 Hz. As wing length has been previously used as a WBF marker,[44] we measured wing lengths from each species and sex. We found that in both sexes *Ae. albopictus* wings were significantly shorter than *Ae. aegypti* wings (Welch's t-tests; $p = 5.414 \times 10^{-7}$ and $p = 4.146 \times 10^{-6}$ for females and males; Figure S1C; Table S2). The shorter wing length of *Ae. albopictus* thus produced a higher WBF in both sexes.

Next, we measured ear mechanical tuning using laser doppler vibrometry (LDV). To estimate the mechanical tuning frequency (the peak frequency at which the ear resonates most), we provided a 1–2000 Hz white noise stimulus and fit a forced-damped oscillator function to the antennal vibrational response data (Figure 1B).[45] Male *Ae. albopictus* ears were found to be mechanically tuned to significantly higher frequencies than male *Ae. aegypti* (Welch's t-test; $p = 0.0004144$ Table 1; Figure 1D), with mean values of 508 Hz and 465 Hz, respectively. In contrast, female *Ae. aegypti* ears were tuned to higher frequencies of sound than *Ae. albopictus* females (Welch's t-test; $p = 0.0002497$; Table 1; Figure S1D), with mean mechanical tuning frequencies of 247 Hz and 231 Hz, respectively.

Taken together, we observed that for both female WBFs and male ear mechanical tuning frequency, *Ae. albopictus* frequency estimates were consistently around 40 Hz higher on average than for *Ae. aegypti*.

Male *Ae. albopictus* respond to higher sound frequencies, while male *Ae. aegypti* are more likely to bend their abdomen and for longer duration in response to sound

Next, using a group-level phonotaxis assay, we quantified male phonotaxis to pure tones for both species to characterize their frequency response profiles (Figure 2A, left).[45]

Both species showed responses to a broad range of pure tones covering 350–850 Hz (normalized frequency response profiles for each species shown in Figure 2B, with bars indicating median of the normalized response per frequency and individual dots representing the
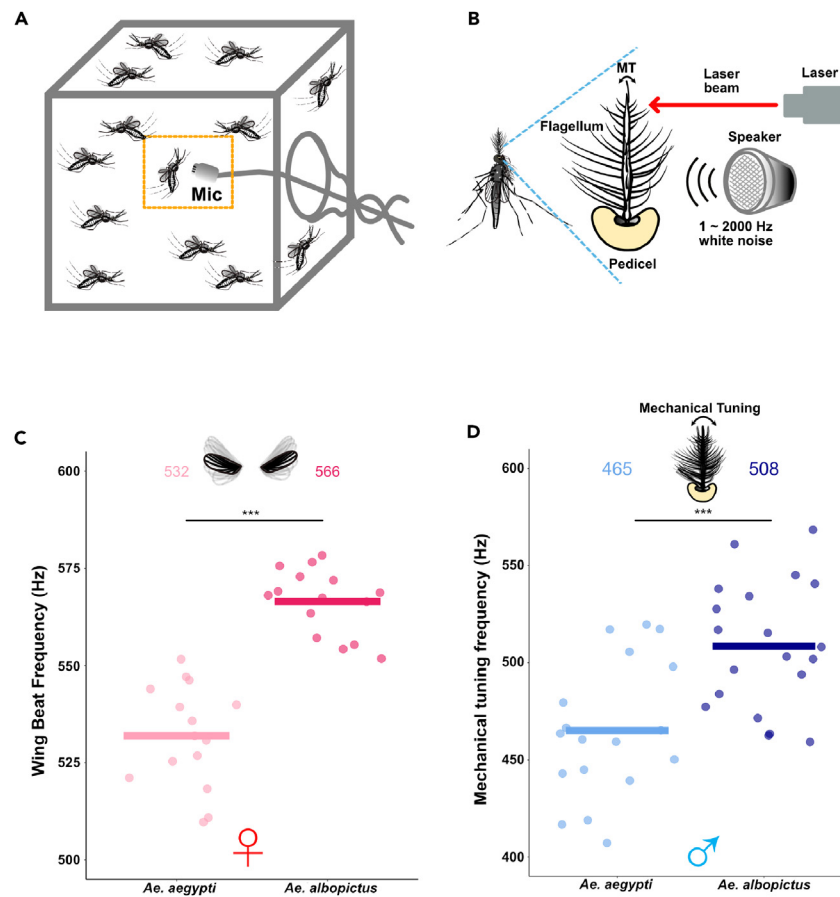
Figure 1. Interspecific differences in female wing beat frequencies and male ear mechanical tuning frequency

(A) WBF measurement assay diagram. Mic, microphone.

(B) LDV assay diagram. MT = mechanical tuning.

(C) WBFs of female *Ae. aegypti* and *Ae. albopictus*, respectively, from this study (Welch's t-test; $p = 9.381 \times 10^{-9}$; $n = 15$ for both groups). Solid lines represent mean values, which are printed at the top of the panel for each group. Individual points represent estimated median WBFs for individual cages. See also Table 1 and Figure S1B. ***, $p < 0.001$.

(D) Mean mechanical tuning frequencies of male *Ae. aegypti* and *Ae. albopictus* respectively during white noise exposure (Welch's t-test; $p = 0.0004144$; $n = 20$ and 17, respectively). Solid lines represent mean values, which are printed at the top of the panel for each group. Individual points represent estimated mechanical tuning frequencies for individual males. See also Table 1 and Figure S1D. ***, $p < 0.001$.

normalized response from a single repeat; individual repeats shown in Figure S2A). However, male *Ae. albopictus* were found to show significantly higher peak phonotaxis response frequencies (estimated from the peak frequency of the loess fit of the frequency response profile from individual repeats) than male *Ae. aegypti* (Welch's t-test; $p = 0.01653$; Table 1; Figure 2C), with mean phonotaxis peak frequencies of 549 Hz and 526 Hz, respectively. We observed no differences in overall phonotactic response level between the species (Welch's t-test; $p = 0.8459$; Figure S2B).

To confirm and deepen our group-level findings, we exposed individually held males to a pure tone of 575 Hz (Figure 2A, right), with a 300 Hz tone played beforehand as a negative control. We scored male phonotaxis as the orientation of males toward the sound source followed by subsequent bending of the abdomen, mimicking copulation.[46] Although at the group level (Figure 2B), males of both species showed attraction to 575 Hz, at an individual level (Table 1) significantly fewer *Ae. albopictus* males showed abdominal bending in response to the pure tone than *Ae. aegypti* (Fisher exact test; $p = 1.562 \times 10^{-7}$; Table 1), with only 22.6% of males showing a bending response in comparison to 87.5% of *Ae. aegypti* males. *Ae. albopictus* males that demonstrated bending behavior did so for a shorter duration than *Ae. aegypti* males (Wilcoxon rank-sum test; $p < 0.0004349$; Figure 2D). No male from either species showed attraction to the 300 Hz tone.

