

Optimal Efficacy in Mosquito-Host Interaction Inhibition by 35-60 kHz Animal Sounds for Malaria Control

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Abstract The haematophagous mated female *Anopheles gambiae sensu stricto*, a malaria vector, continues to develop resistance to synthetic insecticides thus threatening its control. Studies on negative phonotaxis response in mosquitoes showed that the electronic mosquito repellent (EMR) (40-55 kHz) sound, *Odorrana tormota* (35-60 kHz) sound, and winded-EMR sound yielded 68.99%, 45.88%, and 60.70% repellency respectively, proving the feasibility of using non-pollutant sounds in mosquito control. However, studies with *O. tormota* sounds were rudimental and didn't observe protocols for mosquito rearing standard operation procedures (SOPs) and World Health Organisation (WHO) guidelines for efficacy testing of spatial repellents. Also, phonotactic inhibition by natural sounds of *Delphinapterus leucas* and male *A. gambiae s.s* had not been studied. This research, therefore, determined spatial activity index (SAI) and protection index (PI) of mated female *A. gambiae s.s* evoked by 35-60 kHz sounds of *O. tormota*, *D. leucas*, and male *A. gambiae s.s* in adherence to mosquito rearing SOPs and WHO guidelines. Mosquitoes were reared in Kenya Medical Research Institute (KEMRI) Entomology laboratories observing KEMRI SOPs protocols. Sounds of a hundred 3-5-day-old male *A. gambiae s.s* were recorded using an Avisoft recorder whereas the sounds of *O. tormota* and *D. leucas* were acquired through donations. Mosquito exhibited adverse abnormal composure, low flights, antennae erection, open wings, and extension of legs during the bioassays under the treatment with the sound of *O. tormota*. The data obtained were analyzed using Avisoft SASLab and Raven Pro software. Mean SAI evoked by *O. tormota*, male *A. gambiae s.s*, and *D. leucas* were 0.142, 0.318, and 0.206 respectively, indicating negative phonotaxis. The PI elicited by *O. tormota* was 80.06% exceeding male *A. gambiae s.s* and *D. leucas* by 1.65% and 8.24% respectively. Frequency modulated, constant frequency, and pulsating sound of *O. tormota* showed mean bandwidth (mean entire; 13.95 kHz), maximum frequency (mean entire; 41.61 kHz), maximum energy (8.02 Pa²s), entropy (2.87 bits), delta power (24.10 dB), average power (55.60 dB), maximum power frequency (38.09 kHz) and maximum power (75.90 dB). This study provides new acoustic parameters and an effective repellent recommended for mosquito control.

Keywords Spatial Activity Index, Protection index, Entropy, Phonotaxis, Haematophagous

1. Introduction

1.1. Global Malaria Trends and Interventions

Africa experiences the bulk of the malaria global burden because of the presence of the Afro-tropical haematophagous mated female mosquito, *A. gambiae* [72]. The female mosquito, *A. gambiae*, which is a dominant malaria vector species, enjoys favorable climatic conditions which include: annual precipitation of 330-3224 mm, a maximum annual temperature of 25-42°C, and a minimum annual temperature of 5-22°C. [52,70-71]. The malaria

pathogens, *Plasmodium falciparum*, whose vectors are the mated female mosquito, *A. gambiae*, are a major challenge and a threat to human life and accounted for 99.7% of estimated malaria cases in the World Health Organisation (WHO) African Region, 50% of cases in the WHO South-East Asia Region, 71% of cases in the Eastern Mediterranean and 65% in the Western Pacific in 2018 [75,85,89-90]. The *P. falciparum* is also responsible for worldwide economic burden, severe morbidity, low birth weights, impaired physical growth, permanent disability, and mortality worldwide [14,35,40,75,79,85,88-89,93]. However, scale-up of vector control interventions, diagnostic testing, and treatment with artemisinin-based combination therapies (ACT) have led to a slight downward trend in malaria cases and deaths though challenged by the slow rate of decline in Sub-Saharan Africa [38,86-87,89]. Interventions targeting the malaria vector involve the use of

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insecticide-treated nets (ITNs), indoor residual spraying (IRS) and in some specific settings, larval control is a critical component of the multipronged attack on malaria [38]. Protection by ITNs and IRS have yielded a greater impact in reducing malaria cases and deaths in Africa [12,34]. Annual statistics on malaria deaths showed a generally declining trend in the 2010 – 2019 period, with most deaths being young children in sub-Saharan Africa (United Nations Children's Fund [78,82,89]. In 2010, there were an estimated 2.51×10^8 cases of malaria worldwide, slightly declining to an estimated 2.28×10^8 in 2018 [89]. However, there were an estimated 2.31×10^8 malaria cases in 2017, an increase in number by 4.00×10^6 cases from the number of reported cases in 2016 [89]. Further, there were an estimated 5.85×10^5 malaria deaths worldwide in 2010 which reduced to 4.05×10^5 deaths in 2018 with a majority in Africa [11,35,85-89]. There has been a systematic reduction in deaths at an estimated rate of 2.12×10^4 deaths/year based on World Health Organisation statistics between 2010 and 2019 [89].

The effectiveness of the synthetic chemicals currently in use for the control and treatment of malaria has been impeded by the resistance developed in mosquitoes and malaria pathogens. Besides, some synthetic chemicals have had adverse environmental effects [41,73]. Notably, the *Plasmodium* parasites have been reported to exhibit resistance to chloroquine which was the mainstay of antimalarial drug treatment in humans [31,41]. Additionally, electronic mosquito repellents (EMR) currently in use have yielded low repellency in mosquitoes as reported in their recent experimental evaluation [32]. The ineffectiveness of the EMR was attributed to the narrow bandwidth of mimicked animal sounds. However, ultrasound from an EMR fitted with a fan yielded a 60.7% protection index exceeding the protection index yielded when EMR was used per se by 43.4% [63]. Other designed EMR generated ultrasound in the 40-55 kHz frequency band yielding 68.99% repellency [44]. Also, the repellency of 45.88% elicited by the 35-60 kHz recorded sound of *O. tormota* in mated female *A. gambiae* s.s based on observable behavioural responses in recent rudimentary research findings prompted a further study in the natural sounds of the male *O. tormota* [56]. Recent research has shown that minimizing the host-vector interactions was an effective way of reducing malaria cases and deaths [66,89]. It is on this basis that novel interventions were needed to accelerate the rate of decline in malaria transmission and deaths. The study, therefore, determined the efficacy of mosquito-host interaction inhibition by 35-60 kHz animal sounds in malaria control. This study, therefore, used sounds from the male mosquitoes, *A. gambiae* s.s, Chinese frog, *Odorrana tormota*, and Beluga whale *Delphinapterus leucas*. The male *A. gambiae*, *O. tormota*, and *D. leucas* generated sound naturally which had been reported to extend to ultrasonic levels, hence useful in the investigation [36,37,54,68,92]. Additionally, these sounds were presumed to have high acoustic energy resulting in intensified

antennal vibrations of mosquitoes thus improving the effectiveness in startling mosquitoes. The study was anchored on the mosquito's response to ultrasound which included responsiveness, and landing rates on a blood meal. The results of this study provided additional malaria control measures aimed at accelerating the reversal trend in malaria cases and deaths, particularly in Africa.

