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The Startling Effect of the Sound of *C. Afra* and *A. Tormotus* on the Female *A. Gambiae*

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Abstract The female *Anopheles gambiae*, a malaria vector, detects ultrasound by its antenna, initiating an attractive or repulsive response. Modern electronic mosquito repellents exploit this concept in attempt to control malaria, but have shown only 20 % effectiveness. This work determines the startle response of the female *A. gambiae* to recorded sound of *C. afra* and *A. tormotus* and optimum acoustic transmission parameters needed for the design of an effective electronic mosquito repellent. A bioassay involving 3-4 day old female *A. gambiae* bred and reared under standard conditions was conducted in a standard glass cage yielding evasive behavioural responses on exposure to varied frequencies. 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. The evasive response was characterized by 58.5^{o} antenna erection, physical injury, unusual rest and movement, fatigue and falls; attributed to neural stress and fear for predation. The steady increase in signal intensity, maximum and mean acoustic energy in the sound of *A. tormotus* over all frequency ranges yielded greatest startle response in the female *A. gambiae*.

Keywords Startle Response, Optimum Frequency Range, Bioassay, Hardlock Key, Electronic Mosquito Repellent, Insecticide Treated Nets, Indoor Residual Spray

1. Introduction

Malaria, caused by a protozoan parasite of genius *Plasmodium* and transmitted by the female *Anopheles gambiae*, has led to high mortality and morbidity in Africa[6, 11]. The plasmodium parasites kill over a million people per year, and another 500 million people suffer from the clinical disease[12]. The female *A. gambiae* requires blood either from human or animal, for egg development and lays 30-150 eggs in 2-3 days. Control measures such as chemotherapy, chemoprophylaxis, vector control strategies and development of malaria vaccine had been employed with minimal success. Currently malaria vector control-methods preferred include the use of insecticide treated nets, indoor residual spray, destruction of mosquito breeding sites and use of mosquito repellents. However, the use of insecticides to control malaria vectors and drugs to control malaria parasites had failed due to build up of resistance in mosquitoes and the disease agent[10, 22].

The female *A. gambiae* detects ultrasound by its antenna, which can initiate an attractive or repulsive response[14, 16]. Electronic mosquito repellent devices exploit this concept in attempt to control malaria. The African sheath tailed bat; *C. afra* and the Chinese torrent frog; *A. tormotus* which are predators of mosquitoes, generate ultrasound naturally through vocalisation[3, 4, 7]. This work determines the startling effects of the sound of *A. tormotus* and *C. afra* on the female *A. gambiae* and optimum startle frequency range.

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 with electronic mosquito repellents yielded 20% significant repulsion on the female *A. gambiae*, due to a wide bandwidth of the sound rendering it less intense and ineffective[6]. Hence, there was need to investigate the sounds of *C. afra* and *A. tormotus*; determine their startle effect on the female *A. gambiae* and optimum startle frequency range.

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,

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there was need to investigate the natural ultrasounds from the African sheath tailed bat, *C. afra* and the Chinese torrent frog, *A. tormotus*; determine their startle effect on the female *A. gambiae* and the frequency range of optimum startle.

1.2. Objectives

1.2.1. General Objective

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

1.2.2. Specific objectives

i. To determine the startling effect of the sound of *A. tormotus* on the female *A. gambiae*.

ii. To determine the startling effect of the sound of *C. afra* on the female *A. gambiae*.

iii. To determine the sound frequency range of optimum startle on the female *A. gambiae*.

1.3. Justification

Electronic mosquito repellents which mimic ultrasound from animal species are currently in use. 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. Investigation into these sounds on their startle effect on the female *A. gambiae* had not been conducted and reported. It was therefore important to investigate the effect of these naturally generated ultrasounds on the behaviour of the *Anopheles* mosquito. This research was also conducted in order to establish whether there was an improvement on the 20% startle effect on mosquitoes by using ultrasound from *C. afra* and *A. tormotus*. Given that *C. afra* and *A. tormotus* were natural predators of mosquitoes, their sounds ware expected to effectively startle the female *A. gambiae* due to natural fear of predation. The behavioural startle response of the female *A. gambiae* elicited by the sound of *C. afra* and *A. tormotus* was also observed and the significant startle effect noted. The parameters determined from the current research are critical to electronic mosquito repellents designers since effective devices could be realized. Thus, the results provide an additional tool in mosquito control which is environment friendly.

1.4. Hypotheses

1. The sound of *C. afara* and *A. tormotus* do not have any significant startling effect to the female *A. gambiae*.
2. The 10-34 kHz, 35-60 kHz and 61-90 kHz frequency ranges of the sound of *C. afara* and *A. tormotus* do not significantly startle the female *A. gambiae*.

2. Materials and Methods

2.1. Materials

a) The *A. gambiae* mosquitoes

The *A. gambiae* mosquitoes were bred and reared at the Kenya Medical Research Institute Centre for Global Health Research laboratories, Entomology department at 60-80 % humidity, 25±2℃ temperature and light-day cycle of 12 hours light and 12 hours darkness. Three sets of 10 female *A. gambiae*, 3-5 day old were used in the study. Morphologically, the female mosquitoes have a sharp proboscis. They were fed on 10% glucose solution soaked in cotton wool.

b) Sound of *A. tormotus* and Sounds of *C. afra*

Six samples of the sound of *A. tormotus* were recorded individually for varied duration ranging from 1.60 s to 2.90 s using the 702 digital recorders from the Huangshan Hot springs, Anhui Province; China at a sampling frequency of 192 kHz which was converted to 500 kHz. Nine sound samples from *C. afra* were recorded from a colony in Kit-Mikayi caves, Kisumu; Kenya using the Avisoft recorder which consisted of the AUSG (Model 112) individually for a duration which varied from 45.00 s to 300.00 s at a sampling frequency of 500 kHz.

c) Equipment

A computer running on Windows XP and office 2007 mounted with a sound card, hardlock key and sound output ports was used together with the Avisoft recorder during the first stage of the study. The Avisoft recorder, compatible with Windows XP, consisting of the AUSG (model 112) running on specific software RECORDER USG (rec_usg.exe), 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*. During the second stage of the study, two Panasonic 8.0 Ω ordinary external speakers were used to play sound from a single source directed to the bioassay cage housing the female *A. gambiae*. The sound was amplified externally using an amplifier with an output power of 18 W, impedance of 4.0 Ω and separation ≥ 45.0 dB. The stopwatch option in the Samsung cell phone was used to capture activity duration. Softwares which included the Avisoft-SAS LAB Pro Version 5.1 and Batsound were installed in the laptop to facilitate automatic analysis of sound signals. An aspirator was used to transfer the female *A. gambiae* from the rearing cage to the bioassay cage and also remove them from it.