Lack of obvious interspecific differences in the ear gross anatomy of both sexes

We next attempted to find structural differences in the efferent system of both species by conducting immunohistochemistry to visualize the gross ear anatomy of both sexes. The male auditory efferent system is extensive, with numerous pre-synapses identified within the JO to

Table 1. Differences in WBF, hearing function and hearing behaviors between *Ae. aegypti* and *Ae. albopictus* male and female mosquitoes

Group	Mean WBF (Hz)	Mean ear mechanical tuning frequency (Hz)	Mean peak phonotaxis response frequency (Hz)	Percentage of responders to 575 Hz tone (%)	Median abdominal bending duration (s)
Female *Ae. aegypti*	531.9 ± 13.1 (n = 15)	247.0 ± 11.6 (n = 21)	–	–	–
Female *Ae. albopictus*	566.5 ± 8.4 (n = 15)	231.0 ± 14.30 (n = 22)	–	–	–
Male *Ae. aegypti*	887.0 ± 24.9 (n = 17)	465.1 ± 35.2 (n = 18)	525.9 ± 22.6 (n = 10)	87.5 (n = 32)	26 ± 1.1 (n = 28)
Male *Ae. albopictus*	921.0 ± 31.2 (n = 20)	508.4 ± 33.0 (n = 20)	548.7 ± 14.6 (n = 10)	22.6 (n = 31)	15 ± 2.3 (n = 7)

Mean WBF and ear mechanical tuning frequency (in response to white noise stimulation) for male and female *Ae. aegypti* and *Ae. albopictus*, as well as mean peak phonotaxis response frequency for group level phonotaxis and individual level phonotaxis data (percentage of responders and median abdominal bending duration) for male *Ae. aegypti* and *Ae. albopictus*. Estimated mean/median and standard deviations/standard error are shown, with sample sizes given in brackets.

facilitate information transfer from the brain to the ear (Figure 2E). Counter-staining male ear sections with anti-synapsin to localize pre-synapses within the ear found no obvious differences in pre-synaptic site distributions between species (Figure 2F). Equivalent staining of female ears similarly found no clear differences between species (Figures S2C and S2D), though sex-specific differences were still apparent.

Lack of interspecific differences in the antennal nerve electrical tuning of male Johnston's organ

Next, we measured sound-evoked responses from the male antennal nerve to test for interspecific differences in electrical tuning (Figure 3A). By providing large magnitude sweep stimuli varying from 1 to 1000 Hz to the male ears, we calculated peak mechanical and electrical tuning frequencies for both species. Male ear mechanical tuning was once again tested to reconfirm our previous finding (Figure 1D) using a different assay with a distinct stimulus.

While we reconfirmed differences between male *Ae. aegypti* and *Ae. albopictus* at the mechanical tuning level (Welch's t-test; $p = 0.01984$; Figure 3B left), we found no interspecific differences in terms of electrical tuning frequency (Welch's t-test; $p > 0.05$; Figure 3B right). In all males from both species, mechanical tuning frequency was consistently higher than electrical tuning frequency (Welch's t-tests; $p = 7.335 \times 10^{-7}$ and $p = 1.107 \times 10^{-7}$, respectively; Figure S3A).

Mosquito ears have been reported to show both mechanical and electrical level dependent tuning.[36,37] We therefore next tested for differences in frequency tuning between the species at the threshold level. We provided mosquito ears with individual pure tone stimulation ranging from 225 to 625 Hz (in 25 Hz steps) at different attenuations to identify for each frequency the minimum stimulus magnitude necessary to elicit a significant electrical response. Once again, we found no interspecific differences in electrical tuning frequency (Welch's t-test; $p > 0.05$; Figures 3C and 3D), though the tuning frequency for males from both species was significantly greater than that calculated using large sweep stimuli (Welch's t-tests; $p = 0.02852$ and $p = 0.005676$ for male *Ae. aegypti* and *Ae. albopictus*, respectively).

DISCUSSION

Species-specific male hearing system in-tunes to conspecific female WBFs

One way to better understand interspecific mating between *Ae. aegypti* and *Ae. albopictus* is to study their shared precopulatory courtship behaviors, such as male phonotaxis.[16] Corroborating published literature (Figure S1A),[23–28] our results suggest that female *Ae. albopictus* display higher WBFs compared to *Ae. aegypti*, potentially attributed to their smaller wing lengths (Figure S1C).

Ae. albopictus male peak phonotaxis response frequencies matched the higher WBFs of their conspecific females, with a difference of 23 Hz on average between species for male phonotaxis, and 34 Hz on average for female WBFs. This direction and approximate magnitude of change corresponds to the higher ear mechanical tuning frequency of male *Ae. albopictus*, with a difference of 43 Hz on average between heterospecific males. This demonstrates clear interspecific differences across multiple auditory components between the two *Aedes* species.

Frequency matching between sender and receiver has been identified in the acoustic communication systems of other insect species.[32–34] At a molecular level, interspecific differences in ear mechanical tuning could be driven by changes in the mechanotransducers which underlie active hearing, though further molecular and functional work is needed.[34] Within a species, the lower male ear mechanical tuning frequency compared to the estimated female WBFs and male phonotaxis peak frequency could potentially be attributed to the stronger stimulus magnitude we provided when stimulating the ear, a level-dependent mechanical tuning property that has previously been demonstrated across many mosquito species.[34,36] As the magnitude of stimulus increases, a reduction in ear mechanical tuning frequency of about 40% was previously observed in *Ae. aegypti* males.[36] We also saw a reduction in ear electrical tuning when comparing our large magnitude sweep stimulus data to our threshold level recordings (of ~40 Hz), in agreement with previous reports.[37]