1.2. Mosquito Biology, Feeding, Communication, and Mating Behaviour

Mosquitoes have four distinct stages, egg, larva, pupa, and adult in their life cycle recognized by their unique appearance [19]. It is important to understand the lifecycle of the mosquito for effective malaria vector control [91]. The biting female mosquitoes not only irritate people and animals but also transmit malaria [74]. The body parts of the adult stage of the mosquitoes, mainly the antennae serve an important role in communication [61]. The egg, larva, and pupa stages in the lifecycle of the *A. gambiae* are aquatic and last 5-14 days, depending on the species and the ambient temperature [19,33,48]. Both male and female adult mosquitoes feed on plant nectar, but the females which are anautogenous feed on vertebrates' blood, for nutrients required for egg production and maturation [9,10,39,57,58]. Warm-blooded hosts provide mosquitoes with thermal contrast that facilitates the localization of a suitable blood meal [29,60]. An analysis of how the mosquito actually bites, probes for the blood vessels, and finally sucks blood showed that the mean time taken before the mosquito starts probing after landing was 6.5 seconds, the mean probing time was 142 seconds, the mean feeding time was 240 seconds thus giving a total of 389 seconds (6.5 minutes) [20]. The feeding times for the mosquitoes were between 150 and 329 seconds. The female *Anopheles* mosquitoes lay eggs on the surface of the water at night and under favorable conditions, hatching occurs within one or two days and develops within the aquatic habitat [25,62]. Adult mosquitoes have slender bodies consisting of the head, thorax, and abdomen; the head is specialized for acquiring sensory information and for feeding [51]. The mosquito antennae also detect host and breeding sites' odors [81]. The head also has an elongated, forward-projecting proboscis used for feeding and two sensory palps. The adult stages of many mosquito species are feeders of blood, which has given some disease-causing organisms a reliable mode of transmission to animal hosts. During the adult stage of the males and females *Anopheles* rest with their abdomens sticking up in the air and the female *Anopheles* mosquito act as a malaria vector [25]. The adult females *Anopheles* mosquitoes can live up to a month or more in captivity but they don't live more than 1-2 weeks in nature [58]. The mosquito has a pair of large, wraparound eyes, and a pair of long, hairy antennae; its ears project from the front of its face [43]. The antenna, which is sexually dimorphic, detects the particle velocity component of a sound field, which is restricted to the immediate vicinity of the sound source in the acoustic near field [3,28,65,80]. The

Anopheles gambiae mosquitoes generate sound through wing beats and the flight tone is an unusual communication signal in that its production is directly linked to locomotion, only varying the carrier frequency [5,65]. The flight tone is a characteristic of a species and can sometimes be used to identify species or count individuals. Ultrasound generated artificially or naturally is detected by mosquitoes evoking an evasive response [61]. The sound causes neural stress evoking an evasive response. Mosquitoes like many other insects avoid bat ultrasonic sound that the electronic mosquito repellent devices imitate [44,61]. Electromagnetic communication between insects has also been observed in mosquitoes with the antennae playing the role of the receiver or transmitter [1,59]. Mosquitoes use reactive near-field in antennae communication which is defined as a distance less than a sixth of a wavelength from the source (distance, $r < 1/6$ wavelength, λ) [5]. Recent findings have shown that the acoustic interactions between males and females played a role in courtship behavior and gave evidence that the frequency of flight tone stimulus modulated harmonic convergence behavior of the malaria mosquito, *A. gambiae* [76]. Also, variation in conspecific flight tone is perceived by the male and female *A. gambiae* with an average frequency of convergence of 1368.0 ± 165 Hz [17]. Male mosquitoes require about 24 hours before their *terminalia* get rotated and their fibrillae mature enough to become erect and detect females whereas the female mosquitoes need 48-72 hours before they become receptive to males before blood-feeding in the wild [25,64]. *Anopheles* males can mate several times, but females become refractory to re-insemination and re-mating is rare [61]. The male *Anopheles* mosquitoes aggregate before dusk and initiate swarming at the onset of sunset and mating occurs during the early evening, primarily in swarms, a typical time for the mated female mosquitoes to seek blood meal through bites of human beings. [13,21,27,46]. The swarming males use their erect antennal fibrillae to detect a nearby female mosquito's wing beat frequencies, with close-range interactions between males and virgin females established as ~ 2 cm [65]. Also, the auditory system of the male *A. gambiae* is selectively tuned to the female *A. gambiae* in the approximate frequency range of 300-400Hz with a maximum intensity frequency being equal to that of the female *A. gambiae* [45,59].

1.3. The Chinese Frog *Odorrana tormota*, and Beluga Whale, *Delphinapterus leucas*

The *Odorrana tormota* species is a frog restricted to Huangshan in Anhui Province, and Jiande and Anji counties in Zhejiang Province, China [68]. The *O. tormota* frog generates ultrasounds through vocal apparatus and uses the frequency range of up to 128 kHz for communication [11,49,68,69].

The beluga whale, *Delphinapterus leucas* is a medium-sized toothed whale, which becomes completely white when it reaches sexual maturity around seven years of

age [53]. Adult male beluga whale, *D. leucas* attains a length of 4.5 meters and females 3.5 meters and are similar in appearance [23]. Young ones are born dark grey and gradually become paler as they mature spending the summer in coastal and offshore areas [23]. The beluga whale, *D. leucas* have a mean lifespan of between 15 to 30 years though they may live beyond 40 years [24,42]. The beluga whale, *D. leucas* are sexually mature at the ages of 5-7 years and adults are capable of giving birth every 3 years. The beluga whales feed on a variety of fish and invertebrates, yet the polar bears, killer whales, and Inuit hunters are their main predators [24,30]. The Cetaceans produce frequency-modulated sounds and amplitude-modulated sounds [15,24]. The *D. leucas* produced signals with peak frequencies of 40 to 60 kHz in San Diego Bay, California, and 100 to 120 kHz in Kaneohe Bay, Hawaii [6]. The sounds of *D. leucas*, are generated naturally through the movement of air between nasal sacs in the blowhole region [24,50,55]. Recent studies have shown that ultrasound in frequency ranges of 20-30 kHz, 21-29 kHz, 38-44 kHz, and 40-55 kHz from animals played a critical role in malaria vector control by evoking evasive responses [2,44,56,61].

The 35-60 kHz frequency range studied fell within the reported startle and repellency frequency ranges of the mated female *A. gambiae* which included 35-60 kHz, 38-44 kHz, and 40-55kHz and whose sources were of no regard [44,56,61]. The wide band sounds generated by the electronic mosquito repellents (EMR) were reported to have yielded a repellency of 30.3% and 20% in mosquitoes [4,18,32]. Additionally, experiments with an AC-UD under the “fan ON with ultrasonic ON”, “fan ON with ultrasonic OFF”, and “fan OFF with ultrasonic ON” yielded final mortality at 24 hours of 60.7%, 15.3%, and 17.3% in the knockdown tests [63]. This study thus determined the percentage of PI evoked by the sounds of the male mosquito, *A. gambiae*, *O. tormota*, and *D. leucas*.

1.4. The Protection Index (PI) and Spatial Activity Index (SAI)

In this study, the percent protection index (PI) or landing inhibition was determined using the formula given in equation 1.1 as used in past research and the World Health Organisation, (WHO) guidelines for efficacy testing of spatial repellents [4,22,67,84]:

$$PI = \frac{Cl - Tl}{Cl} \quad (1)$$

Where *Cl* is the number of mosquitoes landing in the control chamber (control) and *Tl* is the number of mosquitoes landing (or initiated bites or/and probing) in the treatment chamber (treatment) [84]. The spatial activity index (SAI) of the mosquitoes in a cage whose values vary from -1 to 1 is given by equation 1.2 [84]. The spatial activity index of zero indicates no response, -1 shows that all mosquitoes moved into the treatment chamber (positive

phonotaxis) and 1 designates mosquito movement into the control chamber (negative phonotaxis) [84].

$$SAI = \left(\frac{N_c - N_t}{N_c + N_t} \right) \times \frac{N_m}{N} \quad (2)$$

where N_c is the number of mosquitoes in the control glass chamber (control), N_t is the number of mosquitoes in the treatment glass chamber (treatment), N_m is the number of mosquitoes responding in the entire glass cage (treatment and control i.e. $N_m = N_c + N_t$) and N is the total number of mosquito samples in the entire cage.