d) The bioassay arena (Cage)

A standard bioassay glass cage of dimensions 0.50 m by 0.25 m by 0.25 m was fitted with untreated mosquito net on the 0.25 m by 0.25 m faces and used in the bioassay. A small hole was perforated on both nets to allow the mosquito samples in and out the cage. The cage was divided into three equal sections; A, B and C. Section C was the central region of the bioassay cage. Section A was to the right whereas section B was to the left of the cage. Both the 25 cm by 25 cm faces of the cage were covered with untreated mosquito which had a 1.0 cm diameter hole perforated in the middle of the net. The two holes were covered with cotton wool. Either holes on side A or B could be used as mosquito release points. However, 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. Two speakers were attached to side A of the cage.

2.2. Methods

2.2.1. Recording and Filtering of Sounds

(a). Recording of the Sound of *Coleura afra and A. Tormotus*

The Avisoft recorder which consists of the AUSG (model 112) was used in recording the sound from *C. afra*. The condenser microphone capsule (CM16) which consisted of a thin metalized polyester film and a metal black plate was used in the study. It was connected to the AUSG (model 112) which was then connected to the laptop through one of the universal serial bus (USB) ports. The omnidirectional microphone was selected as a default microphone from the voice recording settings in the laptop. The time domain filter, Finite Impulse Response (FIR) was set to zero for both upper cut-off frequency (f_{uco}= 0 kHz) and lower cut-off frequencies (f_{lco}= 0 kHz). The Fast Fourier transform (FFT) was also set to 512 and the Hamming window selected for the display. The temporal resolution overlap was also set to 50% with the graypal selected for the colour palette. The frame size was set to 100% for real time spectrogram parameters. The black and white box (B/W) was checked for the display option. The Avisoft-SAS LAB Pro, Version 5.1 software was started and the microphone directed to the source of sound. The gain on the AUSG (model 112) was adjusted to an appropriated level to avoid over modulation and the recording level from the computer set to 20 dB. Recording of the sound was started by pressing the record button on the AUSG. Nine sound samples from *C. afra* were recorded separately from a colony in Kit-Mikayi caves, Kisumu at a sampling frequency of 500 kHz for a duration varying from 45.00 s to 300.00 s. The sound samples were saved in the hard disc. The sound sample for the study was obtained by appending four quality sound samples and gave a 1754.07 s playback duration which was saved as "*Coleura* Sample 2.wav" hard disc. Six samples of sounds of *A. tormotus* were recorded individually for a total duration of 12.4 s 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 with a view of increasing playback duration using the AvisoftSASLab Pro Version 5.1 installed in the laptop computer hard disc. The appended sound was further appended to ensure a uniform duration of 1754.07 s and saved as "*A. tormotus*.wav" in the hard disc and the sampling frequency converted from 192 kHz to 500 kHz using AvisoftSASLab Pro Version 5.1 for compatibility. The samples were donated by Feng of Illinois University (USA) to facilitate the study.

(b). Filtering of sounds samples

The high pass filter, band pass filter and low pass filter, inbuilt in the AvisoftSASLab analysis software, were used to segment the appended sounds into appropriate frequency ranges essential for the study. All the sounds were subjected to a high pass filter with a cut-off frequency f_{co}= 10 kHz in order to attenuate noises and a low pass filter with a cut-off frequency f_{co}= 90 kHz. Three sound segments, namely, 10-34 kHz, 35-60 kHz and 61-90 kHz from every sound sample were required for the investigation. In order to obtain the 10-34 kHz the frequency range segment, the sounds saved as "*Coleura* Sample 2.wav" and "*A. tormotus*.wav" were 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. Frequencies below 10 kHz and above 34 kHz were gradually attenuated (amplitude = 0 i.e. off), allowing those in the range of 10-34 kHz (amplitude = 1, or on). Similarly, the band pass filter settings that yielded the 35-60 kHz and 61-90 kHz frequency ranges were f_{lco}=35 kHz, f_{uco}= 60 kHz and f_{lco}= 61 kHz, f_{uco}= 90 kHz respectively. The settings were made from the time domain filter (FIR). These sound segments were also saved in the hard disc.

The predator sounds could not be played by ordinary moving coil speakers hence the need to amplify them. The predator signal was internally amplified and then externally amplified before getting into the external speakers, placed 5 cm from the cage from side A. The speaker was set to face the cage. The amplitude modulation constant of the appended sound of *A. tormotus* was set to n = 0.8 i.e. Normalize at 80% for the entire duration for the *A. tormotus* signal. Similarly, the sound of *C. afra* was internally amplified by setting the 10-34 kHz to volume = 150 %, 35-60 kHz to volume = 130 % and the 61-90 kHz was set to 600 %. The sound was also 50 % amplified using an external amplifier in order to enhance diaphragm vibrations.

2.2.2. Bioassay

The bioassay study involved determination of the effect of sound on the female *A. gambiae* by varying frequency in order to establish the frequency range where startling effect was optimum. Three to five day old female *A. gambiae* were used in the bioassay experiment. The female *A. gambiae* are characterized by large body size and affinity to blood meal. The criteria for the choice of the female *A. gambiae* included the size, status (fed or unfed), activity, mouth parts, resting position and age.

