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Behavioral and molecular responses of *Aedes aegypti* to ultrasound

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ABSTRACT

Mosquito-borne infectious diseases cause mortality and global infectious disease burden worldwide. There are several electronic mosquito repellents (EMRs) based on ultrasound have been developed and commercialized to reduce human-mosquito contacts. However, the efficacy of EMRs against mosquitoes is still unclear. In this study, we present experimental evidence that ultrasound of different frequency and sound pressure differentially affects the host-seeking behavior of *Aedes aegypti* females. Behavioral tests were accompanied by molecular experiments to check whether mosquitoes respond to ultrasound and are there any changes in specific mRNA expression. Experiments in bioassays revealed that the ultrasound of 100 kHz frequency and 90–110 dB pressure significantly disrupted CO_2-oriented olfactory behaviors and blocked indoor invasion. Furthermore, a long time (>24 h) exposure to 100 kHz frequency/90 dB pressure of ultrasound decreased attractive behaviors to human skin. At the molecular level, there was no change in expression of odorant receptor co-receptor (*AaOrco*) in ultrasound treated animals, while one of the CO_2 receptor genes, *AaGr3*, and putative hearing-related gene, *AAEL009258*, were down-regulated and up-regulated, respectively. Our study indicates that high frequency (100 kHz) and pressure (90–110 dB) of the ultrasound has repellent effects to olfactory–driven behaviors of mosquitoes.

Introduction

Aedes aegypti L. (Diptera: Culicidae) is a primary vector of dengue virus, a cause of morbidity and mortality in tropical and subtropical areas of the world (Guzman and Harris, 2015; Kurane, 2007). This virus increases recent decades with 3.9 billion people in 128 countries at risk for infection and 390 million people infected annually (Brady et al., 2012). *Ae. aegypti* also is a vector of yellow fever, chikungunya and zika virus (Epelboin et al., 2017). Global climate change, travel, and migration cause the expansion of *Ae. aegypti* resulting in increased public health threat (Liu-Helmersson et al., 2014). There are several environmental, biological, chemical and electronic methods to reduce human-mosquito contacts (Enayati et al., 2007; World Malaria Report, 2018). Strong demand for personal anti-mosquito equipment is coupled with a supply of chemical and electronic mosquito repellents (EMRs) (Enayati et al., 2007; Tavares et al., 2018). Although chemical repellents demonstrate a comparatively strong anti-mosquito effect (Tavares et al., 2018), they also have a side effect on human health, apparel, and architecture (Barradas et al., 2013; The Medical Letter, 2016). EMRs, in contrast, are a more convenient, safe and eco-friendly method of mosquito control (Enayati et al., 2007; Okorie et al., 2015). However, the efficacy of ultrasound-based EMRs, has not been verified.

Ultrasound is a sound waves with frequencies higher than 20 kHz that are not audible to the human (Heffner and Heffner, 2007). Mosquitoes auditory organ is well described (Albert and Kozlov, 2016; Belton, 1994; Nadrowski et al., 2011; Na et al., 2016; Römer, 2018) and it is suggested that *Ae. aegypti* hears up to 2 kHz sound (Cator et al., 2009; Menda et al., 2019). But the sensitivity of *Ae. aegypti* to high-frequency ultrasound is still unclear. Interestingly, the mosquitoes predators (Gonsalves et al., 2013) bats use ultrasound to navigate and hunt insects (Schnitzler and Kalko, 2001; Simmons et al., 1996). Some insects species observed avoidance behavior against bat-ultrasound cries, due to the coevolutionary arms race with insectivorous (Conner and Corcoran, 2012; Yager, 2012). For malaria vector mosquito *Anopheles gambiae* the startle response to 35–60 kHz cry of African sheath-tailed bat *Coleura afra* was observed (Mang'are et al., 2015; Okorie et al., 2015). We note that *Ae. aegypti* is a day-time mosquito, consequently, its coevolutionary relationship with bats is questionable.

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Meanwhile, this is not a reason to rule out the possibility that *Ae. aegypti* may respond to ultrasound. EMR manufacturers use two rationales to explain the repellency of ultrasound on mosquitoes: (1) inseminated females avoid the high-frequency wing beat of males; (2) mosquitoes avoid the ultrasound cries of bats (Foster and Lutes, 1985). Actually, the mosquitoes flight sound frequency is much lower than ultrasound. This raises serious doubts about the consistency of the first explanation (Chapman, 1982; Michelsen and Larsen, 1985). The second explanation is also disputed by Mankin (Mankin, 2012) and by Enayati et al. (2007).