1.5. Statement of the Problem

Numerous human deaths, disabilities, low birth weight, and global economic burden have been attributed to malaria which is transmitted by mated female *A. gambiae* s.s. Therefore, a multipronged approach including vector control was vital for a successful acceleration of the reversal of the current trend in malaria cases and deaths. Current efforts to address the malaria burden by targeting the malaria vector, mated female *A. gambiae* s.s, using synthetic chemicals had resulted in a slight decline in malaria cases and deaths though slowed by resistance by the malaria vectors and pathogens to the chemicals. Besides synthetic chemicals are electronic mosquito repellents (EMRs) in use which have yielded low mosquito repellency of between 17.3 - 30.3%, attributed to the narrow bandwidth of the sound from most of the ultrasonic transducers. This in turn rendered the signal less intense and ineffective in mosquito repellency. However, recent rudimental research findings based on observable mosquito behavioural responses to the 35-60 kHz recorded natural sound of *O. tormota* yielded an improved repellency of 45.88%. Additional fan to EMR ultrasound yielded a 60.7% protection index compared to the protection index yielded when EMR was used per se. Other designed EMR generating ultrasound in the 40-55 kHz frequency band yielding 68.99% repellency. The challenges experienced in malaria vector and pathogen interventions have impeded the successful realization of good health and well-being as entrenched in the sustainable development goals (SDGs) [77]. However, recent research involving the use of natural sounds and fanned synthetic (EMR) ultrasound in mosquito repellency showed the feasibility of using ultrasound in mosquito control. The effect of the natural sound of the African male *A. gambiae*, and *D. leucas* on the mated African female *A. gambiae*, the malaria vector, had not been studied. Also, bioassays studies on the effect of the natural sound of *O. tormota* on the mated female *A. gambiae* through the determination of spatial activity index (SAI) and protection index (PI) following World Health Organisation (WHO) guidelines had not been conducted. This research, therefore, established the optimal efficacy in mosquito-host interaction inhibition by 35-60 kHz sounds of the male *A. gambiae*, *O. tormota*, and *D. leucas* for malaria control. The study focused on the experimental

determination of the number of mated female *A. gambiae* s.s approaching, landing, and probing the blood meal evoked by 35-60 kHz natural sound of male *Anopheles gambiae*, male *Odorrana tormota*, and male and female *Delphinapterus leucas* through a bioassay under WHO guidelines, and evaluation of SAI and PI. The results from the study were systematically optimised to give optimal parameters in mosquito repellency thus giving additional knowledge in malaria control.

1.6. Objectives

1.6.1. General Objective

To establish the optimal efficacy in mosquito-host interaction inhibition by 35-60 kHz natural sound of male *Anopheles gambiae*, male *Odorrana tormota*, and male and female *Delphinapterus leucas* for malaria control.

1.6.2. Specific Objectives

- (i). Determine the phonotactic behaviour in the mated female *A. gambiae* s.s evoked by 35-60 kHz natural sound of male *Anopheles gambiae*, male *Odorrana tormota*, and male and female *Delphinapterus leucas*
- (ii). Calculate the spatial activity index and protection index (PI) in mated female *A. gambiae* elicited by 35-60 kHz natural sound of male *Anopheles gambiae*, male *Odorrana tormota*, and male and female *Delphinapterus leucas*

2. Methodology

2.1. Study Animals, Rearing, Sound Recording and Filtering

The male *O. tormota*, male and female *A. gambiae* s.s, *D. leucas*, and *T. truncates* were used in the study. Mosquito rearing and feeding were guided by Standard Operation Procedures for *Anopheline* mosquito rearing and maintenance, SOP No. 3005/ENT/014 for KEMRI and the World Health Organisation (WHO) guidelines on the rearing of mosquitoes (WHO, 2013). The male and female *A. gambiae* s.s mosquitoes were bred and reared at KEMRI/CDC entomology laboratories at $80 \pm 10\%$ relative humidity (RH), $27 \pm 2^\circ\text{C}$ temperature, and equal light-darkness hour cycle with one-hour dawn dimming as outlined in SOP No. 3005/ENT/014 and WHO guidelines (WHO, 2013). Larvae were reared on larval pans which were filled with rainwater to a depth of 1.0-2.0 cm and the room temperature was maintained at $30 \pm 2^\circ\text{C}$ as outlined in SOP No. 3005/ENT/014. The larvae were fed on a combination of tetramin baby fish food and Koi's choice premium fish food in the ratio of 1:2 with the quantity of food and feeding frequency determined by the stage, size, and density of larva. The pupae of the *A. gambiae* s.s which do not feed were reared in covered glass vials quarter filled with rainwater at 28°C under standard laboratory conditions. Both male and

female *A. gambiae* s.s were fed on a 10% sugar solution, though the female *A. gambiae* s.s were additionally fed on blood meal 3-7 days post-emergence. The female mosquitoes, *A. gambiae* s.s were separated from the male *A. gambiae* s.s from a swarm of mosquitoes based on their mouthparts and affinity to a blood meal. Twenty-five sets, each of fifty, 3-5 day old mated female *A. gambiae* s.s obtained from emerged mosquitoes were reared separately at KEMRI/CDC Entomology laboratories under controlled conditions. Also, a set of one hundred male *A. gambiae* s.s were reared separately under similar conditions. The bioassays which were sound-based were conducted in a quiet and well-lit room in the KEMRI/CDC entomology laboratory under controlled room conditions.

The beluga whale *D. leucas* were obtained from Vanaqua where their sounds were recorded and supplied for this research by Prof. Herve Glotin of Institut Universitaire de France (Glotin, 2015). The *O. tormota* inhabited the Huangshan Hot Springs in Anhui Province, China and whose sounds were recorded and supplied for this research by Prof. Albert Feng, formerly of the University of Illinois at Urban-Champaign.



Figure 1. The sound recording setup

A computer running on the Windows operating system and office with a mounted sound card was installed with the Avisoft-SAS LAB Pro version 5.2 software for sound recording and playback. A hardlock key interfaced with the computer through universal serial bus enabled the Avisoft-SAS LAB Pro version 5.2 programme software to run. The input and output ports on the computer served as inputs for the signal from the Avisoft recorder. The recorder consisted of the AvisoftUltraSoundGate (model 112) and running on the RECORDER USG (rec_usg.exe) software. The sound was played through a vifa external ultrasonic speaker with frequency range (± 12 dB): 1-120 kHz, impedance: 4 Ω , and sensitivity at 50 kHz: 92 dB / 2.83V / 1m. A set of 100 male and mated female *A. gambiae* s.s obtained from the reared 3-5 days old mosquitoes were separately transferred into a cylindrical glass cage covered at both ends with netting using an aspirator as shown in Figure 1. The sounds of the male and mated female *A. gambiae* s.s were recorded separately using the Avisoft recorder at a

sampling frequency of 500 kHz at 16 bit and saved as a .wav file in the hard disc. The omnidirectional microphone, set to default and connected to the AvisoftUltraSoundGate (model 112), was connected to the computer through the universal serial bus (USB) port. The Avisoft-SAS LAB Pro, version 5.2 software was initiated and the microphone was directed to the source of the sound. With the gain on the AvisoftUltraSoundGate (model 112) adjusted to an appropriate level to avoid over-modulation and the recording level from the computer set to 20 dB, the recording button was pressed to record the sound (Manga're *et al*, 2012).

