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The Combined Predator Ultrasound Acoustic Startle Response in the Female *A. gambiae*

Mang'are P. A.[1,*], Maweu O. M.[2], Ndiritu F. G.[1], Vulule J. M.[3]

[1]Physics Department, Egerton University. P. O Box 536, Egerton, Njoro. Kenya
[2]Physics Department, SEUCO, University of Nairobi. P. O Box 170-90200, Kitui. Kenya
[3]Entomology Department, Kenya Medical Research Institute Centre for Global Health Research. P. O Box 1578, Kisumu. Kenya

Abstract Ultrasound is detected by the female *Anopheles gambiae* using its antenna, evoking either an attractive or repulsive response. Electronic mosquito repellents which exploit this concept in attempt to control malaria, have shown only 20 % effectiveness in repellence. The 112 Avisoft and 702 digital recorders were used to record sounds of *Coleura afra* and *Amolops tormotus* respectively. The sound of *C. afra* and *A. tormotus* were recorded, combined and filtered using the Avisoft software. The startling effect of the combined sound on female *A. gambiae* and the frequency range of optimum startle response were determined in this study. A bioassay was set up with 3-4 day old female *A. gambiae* exposed to 10-34 kHz, 35-60 kHz and 61-90 kHz frequencies of combined sound, total activities and behavioural responses observed and noted. The female *A. gambiae* were significantly startled by the 10-34 kHz combined predator sound triggering evasive behavioural responses in 30 % of the mosquitoes. An antenna erection of 58.5^o besides secondary effects like physical injury, unusual rest and movement, fatigue and falls; attributed to stress on the nervous system and fear of predation was observed. The combined ultrasound effectively repels the female *A. gambiae*.

Keywords Vocalization, Combined Predator Sound, Filters, Signal Power, Colony

1. Introduction

The protozoan parasite of genius *Plasmodium* which is transmitted by the female *Anopheles gambiae,* causes malaria which is the main cause of mortality and morbidity in Africa[5, 12]. Plasmodium parasites kill over a million people per year, and another 500 million people suffer from the clinical disease[13]. The female *A. gambiae* requires blood either from human or animal, for egg development. However, the male *A. gambiae* are incapable of feeding on blood because they lack piercing mouth parts[9, 16]. Malaria being life threatening, many control measures that include: chemotherapy, chemoprophylaxis, vector control strategies and development of malaria vaccine have been undertaken[5, 35]. Preferred vector control-methods include the use of Insect Treated Nets, Indoor Residual Spray, destruction of mosquito breeding sites and use of mosquito repellents[35]. The use of insecticides to control malaria vectors; and the use of drugs to control malaria parasites have failed due to build up of resistance in mosquitoes and the disease agent respectively[9, 35]. An effective prevention requires a combination of factors which address the habits of mosquitoes and their interaction with human beings[35].

* Corresponding author:
philipamuyunzu@gmail.com (Mang'are, P. A.)
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Female mosquitoes produce familiar whining sound whose frequency reflects the wing beat of the species, when searching for a blood meal. The frequency which ranges from 150 to 500 Hz has maximum intensity at 380 Hz for *A. gambiae*[21]. The mosquito sound is frequency modulated and it is transmitted in air activating the antennae of conspecific male besides providing directional indicators [10]. The sexes are brought together by response of the males to flight sound of the females[4]. The Johnstone's organ, located at the base of the antenna, resolves the sound[19]. Female mosquitoes which are mated once in their lifetime store sperms in the spermatheca. The mated female mosquitoes avoid males seeking for a mate by detecting their sounds[23].

Electronic mosquito repellents mimicking the sounds of male mosquitoes produce 38 kHz to repel the female mosquitoes[23]. The African bat, *Coleura afra* and the Chinese frog, *Amolops tormotus*, both insectivorous, generate ultrasound through vocalizations[2, 3, 7]. Bats which belong to order *Chiroptera* inhabit in caves feeding on mosquitoes and other insects. They produce sound by tongue clicks which fall in the frequency range of 20 – 100 kHz purposely for communication and navigation[14, 15, 22, 32, 34]. The aerially hawking bats emit ultrasonic probes and detect flying insect prey by their echoes which return from their bodies[28]. These ultrasonic signals are classified as short clicks, frequency swept pulses and constant frequency pulses[11, 18, 25, 33]. Echoes of high intensity are used to locate and track flying prey. Bats sophisticated echolocation enables them distinguish between mosquitoes and objects [24]. Echolocating bats are able to analyse the returning signal in order to determine the distance from the object, speed and type of object[22]. Since bats change their echolocation based on situation, they are able to approach maximize their ability to detect mosquitoes and other fast moving insects that serve as food[25, 26].

The Chinese frog, *A. tormotus* belongs to sub-order *neobatrachia* and produces countless vocalizations calls of ultrasonic frequency components[2]. Though most amphibians do not hear sound whose frequency is greater than 12 kHz, some frog species have special muscles in the larynx for producing longer glottal pulses giving time for frequency modulation (FM) of the carrier frequency which is simple. The frame-by-frame video analysis of the frog's calling behaviour has shown presence of two pairs of vocal sacs that contribute to the remarkable call-note complexity [6]. The *A. tormotus* are found in hill streams and the surrounding habitats breeding in streams and use ultrasound up to 128 kHz for communication[2, 7, 22, 28]. These ultrasonic communications were observed in the Chinese frog from Huangshan Hot Springs, China and whose males generate diverse bird-like melodic calls[27, 31]. Amphibians are a distinct evolutionary lineage from microchiropterans and cetaceans; hence ultrasonic perception in these animals is a new example of independent evolution[7]. These frogs feed on a wide range of terrestrial and aquatic animals of which insects form the greater part[3].