To date, EMRs efficacy to mosquitoes is not approved (Ahmad et al., 2007; Andrade and Bueno, 2001; Foster and Lutes, 1985; Lewis et al., 1982) and disputed (Enayati et al., 2007; Mankin, 2012). The devices used in these studies had sound frequencies of 2–100 kHz and sound pressure up to 115 dB. Also, constant and random sound patterns at various frequency range were tested. These studies suggest that sound alone is not effective against mosquitoes. Studies on *Ae. aegypti* showed that the blend of stimuli gains higher response than these stimuli alone (Pang et al., 2018; van Breugel et al., 2015). In the wild mosquitoes integrate an array of attracting and startling sensory information while host-seeking. We tested the hypothesis that the ultrasound in combination with wind and CO_2 may gain higher response enough to disorder host-seeking behavior of *Ae. aegypti* females. It was recently shown (Pang et al., 2018; van Breugel et al., 2015) that a combination of different stimuli shifts the ratio of upwind turns of mosquito's host-seeking fly, that also supports our hypothesis. To test this hypothesis we carried out various behavioral experiments with the ultrasound of different frequency and pressure in combination with wind and CO_2. The behavioral experiments were accompanied by molecular tests to check whether mosquitoes respond the ultrasound and are there any changes in host-seeking or hearing-related mRNA expression.

Materials and methods

Mosquito culture

Ae. aegypti non-blood-fed inseminated 5–7 days females were used in all experiments. The stock cultures of *Ae. aegypti* (originated from the National Institute of Health, Korea Centers for Disease Control and Prevention, Seoul, Korea) maintained in temperature-controlled insect rearing rooms of Seoul National University. Larvae were reared in 24 × 35 × 5 cm plastic trays containing 0.5 g of sterilized diet (40-mesh chick chow powder/yeast, 1/1 by weight). Adults were maintained on a 10% sucrose solution and blood-fed on live mice. All stages were held at 27 ± 1 °C and 65–75% relative humidity under a 16:8h light:dark cycle. In all tests, temperature and relative humidity were set up at 25 °C and 40 ~ 50% respectively.

Sound devices

In all experiments, the ultrasound was emitted by a waveform generator (33500B Series, Agilent Technologies, Santa Clara, CA, USA). The sound was recorded and analyzed by the 1/4-inch prepolarized free-field microphone (Type 40BE, GRAS Sound & Vibration A/S, Holte, Denmark) and a pulse multi-analyzer system (Type 3560-C, Brüel & Kjaer, Copenhagen, Denmark).

Survival test

Test equipment

The insecticidal effect of ultrasound was tested by treating 90 dB waves on mosquitoes locked in the 24.5 × 24.5 × 24.5 cm nylon gauze test cage provided by MegaView Science Co. (Fig. 1a). Test cage was covered by a 50 × 50 × 50 cm Plexiglas box to prevent ultrasound emission to outside. The ultrasound speaker was installed to the inner surface of the Plexiglass box.

Test procedure

For each test 50 mosquitoes were released into test cage for 24 h and provided 10% w/w sucrose solution. Tests were performed in pairs, once with the ultrasound speaker switched off (control) and once switched on (case). Three pairs (biological replications) of tests were conducted with 30, 45, 80 and 450 kHz ultrasound treatment cases. After each test, alive mosquitoes in the cage were counted.

Repellency test in the wind tunnel assay

Test equipment

To test whether the ultrasound in combination with the additional stimuli affects mosquito host-seeking behavior we established a wind tunnel assay combining ultrasound, CO_2 and air flow in close to real conditions manner (Fig. 2a). The 280 × 60 × 60 cm wind tunnel was constructed of transparent Plexiglas, four 5 V fans, CO_2 tube (2 L/min), ultrasound speaker and 60 × 70 cm transparent sliding wall. CO_2 tube and ultrasound speaker were located at the end point to attract and repel mosquitoes simultaneously. Fans were located opposite to endpoint to create airflow toward the start point. The sound pressure level was