A male's capability to stay in tune to conspecific female WBFs and accurately discriminate them from heterospecific female sounds could be a favorable trait to select for given the negative costs associated with heterospecific mating.[9] Our results suggest that species-specific
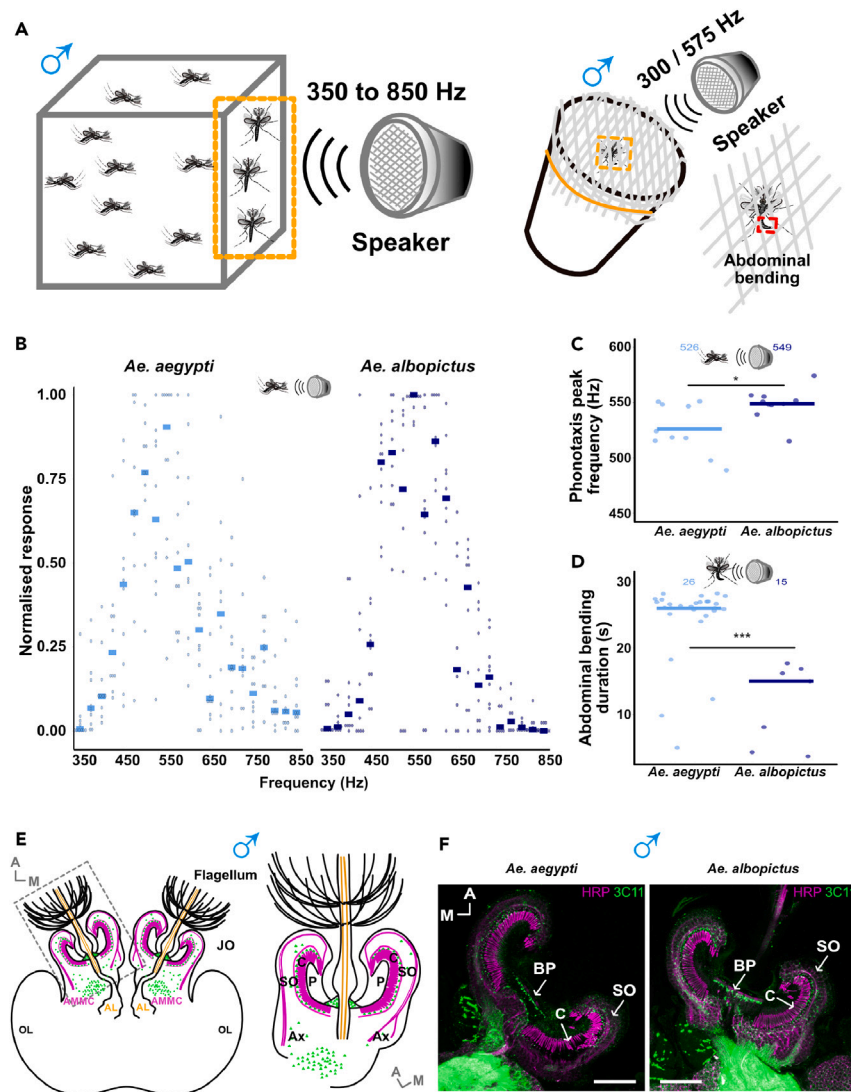
Figure 2. Interspecific differences in male behavioral response to sounds at both group and individual levels and conserved neuroanatomical structures of male ears

(A) Group/cage (left) and individual/cup (right) level phonotaxis assay diagram.

(B) Median normalized frequency response profiles of male *Ae. aegypti* and *Ae. albopictus*, respectively, in the group level phonotaxis assay with males presented with pure tones of different frequencies. Bars represent median normalized response per frequency across all repeats. Individual points represent individual repeat normalized frequency responses.

(C) Phonotaxis peak frequencies (peak frequencies estimated from the loess fits to individual normalized frequency response profiles in Figure 2B) of male *Ae. aegypti* and *Ae. albopictus*, respectively (Welch's t-test; $p = 0.01653$; $n = 10$ for both groups). Solid lines represent mean values, which are printed at the top of the panel for each group. Individual points represent estimated peak frequency responses for individual cages.

See also Table 1 and Figure S2A. *, $p < 0.05$.

(D) Abdominal bending durations for male *Ae. aegypti* or *Ae. albopictus* which showed abdominal bending in response to 575 Hz stimulation (Wilcoxon rank-sum test; $p < 0.0004349$; $n = 28$ and 7, respectively). Solid lines represent median values, which are printed at the top of the panel for each group. Individual points represent bending durations for individual males.

See also Table 1. Note that males that did not show abdominal bending were not included in this bending duration analysis. ***, $p < 0.001$.

(E) Schematic of male ear (flagellum and JO) and brain. (Left) flagellar neurons (orange) project to the antennal lobe (AL), while JO neurons (magenta) project to the antennal mechanosensory and motor center (AMMC). AL, antennal lobe; A, anterior; AMMC, antennal mechanosensory and motor center; JO, Johnston's organ; M, medial; OL, optical lobe. (Right) The location of presynaptic sites within the JO is represented as green dots. Ax, axons; C, cilia; P, prongs; SO, somata.