The sound of the African bat *C. afra* and the Chinese frog *A. tormotus* were played separately through two external speakers attached to the cage at the end labelled A. The sound frequency ranges included 10-34 kHz, 35-60 kHz and 61-90 kHz, obtained by use of filters incooperated in the AvisoftSASLab software. The background noise, which was below 10 kHz, in the sound signals was attenuated using high pass filters. A set of ten, 3-5 day old female *A. gambiae* were released into the cage using an aspirator, one at a time, through the release hole in the net. The bioassay study involved investigation into the behaviour of one mosquito at a time when exposed to 10-34 kHz, 35-60 kHz and 61-90 kHz sound frequency ranges in prose. Investigation into the behavioural startle response in mosquitoes was conducted and the number of mosquitoes exhibiting the traits expressed as a percentage. The behavoural traits considered included directional body movement, jumps, hiding, raising of limbs, raising and lowering of body, body shaking associated with bending of abdomen, wing and limb rubbing, nature of body rest, mosquito movement, spreading of limbs, antennae erection, fatigue, rolling and loss of body parts. Antennae erection from the proboscis was measured from unmodified photo printout of the mosquito and the angle measured using a protractor. The antennae and proboscis were extrapolated for convenience in angle measurement by protractor. The second part of the bioassay involved playing of predator sound for various frequency ranges and the number of activities and duration, in this case flight (F) and rest (R) recorded correspondingly. The predator sound was played and simultaneously starting the stopwatch, observing and recording duration for activities. The observation of each mosquito took a total duration of 7016.28 s with each exposure taking 1754.07 s.

The saved sound files of *A. tormotus* and *C. afra* were played from a laptop. The bioassay study involved the use of ten mosquitoes, one mosquito studied at a time. An initial observation was made on the mosquito without playing any of the predator sounds. That was the control experiment whose results were compared to those obtained when sounds were played. Each mosquito was exposed to sounds of frequency ranges 10-34 kHz, 35-60 kHz and 61-90 kHz, obtained by subjecting them to appropriate filters inbuilt in the AvisoftSASLab filters.

2.2.3. Statistical Analysis

The data collected from the bioassay study was analysed using the SPSS programme.

3. Results and Discussion

3.1. Determination of the Initial Behavioural Response of the Female *A. Gambiae* Elicited by the Sound of *A. Tormotus* and *C. Afra*

In the control experiments the female *A. gambiae* exhibited normal flight and moved freely within the cage and occasionally rested behind barriers. The mosquito samples maintained body rest at 45^o from surface with wings laid along the abdomen as shown in Fig. 1 and Fig. 2 respectively. Normal body movement within the cage and at times none was exhibited. Rubbing of limbs and wings; and raising of limbs was also occasionally observed under the control experiment. Mosquitoes rested their limbs and proboscis on the net surface; the proboscis almost collinear with the antennae. The total number of mosquitoes exhibiting specific behavioural traits at the control was determined and expressed as a percentage. Table 1 shows the percentage of mosquito samples under the control experiment for sounds of *A. tormotus* and *C. afra* different behavioural traits. These behavioural responses of the mosquito samples at the control were an evidence of the use of active mosquito samples in the bioassay study.

Figure 1. The female *A. gambiae* at normal rest

Figure 2. The female *A. gambiae* resting at angle $\beta = 45^o$

Table 1. Percentage of mosquito samples under the control experiment for sounds of *A. tormotus* and *C. afra*

Mosquito Behavior	Percentage of the mosquito samples	
	A. tormotus	*C. afra*
No body movement	10	0
Squeezing/hiding in barriers	10	0
Raised limbs	10	0
Normal movement in the cage	70	80
Rubbing of legs and/or wings	20	20
Normal flight about in the cage	60	70
Rest at 45^o from rest surface ; wings along body	80	70
Limbs and proboscis resting on net or cage	0	10
Antennae and proboscis almost collinear	100	100

The sound in the 10-34 kHz frequency range had not been reported to have any effective startle effect on the female *A. gambiae*[16]. The effect of the sound of *A. tormotus* on *A. gambiae* had also not been reported. However, the 10-34 kHz sound of *A. tormotus* and *C. afra* elicited rubbing of hind limbs, fore limbs and wings in the female *A. gambiae*. The effect was also observed in 30 % of the mosquito samples at this frequency range which increased from 20 % at the control experiment. It was also observed that 30 % of the mosquito samples did not exhibit remarkable body movement on exposure to both sounds of *A. tormotus* and *C. afra* separately. Recent findings with ultrasound from EMR reported immobilization in mosquitoes, an effect also observed in this research[16]. However, the EMR were noted to generate ultrasound that had low degree of repellency on mosquitoes[1, 6]. It was clearly observed that 30 % and 60 % of the mosquitoes exposed to the sounds of *C. afra* and *A. tormotus* respectively moved away from the source of the sounds, an evasive response also reported in earlier findings[16]. This was attributed to greater signal power in the sound of *C. afra* at this frequency range, hence initiating such responses in the female *A. gambiae*. Though the maximum acoustic energy of the sound of *C. afra* in this frequency range was 6.00 Pa^2s above that of *A. tormotus*, the later recorded a mean sound energy which was 11.81 times greater than that of *C. afra*. In both cases, the bandwidth was narrowed. The sound of *A. tormotus* evoked jumps and bounces; raising and lowering of bodies from surface in 50 % of the mosquito samples. Only 20 % of the mosquito samples were seen raising and lowering their bodies on exposure to sound of *C. afra* which was less by 30 % from that due to sound of *A. tormotus* despite the high power and energy.

The sound of *A. tormotus* elicited flapping and opening of wings; weak or exaggerated flights, falls and eventual escape in 30 % of the female *A. gambiae*. Similarly, flapping or opening of wings while resting was observed in 10 % of the sample mosquitoes but 40 % of the mosquitoes displayed weak or exaggerated flights, falls and escape when the sound of *C. afra* was played. The mosquitoes which exhibited flights, falls with some even escaping from the cage due to the powerful sounds of *C. afra* were 10 % more than those observed under the influence of the sound of *A. tormotus*. Raised limbs which were occasionally folded over the body was observed in 60 % of mosquitoes which were exposed to the sounds of *A. tormotus* and the sound of *C. afra*, with the later recording 20 % less. Another evasive behaviour was observed in 90 % and 30 % of sample mosquitoes which were exposed to sounds of *A. tormotus* and *C. afra* respectively; which included squeezing of body and proboscis through barriers and surfaces besides exhibiting hiding tendencies as shown in Fig. 3. Body shaking associated with abdomen curving towards thorax, a behaviour not observed at the control study, was observed in 60 % and 30 % mosquito samples under the influence of the sound of *A. tormotus* and *C. afra* respectively. Unusual forward and backward or sideways body movement was observed in 10 % of mosquito samples on exposure to both predator sounds in this frequency range. Due to the exhaustion, fear of predation and stress, 40 % and 20 % of sample mosquitoes exposed to sounds of *A. tormotus* and *C. afra* respectively rested by their abdomen and thorax[8, 16, 21].