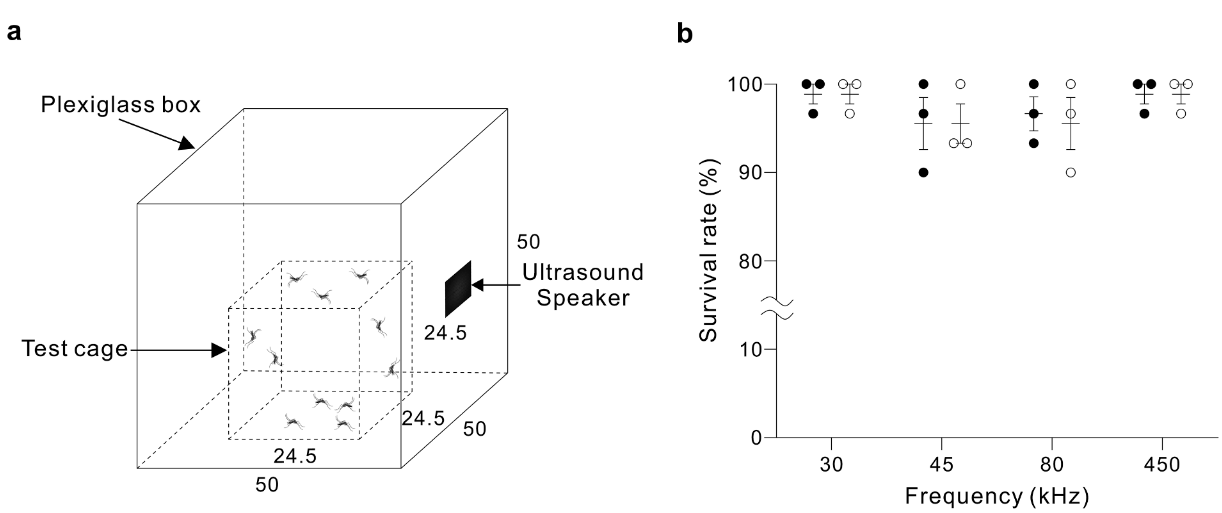

Fig. 1. The scheme (a) in cm and the results (b) of the survival test of *Ae. aegypti* females treated by 90 dB ultrasound for 24 h. Black (case) and white (control) data points represent mean ± SEM values from biological replicates (n = 3). All schematic figures in this paper were created with CorelDRAW Graphics Suite 2019 (Corel Corporation, Canada) by S. Lim.

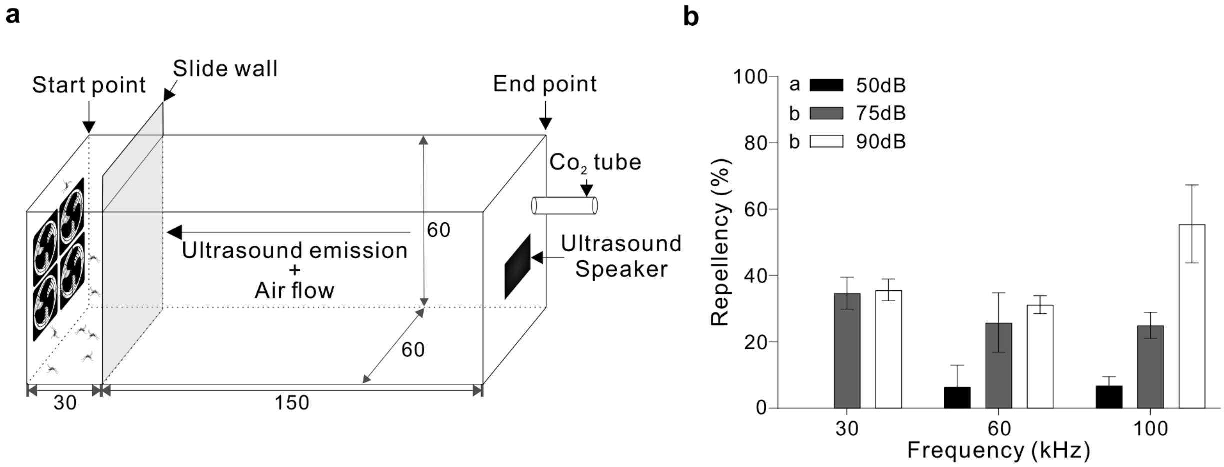

Fig. 2. Repellency test in the wind tunnel assay. (a) Scheme of assay in cm. (b) The repellency of ultrasound of different frequencies and pressure in combination with wind to *Ae. aegypti*. Each bar represents the mean ± SEM values from biological replicates (n = 3).

measured at the end point and at 50, 100, 150, 180 cm away from the speaker (Table S1.1, 1.2).

Test procedure

For each test we released 20 mosquitoes to the start point, then opened the sliding wall, turned on CO_2 tube, and fans at the same time. Only mosquitoes reached the endpoint were counted as control score and used for the corresponding case test. 15–20 mosquitoes were used in our case tests. The case tests were carried out in the same conditions but using ultrasound. Each case test was operated by a distinct pair-wise combination of 30, 60, 100 kHz frequency and of 50, 75, 90 dB pressure. Each test was performed for 5 min in triplicate. Repellent rate (R) was calculated as follows: $R = ((C - T) / C) \times 100$, where C - number of released mosquitoes, T - number of mosquitoes found in the endpoint.