The sounds of the male *O. tormota* recorded using the 702 digital recorder from the Huangshan Hot Springs at a sampling frequency of 192 kHz were acquired through Prof. Albert Feng, formerly of Illinois University. The sounds of the mixed male and female *D. leucas* were recorded using the the Wavshark system, C75, and C55 hydrophone at a sampling frequency of 128 kHz as they swam in the tank of the Vanaqua, and were acquired from Prof. Herve Glotin of Institut Universitaire de France (Glotin, 2015). The entire spectrum of the sound of the male *A. gambiae*, male *O. tormota*, and mixed male and female *D. leucas* were saved as malegambiasound. wav, tormotasound. wav, and leucassound. wav respectively were saved in the hard disc. The sounds of male *A. gambiae*, male *O. tormota*, mixed male and female *D. leucas* were subjected to 35-60 kHz band-pass filter incorporated in both the Avisoft and Raven Pro software to yield the 35-60 kHz frequency range. The Avisoft software was set to time domain filter (Finite Impulse Response -FIR) option with the filter type, upper cut-off frequency $f_{uco}= 140.00$ kHz and $f_{lco}= 0.00$ Hz for recording the sound of the male *A. gambiae*. Filtering of the sound of the male *A. gambiae*, male *O. tormota*, and mixed male and female *D. leucas*, time domain filter (Finite Impulse Response -FIR) option with the filter type was selected with upper cut-off frequency $f_{uco}= 60.00$ kHz and $f_{lco}= 35$ Hz. General settings of the Avisoft software were made from the tools option, with the calibration was set to sound pressure level(SPL) with reference to sound, and the SPL reference was set to 20 μ Pa. Also, the Fast Fourier transform (FFT), an option under the spectrogram parameters was set to 512, and the hamming window was selected for the display. The temporal resolution overlap was set to 50% with the colour palette set to graypal. The frame size was set to 100% for real-time spectrogram parameters and the black and white box (B/W) checked for display. Besides, the envelope was also set to the original waveform whereas the pulse detection was set to gate function. The same settings were made to the Raven Pro. 1.4 software for accuracy.

2.2. The Phonotactic Behavior, Spatial Activity Index (SAI) and the Protection Index (PI) in the Mated Female *A. gambiae* s.s Evoked by the 35-60 kHz Sound of the *O. tormota*, *D. leucas*, and Male *A. gambiae* s.s

Feeding and maintenance of both male and female *A.*

gambiae s.s was guided by the Standard Operating Procedures, SOP No. 3005/ENT/014 for KEMRI and WHO guidelines on mosquito rearing (WHO, 2013). The cow (*Bos taurus*) blood meal which is preferred by the *A. gambiae* s.s was obtained from a slaughterhouse, processed, and stored in Kenya medical research institute as per the Kenya Medical Research Institute (KEMRI)/ Centre For Global Health Research (CGHR) Standard Operating Procedures (SOP) for collecting blood for blood-feeding insects in the laboratory given. Active 3-5 day old mated female *A. gambiae* s.s which had been starved for 24 hours and of high affinity to the blood meal were selected for the bioassay. The 35-60 kHz filtered natural sounds of the male *O. tormota*, male *A. gambiae*, and the sound of the male and female *D. leucis* were used in the bioassay as the treatment. The 35-60 kHz sounds were broadcasted into the bioassay cage as a treatment to the blood meal and behavioural responses of the mated female *A. gambiae* s.s observed and recorded. A 1.0 m long modified standard Y cage called fighto-Y bioassay glass cage fitted with a mosquito netting on the three cross-section areas A, B, and C as shown in Figure 2 was used in the bioassay (Rodriguez *et al.*, 2015; WHO, 2013). Cotton wool was used to seal the entry/ exit hole on the net placed on face C of the fighto-Y glass cage. The cage was divided into three sections, A, B, and the Neutral chamber (C). The open ends of chamber A, B, and C were covered with mosquito netting with net A and B being in contact with the cellulose membrane covering the warm blood contained in the feeding chamber. The feeding chamber connected to the Hemotek membrane feeding apparatus was used to feed the blood-sucking mated female *A. gambiae* s.s through an artificial membrane as described in the SOP No. 3005/ENT/014. The blood chamber which was an aluminium cylindrical container was loaded with fresh blood using a Pasteur pipette through the ports at its back. The ports were covered with a removable rubber material. The loaded blood in the chamber was covered with an artificial cellulose membrane interfaced with the cage netting on face A and B. The cellulose membrane allowed for mosquito landing, probing (bites), and sucking of the blood meal (cow) which was maintained at the body temperature of a healthy cow of 38.60°C by the Hemotek membrane feeding apparatus. The duration of the bioassay study was measured using a digital timer.

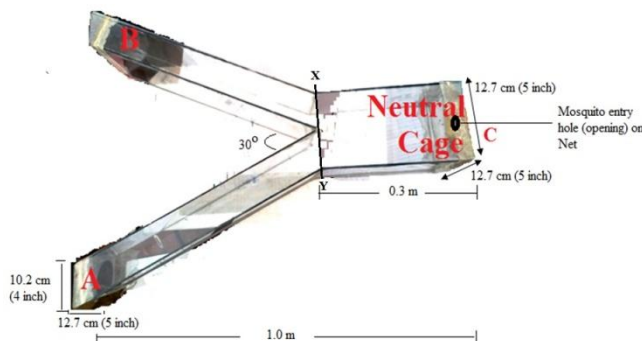


Figure 2. Fighto-Y glass cage for bioassay study

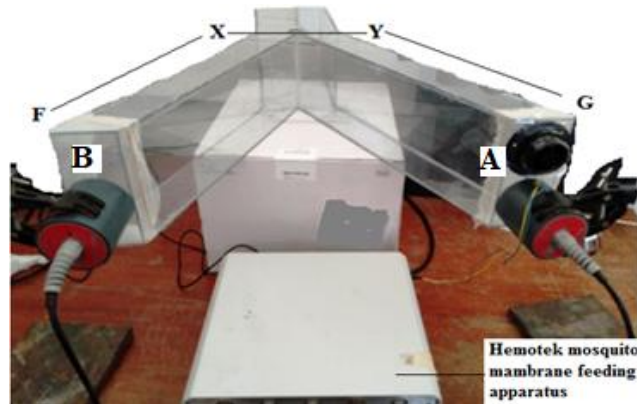


Figure 3. Bioassay set-up with A as a treatment chamber

The 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucis* which were used as the treatment on the blood meal in the bioassay study were played through the netting on side A and B of the fighto-Y cage interchangeably to avoid bias. The blood meal in the treatment chamber was placed 2.0 - 3.0 cm from the source of the natural sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucis* (Arthur *et al.*, 2014). Two sets of bioassays, the treatment, and control experiments were performed simultaneously by exposing fifty mated and starved female *A. gambiae* to cow blood meal in the fighto-Y cage under controlled laboratory conditions of 25±2°C and 70±10% relative humidity. The bioassay study conducted in the chamber in which the starved and mated female *A. gambiae* s.s were exposed to blood meal with no sound was the control whereas the bioassay study in the chamber in which the starved and mated female *A. gambiae* s.s were exposed to blood meal with sound was the treatment as given in Figure 3. The study was based on the in vitro method ("in the glass") and the ASTM E951-94 repellent procedures with the treatment being the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucis* (Barnard, 2006; Buescher *et al.*, 1983). In these bioassay procedures, a stimulus is applied and the response observed repeatedly for a population and the response is estimated with the desired level of precision (Kröber *et al.*, 2010). In repellent bioassays, the stimulus is normally a dosage of repellent applied to human skin, to the skin of an animal subject, or an inanimate object such as fabric, membrane, or filter paper (Barnard, 2005). Fifty laboratory-reared mated and starved female *A. gambiae* were allowed into the neutral chamber using an aspirator through a 1.0 cm diameter opening on the netting at the neutral chamber of the fighto-Y glass cage. The hole was covered using a piece of cotton wool. The number of starved and mated female *A. gambiae* s.s that occupied Chamber B from point X to F and the number of starved mated female *A. gambiae* s.s occupying chamber A from point Y to G as shown in Figure 3 were considered to be responsive (activity) in chamber B and A respectively.