Spreading of limbs on the resting surface and antennae erection was observed in 30% and10 % of the mosquito samples respectively on exposure to the sound of *A. tormotus* only. The mosquito antennae erection angle increased to 18.5^o from its normal rest position as shown in Fig. 4. The antennae which are ultrasound sensors responded to the 10-34 kHz sound by erecting above normal, a verification of its essentiality in communication through oscillations as earlier reported[2, 5, 13, 14, 15, 18, 20, 24]. Such intense response was not observed with the sound of *C. afra* partly because the sound consisted of both sonar and social calls[9, 19].

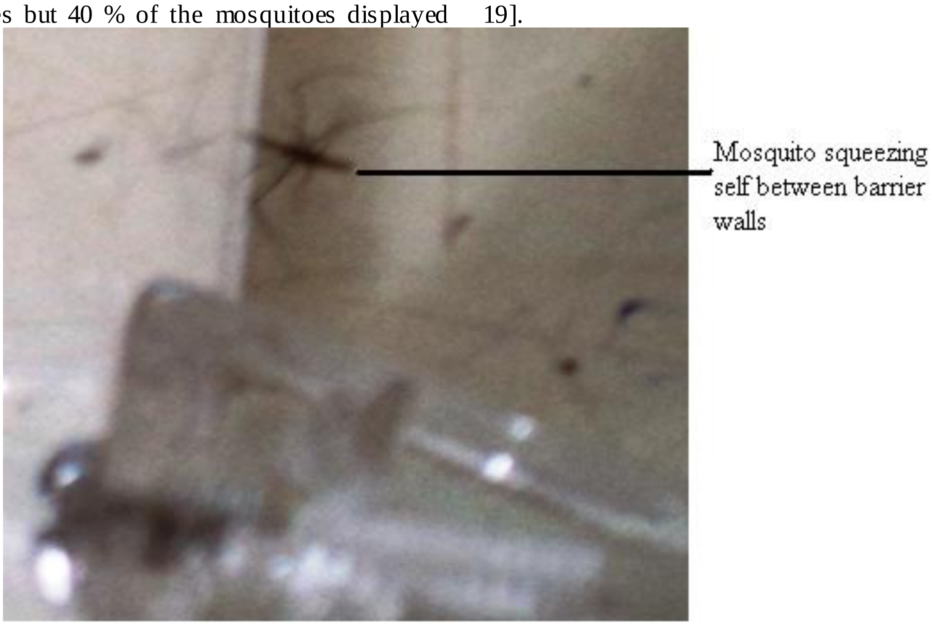

Figure 3. The female *A. gambiae* squeezing through barrier

Figure 4. Mosquito antennae erection at 18.5o due to the 10-34 kHz sound of A. tormotus

The 35-60 kHz ultrasound from EMR had been reported to startle the female *A. gambiae* in recent research findings, yielding only 20 % effective repellency[1, 6, 16]. However, the repellency due to the sound of *A. tormotus* on *A. gambiae* had not been reported. This research established that the sound of *A. tormotus* and *C. afra* elicited rubbing of hind and fore limbs, and wings in 20 % and 10 % of the mosquitoes respectively. However, the effect was observed to decline by 10 % for the sound of *A. tormotus* and 20 % in the sound of *C. afra* in the 10-34 kHz frequency range. There was a 20 % and 70 % of the mosquito samples which did not exhibit remarkable body movement on exposure to both sounds of *A. tormotus* and *C. afra* respectively. There was a remarkable increase in the number of mosquitoes by 40 % on exposure to the sound of *C. afra* with that of *A. tormotus* declining by 10 % which did not display remarkable body movement. This immobilization in mosquitoes due to neural stress and fear of predation, observed in recent research findings, was greatest with the sound of *C. afra*[8, 16, 21]. The percentage of the mosquitoes which avoided the source of sounds on exposure to the sounds of *C. afra* remained constant at 30 %. However, the percentage of the mosquitoes that avoided the source reduced by 10 % on exposure to the sounds of *A. tormotus*. The mosquito samples were observed moving away from the sound source. The sound of *A. tormotus* evoked jumps and bounces; raising and lowering of bodies from surface of rest in 50 % of the mosquito samples, a value similar to that in 10-34 kHz range. The percentage of mosquitoes raising and lowering their bodies on exposure to sound of *C. afra* increased by 20 %. The sound of *C. afra* did not evoke jumps and bounces at all in any mosquito samples under study, behaviour similar to that in 10-34 kHz range. The number of mosquito samples which exhibited antennae erection increased by 70 % in response to the sound of *A. tormotus*, a response not observed with the sound from *C. afra*. The antennae erection shown in Fig. 5, increased gradually by 40.0^{o} above the erection elicited under the 10-34 kHz sound range. The pronounced behavioural change in this frequency range had also been reported[16].

Figure 5. The female *A. gambiae* resting by side with antennae erection at 58.5^{o}

The percentage of mosquitoes which were seen to rest on their abdomen with limbs on surface was 40 % and 20 % under exposure to the sound from *A. tormotus* and *C. afra* respectively. The sound of *A. tormotus* elicited flapping and opening of wings which increased remarkably in 40 % of the female *A. gambiae* and 60 % displayed weak or exaggerated flights, falls and eventual escape from cage. Similarly, flapping or opening of wings when resting was noted in 20 % of the mosquito samples and 30 % of the mosquitoes, reduced from the number in 10-34 kHz, displayed weak or exaggerated flights, falls and escape when the sound of *C. afra* was played. There was a reduction in the number of mosquito samples which exhibited raised limbs when exposed to the sound of *A. tormotus* and *C. afra* by 20 % and 10 % respectively from the number under the 10-34 kHz frequency range. The number of mosquito samples which squeezed their bodies and proboscis through barriers and surfaces besides exhibiting hiding tendencies were also reduced by 20 % from the number reported in the previous frequency range when they were exposed to both predator sounds. There was a 20 % and 30 % increase in the number of mosquito samples under the influence of the sound of *A. tormotus* and *C. afra* respectively which exhibited body shaking which was associated with abdomen that curving towards thorax as shown in Fig. 7. The forward and backward movement, or sideways body movement increased from 10 % of mosquitoes under the 10-34 kHz range for both predator sounds to 40 % on exposure to the sound of *A. tormotus*, with none for the sound of *C. afra*. This was mainly due to the decline in the mean acoustic energy by 0.05 Pa^2s of the sound of *C. afra* besides the presents of both sonar and social calls[9, 19,]. The mean acoustic energy of the sound of *A. tormotus* increased progressively from the energy in the 10-34 kHz by 0.12 Pa^2s. It was this frequency range which evoked new behavioural traits which included resting by back or side as shown in Fig. 6, and rolling on surfaces. These new behavioural traits were observed in 60 %and 20 % of the mosquito samples exposed to sounds of *A. tormotus* and *C. afra* respectively. Other behavioural responses such as spreading of limbs when resting on surface was observed in 50 % and 10 % of the mosquito samples exposed to the sounds of *A. tormotus* and *C. afra* respectively. Severe secondary responses which entailed exhaustion and loss of limbs was observed in 20 % and 10 % of mosquito samples which were exposed to the sound of *A. tormotus* only. These physical injuries were caused by mosquitoes knocking themselves on cage walls and net hence resulting to loss of limbs. The difference in response to predator ultrasound in this frequency range was attributed to slightly broadened bandwidth than that in the 10-34 kHz range in both predator ultrasounds.