(F) Male *Ae. aegypti* and *Ae. albopictus* JO anatomy visualized using immunohistochemistry. JOs were stained with anti-synapsin 3C11 (green) and anti-HRP (magenta). A, anterior; BP, basal plate; C, cilium; M, medial; SO, somata. Scale bar, 50 μm.
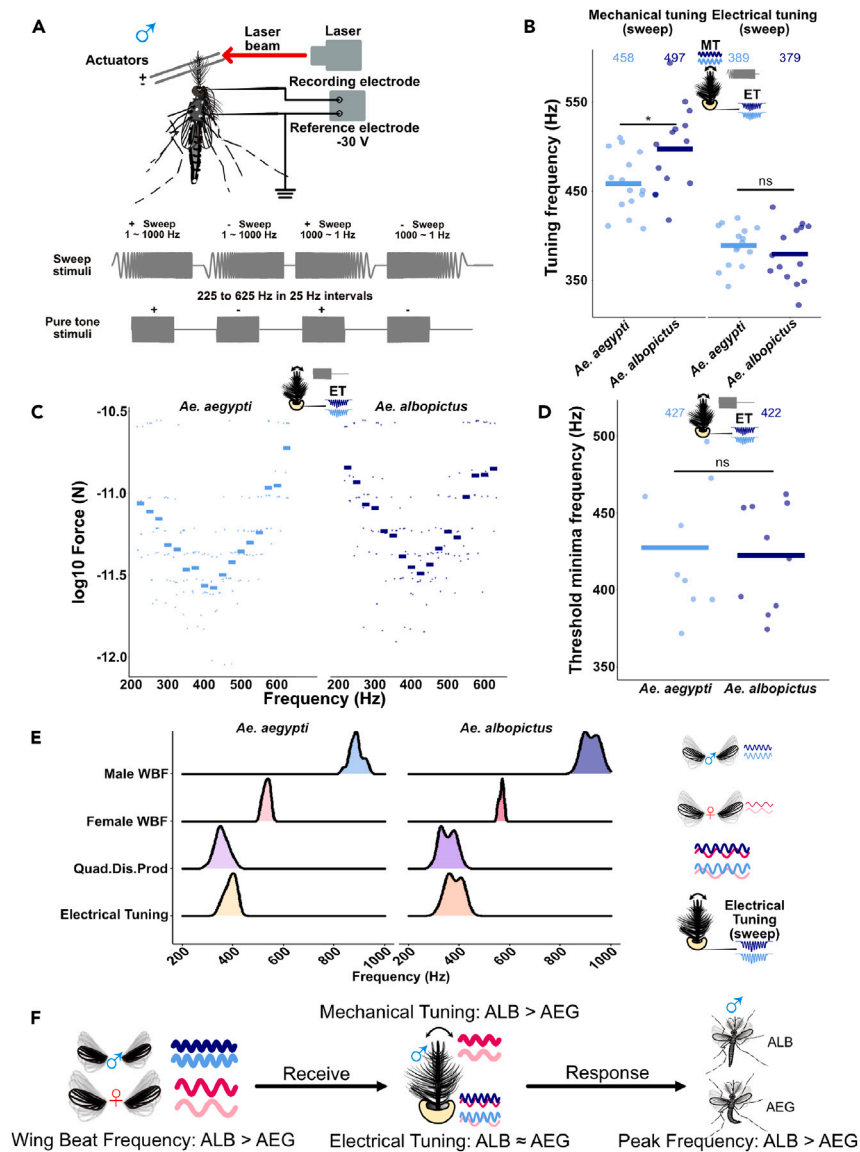
Figure 3. Lack of interspecific differences in male ear electrical tuning

(A) Electrophysiology assay diagram.

(B) Tuning frequencies of male *Ae. aegypti* and *Ae. albopictus* ears in response to large stimulus (Welch's t-tests; $p = 0.01984$ and $p > 0.05$; $n = 15$ and 14, respectively). Solid lines represent mean values of (left) mechanical tuning (MT) and (right) electrical tuning (ET) frequencies, which are printed at the top of the panel for each group. Individual points represent tuning frequencies for individual males.

See also Figure S3A and Table S3. For analysis pipeline of sweep stimuli electrophysiological recordings.

See Figure S3B. ns, $p > 0.05$; *, $p < 0.05$.

(C) Threshold electrical responses of male *Ae. aegypti* and *Ae. albopictus* ears across a range of pure tone frequencies. Bars represent mean minimum force necessary to elicit significant response per frequency across all repeats. Individual points represent individual repeat data. For analysis pipeline of pure tone stimuli electrophysiological recordings.

See Figure S3C.

(D) Estimated minima threshold frequencies for male *Ae. aegypti* and *Ae. albopictus* ears (Welch's t-test; $p > 0.05$; $n = 9$ and 10, respectively). Solid lines represent mean frequencies, which are printed at the top of the panel for each group. Individual points represent frequencies for individual males.

See also Table S3. ns, $p > 0.05$.

(E) Male ear electrical tuning (ET) overlaps with predicted QDP frequency ranges for both male *Ae. aegypti* and *Ae. albopictus*. Density plots based on data collected here showing male WBF, female WBF, predicted quadratic distortion product (QDP; male WBF—female WBF) and ET frequency of the male ear, for *Ae. aegypti* and *Ae. albopictus*, respectively. WBF, wing beat frequency; Quad. Dis. Prod, quadratic distortion product.

(F) Differences in female WBFs drive separation in the male hearing system across species. Schematic diagram summarising the inter-species differences between *Ae. aegypti* and *Ae. albopictus* in terms of male and female WBFs, male ear mechanical tuning frequency and male phonotaxis peak frequency with

Figure 3. Continued

Ae. albopictus males displaying higher frequencies for all these components compared to *Ae. aegypti*. Interspecific differences were not found at the male ear electrical tuning level. The overlap of two waveforms at the electrical tuning level represents the non-linear interference of two tones (male WBF and female WBF). Note that abdominal bending behavior in response to female-like sound stimulation was more readily observed in *Ae. aegypti* than *Ae. albopictus*, at an individual level. AEG, *Ae. aegypti*; ALB, *Ae. albopictus*.

selectivity of mosquito hearing systems may influence premating barriers by sharpening the mechanical tuning of male ears to conspecific female WBFs. The evolution of reproductive character displacement could be important considering the chances of encountering a mate in the wild and the energy costs associated with mating, potentially prompting males of either species to reduce their likelihood of cross-inseminating heterospecific females.[47–51] The rapid evolution of satyrization resistance in *Ae. aegypti* females has recently been observed under a lab-based setting.[8,9,13,14] It is possible that equivalent reproductive character displacement could also co-evolve on the male side (via modulating their hearing systems) to ensure reproductive success in the wild.[52]

Interspecific differences in female tone-induced precopulatory behavior

Mosquito courtship consists of a number of distinct components, including several acoustic related behaviors. One such precopulatory acoustic interaction between male and female mosquitoes, rapid frequency modulation (RFM), has previously been observed in multiple mosquito species, including the major disease vectors *Culex quinquefasciatus*,[53] *An. gambiae* and *An. coluzzii*,[54] and *Ae. aegypti*.[55] However, the exact role played by RFM, which is characterized by rapid changes in male WBF which can be elicited even by artificial tones, during mosquito courtship remains unclear.[55] Beyond intersexual acoustic interactions between males and females, intrasexual interactions between males have also been reported which may be relevant in the context of male swarming behavior driving mating.[56]

Here, we observed that both *Ae. aegypti* and *Ae. albopictus* males show strong phonotaxis to a range of pure tones mimicking female WBFs. However, closer observations at an individual level found that *Ae. albopictus* males were significantly less likely to show abdominal bending behaviors than *Ae. aegypti* and the bending duration was also significantly shorter. Our findings suggest that abdominal bending is another feature of mosquito precopulatory acoustic behavior, which appears different between species. Furthermore, previous reports claiming *Ae. albopictus* males lack phonotaxis behavior under lab-based settings may have come to those conclusions due to different definitions of phonotaxis.[27,57]