The number of starved mated female *A. gambiae* s.s whose

legs made contact with the blood meal or proboscis in contact with blood meal or both in chamber A or B were considered to have landed, probed (bit), or landed and probed the blood meal respectively and thus described as landed. However, the number of starved mated female *A. gambiae* s.s that remained in the neutral chamber were considered non-responsive. Position XY on Figures 2.2 and 2.3, which was the decision point for starved mated female *A. gambiae* s.s was 0.30 m from the point of release of the starved and mated female *A. gambiae* s.s into the fighto-Y cage (Rodriguez *et al.*, 2015; WHO, 2013). The mated female *A. gambiae* s.s had an equal likelihood in the choice of chamber A or B at point XY of the fighto-Y cage as shown in Figure 2.3. The 35-60 kHz natural animal sounds were played separately for duration of 1,200 s, and the number of female *A. gambiae* that approached the blood meal; landed on blood meal; landed and probed the blood meal in chamber A and B for duration of 120 s determined and recorded (Barnard, 2005).

2.2.1. Phonotactic Behaviour in the Mated Female *A. gambiae* s.s

Phonotaxis behavioural parameters of the mated female *A. gambiae* s.s evoked by the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas* on exposure to a blood meal included and not limited to the level and nature of flight, activeness, nature of movement along the surface of rest, postural adjustments, steering from the ultrasound, nature of landing on the floor surface of the cage, engorgement of the abdomen, mobility, composure, wing-beat frequency, and the hind-leg extension and deflection. Through the bioassay studies, the observable physical startle behaviour of the starved and mated female *A. gambiae* s.s on blood meal elicited by the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas* were observed and recorded. To study the phonotactic behaviour in the mated female *A. gambiae* s.s, fifty 3-5 day old starved and mated female *A. gambiae* s.s were allowed into the neutral chamber using an aspirator and allowed 10.0 s to settle and decide on either to enter the treatment chamber or the control chamber or remain in the neutral chamber voluntarily. The starved and mated female *A. gambiae* s.s in the control chamber were only exposed to warm blood meal whereas the starved and mated female *A. gambiae* s.s in the treatment chamber were exposed to a warm blood meal and the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas* sounds. The starved and mated female *A. gambiae* s.s in the neutral chamber were neither exposed to the warm blood meal nor the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas* sounds. The behavioural responses in the control chamber and treatment chamber were simultaneously observed and electronically captured using a high definition Canon 1300D camera at an interval of 120 s for 1200 s.

2.2.2. Protection Index (PI) and Spatial Activity Index (SAI)

The protection index (PI) and spatial activity index (SAI) were determined using equation 1 and 2 respectively based on the number of starved and mated female *A. gambiae* s.s that responded, and landed on the blood meal in chamber A and B which was a measure of the extent of protection of the blood meal against the mated female *A. gambiae* s.s and the nature of phonotactic reactions of the starved and mated female *A. gambiae* s.s to sound.

3. Results and Discussion

3.1. Comparative Analysis of the SAI and the PI in the Mated Female *A. gambiae* Evoked by the 35-60 kHz Individual Sound of the Male *A. gambiae* s.s, *O. tormota*, and *D. leucas*

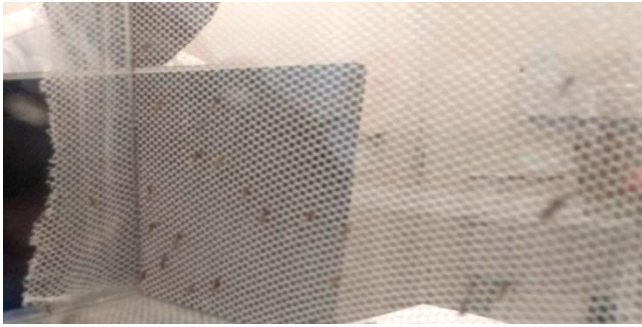
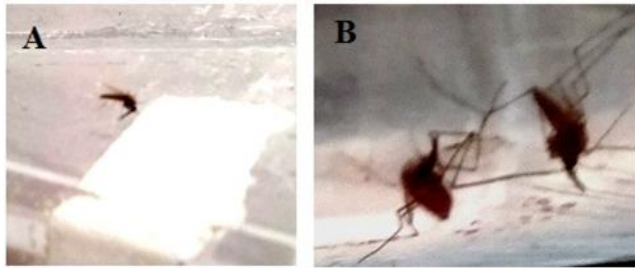
3.1.1. Phonotactic Behaviour in the Mated Female *A. gambiae* s.s

The mated female *A. gambiae* s.s in the control and neutral bioassay chamber rested in the normal posture and composure projecting their abdomen in the air at 45° under the bioassay study as given in Figure 4. A swarm of mosquitoes flew lowly in and out of the control chamber with some mosquitoes flying about freely, landing with ease and at times rested around the blood meal which was not treated with the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas*. During the 360-480s duration, only three fully fed mosquitoes rested on the cage walls in the control chamber under the 35-60 kHz sound of *O. tormota* bioassay study. The number of female *A. gambiae* s.s landing, probing, and feeding in the control chamber increased steadily up to the 840th second in the bioassay involving the 35-60 kHz sound of *D. leucas*. Low flights exhibited by the mated female *A. gambiae* s.s were attributed to increased weight resulting from feeding on blood meal. The fully fed mated female *A. gambiae* s.s were characterised by red engorged abdomen. Normal wing-beat for searching blood meal associated with unsteered flight was observed in the mated female *A. gambiae* s.s. Notably, 17 mated female *A. gambiae* were observed approaching the control chamber of which eleven were seen landing, probing, and feeding during the first 120 s for the bioassay study involving the sound of 35-60 kHz sound of the male *A. gambiae* s.s. Fully fed mated female *A. gambiae* s.s exited the control chamber to the neutral chamber leading to a reduction in the number of the female *A. gambiae* in the control bioassay chamber.

Generally, the number of mated female *A. gambiae* approaching blood meal in the control chamber increased with time compared to the number approaching the blood meal in the treatment chamber as shown in Table 1 under the bioassays involving the 35-60 kHz sounds of the male *O. tormota*, male *A. gambiae*, and male and female *D. leucas*.