The 35-60 kHz sound of *A. tormotus* and *C. afra*, the optimum frequency range, evoked evasive responses in an average of 46 % and 23 % of the mosquitoes, higher than the reported 20 % effective repulsion of EMR sound[5, 17]. Earlier experiments electronic mosquito repellents mimicking calls from bats and male *A. gambiae* in the frequency range of 125 Hz to 74.6 kHz showed that 12 out of 15 field experiments yielded higher landing rate on the human bare body parts than the control experiments, translating to 20 % effectiveness, hence, considered insignificant to justify their use[5]. The repulsion of mosquitoes due to the combined sound of *A. tormotus* and *Coleura afra* had not been investigated. Due to the low effectiveness in mosquito repulsion, the current study determines startle effect and optimal startle frequency on the female mosquitoes through combined sounds of *A. tormotus* and the *C. afra* with a view to increase effectiveness.

1.1. Statement of the Problem

Electronic mosquito repellents (EMR) that mimic ultrasonic calls from bats and male mosquitoes, *A. gambiae* have been designed and used in startling the female mosquitoes, *A. gambiae*. Earlier studies showed that the electronic mosquito repellents yielded only 20 % significant repulsion on the female *A. gambiae* due to a wide bandwidth of the sound rendering it less intense and ineffective. Hence, there was need to investigate the combined natural sounds from *C. afra* and *A. tormotus*; determine their startle effect on the female *A. gambiae* and optimal startle frequency. Combining the predator sounds would narrow the average bandwidth and intensify the sound, thus improving on the effectiveness in the startling of the female mosquito *A. gambiae*.

1.2. Objectives

1.2.1. General Objective

To determine the startling effect and optimal startle frequency range of the combined sound of *C. afra* and *A. tormotus* on the female *A. gambiae,* a malaria vector.

1.2.2. Specific Objectives

i. To determine the startling effect of the combined sound of *C. afra* and *A. tormotus* on the female *A. gambiae.*

ii. To determine the optimal mosquito startle frequency range of the combined sound of *C. afra* and *A. tormotus.*

1.3. Justification

Electronic mosquito repellents which mimic ultrasound from animal species are currently in use for mosquito's repellence. However, these electronic mosquito repellents which generated wide bandwidth sound, yielded only 20% startle response in the female *A. gambiae* rendering them less effective. The African bat *C. afra* and the Chinese frog *A. tormotus* generated ultrasound naturally through vocalisation. It was therefore important to investigate the effect of this naturally generated combined ultrasound from *C. afra* and *A. tormotus*. Given that *C. afra* and *A. tormotus* were natural predators of mosquitoes, a combination of their sounds was expected to effectively startle the female *A. gambiae* due to natural fear of predation. The startle response of the female *A. gambiae* and the optimum startle frequency range elicited by the combined sounds of *C. afra* and *A. tormotus* was observed and noted. These results determined from the current research are critical to electronic mosquito repellents designers since effective devices could be realized. Also, the results provide an environment friendly additional tool in mosquito control.

1.4. Hypotheses

1) Combining the sound of *C. afra* and *A. tormotus* did not have any significant startling effect on the female *A. gambiae.*

2) The 10-34 kHz, 35-60 kHz and 61-90 kHz frequency range of the combined sound did not startle the female *A. gambiae* significantly.

2. Materials and Methods

2.1. Materials

2.1.1. The Female Mosquitoes

The *A. gambiae* mosquitoes were bred and reared at Kenya Medical Research Institute Centre for Global Health Research laboratories, Entomology department at 60 − 80 % humidity, 25 ± 2 °C temperature and twelve hours of day and night. The female *A. gambiae* mosquitoes were fed on 10 % glucose solution soaked in cotton wool. The female mosquitoes were identified by their sharp proboscis, large body size and affinity to blood meal. Ten samples of female *A. gambiae*, 3-5 day old were used in the study.

2.1.2. The Sound of *A. Tormotus* and *C. Afra*

Sound samples of *A. tormotus* and *C. afra* were recorded using the 702 digital and Avisoft recorder which consisted of the Avisoft Ultrasound Gate (Model 112) recorders respectively. The sound of *A. tormotus* was recorded from the Huangshan Hot springs, Anhui Province; China at a sampling frequency of 192 kHz. Also, the sound of *C. afra* was recorded from a colony in Kit-Mikayi caves, Kisumu; Kenya at a sampling frequency of 500 kHz.

2.1.3. The Equipment

A computer operating on Windows XP and office 2007 mounted with a sound card, hardlock key and sound output ports was used in sound recording. The Avisoft recorder, which consists of the Avisoft Ultrasound Gate (model 112) running on rec_usg.exe software, ultrasound microphone with high pass filter with cut-off frequency of 10 kHz was used in the recording of ultrasounds from the African bat, *C. afra*. Two Panasonic 8.0 Ω ordinary external speakers were used to play the predator sounds. The sound was amplified externally using an amplifier with output power = 18 W, impedance = 4.0 Ω with separation ≥ 45.0 dB. The stopwatch option in the Samsung cell phone was used to capture activity duration.

A bioassay investigation involving the recorded predator sounds was conducted in a glass cage, covered at the two ends with mosquito netting and whose dimensions were 50 cm long, 25 cm width and 25 cm in height. An aspirator was used to transfer the mosquitoes from the rearing cage to the bioassay cage and also remove them.

2.1.4. The Bioassay Arena

A bioassay glass cage of dimensions 0.50 m by 0.25 m by 0.25 m fitted with untreated mosquito net on the 0.25 m by 0.25 m faces was used. The cage was divided into three equal sections; A, B and C. Section C was the central region of the bioassay cage whereas section A was to the right and section B to the left. Both faces of dimension 25 cm by 25 cm were covered with untreated mosquito net and a small hole of 1.0 cm diameter hole perforated at their centres to allow the mosquito samples in and out of the cage. The two holes were covered with cotton wool and either the hole on side A or B could be used as a mosquito release point. The hole on side B of the net was used as the mosquito release point for consistence whereas the hole on side A was closed permanently using a piece of cotton wool.