There was a progressive increase in the maximum value of acoustic energy in the sound from *A. tormotus* by 1.99 Pa^2s. However, the sound of *C. afra* recorded a progressive decline in this acoustic energy, though remaining it above the energy in the sound from *A. tormotus*. The power also declined significantly in both predator sounds.

The 61-90 kHz frequency range had not been reported to repel mosquitoes in recent findings by[16] yet this study observed its repulsive effect on mosquitoes. The number of mosquito samples that exhibited tiredness, weakness, loss of limbs, rested by side or back, rolled on surface, flapped wings, occasionally collapsing and opening wings on exposure to sound of *A. tormotus* increased by 10 % in this frequency range. Similar sound evoked rest by abdomen with limbs on surface in 70 % of the mosquito samples, an increase by 30 % from the number in the 35-60 kHz range. For the sounds of *A. tormotus*, the number of mosquitoes which raised their limbs and sometimes folded them over the body, shook their bodies and curved their abdomen towards the thorax as shown in Fig. 7 were $\geq$ 40 %, a number maintained from the 35-60 kHz frequency range. However, the number of mosquitoes exhibiting similar behaviour was reduced by 40 % when subjected to the sound of *C. afra*. The intensified evasive response to this sound frequency range was attributed to variation in call bandwidth, acoustic energy and power variation; and presence of both predation and neural stress causing calls[8, 16, 21]. The minimum total energy for *C. afra* and *A. tormotus* was reduced by 1.3 x 10^{-4} Pa^2s and 4.77 x 10^{-03} Pa^2s respectively. Similarly, the sound signal of *A. tormotus* and *C. afra* in this range recorded a reduced maximum total energy by 3.1445 Pa^2s and 6.73 Pa^2s respectively from the energy in the 35-60 kHz frequency range. The maximum and minimum acoustic energy of *C. afra* was greatly reduced compared to that of *A. tormotus*. Due to this change in energy, the number of mosquito samples which did not show any body movement on exposure to the sound of *A. tormotus* and *C. afra* reduced by 10 % and 50 % respectively. The mosquito samples earlier immobilized recovered by 50 % on exposure to the sound of *C. afra*. The 61-90 kHz sound of *C. afra* did not evoke raising and folding limbs over the body, raising and lowering body, resting by abdomen, flapping or opening wings, spreading limbs, resting by back or side or rolling on surface in any mosquitoes. The deviation in the number of mosquito samples was between 10 % and 40 % from the number of mosquito samples in the 35-60 kHz range.

Figure 6. The female *A. gambiae* rests by side with erected antennae

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

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

Figure 7. Mosquito resting on spread limbs with abdomen curved towards thorax

The mosquito samples observed rubbing their limbs and wings on exposure to the sound of *A. tormotus* was reduced by 20 % from the number recorded in the 35-60 kHz frequency range. In this frequency range, the number of mosquito samples jumping, bouncing and squeezing of bodies and proboscis in barriers were reduced by 50 % on exposure to the sound of *A. tormotus*. Similarly, the number of mosquitoes exhibiting erection of antennae, spreading of limbs and moving away from the sound of *A. tormotus* reduced by 50 %, 20 % and 10 % respectively; though the movement away from the sound of *C. afra* was unchanged. The antennae erection in mosquitoes was maintained at 58.5^{o} as indicated in Plate 5. The total number of mosquito samples that had previously shown weak or exaggerated flights, falls and directional body movement was reduced by 30 %. The significant reduction in the number of mosquito sample was attributed to the maximum signal power which was greatest in the sound of *C. afra* than that of *A. tormotus* which has a wide energy range compared to its power. This response was also due to the mosquitoes continuously being subjected to high energy ultrasonic sounds which evoked fear of predation and stress on neural system[8, 16, 19, 21]. Similarly, there was a widened mean bandwidth (maximum entire) for the sound of *A. tormotus* and *C. afra* than the bandwidth for the sounds in the 35-60 kHz, though narrower than the reported EMR bandwidths[1, 6].

The number of the female *A. gambiae* which exhibited behavioural response to 10-34 kHz, 35-60 kHz and 61-90 kHz sound frequency ranges for *A. tormotus* and *C. afra* was determined and expressed as a percentage. The percentage mosquito showing varied distinct behaviour under varied sound frequencies of *A. tormotus* and *C. afra* are indicated in Table 2.