Table 1. The number of mosquitoes approaching the control and treatment chamber for the 35-60 kHz individual animal sounds

Time (s)	35-60 kHz sound of <i>O. tormota</i>		35-60 kHz sound of <i>A. gambiae s.s</i>		35-60 kHz sound of <i>D. leucas</i>	
	Warm blood meal with Sound (N _i)	Warm blood meal without Sound (N _c)	Warm blood meal with Sound (N _i)	Warm blood meal without Sound (N _c)	Warm blood meal with Sound (N _i)	Warm blood meal without Sound (N _c)
120	8	19	10	17	7	13
240	5	16	8	21	5	15
360	9	20	5	22	6	13
480	12	20	3	18	6	15
600	11	17	3	17	4	16
720	7	16	6	18	4	17
840	12	13	3	30	6	18
960	8	14	2	22	5	17
1080	8	12	3	21	2	14
1200	11	15	4	20	2	12

**Figure 4.** The female *A. gambiae* resting on the netting from the neutral chamber**Figure 5.** Unusual posture and composure in the female *A. gambiae s.s* under treatment of the 35-60 kHz sound of *O. tormota* in the treatment chamber

The 35-60 kHz sounds of the male *A. gambiae s.s*, *O. tormota*, and *D. leucas* were used as a treatment in the treatment chamber. Ten female *A. gambiae* mosquitoes approached the blood meal with 90% of the mosquitoes successfully landing, probing, and feeding during the first 120 s in the treatment bioassay chamber under exposure to the 35-60 kHz sound of the male *A. gambiae s.s*. For the 35-60 kHz sound of *O. tormota* bioassay study, the number of female *A. gambiae s.s* in the treatment chamber flew about uncomfortably and rested in unusual posture and composure as shown in Figure 5. Additionally, adverse behavioural

responses included significant antennae erection, the extension of hind-legs and deflection, and open wings as shown in Figure 5 A and B due to neural discomfort and fear of predation.

There was increased flight activity in the treatment chamber with some mated female *A. gambiae* resting on the floor of the treatment chamber the first 240 seconds under exposure to the 35-60 kHz sounds of *D. leucas*. Similar observations were observed with the 35-60 kHz sounds of the male *A. gambiae s.s*, and *O. tormota*. Uniquely, some mosquitoes in the treatment chamber flew to the blood meal and unsuccessfully fed then exited to the neutral chamber due to the 35-60 kHz sounds of the male *A. gambiae s.s*, and *O. tormota* that evoked neural stress. In the treatment chamber of the 35-60 kHz sounds of *D. leucas* and 35-60 kHz sounds of *O. tormota*, unfed mosquitoes were seen flying lowly in a bouncing manner along the floor, with some exhibiting minimal movement within the chamber. Further, some mated female *A. gambiae s.s*, shown in Figure 6 were seen squeezing through the edges of the chamber with one resting on its belly with spread legs due to the 35-60 kHz sounds of *O. tormota*. Unlike the mosquitoes in the control chamber which appeared relaxed while feeding, the mosquitoes in the treatment chamber under the treatment of the 35-60 kHz sounds of the male *A. gambiae s.s*, *O. tormota*, and *D. leucas* appeared disturbed by exhibiting body shakes. The number of mated female *A. gambiae s.s* in the treatment chamber declined drastically with time. The mated female *A. gambiae s.s* displayed steered fast flights associated with bounces on the walls and floor in the treatment chamber under all sounds studied. In the 240-360 s duration, six mated female *A. gambiae s.s* out of nine were seen flying about while one fully fed *A. gambiae s.s* rested on the floor with a shaky body, a behaviour elicited by the 35-60 kHz sounds of *O. tormota*. A total of 12 unfed mosquitoes in the treatment chamber rested near the neutral chamber with one bouncing

along the wall and floor under exposure of the 35-60 kHz sounds of *O. tormota* during the 960-1200 s period. Similarly, low exit flights into the neutral chamber, rest on the floor and general immobilisation was observed in the mated female *A. gambiae s.s* in the 0-960 s duration under exposure to the 35-60 kHz sounds of *O. tormota* and *D. leucas*. The confusion and immobilisation observed in the mated female *A. gambiae s.s* were attributed to neural stress and fear of predation caused by the ultrasound.



Figure 6. The mated female *A. gambiae s.s* under treatment of the 35-60 kHz sounds of *O. tormota* squeezing through the edges of the chamber

Figure 7 gives the instantaneous SAI of the response of the female *A. gambiae s.s* elicited by the 35-60 kHz animal sounds with the sound of the male *A. gambiae s.s*, and *D. leucas* yielding a positive trend in SAI of 0.016/s and 0.008/s respectively. However, the sound of *O. tormota* in the 35-60 kHz frequency range which was highly pulsating in nature yielded a declining trend in SAI with a slope of -0.015/s. The 35-60 kHz sounds of *O. tormota* sustained a slightly declining trend in the instantaneous SAI response in the female *A. gambiae s.s* though the number of the female *A. gambiae s.s* that approached the control chamber was greater than the number of the mosquitoes that approached the treatment chamber as shown in Figure 7. Notably, the entire measurements of the instantaneous SAI of in the female *A. gambiae s.s* evoked by the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* was a negative phonotaxis response, with the magnitude of repellency being determined by the nature and acoustic spectral properties of the animal sounds. During the first 120 s, the sounds of the male *A. gambiae s.s*, and *D. leucas* yielded the least SAI of 0.14 and 0.12 respectively attaining a maximum of 0.54 at 840 s and 0.26 at 720s respectively. Beyond the 840th s and 720th s, the SAI of the female *A. gambiae s.s* evoked by the sounds of the male *A. gambiae s.s*, and *D. leucas* respectively declined yielding 0.32 and 0.20 between 1080-1200 s duration. However, the sound of *O. tormota* recorded the highest SAI of 0.22 in the response of the female *A. gambiae s.s* at 120s, yielding the least SAI of 0.02 during the 840th second and the trend reversed. The greatest instantaneous SAI evoked by the sound of the male *A. gambiae s.s* was attributed to neural stress and the refractory response of the female *A. gambiae s.s* to male ultrasound in avoidance of further mating. The activity of the mated female *A. gambiae s.s* in the control chamber was based on

the number of responding mated female *A. gambiae s.s* in the bioassay study. The responding mosquitoes included the mated female *A. gambiae s.s* that entered the treatment and the control chamber under the treatment of the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas*. The rate of activity in the mated female *A. gambiae s.s* due to the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* were 8.10 activity/minute, 10.30 activity/minute and 7.50 activity/minute respectively in the control bioassay chamber. The paired sample T-test comparison of the number of female *A. gambiae s.s* that approached the blood meal in the treatment chamber to ones that approached the blood meal in the control chamber in a bioassay study involving the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* showed a highly significant difference in the activities with significance values $p = 1.1752 \times 10^{-4}$, 5.5459×10^{-6} , and $2.3148 \times 10^{-7} < 0.05$. The number of female *A. gambiae s.s* that approached the blood meal in the treatment chamber to the ones that approached the blood meal in the control chamber independently in a bioassay study involving the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* correlated lowly with Pearson's correlation values $p = 0.0980$, -0.3386, and 0.1957.

The overall mean SAI in the female *A. gambiae s.s* evoked by the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* were 0.142, 0.318, and 0.206 respectively as shown in Figure 9. This was generally a repellent response in the mated female *A. gambiae s.s* initiated by the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas*.