3. Methods

3.1. Recording, Combining and Filtering of Sounds

3.1.1. Recording of the Predator Sounds; *C. Afra* and *A. Tormotus*

The sound of *C. afra* was recorded using Avisoft recorder which consisted of the Avisoft Ultrasound Gate (model 112) and a condenser microphone capsule (CM16). They were connected to the computer through one of the universal serial bus (USB) port. The omnidirectional microphone, selected from the voice recording settings was set as default in the computer. The Finite Impulse Response (FIR) was set to zero for both upper and lower cut-off frequencies. Also, the Fast Fourier transform (FFT) was set to 512 whereas the Hamming window selected for the display. Temporal resolution overlap was set to 50 % with colour palette set to gray pal. The 100% frame size was set for real time spectrogram parameters, checking the black and white box (B/W) in the display option. Avisoft-SAS LAB Pro, Version 5.1 software was open and the microphone directed to the source of sound. The gain was adjusted to an appropriate level to avoid over modulation. The recording level was set to 20 dB from the computer and the recording of the sound was started by pressing the record button on the Avisoft Ultrasound Gate. Sound samples of *C. afra* were recorded from a colony in Kit-Mikayi caves, Kisumu at a sampling frequency of 500 kHz for varying duration, the minimum being 45.00 s and the maximum being 300.00 s. The sound sample for the study was obtained by appending quality sound samples for a total duration of 1754.07 s playback duration, saved in the hard disc as “*Coleura* Sample 2.wav”.

Samples of sounds of *A. tormotus* were recorded using the 702 digital recorder from the Huangshan Hot springs, Anhui Province; China at a sampling frequency of 192 kHz. The sound samples were appended using the Avisoft SASLab Pro Version 5.1 to ensure a uniform duration of 1754.07 s and saved as “*A. tormotus*.wav” in the hard disc. The sampling frequency was converted from 192 kHz to 500 kHz using Avisoft SASLab Pro Version 5.1 for compatibility.

3.1.2. Combination of the Appended Sounds of the *A. Tormotus* and *C. afra*

The sound of *A. tormotus* and *C. afra* which were at a sampling rate of 500 kHz and each of duration 1754.07 s were combined using the Avisoft SASLab Pro Version 5.1 installed in the computer. The sound file “*A. tormotus*.wav” was open using the Avisoft SASLab analysis software and copied. The sound of “*Coleura* Sample 2.wav” was also open with the same software and mixed with the copied sound of *A. tormotus* to give the combined sound. This was achieved by using the mix option in the edit menu in Avisoft SASLab Pro Version 5.1 software. The combined sound was saved as "Combined sound.wav" in the hard disc.

3.1.3. Filtering of the Combined Sound Sample of *C. Afra* and *A. Tormotus*

The high pass filter, band pass filter and low pass filter, inbuilt in the Avisoft SASLab analysis software were used to segment the sounds into 10-34 kHz, 35-60 kHz and 61-90 kHz frequency ranges. Filter settings were made from the time domain filter (FIR) from the analysis software. The combined sound of *C. Afra* and *A. tormotus* was subjected to a high pass filter at a cut-off frequency, f_{co} = 10 kHz to attenuate noises. Also, a low pass filter set to a cut-off frequency, f_{co} = 90 kHz was used to allow frequencies of the three sounds which fell below 90 kHz. The combined sound of *C. Afra* and *A. tormotus* was also subjected to a band pass filter with an upper cut-off frequency, f_{uco}= 34 kHz and a lower cut-off frequency, f_{lco}=10 kHz. The 35-60 kHz and 61-90 kHz frequency ranges obtained by using settings f_{lco}= 35 kHz, f_{uco}= 60 kHz and f_{lco}= 61 kHz, f_{uco}= 90 kHz respectively.

3.1.4. Bioassay

The bioassay investigation involved determination of the startle response of the female *A. gambiae* to varied frequencies of predator sound and establishment of the frequency range with optimum frequency range. The criteria for the selection of the female *A. gambiae* included size, feeding status, activity, mouth parts, resting position and age. The combined sound was played through two external speakers attached to the cage. A set of ten, 3-5 day old female *A. gambiae* were released into the cage using an aspirator and observed one at a time. The behavioural startle response of the female *A. gambiae* to the 10-34 kHz, 35-60 kHz and 61-90 kHz combined sound frequency ranges was observed and the number of mosquito samples exhibiting the traits expressed as a percentage. The angle of antennae erection from the proboscis was measured from unmodified photo printout of the mosquito using a protractor. Also, the number of activities which included flights (F) and rests (R) were recorded and the duration recorded from the stopwatch correspondingly. The saved combined sound was played using a computer. Observation was made on the mosquito without playing the combined sound under the control experiment whose results were compared to those obtained when sounds were played.

3.2. Statistical Analysis

The data obtained from the study was statistically analysed using ANOVA.

4. Results and Discussion

4.1. Initial Behavioural Observations in the Female *A. Gambiae* Elicited by the Combined Sound of *A. Tormotus* and *C. Afra*

The percentage of mosquito samples that rested with their bodies inclined at 45^{o} from surface of rest with wings laid along their bodies and moved normally within the cage was 90 % and 60 % respectively. In all the mosquito samples, the antennae and proboscis were almost collinear at the control. None of the mosquito samples under study squeezed themselves between barriers, hid behind barriers, raised their limbs, nor rested the limbs and proboscis on net or cage surface in the control experiment; responses observed in individual predator sounds. Only 10 % of the mosquito samples displayed normal flight within the cage. An equal number of mosquito samples were seen rubbing their limbs, wings or both under similar conditions as shown in Table 1.