3.2. Determination of the Mosquito Activity and Optimal Startle Frequency Range under the Influence of the sound of *A. tormotus* and *C. Afra*

Recent findings based on mosquito landing rates on bare human body parts with ultrasound from functioning EMR yielded 20 % effective repellency[1, 6]. The mosquito activities in this section of the research was limited to flight and rest besides the behavioural response. All the mosquitoes exposed to predator sounds separately, had their flight time above the control. However, 6.7 % and 33.3 % of these mosquito samples displayed their flight time below the control when exposed to the sounds of *A. tormotus* and *C. afra* respectively. Fig. 8, Fig. 9, Table 3 and Table 4 show the relationship between flight duration with frequency for ten mosquitoes exposed to the sounds of *A. tormotus* and *C. afra*. When the mosquitoes were exposed to 10-34 kHz, 35-60 kHz and 61-90 kHz sound frequencies of *A. tormotus*, the flight duration increased by an average duration of 433.52 s, 352.52 s and 654.88 s respectively, above the control experiment as shown in Table 3. The mosquito samples exhibited a decline in the average flight duration by 18.249 s from the control in the 10-34 kHz of the sound of *C. afra* as shown in Table 4. This response was because of acoustic energy for the sound of *C. afra* declining by 2.534 Pa^2s and the signal power also declining uniformly from -55 dB to -59 dB in the 35-60 kHz frequency range. The mosquitoes, under the influence of the sound of *C. afra* and *A. tormotus* spent most time in air in the 35-60 kHz and 61-90 kHz range as shown in Table 5 and Table 6 respectively. The mosquitoes exposed to sound of *A. tormotus* also yielded remarkable suspension time in the 10-34 kHz sound range. Ultrasound in the 35-60 kHz range, yielded significant acoustic energy as earlier reported with the sound from bats[19]. The sound of *A. tormotus* recorded progressive increased in energy from 10-34 kHz to 35-60 kHz. However, the energy declined as the frequency changed from 35-60 kHz to 61-90 kHz by 3.1445 Pa^2s, recording an energy of 7.699 Pa^2s, which was still higher than that of *C. afra*. The signal power maintained the maximum value at -100 dB with the minimum value less by -10 dB from the minimum power in the 35-60 kHz frequency range. Energy and power variation in predator sounds enhanced both flight and rest activities above the control for sample mosquitoes investigated. The mosquitoes' behaviour in the 10-34 kHz and 35-60 kHz under the sound from *C. afra* and *A. tormotus* respectively was attributed to search for safe conditions within the cage. The mosquitoes were characterized by immobilization though they struggled to escape; occasionally taking long flights. The mosquitoes' evasive behaviour was attributed to the stress caused on the nervous system and fear of predation[8, 16, 21].

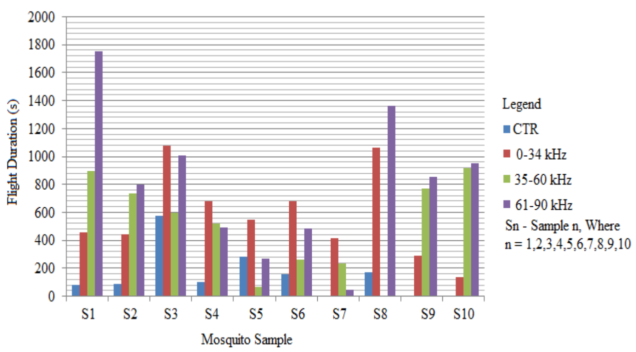

Figure 8. The relationship between mosquito flight duration with frequencies of A. tormotus

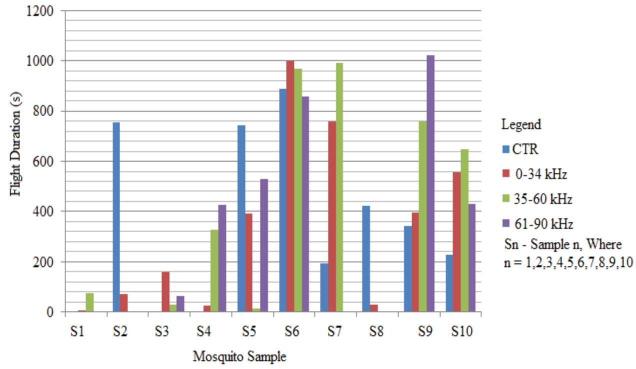

Figure 9. The relationship between mosquito flight duration with frequencies of *C. afra*

Table 3. Average flight duration per mosquito due to sound frequencies of A. tormotus

Frequency Range	Flight Duration (s)
CTR	146.408
0-34 kHz	579.932
35-60 kHz	498.931
61-90 kHz	801.283

Table 4. Average flight duration per mosquito due to sound frequencies of *C. afra*

Frequency Range	Flight Duration (s)
CTR	357.773
0-34 kHz	339.524
35-60 kHz	381.798
61-90 kHz	333.803

Table 5. Total flight time of mosquito under the sound frequencies of A. tormotus

Frequency (kHz)	Total Flight time (s)
Control	1464.08
10-34	5799.32
35-60	4989.31
61-90	8012.83

Table 6. Total flight time of mosquito under the sound frequencies of C. afra

Frequency (kHz)	Total Flight time (s)
Control	3577.73
10-34	3395.24
35-60	3817.98
61-90	3338.03

Fig. 10 and Fig. 12 show the distribution of flight and rest activities of the female mosquitoes under the influence of the sound of *A. tormotus* and *C. afra* respectively. The mosquitoes displayed normal activity under the control experiment. All the frequency ranges in the sounds of *A. tormotus* and *C. afra* initiated activities above the control. On exposure to energetic ultrasound, this research revealed that the mosquitoes exhibited startle response in all frequency ranges. The female *A. gambiae* were excited on exposure to the sound of the *A. tormotus* with an increase of 583 activities under the 10-34 kHz frequency range from the control as shown in Fig. 11. The number of mosquito activities increased further at higher frequency band of 35-60 kHz, which then remained almost constant at 61-90 kHz. The average mosquito activity at 10-34 kHz sound of *A. tormotus* was 3.52 times the average activities under the control experiment, shown in Table 5. However, the sound of *C. afra* initiated 4.48 times the average activities under the control experiment, shown in Table 6. This was due to high onset maximum acoustic energy of the sound of *A. tormotus* which was 8.8568 Pa^2s and signal power which was steady and maintained at -118 dB. The acoustic energy of the sound of *C. afra* was greater than the acoustic energy of *A. tormotus*, though it declined greatly with increase in frequency. These findings were in agreement with the results from previous studies[1, 6, 17, 19, 23]. The onset frequency range of 10-34 kHz initiated the greatest number of activity under the influence of ultrasound from *C. afra*, shown in Fig. 12 and Fig. 13. The 61-90 kHz frequency range of the sound of *C. afra* initiated almost an equal amount of activity with the 35-60 kHz, with a lag of twelve due to exhaustion. Therefore, the female *A. gambiae* were startled by the sound of *C. afra*, with maximum number of activities occurring in the 10-34 kHz range. The mosquito activities were sustained below 18 for all the ten mosquito samples under the influence of the sounds of *C. afra* as shown in Fig. 12.