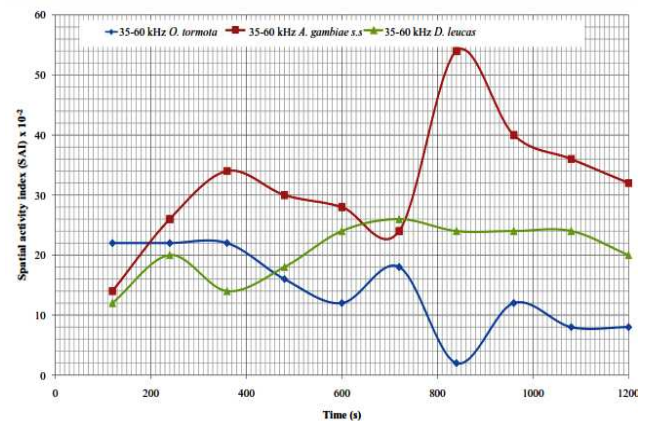


Figure 7. The SAI yielded by the 35-60 kHz animal sound in the female *A. gambiae s.s*

3.1.2. Protection Index (PI) and Spatial Activity Index (SAI)

The number of mosquitoes that landed, on the blood meal treated with the 35-60 kHz sounds of *O. tormota*, male *A. gambiae s.s*, and *D. leucas* in the treated chamber was lower compared to the number of mosquitoes that landed on the blood meal in the control chamber as given in Table 2 and Figure 8. The least instantaneous protection indices yielded due to the sounds of the male *A. gambiae s.s* and *D. leucas* were 18.18% and 33.33%, with corresponding maximum instantaneous protection indices of 100% and 92.31%

respectively as shown in Figure 8. A positive general trend of 0.043%/s and 0.046%/s in the instantaneous PI was yielded by the 35-60 kHz sound of the male *A. gambiae* s.s, and *D. leucas*. However, the 35-60 kHz sounds of *O. tormota* recorded a negative trend of -0.016%/s in the PI against the female *A. gambiae* s.s as shown in Figure 8. The 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* elicited 0.75 landings/minute, 1.05 landings/minute, and 1.25 landings/minute respectively in the treatment bioassay chamber, whereas the control bioassay chamber yielded 4.15 landings/minute, 4.50 landings/minute and 5.1 landings/minute respectively as given in Figure 9 and Table 2.

Table 2. The number of mosquitoes landing on blood meal in the control and treatment chamber for the 35-60 kHz individual animal sounds

Time (s)	35-60 kHz Sound Source					
	<i>O. tormota</i>		<i>D. leucas</i>		Male <i>A. gambiae</i>	
	TI	CI	TI	CI	TI	CI
120	2.00	11.00	4.00	6.00	9.00	11.00
240	2.00	13.00	3.00	7.00	5.00	13.00
360	1.00	12.00	4.00	8.00	2.00	9.00
480	2.00	12.00	4.00	10.00	1.00	11.00
600	2.00	9.00	1.00	12.00	0.00	10.00
720	2.00	7.00	1.00	13.00	2.00	8.00
840	0.00	5.00	3.00	16.00	1.00	7.00
960	1.00	5.00	3.00	12.00	0.00	10.00
1080	1.00	5.00	1.00	8.00	0.00	7.00
1200	2.00	4.00	1.00	10.00	1.00	4.00

The number of mosquitoes that landed on the blood meal in the treated chamber, where the blood meal was treated with the 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* differed significantly from the ones that landed, probed, and fed on the untreated blood meal in the control chamber with significance value $p = 9.9523 \times 10^{-5}$, 2.7808×10^{-5} and 1.0429×10^{-4} respectively, determined through the paired sample T-test comparison. The 35-60 kHz sounds of *O. tormota* correlated positively low with the 35-60 kHz sounds of the male *A. gambiae* s.s with a Pearson's correlation value $r = 0.3589$. However, the 35-60 kHz sounds of *O. tormota* correlated negatively low with the 35-60 kHz sounds of *D. leucas* with a Pearson's correlation value $r = -0.1741$.

The overall mean PI against the mated female *A. gambiae* s.s of 80.06%, 78.41% and 71.82%, shown in Figure 9 were yielded by the 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* respectively. The PI of the sound of the 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* based on the number of mosquitoes that landed, probed, and fed on the blood meal in the treatment and control chamber exceeded the reported PI based startle responses due to the 35-60 kHz recorded sound of *O. tormota*, 40-55kHz EMR ultrasound, repellency due to the AC-UD and the Anti-Pic® respectively. The optimised PI of

80.06% yielded by the 35-60 kHz sounds of *O. tormota* exceeded the reported landing inhibitions in behavioural based startle response due to the 35-60 kHz recorded sound of *O. tormota*, 40-55kHz EMR ultrasound, repellency due to the AC-UD and the Anti-Pic® by 34.18%, 11.07%, 19.36%, and 49.76% respectively. The difference in PI determined through the one-sample T-test comparison for the 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* from the reported repellency based on observable behavioural responses in mosquitoes elicited by the 35-60 kHz recorded sound of *O. tormota* was significant with significance values, $p = 1.7523 \times 10^{-5}$, 0.0025 and 0.0013 respectively. Additionally, the PI of the 35-60 kHz sounds of *O. tormota* from the reported repellency based on observable behavioural responses in mosquitoes elicited by the designed EMR which generated ultrasound in the 40-55 kHz range, and determined through the one-sample T-test comparison was significant with significance value, $p = 0.0257$. However, the PI of the 35-60 kHz sounds of the male *A. gambiae* s.s, and *D. leucas* from the reported repellency based on observable behavioural responses in mosquitoes elicited by the designed EMR which generated ultrasound in the 40-55 kHz range, and determined through the one-sample T-test comparison was not significant, with significance values, $p = 0.2606$ and 0.6406 respectively. Further, the difference in PI elicited by 35-60 kHz sounds of *O. tormota*, and male *A. gambiae* s.s with the reported repellency evoked by the AC-UD, determined through the one-sample T-test comparison was significant with significance values, $p = 0.001$ and 0.050 respectively. Conversely, the difference in PI elicited by 35-60 kHz sounds of *D. leucas* with the reported repellency evoked by the air conditioner (AC) with the AC-UD, determined through the one-sample T-test comparison was not significant with significance value, $p = 0.088$. The PI determined through the one-sample T-test comparison of the 35-60 kHz sounds of *O. tormota*, male *A. gambiae* s.s, and *D. leucas* differed significantly from repellency reported yet disputed for the ultrasound generated by the Anti-Pic® with significance values, $p = 7.7511 \times 10^{-7}$, 1.7280×10^{-4} and 3.4454×10^{-5} respectively.

The minimum, maximum and mean acoustic energy of the highly pulsating 35-60 kHz sound of *O. tormota* for the 606 calls were $0.00058\text{Pa}^2\text{s}$, $8.01775\text{Pa}^2\text{s}$, and $1.4605\text{Pa}^2\text{s}$, exceeding the sound of the 35-60 kHz male *A. gambiae* of 30,223 calls by $0.00045\text{Pa}^2\text{s}$, $7.03576\text{Pa}^2\text{s}$, and $1.45235\text{Pa}^2\text{s}$ respectively. Also, the 35-60 kHz sound of *D. leucas* which consisted of 11,825 calls yielded lower minimum, maximum, and mean acoustic energy by $0.00047\text{Pa}^2\text{s}$, $6.87681\text{Pa}^2\text{s}$, and $1.42936\text{Pa}^2\text{s}$ from the corresponding measurements of the 35-60 kHz sound of the male *A. gambiae* s.s. Acoustic power for the sound of the 35-60 kHz sounds of the *O. tormota*, male *A. gambiae* s.s, and *D. leucas* as given in Figures 10 and Table 3 also were dependent on call duration. The 35-60 kHz sound of the male *A. gambiae* s.s yielded maximum and mean call duration of 0.1392 s and 0.00312 s, which were less than the corresponding measurements of the 35-60 kHz sound of *O. tormota* by 0.2693 s and 0.1159 s respectively.

Similarly, the call duration for the 35-60 kHz sound of *D. leucas* was 0.3911 s and 0.11843 s less than the corresponding measurements of the 35-60 kHz sound of *O. tormota*.