Table 1. Percentage of mosquito samples under the control experiment

Mosquito behaviour	Combined Sound
No body movement	0
Squeezing/ hiding in barriers	0
Raised limbs	0
Normal movement in the cage	60
Rubbing of legs and/or wings	10
Normal flight about in the cage	10
Rest at 45^{o} from rest surface ; wings along body	90
Limbs and proboscis resting on net or cage	0
Antennae and proboscis almost collinear	100

The effect of the combined sound of *A. tormotus* and *C. afra* on the *A. gambiae* had not been reported in recent findings for the 10-34 kHz, 35-60 kHz and 61-90 kHz sound frequencies. Contrary to recent findings involving the 10-34 kHz ultrasound generated by EMR by[23] the current study observed that the combined natural sound from the mosquito predators evoked behavioural response. New responses not observed with individual predator ultrasound included; rest by back or side or rolling on surface; and exhaustion or collapsing in mosquitoes which were noted in 30 % and 10 % of the mosquito samples studied respectively[17]. In this frequency range, 50 % of the mosquito samples studied exhibited spread of limbs on rest surface and erection of antennae which was an increase from the number of mosquitoes affected by individual predator sound[17]. The number of mosquitoes that did not show any significant body movement under the influence of the combined sound was reduced by 30 % from the number recorded in individual predator sounds[17]. However, the number of mosquitoes that displayed jumping or bouncing due to exposure to the combined sound was 50 %. These behavioural traits confirmed the startling of mosquitoes by ultrasound as reported in recent studies[23, 30].

The number of mosquitoes that squeezed their bodies and proboscis in barriers when subjected to combined sound reduced considerably by 50 % from that observed under the sound of *A. tormotus*. However, the number increased by 10 % from that noted under influence of the sound of *C. Afra*[17]. Also, the number of mosquitoes raising or folding of limbs, or both, was reduced by 10 % from the number under the sound of *A. tormotus* but increased by an equal margin with the sound of *C. afra*[17]. Raising and lowering of mosquito body from the resting surface was observed in 20 % of the mosquitoes, a number that was maintained for the sound of *C. afra* but reduced by 30 % from the number under *A. tormotus* sound. Directional mosquito movement was not observed in this frequency range for the combined sound though 40 % of the mosquito samples rubbed their hind limbs or wings. The number of mosquito samples exhibiting body shaking and abdomen curving were 70 % of mosquito samples.

Out of the ten *A. gambiae* mosquitoes studied, 40 % exhibited weak or exaggerated flights associated with occasional falls when exposed to the combined predator sound. The number of mosquitoes moving away from the combined sound source or rested most in corners or behind barriers was 10 %, an evasive response observed in higher frequencies emitted from EMR[23]. Flapping of wings was prominent in 30 % of the sample mosquitoes when the samples were exposed to combined ultrasound. A percentage mean of 30 % of the total mosquito samples studied in this frequency range was startled by the combined sound which was greater of than the mean percentage affected by the sound of *C. afra* by 12.94 %, but less than that due to the sound from *A. tormotus* by 4.12 %[17]. The difference in response in mosquitoes to predator ultrasound was due to reduced maximum and mean acoustic energy in this frequency range. This is evidenced in a reduction of maximum acoustic energy by 2.78 kPa^2s and 8.78 kPa^2s in sounds of *A. tormotus* and *C. afra* respectively[18]. Similar reduction was observed in the mean acoustic energy by 1.13 kPa^2s and 0.0085 kPa^2s in the sounds of *A. tormotus* and *C. afra* respectively[18]. More so, the maximum and a minimum signal power of the combined sound fluctuated between -98 dB and -136.67 dB respectively referenced to -20 dB. The signal power in *C. afra* was the greatest of all[18]. The closeness in mosquito behavioural response to the sound of *A. tormotus* and the combined sound was attributed to equal power range with minimum deviation in acoustic energy[17, 18]. Similarly, the difference in mosquito response to combined sound from individual predator sound was because the mean bandwidth for the combined sounds which was broader than that of *A. tormotus* but narrowed from that of *C. afra* by 5.44 kHz and 1.29 kHz respectively[18].

In the 35-60 kHz, the optimum startle frequency range for individual predator sound, characterised by fatigue, loss of limbs and collapsing was seldom in any mosquitoes on exposure to the combined sound[17]. This frequency range had also been reported to startle mosquitoes optimally in recent findings[1, 5, 23]. Fifteen out of seventeen (88.23 %) behavioural traits were observed in mosquito samples which were exposed to ultrasound from the combined sound as indicated in Table 2.

This frequency range elicited the least number of mosquito samples, 10 %, which either rubbed their limbs or showed no body movement at all. Antennal erection of up to 58.5^o, observed in 70 % of the mosquito samples, was elicited by the combined sound. This number was 10 % less than that observed in the sound of *A. tormotus*[17]. The combined sound also elicited spreading of limbs, movement away from sound source, flapping and opening of wings in 30 % of the mosquitoe samples, a number that was less than that observed in the sound of *A. tormotus* in the same frequency range[17]. The number of mosquitoes spreading limbs, moving away from sound source, flapping and opening of wings in the combined sound was higher or equal to the number under the influence of *C. afra*. In this frequency range, 90 % of the sample mosquitoes were noted to squeeze their bodies and proboscis between barriers, 20 % and 40 % above the number noted under the sounds of *A. tormotus* and *C. afra* respectively[17].