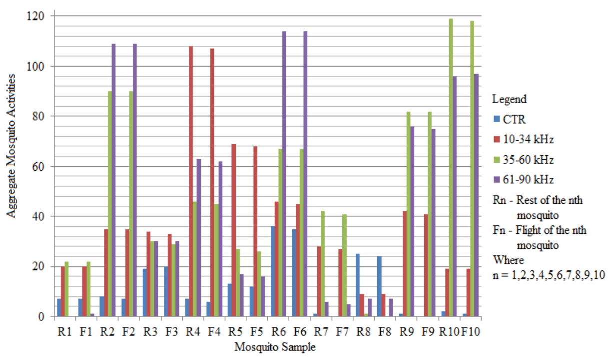

Figure 10. The number of mosquito activities under varied sound frequencies of *A. tormotus*

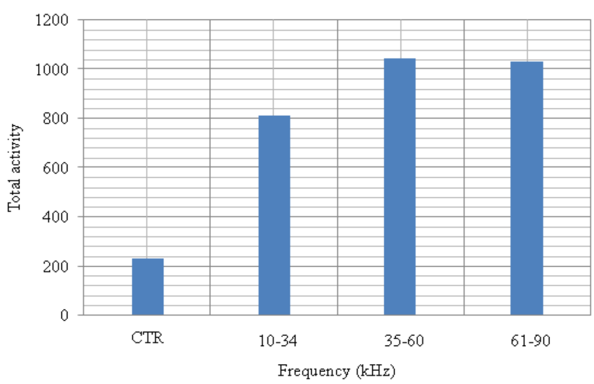

Figure 11. The total mosquito activity under varied sound frequencies of *A. tormotus*

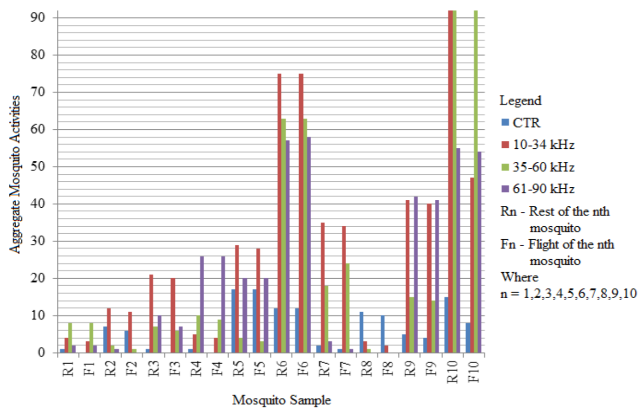

Figure 12. The number of mosquito activities under varied sound frequencies of *C. afra*

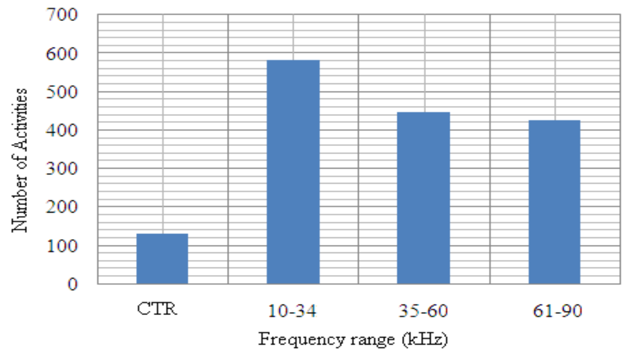

Figure 13. The total mosquito activity under varied sound frequencies of *C. afra*

Table 7. The average mosquito activities elicited by varied sounds of *A. tormotus*

Frequency range	Average activities per Mosquito
CTR	11.55
0-34 kHz	40.7
35-60 kHz	52.3
61-90 kHz	51.7

Table 8. The average mosquito activities elicited by varied sounds of *C. afra*

Frequency range	Average activities per Mosquito
CTR	6.5
0-34 kHz	29.1
35-60 kHz	22.3
61-90 kHz	21.25

The greatest mosquito activities was recorded when the female *A. gambiae* were exposed to the 10-34 kHz sound of *C. afra*. A decline in mosquito activity as shown in Fig. 16 was displayed on exposure to higher frequencies of the sound of *C. afra*. However, the decline in mosquito activity on exposure to the sound of *A. tormotus* began after 35-60 kHz, a frequency slightly higher than that in *C. afra*. The mosquito activities were sustained below 18 for all the ten mosquito samples under the influence of the sounds of *C. afra* as shown in Fig. 12. The decline in maximum energy of the sound of *C. afra*, shown in Fig. 14, and the uniform decline in acoustic power resulted to progressive decline in mosquito activity as shown in Fig. 16. However, the maximum energy of the sound of *A. tormotus* increased slightly resulting to increased activity in mosquito samples up to 35-60 kHz frequency range, as shown in Fig 7 and Fig 8. A decline in acoustic energy in the 61-90 kHz frequency range of the sound of sound of *A. tormotus* resulted to a corresponding decline in activity as shown in Fig. 15. Though low, the power in the sound of *A. tormotus* remained almost constant in this frequency range.

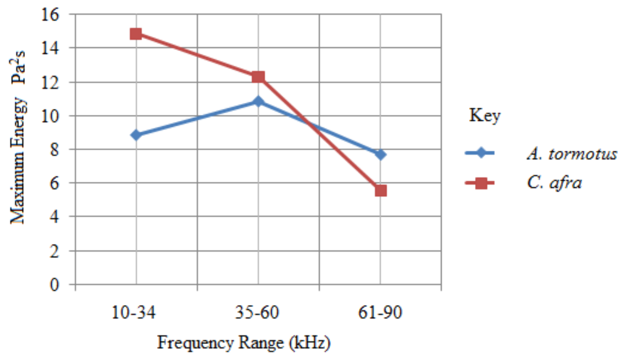

Figure 14. The variation of predator maximum energy with frequency

The sound of *A. tormotus* and *C. afra* is detected by the antennae of which the ultrasound component causes neural stress on *A. gambiae*. The sounds of *A. tormotus* and *C. afra* which predate on *A. gambiae* also evoke natural fear of the animals emitting it. The sounds of *A. tormotus* and *C. afra* initiate avoidance response in mosquitoes, as reported in recent findings[8, 16, 21].