Repellency test in the two-part chamber

Test equipment

To examine whether ultrasound in combination with the CO_2 reduces mosquito invade indoors from outdoors we used a 420 × 150 × 210 cm chamber divided into 'indoor' and 'outdoor' parts by a sound-proof wall with a 40 × 40 × 40 cm window (Fig. 3a). A 30 × 30 × 30 cm mosquito cage was placed in each side of the chamber. Both cages and a 200 cm length tunnel connecting them were covered by polyester netting. The sound pressure was measured within each cage and within the tunnel between cages (Table S2). The Carbon dioxide sensors (IAQ-Calc™ Indoor Air Quality Meter 7535, TSI, MN, USA) were set up the center of the chambers and the tunnel. In all case tests the concentration of CO_2 in the 'indoor' chamber was set to 1,500 ± 200 ppm. The corresponding concentration in 'outdoor' cage was maintained at <200 ppm.

Test procedure. 50 female mosquitoes were released into the 'outdoor' cage and the tunnel was tied by a rubber band to prevent the access to 'indoor' cage. Then, mosquitoes were allowed to acclimatize in the environment for 30 min before tests. Next, the concentration of CO_2 in the 'inside' chamber was set to 1,500 ppm and the rubber band was removed to allow the free flight of mosquitoes. The number of mosquitoes introduced into the 'indoor' cage was counted after 1 h. The control tests were carried out without ultrasound. The case tests were carried out in the same conditions but using ultrasound. Each case test was operated by a distinct pair-wise combination of 30, 40, 45, 60, 70, 80, 90, 100 kHz frequency and of 90, 110 dB pressure. Each test was performed in triplicate. The sound of 100 kHz frequency and 110 dB

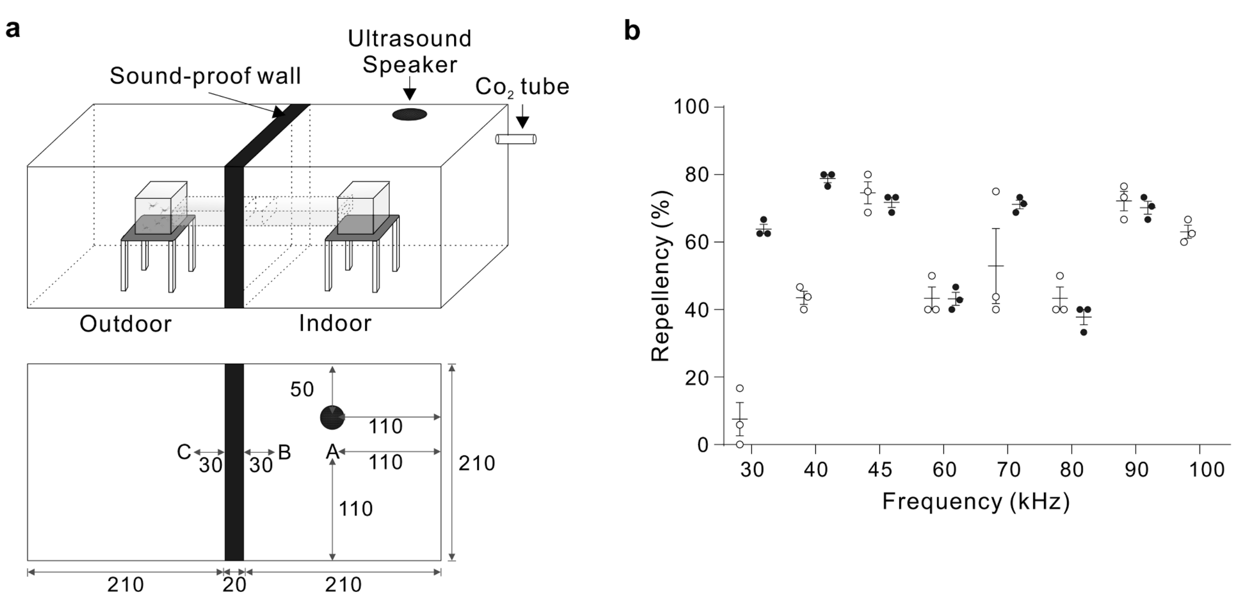

Fig. 3. Repellency test in the two-part chamber assay. (a) Scheme of assay in cm. A - 'indoor' cage, B, C - tunnel between cages. (b) The repellency of ultrasound of different frequencies and pressure (white – 90 dB, black – 110 dB) to *Ae. aegypti*. Each bar represents the mean ± SEM values from biological replicates (n = 3).

pressure was out of the limitation of speaker output. Repellent rate (R) was calculated as follows: R = ((C – T) / C) × 100, where C - number of mosquitoes found in the 'indoor' cage in control test (without ultrasound), T - number of mosquitoes found in the 'indoor' cage in case test (with ultrasound).