Species-specific differences in courtship rituals and asymmetries in copulatory behavior have been reported extensively in *Drosophila*,[34,58] which possess a rich genetic toolbox for dissecting the central processing of external cues. The differences we observed here for the female tone-induced precopulatory behavior between two *Aedes* species could be related to altered central processing pathways for female acoustic cues, or divergence in the neural circuits responsible for the execution of motor responses.[59]

Distortion product-based acoustic communication system may form the basis of primary auditory signal processing in males from both aedes species

In a DP-based acoustic communication system, the flagellar ear of a flying male which encounters a flying female nearby will detect and resonate strongly at two frequencies: his own WBF and the female's.[35,36] The non-linear interference of these two tones in the male ear will generate additional mathematically predictable distortions whose tones fall within the sensitive frequency range of male ear electrical tuning. To achieve "super distortion" of these two WBFs (creating a maximal magnitude distortion product) requires a male-to-female WBF ratio of 1.5; at this ratio, the quadratic distortion product (QDP: male WBF - female WBF) and the cubic distortion product (CDP: 2 × female WBF - male WBF) should overlap.[35]

Here, the male-to-female WBF ratios based on the WBF estimates are, respectively, 1.67 and 1.62 for *Ae. aegypti* and *Ae. albopictus* (Table S3), similar to previous reports for *Ae. aegypti* and higher than the golden ratio of 1.5.[35] It is worth noting that WBF ratios themselves are not consistent across environmental conditions.[35] Here, we calculated an identical QDP of 355 Hz for both species (Table S3), close to the saturated male ear electrical tuning frequency calculated using large sweep stimulation (*Ae. aegypti*: ~390 Hz, *Ae. albopictus*: ~380 Hz). While there are significant overlaps between QDP estimates and electrical tuning estimated using large sweep stimulation (Figure 3E), these overlaps are not complete, potentially resulting from variations in environmental conditions between assays. Furthermore, the electrical tuning frequency calculated from threshold experiments using pure tones (both species: ~425 Hz) does not overlap greatly with QDP estimates, potentially implying the overlap occurs only when males are close enough to females for their flight tones to be of sufficient magnitude.

Despite differences at the male ear mechanical tuning level between species, the similar predictions of QDP and male ear electrical tuning frequency (calculated using large sweep stimulation) between species suggest that while differences in male ear mechanical tuning are essential in tuning into and amplifying conspecific female WBFs, the resulting primary auditory signal generated remains conserved across species. Collectively, our findings support a DP-based acoustic communication system in these two *Aedes* species, with future work now needed to investigate male auditory responses in the primary auditory center in the brain, the antennal mechanosensory and motor center (AMMC).[45,58]

Profiling distinct mosquito hearing properties may shed light on understanding the importance of these hearing systems

Ae. aegypti and *Ae. albopictus* exhibit similar circadian activity patterns, meaning their activity profiles overlap throughout the day.[60] The high ecological plasticity and behavioral adaptability of both species also mean that sharing of ecological habitats and niches could occur in the

field.[61,62] Such species-interactions are linked to the displacement of *Ae. aegypti* by *Ae. albopictus*, a phenomenon observed in many regions worldwide, with exceptions in certain regions.[7] The mechanisms giving rise to such unidirectional displacement, and in some cases, region-specific displacement remain unclear.[7]

Apart from interspecific differences at the male ear mechanical tuning level, we also observed a significant, albeit small, interspecific difference in female ear mechanical tuning frequency (Table 1; Figure S1D). The direction of difference is the opposite of males, with female *Ae. aegypti* ears displaying a higher mechanical tuning frequency than *Ae. albopictus*. Species-specific differences in female ear mechanical tuning frequency were previously observed between *Aedes*, *Culex*, and *Anopheles*, though their biological significance warrants further investigation.[36] Females could potentially use hearing to detect predator flight tones, a behavior also identified in males, suggesting potential conservation of some hearing mechanisms between sexes.[63–66] Previous reports have also suggested that female *Anopheles albimanus* may select males during flight and alter their WBFs to reject undesirable males, a process potentially involving hearing.[67]

Furthermore, at a macroscopic level, we observed a lack of interspecific differences in the auditory efferent system for both sexes (though the extent of sexual dimorphisms remains conserved across species), thus the potential implication of the auditory system in defining species-specific properties of mosquito hearing function still warrants further investigation.[29,36,68]

Our work suggests that future acoustic-based mosquito control tools such as sound lures may need to account for species-specific variations in hearing systems to achieve maximal capture efficiency in the field, by tailoring acoustic stimuli to the target species. In the future, broader cross-species comparisons could provide insights into the evolutionary processes that shape the complex and diverse hearing systems of different mosquito species, with species-specific differences possibly contributing to speciation. Despite the clear species-specific separation observed across different auditory components, such differences were not found at the male ear electrical tuning level (Figure 3F), potentially indicating strong conservation of primary auditory processing pathways in males across mosquito species.

Limitations of the study

Previous research into the auditory processing pathway in *Drosophila melanogaster* has identified multiple stepwise neural circuits innervating distinct brain regions.[58,69] Our electrophysiological experiments suggest a potentially conserved primary auditory processing pathway between males of both species. However, further investigations via different approaches such as calcium-imaging, are highly important to lend support for our current findings and hypotheses. We also recorded only compound responses to stimulation, rather than collecting recordings from individual neurons in the JO, which may have allowed for greater resolution of peak responses.