Table 2. Percentage of mosquito samples under varied frequency ranges of the combined sound of *A. tormotus* and *C. afra*

Observable mosquito behavioural traits	Percentage of Mosquitoes under Combined sound frequency (kHz)		
	0-34	35-60	61-90
No body movement	0	10	10
Jumping and/or Bouncing	50	50	40
Squeezing body and proboscis/ hiding in barriers	40	90	30
Raised limbs/ folded limbs	50	70	70
Raising and lowering of body	20	30	10
Forward/ backwards or sideways body movement	0	30	0
Body shaken/ Abdomen curving thorax	70	50	40
Rubbing of limbs or wings	40	10	10
Rest by abdomen/ thorax with limbs on surface	30	70	50
Flapping or opening of wings	30	30	40
Weak or exaggerated flights, falls and escape	30	40	40
Movement away from sound source	10	30	20
Spreading of limbs when resting	50	30	20
Erect antennae	50	70	80
Tired or weak or collapsed mosquito	10	0	20
Rest by back/ Sideways rest / Rolling on surfaces	30	50	90
Loss of limbs	0	0	10
Average Number of Mosquitoes	30.00	38.82	34.12

Jumping and/or bouncing was observed in 50 % of the sample mosquitoes whereas 70 % raised and folded their limbs and also rested by their abdomen. The number of mosquito samples showing shaken body, abdomen curved towards thorax; rested by side, back or rolled on surface was observed in 50 % of the sample mosquitoes. There were 40 % of the mosquito samples which displayed either weakened or exaggerated flights and falls on exposure to the combined sound.

The average percentage of the mosquitoes affected by the combined sound in the 35-60 kHz frequency range was 38.82 %, which was greatest. The maximum acoustic energy for the combined sound, which progressively increased from the energy in the 10-34 kHz, was 0.5009 Pa^2s and 1.9804 Pa^2s less the maximum acoustic energy in the sound of *A. tormotus* and *C. afra* respectively[18]. However, the mean acoustic energy in the combined sound, which was higher than that for the sound of *C. afra* in this frequency range reduced by 0.0427 Pa^2s from its energy recorded in 10-34 kHz. The signal power for the combined sound sustained a constant power trend with a narrowed mean bandwidth (maximum entire) from both sounds of *A. tormotus* and *C. afra* by 0.079 kHz and 0.5312 kHz respectively[18].

The study observed that the 61-90 kHz frequency range startled the female *A. gambiae* considerably. The mosquitoes erected and opened their antennae and sustained it at 58.5^o in 80 % of the mosquito samples studied in this combined sound frequency range. The number of mosquitoes exhibiting this antennal behaviour was 50 % more than those noted in *A. tormotus*[17]. Shaking in mosquito bodies, rest by back or side or rolled, weak flights with several falls and resting by abdomen or side was observed in 40 %, 90 %, 40 % and 50 % of the mosquito samples respectively on exposure to the combined sound. Only 50 % of the mosquitoes investigated rested by the abdomen on the net and also 10 % of the mosquito samples rubbed wings and legs. Occasionally the mosquitoes hang on the net weakly with their abdomen curved towards thorax. The mosquitoes had either one or both wings open. There were loses of limbs, five remaining in 10 % of the sample mosquitoes, a drop of 10 % from the number observed with the sound of *A. tormotus*[17]. The 20 % of mosquito samples which rested by abdomen also spread their limbs. An equal number tended to move away from the source of the combined sound. Bouncing and jumping on the surface was also observed in 40 % of the sample mosquitoes, occasionally, raising their legs and wings simultaneously. The mosquitoes appeared weak and displayed no body movement in 20 % and 10 % of the sample mosquitoes respectively, as observed in the 35-60 kHz frequency range. The limbs in 70 % of mosquito samples were occasionally raised and folded backwards while resting by the abdomen. The 20 % of the sample mosquitoes appeared exhausted though they flew about weakly. Only 10 % of mosquito samples were observed moving up and down from the rest surface as they jumped and rolled severally. The combined sound of *C. afra* and *A. tormotus* evoked squeezing of their bodies and proboscis in barriers in 30 % of the mosquitoes. Similarly, flapping and opening of wings was observed in 40 % of the sample mosquitoes. The percentage of mosquitoes startled by the combined sound was 34.12 %, equal to the number evoked by the sound of *A. tormotus* but greater than that of the sound of *C. afra*[17]. The increase in the number of mosquitoes disturbed by the combined sound was due to the steady signal power that stretched between -108.75 dB and -130 dB at a -20 dB threshold and referenced to 1 pW[18]. It was also noted that the mean bandwidth (maximum entire) for the combined sound was greatly narrowed from that of the sound of *A. tormotus* and *C. afra* by 23.9415 kHz and 8.3566 kHz respectively[18]. However, combining the sounds of *A. tormotus* and *C. afra* lowered the maximum acoustic energy from that of individual predator sounds by 2.104 Pa^2s and 0.0775 Pa^2s respectively[18]. Similarly, the combined sound energy was less than the energy in the 35-60 kHz by 4.8251 Pa^2s, hence a reduction in the average number of mosquitoes affected by the sound[17, 18].