Repellency test in the arm-in-cage

Test equipment

To examine whether ultrasound decreases landing rate, mosquitoes were pre-treated with ultrasound and then tested in cubic nylon gauze arm-in-cage with 45-cm sides (Fig. 4a). Volunteers wore nitrile rubber gloves with 4 × 5 cm rectangular opening for mosquitoes to land on the skin.

Test procedure

For each test, 70 blood starved mosquitos group was pre-treated for 24 h with the ultrasound of 90 dB pressure. These pre-treatments were operated by sound of 30 kHz and 100 kHz frequency. The groups not pretreated with ultrasound served as the control. Each volunteer inserted his arm into the test cage for 1 min and counted the mosquitoes that landed on the skin for > 2 s. The number of landed mosquitoes was counted at 12, 17, 24, 48 h after ultrasound pre-treatment. None of the volunteers were bitten by mosquitoes in the experiment. Each test was performed 4–5 times. Landing rate of treatment (RT) was calculated as follows: RT = (A / B) × 100, where A - number of mosquitoes that landed on the skin, B - number of mosquitoes in the arm-in-cage. Relative landing rate was calculated by the following equation: R = (RT / RC) × 100, where RT - landing rate of a treatment, RC - landing rate of control. The tests were performed with three male human volunteers of 20–40 years old recruited from volunteers living in Seoul and Suwon city, Korea. Before volunteers involved in this test, they were informed specific precedures and remedial arrangements for any discomforts that might occur. All volunteers signed an informed consent form after having received a full explanation of the test objectives. The protocol for this study received formal approval from the Instituitional Review Board of Seoul National University (approval number: 1108/001-002). All methods were performed in accordance with the relevant guidelines and regulations.

Quantitative real-time PCR

Total RNA was isolated from the mosquito head using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Using 1 μg of total RNA, cDNA was synthesized with oligo-dT with Invitrogen Superscript III enzyme (Grand Island, NY, USA). Quantitative real-time PCR (qRT-PCR) was carried out with StepOne Plus (Applied Biosystems, Foster City, CA, USA) using SYBR green qRT-PCR Master Mix (Fermentas, Ontario, Canada). Quantitative analysis was employed by StepOne plus Software V. 2.0 (Applied Biosystems, Foster City, CA, USA). Results were normalized to a validated control gene *Aarps7*, using the ΔΔCt method. Primer information for qRT-PCR is described in Table S3.

Statistical analysis

Repellency in wind tunnel assay was analyzed using two-way analysis of variance (ANOVA). Relative landing rate in arm-in-cage was analyzed using a one-way ANOVA test. Repellency of different soud pressure levels in two-part chamber assay and the gene expression level were compared with a Student's *t*-test. All statistical analyses were performed using SPSS® Statics 25 (IBM, Armonk, NY, USA). All data were shown as means ± standard error.

Result

Ultrasound has no effect on mosquitoes survival rate

Before behavioral experiments, we checked whether the ultrasound of high pressure has an insecticidal effect on *Ae. aegypti*. Using ultrasound of 90 dB that is the human-safe pressure we compared the survival rate of mosquitoes treated for 24 h at a different frequency. Our data demonstrate that high-pressure ultrasound of any frequency doesn't have an insecticidal effect on *Ae. aegypti* females (Fig. 1b).A subtle effect of 45 and 80 kHz waves was observed on mosquitoes survival rate, but the shifts were not statistically significant. Thus, ultrasound cannot remove *Ae. aegypti* from human environment. But it can significantly reduce the host-seeking activity of *Ae. aegypti* females. This highly debatable suggestion was carefully tested in the following experiments.