In-depth exploration of central processing will be vital for determining differences in downstream behavioral outputs such as male abdominal bending in response to acoustic stimulation. Furthermore, the strains of *Ae. aegypti* and *Ae. albopictus* tested here have been kept as lab colonies for numerous generations without interspecific sexual selection pressures. The influence of these pressures on mosquito hearing systems as such remains to be tested.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.M.L., Y.YJ.X., M.P.S., and A.K.; methodology, Y.M.L., Y.YJ.X., T.T.L., T.S.O., Y.D.Z., D.F.E., and M.P.S.; investigation, Y.M.L., Y.YJ.X., M.P.S., and A.K.; resources, D.F.E., M.P.S., and A.K.; writing, Y.M.L., Y.YJ.X., D.F.E., M.P.S., and A.K.; funding acquisition, D.F.E., M.P.S., and A.K.; supervision, M.P.S. and A.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-SYNORF1 3C11	Developmental Studies Hybridoma Bank	RRID: AB_528479
Cy™3 AffiniPure™ Goat Anti-Horseradish Peroxidase	Jackson Immuno Research	RRID: AB_2338959
Goat anti-mouse IgG Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher	#A-11029; RRID: AB_2534088
Deposited data		
Wing Beat Frequency data	This paper	Mendeley Data: https://doi.org/10.17632/g79w8wxpr7.1
Laser Doppler vibrometry data	This paper	Mendeley Data: https://doi.org/10.17632/g79w8wxpr7.1
Immunohistochemistry data	This paper	Mendeley Data: https://doi.org/10.17632/g79w8wxpr7.1
Electrophysiology data	This paper	Mendeley Data: https://doi.org/10.17632/g79w8wxpr7.1
Wing length data	This paper	Mendeley Data: https://doi.org/10.17632/g79w8wxpr7.1
Phonotaxis video data	This paper	Shared upon request by the lead contact
Experimental models: Organisms/strains		
Aedes aegypti (Higgs strain)	Li et al.[70]	N/A
Aedes albopictus (Foshan strain)	Liu et al.[71]	N/A
Software and algorithms		
Audacity (version 3.0.3)	Audacity Team	https://www.audacityteam.org/
IC Capture – Image Acquisition (version 2.5)	The Imaging Source	https://www.theimagingsource.com/en-us/support/download/
ImageJ (version 1.54g)	ImageJ[72]	https://imagej.net/ij/
Picoscope (version 6.14)	Pico Technology	https://www.picotech.com/
VibSoft (version 6.0)	Polytec	https://www.polytec.com
R (version 4.3.1)	R Core Team[73]	https://www.R-project.org/
Spike2 (version 10.08)	Cambridge Electronic Design	https://ced.co.uk/products/spkovin

RESOURCE AVAILABILITY

Lead contact

Further information on all experiments conducted as part of this report and requests for resources should be directed to and will be fulfilled by the Lead Contact, Matthew P. Su (su.matthew.paul.y3@f.mail.nagoya-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All original, unprocessed Wing Beat Frequency, laser Doppler vibometry, immunohistochemistry, electrophysiology and wing length data used for this study has been deposited at Mendeley Data. The relevant accession number is listed in the key resources table and all data is publicly available as of the date of publication. Phonotaxis data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Additional information regarding analysis protocols/data collection is available via from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mosquitoes

Ae. aegypti (Higgs strain) and *Ae. albopictus* (Foshan strain) mosquitoes were reared at 28°C and 60–70% relative humidity in a light, temperature and humidity-controlled insectary under a 12 h:12 h Light:Dark [LD] cycle. Larvae were provided with fish food throughout larval stages and pupae were sexed to ensure adult virgin status. Adults were provided with 10% glucose water as a food source. Unless otherwise noted, experiments used virgin adult female or male mosquitoes from both species aged 2–3 days old at the start of experimental entrainment period (2 days of entrainment). Male mosquitoes from both species were used for all types of experiment conducted. Female mosquitoes from both species were used for all types of experiment conducted apart from phonotaxis and electrophysiology assays.

METHOD DETAILS

Ae. aegypti and *Ae. albopictus* WBF and male phonotaxis literature search

Electronic literature databases were utilized to search for publications related to *Ae. aegypti* and *Ae. albopictus* WBFs of either sex. The search was conducted during November 2023. Pubmed was used to search for peer-reviewed publications while gray literature sources were identified from Scopus, Web of Science, Google Scholar and OpenGrey. Non-peer reviewed articles identified from bioRxiv were also included in this review. For WBFs, the following search terms were used in combination with the appropriate Boolean Operators and truncated form of each keyword: "flight tone", "wing beat", "sound", "frequency", "mosquito", "aedes", "aedes aegypti" and "aedes albopictus". For male phonotaxis, search terms: "phonotaxis", "attraction", "sound", "acoustic", "trap", "lure", "mosquito", "aedes", "aedes aegypti" and "aedes albopictus" were utilized. Article duplicates identified from different electronic databases were removed.

To allow for quantitative comparison, data were extracted only from publications that simultaneously reported WBFs of both species under the same conditions. In total, 6 and 3 matching papers were identified that reported on female and male WBFs, respectively.

Due to the scarcity of studies that simultaneously studied male phonotaxis of both species under the same conditions, data from identified papers were not extracted numerically, but their findings were highlighted throughout the study.

Experiment entrainment paradigm

2-3 day old adult female or male mosquitoes were entrained for 2 days in incubators at $28 \pm 1°C$ under a 12:12 LD regime, except for the cage phonotaxis assay where a 13:11 LD regime with 1 h of light ramping at the beginning and end of each scotophase was adopted. On the third day of entrainment mosquitoes were tested between Zeitgeber Time [ZT] 11 – ZT13, corresponding to the 'dusk' phase of their entrainment. 10% glucose was provided *ad libitum* to mosquitoes throughout this time. All experiments were either carried out in the entrainment incubator, or performed in temperature-controlled room, at 28°C ($\pm 1°C$).

Wing beat frequency measurements

Cages containing either 50 virgin male or female mosquitoes were entrained as described above. During the 30 min prior to lights off (ZT 11.5) on the third day of entrainment, a microphone (EK series, Knowles) was inserted into the cages and 30-min recordings were made of mosquito flight sounds using a Picoscope 2408B and the Picoscope 6.14 software (Pico Technology) at a 50 kHz sampling rate (Figure 1B).[74] All recordings were conducted at $28 \pm 1°C$ as the entrainment temperature. Recordings were analyzed using the simbaR package[35] in R, with only flight recordings longer than 150 ms included in the final analysis.

In total, 15 female and 20 male *Ae. aegypti* cages, as well as 15 female and 18 male *Ae. albopictus* cages were analyzed.

Wing length measurements

5-6 day old virgin adult mosquitoes were sedated on ice, before having one wing removed. Wings were mounted on a slide glass and imaged under a confocal microscope (FV3000) using a 2× lens (UPLSAPO2x) with 2-fold zoom magnification. Wing length of each individual mosquito were then estimated using ImageJ 1.54g.[72] Wing lengths were measured as the distance from the distal side of the alula to the axillary incision site.[75]

Wing measurements were collected for 25 female and 35 male *Ae. aegypti* and 29 female and 33 male *Ae. albopictus*.

Laser doppler Vibrometry: Recordings

Entrained mosquitoes were sedated on ice before the dorsal side of their abdomens were glued to the tip of a plastic rod between ZT 11 – ZT 13. Glue (Norland Products Inc., 81) was applied to the entirety of the mosquito body (avoiding spiracles), as well as the neck, to reduce movement as much as possible. The right flagellum of the mosquito was glued to the head while the left flagellum was left intact. A small blob of glue was finally applied in between the pedicels to minimise movement of the antennae.