4.2. The Influence of Combined Sound of *A. Tormotus* and *C. Afra* on Mosquito Activities

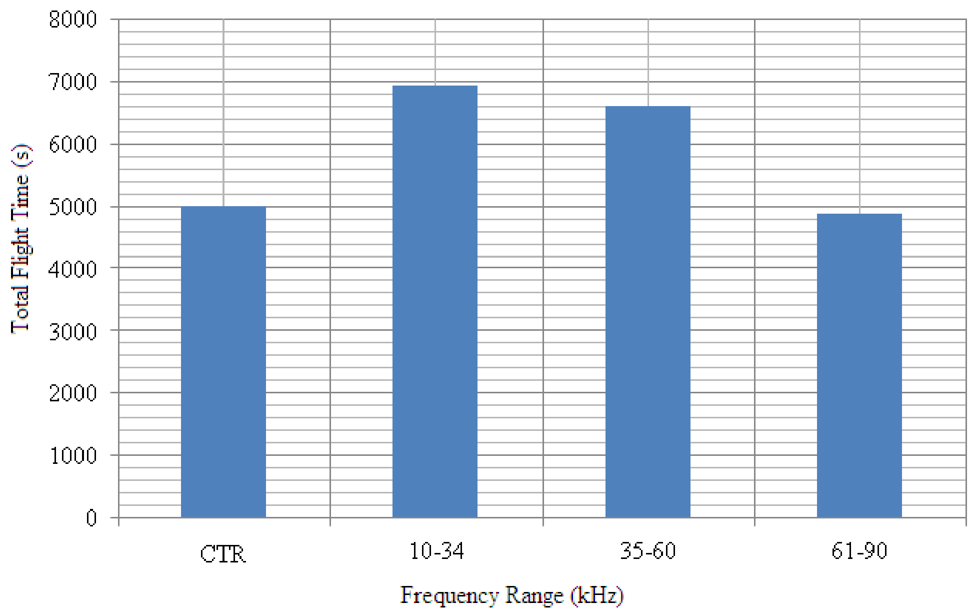

Figure 1. The total mosquito flight time in relation to varied frequencies of the combined sound

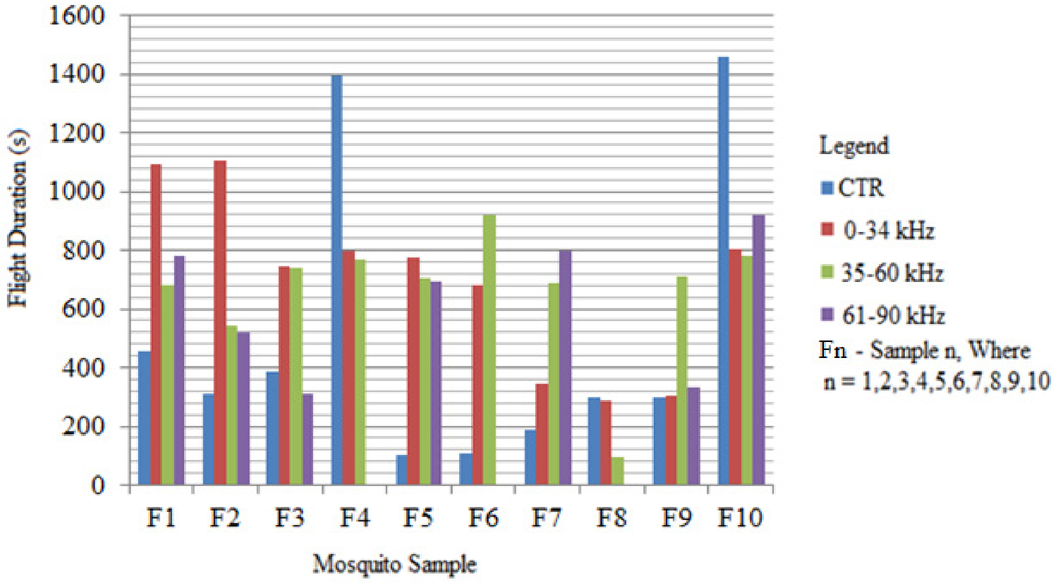

Figure 2. Variation of mosquito flight duration with varied frequencies of the combined sound

The female *A. gambiae* remained suspended in air in the 10-34 kHz and 35-60 kHz frequency range; with their flight time distinctly above the control as shown in Fig. 1. There was an increase in total flight time from the control by 1926.10 s, an indication of the excitation due to ultrasound. In 60% of mosquito samples exposed to the 10-34 kHz combined sound, the total flight time was distinctly above all the total flight time recorded in 36-60 kHz, 61-90 kHz and the control as shown in Fig. 2. The samples displayed a docile behaviour in the range of 61-90 kHz with the total flight time for 40 % of the mosquito samples being least due to drastic drop in energy. Earlier studies reported that the mosquitoes' evasive behaviour was due to the stress caused on the nervous system and fear of predation[8, 23, 30].

The rest time for the mosquitoes exposed to combined sound in the 10-34 kHz and 35-60 kHz frequency range was below the control. The mosquitoes were disturbed with the onset of the 10-34 kHz of the combined sound as noted in the sudden increase in flight time which was above the control. Other studies had reported that insects became dormant with some getting immobilized due to fatigue and stress[8, 23, 30].

4.3. Mosquito Activities under the Influence of Different Frequencies of Combined Sound

The number of activities of the mosquitoes under the influence of different frequencies of the combined sound was critical in establishing the frequency range that evoked effective response in mosquitoes. The mosquitoes were considered to exhibit normal activity under the control experiment as shown in Fig. 3 and Fig. 4. It was noted that 60 % of the sample mosquito activities in the 10-34 kHz range were above all the rest time in other frequency ranges. All frequency ranges in the combined sound elicited activities in mosquitoes which were above the control experiment. More activities were exhibited by the female *A. gambiae* in the 10-34 kHz frequency range with minimum activities being exhibited at the control. All the total mosquito activities in 10-34 kHz, 35-60 kHz and 61-90 kHz frequency ranges were above the control, an evidence for the startle response to the combined sound on the female *A. gambiae*. The total number of activities of the mosquitoes in the 10-34 kHz frequency range increased greatly by 451, above the control. There was a slight decline in the activities by 181 as the frequency range changed from 10-34 kHz to 35-60 kHz. However, the activities increased to 755 in the 61-90 kHz, though still less than the activities recorded in the 10-34 kHz frequency range illustrated in Fig. 5. The sampled mosquitoes displayed 92.81 activities per hour at the control experiment also shown in Fig. 5, which drastically rose by 185.42 activities per hour when the first sound of 10-34 kHz was played. The rate of activities per hour declined as the mosquitoes were exposed to 35-90 kHz, later increasing slightly to 155.05 activities per hour in the 61-90 kHz frequency range. The activities exhibited in various frequency ranges were associated with respective behavioural responses discussed in 4.1.