Ultrasound in wind tunnel breaks up mosquitoes CO_2-oriented behaviors

We asked whether the ultrasound combined with a wind disorders *Ae. aegypti* female's CO_2-oriented flight. To test this hypothesis we created bioassay (Fig. 2a) combining CO_2, ultrasound, and wind in close to real manner. Our results show that 50 dB ultrasound has a subtle effect on *Ae. aegypti* females. The moderate repellency observed in all other tests with higher sound pressure. It is notable that the test with the

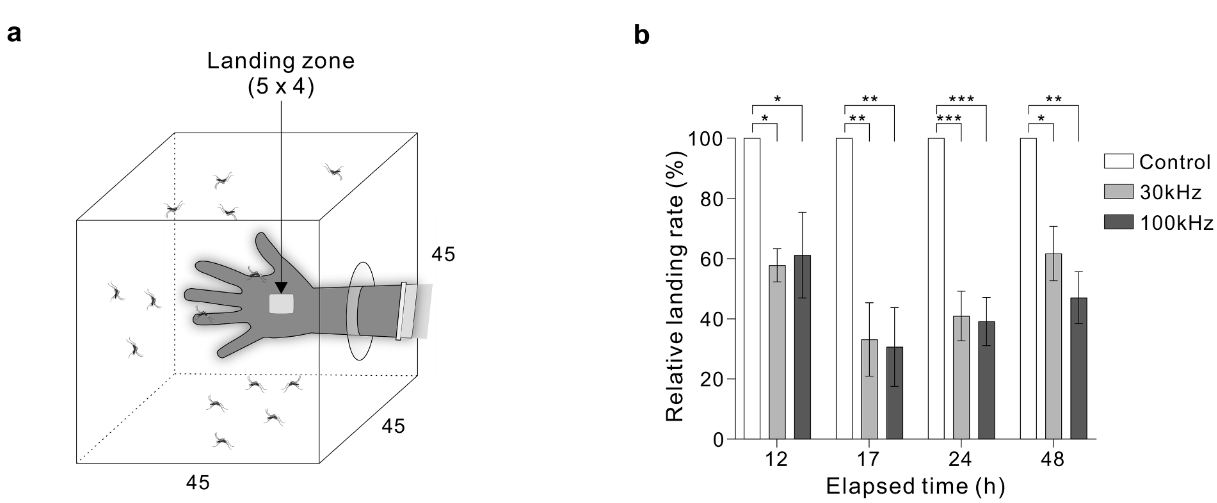

Fig. 4. Repellency test in the arm-in-cage. (a) Scheme of the arm-in-cage. (b) Relative landing rate of *Ae. aegypti* at a different time elapsed after pretreatment to ultrasound for 24 h. Each bar represents the mean ± SEM. Statistical significance was determined by one-way ANOVA with Bonferroni's test (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

ultrasound of the highest acoustic energy (100 kHz and 90 dB) demonstrated best repellency. Our results showed that the sound pressure level is positively correlated with the repellency under the same frequency (Fig. 2b). However, frequency and repellency under the same sound pressure are not correlated (two-way ANOVA, $F_{2,6} = 20.588$, $P < 0.001$ for sound pressure, $F_{2,6} = 1.112 P = 0.345$ for frequency and $F_{4,18} = 1.964$, $P = 0.152$ for their interaction).

Ultrasound reduces mosquito invade indoors

The experiment demonstrates whether ultrasound reduces mosquito invade indoors from outdoors through the open window while indoors treated to ultrasound and CO_2. Experiment was operated by eight specific frequencies from 30 to 100 kHz. Hence, the previous experiment revealed comparative efficacy of high-pressure ultrasound here we focused on 90 and 110 dB. Our results show that the ultrasound of all tested parameters, except 30 kHz / 90 dB significantly reduce mosquito invade indoors (Fig. 3c, Table S4). For 30 and 40 kHz ultrasound, the repellency was positively correlated with sound pressure level.

Ultrasound pretreatment decreases landing rate

To test whether ultrasound affects mosquito attraction to a live human skin we assessed the landing rate of ultrasound pretreated mosquitoes in arm-in-age. The relative landing percentages of mosquitoes pre-treated with ultrasound for 24 h were evaluated 12, 17, 24 and 48 h after exposure to ultrasound. The results showed that 30 and 100 kHz of ultrasound significantly decreased the landing behaviour of mosquitoes even 48 h after the expose of ultrasound (Fig. 4b, one-way ANOVA: 12 h, $F_{2,12} = 7.093$, $P = 0.009$; 17 h, $F_{2,9} = 14.553$, $P = 0.002$; 24 h, $F_{2,9} = 27.404$, $P < 0.001$; 48 h, $F_{2,9} = 14.290$, $P = 0.002$), suggesting that ultrasound causes a change in host-seeking behavior through a physiological effect on the mosquitos.