The mosquito was transferred to the recording room preset at $28 \pm 1.5°C$. For recording, the mosquito was held in a micromanipulator placed on a vibration isolation table. The mosquito flagellum was positioned perpendicular to the laser beam emitted from a laser Doppler vibrometer (Vibroflex, Polytec) placed on the same vibration isolation table. Stimulated recordings were made by measuring the mechanical vibrational response of the mosquito flagellum to white-noise (1–2000 Hz, made in Audacity 3.0.3) playback provided by a speaker (FF85WK,

Fostex) placed 1.5 cm from the mosquito. Speaker playback was calibrated to a Particle Velocity Level [PVL] of $1.58 \times 10^{-4}\,ms^{-1}$, equivalent to 70dB Sound Pressure Level [SPL] following conversion via the formula provided in Menda et al.[63] The second flagellomere from the tip was chosen as the laser focal point for the males while the third from the tip was chosen for the females. All data collection was performed using the VibSoft 6.0 software (Polytec).

Laser doppler Vibrometry: Data analysis

Fast Fourier transformation of the raw time-domain data were conducted in the VibSoft software (Polytec) for frequencies between 1 Hz and 10 kHz, with frequency values below 125 Hz and above 2000 Hz excluded from further analyses due to significant noise levels in our recording system.

A forced damped oscillator function was fit to all transformed data using the lme4 package (version 1.1–33) in R (version 4.3.1):

$$\dot{X}(\omega) = \frac{F_0/m}{\sqrt{\omega^2 \cdot \left(\left(\omega_0^2 - \omega^2 \right)^2 + \left(\omega \cdot \frac{\omega_0}{Q} \right)^2 \right)}}$$

With F_0 = external force strength, m = flagellar apparent mass, ω = angular frequency, ω_0 = natural angular frequency and Q = quality factor = $m\omega_0/\gamma$ (γ = damping constant).

This function enabled calculation of the natural angular frequency (ω_0) of each individual recording, thereby allowing for the estimation of the flagellar best frequency, f_0 (as $f_0 = \omega_0/2\pi$), hereafter referred to as the flagellar ear mechanical tuning frequency.[36,45]

In total, 21 female and 18 male *Ae. aegypti*, as well as 22 female and 20 male *Ae. albopictus* recordings were made and analyzed.

Group level phonotaxis paradigm

Groups of 50 virgin male mosquitoes were aspirated into $30 \times 30 \times 30$ cm insect cages (4S3030 Insect Rearing Cages, Bugdorm) and entrained at $28 \pm 1°C$ under a 13:11 LD regime (Figure 2A). A speaker (FF225WK, Fostex) was placed next to the cage for pure tone playback for each pure tone (generated in Audacity 3.0.3). Speaker playback was again calibrated to a PVL of $1.58 \times 10^{-4}\,ms^{-1}$, equivalent to 70dB SPL following conversion.[63] During dusk (ZT 12.5) on the third day of their entrainment, males in the incubator were exposed to pure tones of 1-min length each (with a 1-min gap between playbacks). Tones ranging in frequency from 350 to 850 Hz in 25 Hz steps were provided in a pseudo-randomised order. The sample function in R[73] was utilised to randomise the playback tone order for each repeat. Male phonotactic response to pure tones were recorded with a video camera (DMK33UP1300, ARGO) using IC Capture – Image Acquisition software (version 2.5) for offline analysis.

Video analyses were conducted via manual counting of males which landed on the netting directly next to the speaker during playback of each tone. The number of males already resting on the netting before tone playback was then subtracted from this count. Counting was conducted while blinded to the specific frequency order for each repeat. Normalization of counts within each repeat was conducted to reduce the variations in overall activity level between different repeats. To estimate peak phonotaxis response frequency, a loess fit with a 1 Hz resolution was applied to the raw counts for each individual repeat and the peak phonotaxis frequency was estimated as the frequency corresponding to the highest point of the loess fit. The Area Under the Curve (AUC) for the raw count data were estimated using the AUC function (DescTools package) in R.[76]

In total, data from 10 male *Ae. aegypti* and 10 male *Ae. albopictus* cages was analyzed.

Individual level phonotaxis paradigm

Males entrained in groups for two full days were aspirated into individual cups prior to dusk (ZT 12) on the third day of their entrainment. Individual level phonotaxis assay was conducted in a temperature-controlled room at $28 \pm 1.5°C$. Specifically, an attractive tone of 575 Hz was provided to each individual stationary male for 30 s by a speaker (FF85WK, Fostex). Speaker playback was calibrated to a PVL of $5.0 \times 10^{-4}\,ms^{-1}$, equivalent to 80dB SPL following conversion.[63] As a negative control, a 300 Hz pure tone was provided to each male prior to the 575 Hz stimulation. A video camera (GoPro) was used to record all behavioral responses for later analysis.[46,74]

Male responses to sound were scored as either 0 (did not approach sound source or approached sound source but did not display abdominal bending) or 1 (approached sound source and demonstrated abdominal bending). If a male displayed abdominal bending, the bending duration was measured via watching video playback.

In total, 32 male *Ae. aegypti* and 31 male *Ae. albopictus* were analyzed.

JO immunohistochemistry

Following previous protocols,[45] male and female mosquitoes were sedated on ice and their heads removed and fixed for 1 h in 4% paraformaldehyde [PFA] in Phosphate-buffered saline [PBS] with 0.25% Triton X-100 [PBT]. Fixed heads were next embedded in 6% albumin solution. Albumin blocks were kept at 4°C for 10 min prior to overnight fixation in 4% PFA at 4°C. On the following day, albumin blocks were washed in 100% methanol for 10 min, then transferred to PBS for 30 min before being sectioned using a vibratome (Leica VT1200S).

40 μm thick sections were washed in PBT containing 0.5% Triton X-100 three times at room temperature followed by blocking in a 10% normal goat serum [NGS] (Vector Laboratories, Inc.)/PBT solution for 1 h. After blocking, the sections were incubated at 4°C with primary antibodies in 10% NGS overnight.

Samples were washed three times with 0.5% PBT before incubation at room temperature with secondary antibodies in 10% NGS for 2 h. Samples were then washed three times with PBT, then once with PBS. Sections were transferred to microscope slides and imaged with a laser-scanning confocal microscope (FV3000, Olympus) equipped with a 20× air objective (UPlanSApo, NA = 0.75) or silicone-oil immersion 30× or 60× Plan-Apochromat objective lenses (UPlanSApo, NA = 1.05 and 1.3, respectively).