Though there was a decline in acoustic energy, the steady signal power of the combined sound in the 61-90 kHz frequency range yielded increased activity in mosquitoes. However, the signal power in the sound of *A. tormotus* fluctuated over time whereas the signal power of the sound of *C. afra* declined; lowering the mosquito activities[17, 18].

The comparison of the mosquito activities in the10-34 kHz, 35-60 and 61-90 kHz combined sound ranges by activities under the control was determined and shown in Table 3. The combined sound in the 61-90 kHz frequency range yielded significance value, $p = 0.5343 > 0.05$. The combined sound under 10-34 kHz and 35-60 kHz yielded significance values, $p = 2.5657 \times 10^{-5} < 0.05$ and $0.0128 < 0.05$ respectively.

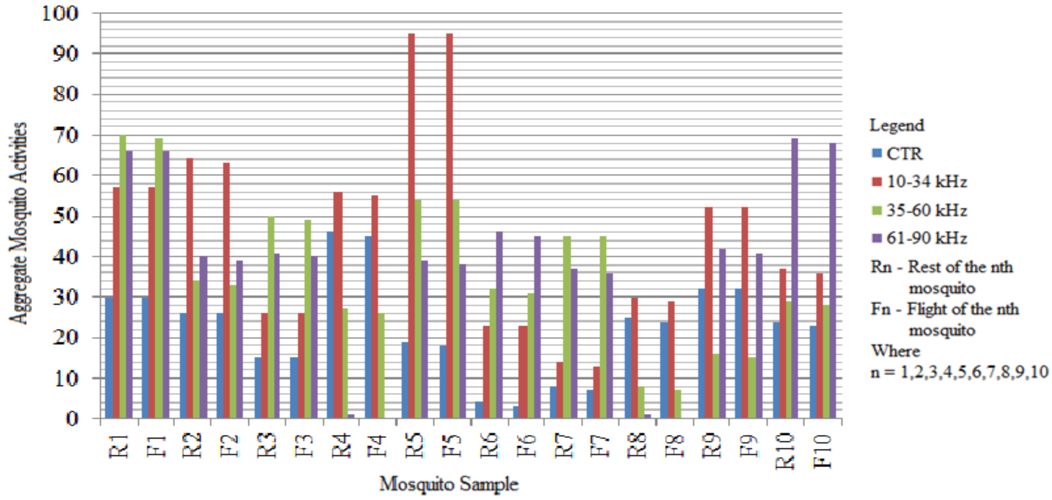

Figure 3. Distribution of aggregate mosquito activities with varied combined sound frequencies

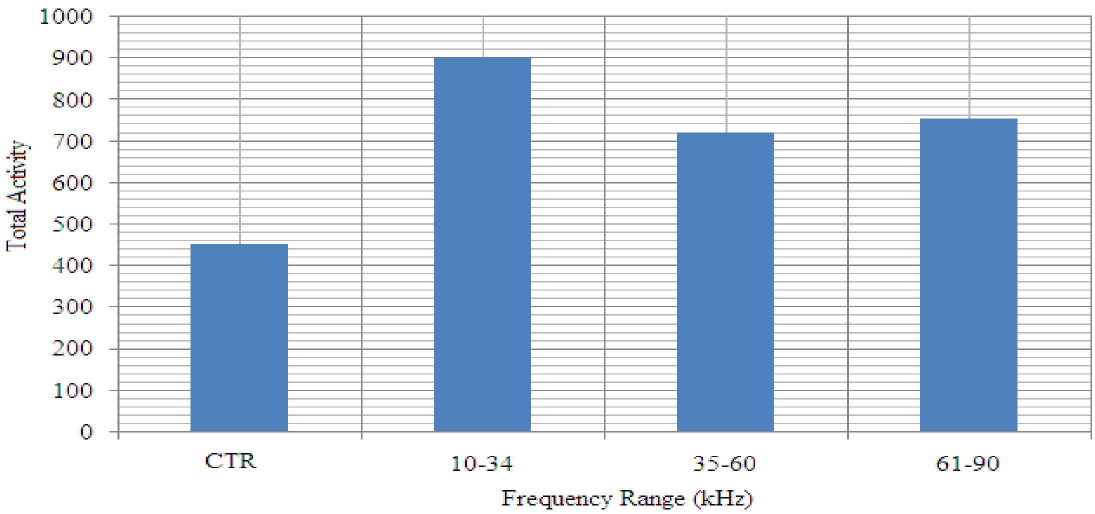

Figure 4. Distribution of the total mosquito activity over different combined sound frequency ranges