Ultrasound changes the expression of host-seeking related genes

To investigate whether host-seeking disorder revealed from the previous experiments caused by physiological changes, we examined the expression levels of olfactory, CO_2 sensing, and auditory related genes of *Ae.aegypti* exposed to 100 kHz / 90 dB ultrasound for 24 h (Fig. 5). The expression of odorant receptor co-receptor of *Ae.aegypti* (*AaOrco*) was not significantly changed after exposed to ultrasound (P = 0.569). The gustatory receptor 3 (*AaGr3*) known as a carbon dioxide receptor, was significantly decreased (P = 0.015). Interestingly, AAEL009258, homolog to *Inactive* (*Iav*) of *drosophila melanogaster* required for hearing, was significantly increased (P = 0.037).

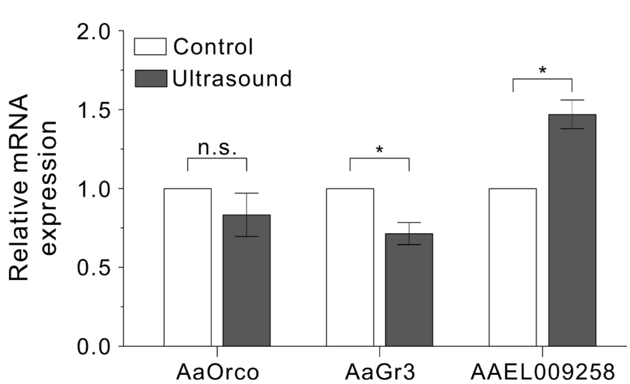

Fig. 5. Relative mRNA expression of odorant receptor co-receptor (*AaOrco*), gustatory receptor 3 (*AaGr3*) and putatively hearing related gene *AAEL009258*, in samples isolated from the heads of the *Ae. aegypti* females treated to 100 kHz / 90 dB ultrasound for 24 h. Each bar represents mean ± SEM. Statistical significance was determined by student's *t*-test (n.s. = non-significant, * = P < 0.05).

Discussion

Several studies demonstrated a frustrating inability of commercial ultrasound based EMRs to repel mosquitoes (Ahmad et al., 2007; Enayati et al., 2007; Foster and Lutes, 1985; Jensen et al., 2000; Mankin, 2012; Schreck et al., 1984). In contrast to them the data provided by Hadi et al. (2009) and Okorie et al. (2015) indicates that ultrasound has a significant repellent effect on mosquitoes. These contradictory findings tell us that the ultrasound of different frequency and pressure in different environmental conditions affects mosquitoes differentially. But the molecular and neural mechanisms underlying these contradictory behavioral responses to ultrasound remain unclear.

It is time to re-examine the current assumptions on ultrasound role in mosquitoes. The first challenging question is: does mosquito hear the ultrasound? Recently Menda et al. (2019) showed that Johnston's organ of *Ae. aegypti* detects the sound with frequency up to 2 kHz. Our behavioral and molecular experiments tell us that *Ae. aegypti* respond to ultrasound up to 100 kHz. We found that *Ae. aegypti*'s AAEL009258, homolog to Drosophila's hearing related *Inactive* gene (Gong et al., 2004; Lehnert et al., 2013), was upregulated in the head tissue after 24 h exposure of ultrasound. The AAEL009258 is abundantly expressed in Johnston's organ of *Ae. aegypti* and less in whole body tissue (Na et al., 2016). Interestingly, malaria mosquito *Anopheles gambiae* showed startle response to ultrasound like antenna erection, unusual rest and movement, fatigue and falls attributed to neural stress (Mang'are et al., 2015). Thus, taking into account the above mentioned findings, we can suggest that mosquitoes respond to ultrasound but ultrasound hearing mechanism remain unclear.

The second question is: does ultrasound repel mosquitoes? Several independent studies have failed to show the efficacy of ultrasound devices against mosquitoes (Ahmad et al., 2007; Enayati et al., 2007; Foster and Lutes, 1985; Jensen et al., 2000; Mankin, 2012; Schreck et al., 1984). In these studies, the devices had comparatively lower sound frequency or pressure. Our data agrees with these previous findings that ultrasound of low frequency and pressure had no repellent effect on mosquitoes. There was a study indicating that ultrasound of high frequency and pressure also did not repel mosquitoes (Ahmad et al., 2007). The misconception is the mentioned study is that ultrasound of high energy has an insecticidal effect and must force mosquitoes to fly away from the ultrasound source. Our survival test (Fig. 1) also proved that ultrasound of high energy doesn't have an insecticidal effect for mosquitoes. Another conception by Foster and Lutes (1985) is that ultrasound must disorder oriented flight of mosquitoes toward the source of human breath. Despite Foster's and Lutes's experiment didn't show a significant repellent effect on mosquitoes, we used this concept in our wind tunnel assay. We improved Foster's and Lutes's experiment by adding the wind produced by four 5 V fans (Fig. 2a). We put forward 3 rationals to use wind in combination with ultrasound: (1) The wind is considered to be a key stimulus while host-seeking in the presence of CO_2 (Dekker and Carde, 2011). (2) Recent studies in *Ae. aegypti* showed that the blend of stimuli gains higher response than each of these stimuli alone (Pang et al., 2018; van Breugel et al., 2015). (3) Mosquitoes integrate an array of sensory information like CO_2, heat, odorants, wind while host-seeking (McMeniman et al., 2014; Pang et al., 2018; van Breugel et al., 2015). Combination of these different stimuli shifts the ratio of upwind turns of mosquito's host-seeking flight and changes host-finding rate (Pang et al., 2018; van Breugel et al., 2015). According to this rationals we hypothesized that the ultrasound in combination with wind may gain higher repellent effect.