Anti-SYNORF1 3C11 monoclonal antibody (RRID: AB_528479, 1:30, Developmental Studies Hybridoma Bank (DSHB), University of Iowa) was used as primary antibody. Secondary antibodies used were Alexa Fluor 488-conjugated anti-mouse IgG (#A-11029; RRID: AB_2534088, 1:300, ThermoFisher), and Cy3-conjugated goat anti-horseradish peroxidase (anti-HRP, RRID: AB_2338959, 1:150, Jackson Immuno Research).

Electrophysiology: Sweep stimulus recordings

All electrophysiological recordings were conducted using two-days entrained mosquitoes at ZT 11- ZT 13 of their entrainment circadian time, in a temperature-controlled room set at $28 \pm 1.5°C$. Mosquitoes were mounted as described above for laser Doppler vibrometry experiments and held in a micromanipulator placed on a vibration isolation table.[77,78]

For recordings, an electrolytically sharpened tungsten reference electrode was inserted into the mosquito thorax and the mosquito was charged to -30 V relative to ground. Electrostatic actuators were positioned equidistant around the mosquito flagellum to enable provision of electrostatic stimulation. A tungsten recording electrode was then inserted into the base of the pedicel to allow for electrical recordings from the antennal nerve.[35,36] A laser Doppler vibrometer was focused on the antennal tip to enable simultaneous measurements of flagellar displacement. Prior to sweep stimulation, a calibrated force-step stimulus was provided to each mosquito to calibrate the flagellum displacement to approximately ±3.5 μm via use of a CED1401 (CED). Sweep stimuli, comprising 1-second-long chirps of linearly increasing (forward, 1 to 1000 Hz) or decreasing frequency (backward, 1000 to 1 Hz), were provided to the mosquito (Figure 3B).

Each loop of sweep stimulation consists of 10 sets of sweeps, with each set comprising 4 different sweeps: forward phasic, forward anti-phasic, backward phasic and backward anti-phasic. Between phasic and anti-phasic sweeps of the same frequency orientation, a 0.1 s of silence was provided. A 0.4s of silence was provided between sweep sets. Finally, a 45s of silence were provided between two loops of sweep stimulation. Flagellum displacement and nerve recording data, as well as laser quality data, were recorded simultaneously using the Spike2 software 10.08 (CED). Laser and stimulus channels were recorded at 100 kHz resolution, while the nerve channel was recorded at 20 kHz resolution.

Electrophysiology: Sweep stimulus data analysis

For flagellar ear mechanical tuning analyses, data for which the laser quality data were recorded as zero was excluded, as during these times flagellar displacements could not be measured. A DC remove function with a time constant of 0.01s was applied to the laser channel data, followed by rectification and smoothing with a time constant of 0.0005s to define the envelope of the response. A slope function with 0.1s time constant was then applied, enabling identification of when the channel slope was equal to zero (i.e., the turning point at which the magnitude of the flagellar vibration reached its maximum) (Figure S3B). Calculation of the time at which the slope was equal to zero allowed for estimation of the stimulus frequency at that time, referred to as flagellar ear mechanical tuning (MT) frequency.

For electrical tuning analyses, sequential phasic and anti-phasic nerve responses were first averaged to cancel out artifacts recorded in the nerve channel due to electrostatic actuation (Figure S3B). The resulting nerve signal was then analyzed as for the laser signal; a DC remove was applied, followed by rectification and smooth application, and a final slope function applied to the nerve signal. Identification of the time at which the slope was equal to zero facilitated calculation of the corresponding stimulus frequency, also referred to as ear electrical tuning (ET) frequency.

For the mechanical tuning frequency of an individual ear, the average was first taken between the phasic and anti-phasic sweeps of the same orientation and the median was then taken for these averages. As the laser focus on the mosquito flagellomere could occasionally be lost during the recording, if a frequency difference of more than 50 Hz was observed between the phasic and anti-phasic sweeps or between the forward and backward averages, the mechanical response for such set of sweeps was excluded from the analysis. For electrical tuning frequency, the average between the forward and backward averaged sweeps were first computed and the median was then taken across the averages.

In total, 15 *Ae. aegypti* and 14 *Ae. albopictus* male ear mechanical and electrical recordings were made and analyzed.

Electrophysiology: Threshold (pure tone stimulus) recordings

For pure tone experiments, male mosquitoes were mounted and charged as described above for sweep stimulus experiments. A series of calibrated force-step stimuli was initially provided to each mosquito to calibrate the flagellum displacement to approximately ±3.5 μm and facilitate later force conversion.

Pure tone stimuli, consisting of 500 ms length tones ranging from 225 to 625 Hz in 25 Hz steps, were then provided at different levels of attenuation. Each series of pure tones at each attenuation consisted of 4 sets of phasic and anti-phasic stimuli of a single frequency. Between each stimulus 500 ms of silence was provided, and between each attenuation approximately 1 min of silence was provided.

Electrophysiology: Threshold (pure tone stimulus) data analysis

As for the sweep stimulus analysis, sequential phasic and anti-phasic nerve responses were averaged to cancel out artifacts in the nerve channel resulting from electrostatic actuation (Figure S3C). A DC remove was then applied to the nerve channel, followed by rectification.

The AUC was calculated for the 250 ms prior to stimulus onset, as well as the final 250 ms of stimulus playback, for all stimuli. By testing for significant differences between these values for each frequency, we identified the smallest amplitude stimulus necessary to elicit a significant electrical response for each mosquito. To adjust for outliers, we applied an additional criterion that all stimuli of a greater amplitude for this frequency must also elicit a significant response. Finally, we converted the stimulus unit from Volts to Newtons to facilitate comparisons between individuals.

In total, 9 *Ae. aegypti* and 10 *Ae. albopictus* male recordings were analyzed.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data analysis was conducted using R. Asterisks for statistical significance in figures use the following format; ns, not significant; *, $p < 0.05$; ***, $p < 0.001$.

As Shapiro-Wilk tests for normality found that all WBF, LDV, electrophysiology, wing length and cage phonotaxis data were normally distributed, statistical comparisons for these datasets were made using Welch's t-tests. Differences in abdominal bending responses between species were calculated using Fisher's exact tests. Differences in the abdominal bending duration were calculated using Wilcoxon rank-sum test, as these data was found to be not normally distributed using Shapiro-Wilk tests.