Table 3. Comparative power parameters for the 35-60 kHz natural animal sounds

Parameter	Sound Samples		
	<i>O. tormota</i>	<i>A. gambiae</i>	<i>D. leucas</i>
Aggregate entropy (Bits)	2.87	4.75	4.63
Average entropy (Bits)	2.87	4.75	4.63
Average power (dB)	55.60	49.70	39.80
Delta power (dB)	24.10	43.70	28.00
Maximum entropy (Bits)	2.87	4.75	4.63
Maximum power frequency (kHz)	38.09	37.11	42.97
Maximum power (dB)	75.90	60.50	51.70
Minimum entropy (Bits)	2.87	4.75	4.63
Peak power (dB)	75.90	60.50	51.70

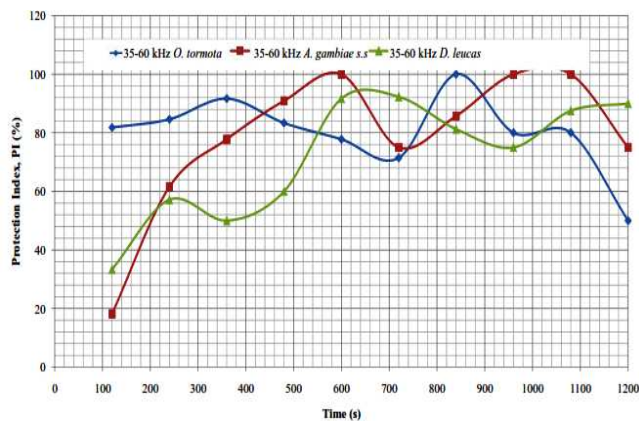


Figure 8. The instantaneous PI evoked by the 35-60 kHz sound of *O. tormota*, male *A. gambiae* s.s., and *D. leucas*

The 35-60 kHz sounds of *O. tormota* yielded equal aggregate entropy, average entropy, maximum entropy, and minimum entropy of 2.87 bits which was less than the corresponding parameters of the 35-60 kHz sounds of male *A. gambiae* s.s. and the 35-60 kHz sounds of *D. leucas* by 1.88 bits and 1.76 bits respectively as indicated in Table 3. However, the delta power (ΔP) of 35-60 kHz sounds of *O. tormota* was 24.1 dB, which was less than the corresponding parameters of the 35-60 kHz sounds of male *A. gambiae* s.s. and 35-60 kHz sounds of *D. leucas* by 19.6 dB and 3.9 dB respectively. The average power of the 35-60 kHz sounds of *O. tormota* was 55.60 dB, exceeding the corresponding measurements of the 35-60 kHz sounds of male *A. gambiae* s.s. and *D. leucas* by 5.90 dB and 15.80 dB respectively. The maximum power frequency of the 35-60 kHz sounds of *O. tormota* was 38.09 kHz which exceeded the corresponding measurements of the 35-60 kHz sounds of male *A. gambiae* s.s. by 0.98 kHz but was less than the respective measurements of the 35-60 kHz sounds of *D. leucas* by 4.88 kHz. The peak power (equal to maximum

power) of the 35-60 kHz sounds of *O. tormota* was 75.9 dB which exceeded the corresponding measurements of the 35-60 kHz sounds of male *A. gambiae* s.s. and 35-60 kHz sounds of *D. leucas* by 15.4 dB and 24.2 dB respectively.

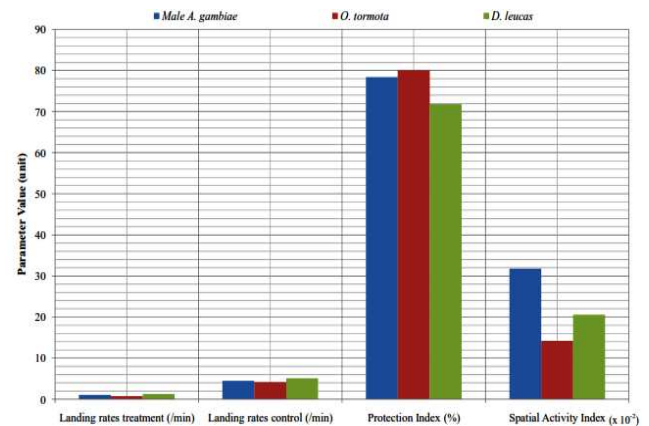


Figure 9. Overall female *A. gambiae* s.s. behavioural response evoked by the 35-60 kHz natural animal sounds

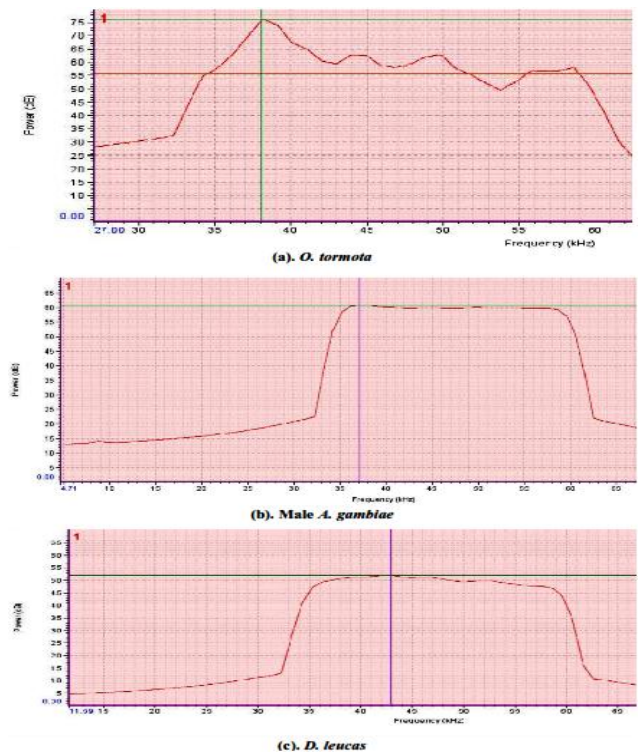


Figure 10. The 35-60 kHz frequency band power spectra for Animal sounds

4. Conclusions

1. The 35-60 kHz sounds of the male *A. gambiae*, *O. tormota* and *D. leucas* inhibited the interaction between the mated female *A. gambiae* s.s. (vector) and the blood meal mimicking host (host) condition, as evidenced in the negative phonotactic behavior.
2. The 35-60 kHz sounds of the male *A. gambiae*, *O. tormota* and *D. leucas* yielded positive spatial activity

index in the mated female *A. gambiae* s. s (vector), indicating repellency or negative phonotaxis. Additionally, the 35-60 kHz sounds of the male *O. tormota* evoked the greatest protection index (PI) against the mated female *A. gambiae*.

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Declaration

Ethical Approval

This research was vetted for Ethical Approval by Masinde Muliro University of Science and Technology Institutional Ethics and Review Committee (IERC). The approval was awarded vide Ethical MMU/COR: 403012 Vol 5(01). Additionally, Kenya Medical Research Institute committed to supply research materials which included mosquitoes and blood meal (for *Bos taurus*) vide letter CGHR/CORR/005/132. The research was approved by NACOSTI, Kenya vide License number NACOSTI/P/21/14342. The study was conducted in conformity with the standard operation procedures (SOPs) for mosquito rearing and World Health Organisation (WHO) guidelines for efficacy testing of spatial repellents. No consent for participation (did not involve human beings) and publishing was required.

Competing interests

There were No competing interests whatsoever in this study, whether of financial or personal nature.

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Availability of data and materials

All data used in this study is available within the Manuscript under Results and Discussion as Table 1-3.

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