Foster and Lutes (1985) showed that 5% (not significant) of *Ae. aegypti* females exposed to 75 kHz / 92 dB ultrasound failed to find CO_2 source in the 120 cm × 30 cm^2 tunnel assay. In our experiment in the 180 cm × 60 cm^2 tunnel assay with the wind and 60 kHz / 90 dB ultrasound, more than 30% (significant) of the same species animals failed to find CO_2 source. When we set ultrasound emitter to 100 kHz and 90 dB, more than 50% of mosquitoes failed. Interestingly, another mosquito species *A. gambiae* also showed higher response to ultrasound combined with the wind (Okorie et al., 2015). With previous findings (Foster and Lutes, 1985; Okorie et al., 2015; Ahmad et al., 2007; Albert and Kozlov, 2016) and present our study suggest that ultrasound in combination with wind gains higher repellent effect on mosquitoes than ultrasound alone. Also, wind tunnel experiment tell us that the frequency and pressure of an ultrasound are positively correlated with the repellency.

The third question is: does ultrasound prevent mosquitoes invade to indoor? Although EMR producers claim that ultrasound prevent mosquitoes invade to indoor, there is no published experimental data about this. Our two-part-chamber allows to test how mosquitoes from outdoor invade to the indoor through the open window when indoors treated by CO_2 and ultrasound simultaneously. Our results show that the ultrasound of high energy, except 30 kHz / 90 dB (lowest one) significantly reduce mosquito invade indoors (Fig. 3c, Table S4). Here we mention that the similar experiment by Foster's and Lutes,'s (1984) didn't find repellent effect of 25 kHz (peak) / 92 dB ultrasound. It is obvious that ultrasound with parameters lower than 30 kHz and 90 dB is not effective to prevent mosquitoes invade to indoor. Higher than 40 kHz and 90 dB ultrasound is expected to be effective to protect indoors from mosquitoes invade.

The fourth question is: does ultrasound reduce landing rate? Previously Schreck et al. (1984) demonstrated that 10 msec puls train ultrasound with a peak frequency at 30, 43 and 53 kHz and 96 dB pressure did not change landing rate when alive human skin and ultrasound introduced together into the chamber. In our arm-in-cage experiment we pretreated mosquitoes to harmonic 100 kHz / 90 dB ultrasound for 24 h and then introduced the alive human skin without ultrasound. This experiment showed that high energy ultrasound pretreatment significantly decreased the landing rate of *Ae. aegypti* females on human skin. We isolated the mRNA from the heads of these ultrasound pretreated animals and found that the expression level of CO2-receptor (*AaGr3*) was downregulated. Further tests in arm-in-cage showed that residual effect from ultrasound pretreatment was prolonged for at least 48 h. McMeniman et al. (2014) demonstrated that the *AaGr3*-mutants use other sensory cues to find live human skin in close distance. In our arm-in-cage test significant number of pretreated mosquitoes failed to find a live human skin. Therefore, it is possible that 24 h pretreatment to high pressure ultrasound reduces the expression of different receptors involved to mosquito's attraction to humans. To clarify this hypothesis, it will be interesting to investigate how ultrasound changes the expression of heat, human odor and visual receptors.

Taken together, the current study is significant to show that the ultrasound of high pressure (1) disorders host-seeking behavior and (2) changes the expression of host-seeking related genes in *Ae. aegypti*. Therefore, a better understanding of molecular and neural mechanisms underlying the effect of ultrasound on mosquito behavior will be important to solve the current problems of EMR efficacy.

Author contributions

D.K., R.I. and S.L. and H.W.K. designed the experiment. D.K. performed overall experiments. D.K. and U.Y. carried out data analysis. D. K., R.I., U.Y., and H.W.K wrote the main manuscript text. All authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2020.12.016.

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