The rate of mosquito activity caused by the sounds of *A. tormotus* and *C. afra* based on a total time of 4.87 hr and shown in Fig. 15 and Fig. 16 respectively was greatest in the 35-60 kHz and 10-34 kHz frequency respectively. The rate of activities in the sampled mosquitoes rose to a maximum value of 92.62 activities per hour above the control on exposure to the 10-34 kHz sound frequency range of *C. afra*. The greatest rate of activity in mosquitoes due to the sound of *C. afra* was recorded in the 10-34 kHz sound frequency range with a slight decline of 27.83 activities per hour as it tended towards the 35-60 kHz and then 4.46 activities per hour towards 61-90 kHz. There was a slight decline by 2.45 rate of activity per hour as the frequency increased towards the 60-91 kHz for *A. tormotus*. The maximum rate of mosquito activity in *A. tormotus* was recorded in the 35-60 kHz frequency range. The trend line in Fig. 15 showed that the rate of activity per hour of the female mosquito increased at the rate of 54.20 Activities/hr under the sound of *A. tormotus*. However, the rate of mosquito activities per hour for *C. afra* in the same frequency range, shown in Fig. 16, declined on exposure to higher frequencies.

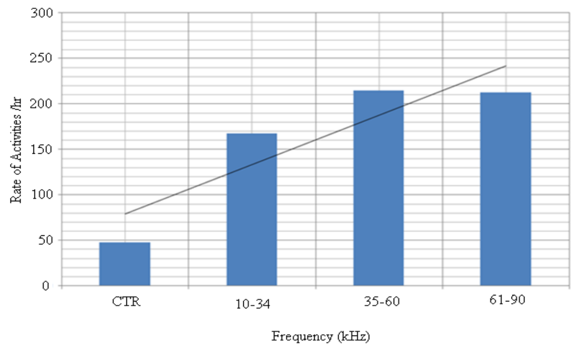

Figure 15. The trend of rate of activity per hour with sound frequency ranges of *A. tormotus*

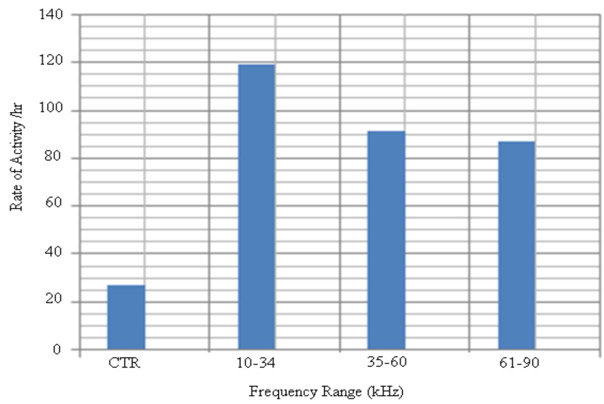

Figure 16. The trend of rate of activity per hour with sound frequency ranges of *C. afra*

The one-way ANOVA comparison of the mosquito activities elicited by the 10-34 kHz, 35-60 and 61-90 kHz sound of *A. tormotus* by the mosquito activities under control was determined. The significance values obtained in this comparison was greater than 0.05 ($p > 0.05$) in all the frequency ranges. On the other hand, the comparison of the mosquito activities elicited by 10-34 kHz, 35-60 and 61-90 kHz sound of *C. afra* by the activities under the control yielded significance values less than 0.05 ($p < 0.05$). In comparison of mosquito activities in varied frequency ranges of individual predator sounds by the control, the significance values determined are shown in Table 9.

At 5 % significance level, there was no significant deviation in mosquito activities elicited by the 10-34 kHz. 35-60 kHz and 61-90 kHz sound of *A. tormotus* from the mosquito activities under the control. However, the deviation in mosquito activities elicited by 10-34 kHz, 35-60 kHz and 61-90 kHz sound of *C. afra* was significant from that of the mosquito activities under the control. Hence, the startle response, based on mosquito activity differed significantly in the 35-60 kHz for the sound of *C. afra* from the activities under the control. The optimum startle frequency for the individual sound of *A. tormotus* and *C. afra* on the female *A. gambiae* was 35-60 kHz. The average percentage of the mosquitoes affected by sound of *A. tormotus* and *C. afra* in the 35-60 kHz frequency range was 45.88 % and 22.94 % higher than the reported 20.0 % effective repulsion by EMR sound.

Table 9. Significance values of the comparison of mosquito activities in varied frequency ranges of individual predator sounds by the control

Sound source	Frequency Range	F	p
A. tormotus	Comparison of mosquito activities under the 10-34 kHz by the activities under the control	0.744	0.693
	Comparison of mosquito activities under the 35-60 kHz by the activities under the control	1.144	0.461
	Comparison of mosquito activities under the 61-90 kHz by the activities under the control	0.639	0.766
C. afra	Comparison of mosquito activities under the 10-34 kHz by the activities under the control	9.409	0.003
	Comparison of mosquito activities under the 35-60 kHz by the activities under the control	52.927	0.000
	Comparison of mosquito activities under the 61-90 kHz by the activities under the control	6.921	0.008

4. Conclusions

The average percentage of the mosquitoes affected by sound of *A. tormotus* and *C. afra* in the 35-60 kHz frequency range was 45.88 % and 22.94 % higher than the reported 20.0 % effective repulsion by EMR sound. The high response in the sound of *A. tormotus* was due to progressive increase in maximum and mean acoustic energy from the 10-34 kHz frequency range. Similarly, the signal intensity for *A. tormotus* in 35-60 kHz was greater than that of the 10-34 kHz frequency range. However, the energy and signal power for *C. afra* declined from their value in 10-34 kHz rendering it weak compared to the constant power of *A. tormotus*. The decline in the energy and power in *C. afra* yielded a reduced number of the mosquitoes affected by the ultrasound in this range. The sound of *A. tormotus* significantly startled the female *A. gambiae* compared to the sound of *C. afra*. The startle response in the female *A. gambiae* due to the 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. Secondary effects of the ultrasound on the mosquitoes included physical injury, fatigue and falls. The optimum startle frequency for the individual sound of *A. tormotus* and *C. afra* on the female *A. gambiae* was 35-60 kHz.

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