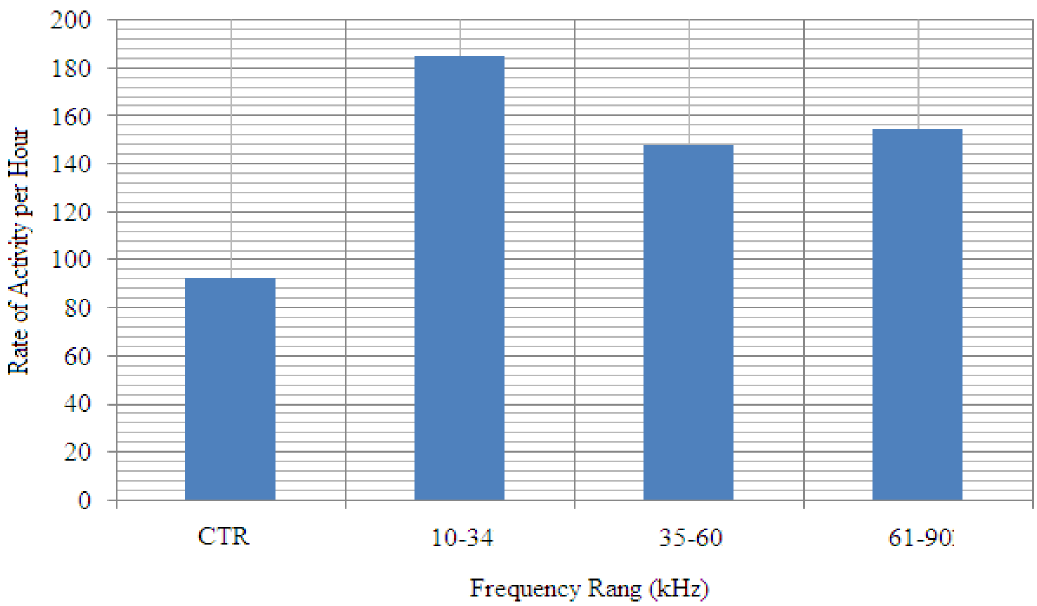

Figure 5. Variation of the rate of mosquito activities with frequencies of combined sound

Table 3. Significance values of the comparison of mosquito activities under varied frequency ranges of the combined sound by the mosquito's activity under the control

Parameter	F	p
Comparison of mosquito activities in 10-34 kHz of the combined sound by the mosquito activity under the control	116.045	2.5657×10^{-5}
Comparison of mosquito activities in 35-60 kHz of the combined sound by the mosquito activity under the control	8.767	0.0128
Comparison of mosquito activities in 61-90 kHz of the combined sound by the mosquito activity under the control	1.025	0.5343

There was sufficient evidence at 5 % significance level to show that the activities exhibited by mosquitoes due to the combined sound in the 10-34 kHz frequency range differed significantly from that at the control. The mosquito activities in 35-60 kHz frequency range also differed significantly from the control though the difference was less compared to that in 10-34 kHz. However, the mosquito activities in the 61-90 kHz did not differ significantly from the activities at the control. The greatest deviation in one-way ANOVA comparison of the mosquito behavioural activities in the sound of *A. tormotus* and *C. afra* by the mosquito activities under their respective control was in the 35-60 kHz range, yielding a significance value $p = 0.461 > 0.05$ and $0.000 < 0.05$ respectively[16]. The 10-34 kHz frequency range combined predator sound compared by the activities under the control yielded significance value, $p = 2.5657 \times 10^{-5} < 0.05$ in mosquito activities under one-way ANOVA comparison. The 10-34 kHz combined sound evoked behavioural response with mean percentage of 30.00 %. The maximum and a minimum signal power of the combined sound in this range fluctuated between -98 dB and -136.67 dB respectively. Also, the one-way ANOVA comparison of activities under the influence of the 10-34 kHz of the combined sound by the activities in the 35-60 kHz of the sound of *A. tormotus* yielded a significance value, $p = 0.000 < 0.05$[17]. Similarly, comparison of activities due to 10-34 kHz combined sound by the activities in the 35-60 kHz of the sound of *C. afra* yielded a significance value, $p = 0.067 > 0.05$[17]. Hence, at 5 % significance level, there was no significant deviation in mosquito activities elicited by the 10-34 kHz frequency range for the combined sound from the mosquito activities elicited under the 35-60 kHz range for sound of *C. afra*[17]. However, the deviation in mosquito activities elicited by 10-34 kHz for the combined sound from the mosquito activities elicited under the 35-60 kHz frequency range for *A. tormotus* was highly significant[17]. The two sounds; 10-34 kHz frequency of combined sound and 35-60 kHz frequency range for *A. tormotus* showed great variation in both behavioural response and rates of activities[17, 18]. The total mosquito activities and the number of mosquitoes affected under initial behavioural response reduced considerably by combining the sounds of *A. tormotus* and *C. afra*[17]. The 10-34 kHz combined sound was characterised by reduced maximum acoustic energy by 2.77507 Pa^2s from the energy in this range for *A. tormotus*[18]. The maximum and a minimum signal power of the combined sound fluctuated between -98 dB and -136.67 dB respectively which were equal to the power in the sound of *A. tormotus*. The sound of *A. tormotus* recorded progressive increase in acoustic energy by 1.9867 Pa^2s. The mean acoustic energy also increased correspondingly[18]. The sound of *C. afra* recorded the greatest maximum acoustic energy in the 10-34 kHz and 35-60 kHz frequency range, above the combined sound and the sound of *A. tormotus*[18]. The sound of *A. tormotus* significantly startled the female *A. gambiae* compared to the combined sound in the 35-60 kHz frequency range[16]. In combining the sound of *A. tormotus* and *C. afra*, the mean acoustic energy reduced significantly from the energy of individual predator sounds[17]. However, the startle effect on the mosquitoes for the combined sound was greater than that of the sound of *C. afra*, but very close to that of the sound of *A. tormotus*. Combining the sound of *A. tormotus* and *C. afra* did not yield any significant startle effect to the female *A. gambiae* compared to single animal species sound earlier studied.

5. Conclusions

The combined predator sound of *C. Afra* and *A. tormotus* evoked evasive behavioural responses in 30.0 % of the mosquitoes in the 10-34 kHz, the optimum startle range, higher than the reported 20.0 % effective repulsion by EMR sound. The startle response in the female *A. gambiae* due to the combined sound of *A. tormotus* and *C. afra* was predominantly evasive, characterized by 58.5^o antenna erection, unusual rest and movement, attributed to stress on nervous system and fear of predation. The secondary effects of the sound on the mosquitoes included physical injury, fatigue and falls.

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