BIOACOUSTICS AND MOLECULAR TOOLS FOR MOSQUITO IDENTIFICATION AND SURVEILLANCE

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

By

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DECLARATION

I hereby declare that the presented work in the thesis entitled **"Bioacoustics and molecular tools for mosquito identification and surveillance"** in fulfillment of the degree of Doctor of Philosophy (Ph. D.) is the sole outcome of the research work which was carried out under the supervision of Dr. Joydeep Dutta, Professor & Head, Department of Zoology and Botany, School of Bioengineering and Biosciences, Lovely Professional University Punjab, India and Dr. Devi Shankar Suman, Scientist E, Vector Biology Lab, Zoological Survey of India. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever the work described here has been based on the experimental findings of other investigators. This thesis has never been fully or partially submitted to another University or any institute for the award of any type of academic degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **"Bioacoustics and molecular tools for mosquito identification and surveillance"** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Zoology, School of Bioengineering and Biosciences, is a research work carried out by Udita Mandal, 12014210, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Mosquito-borne diseases cause millions of deaths and severe health issues worldwide. Conventional mosquito surveillance and identification measures require major expenses, prolonged time, and technical expertise. At the same time, bioacoustics has proven to be an efficient, eco-friendly way to monitor and conduct surveillance of mosquitoes. This study evaluates the effectiveness of tools for the surveillance and detection of mosquitoes based on acoustics and molecular tools, along with the effects of environmental factors and morphometric features. The mosquitoes were collected from various locations in different states of India, i.e., ZSI-Kolkata, Jalpaiguri, Neora Valley, Batanagar, Budge Budge, Eden City, Nalban East, Kolkata of West Bengal, Bhubaneswar of Odisha, Baroda of Gujarat, Sheopur of Madhya Pradesh, Sundaranjanpatti Annamalai, Madurai, Velaypatti, Sirkurmundra, and Thirkurmundaram of Tamil Nadu, with the help of CDC-LT trap and BG-Sentinel trap. Mosquito larvae were also collected using ladles from different aquatic habitats. Rearing was done up to the adult stage under standard laboratory conditions, i.e., 26 ± 1 °C, 12:12 (L:D), and 65 ± 5% relative humidity. Field survey and acoustic data collection were conducted from November 2021 to December 2022. Fundamental frequency, higher harmonic frequency, delta frequency, high & low frequency, delta time, peak frequency, median amplitude envelopes, amplitude & frequency modulation, acoustic entropy, and bioacoustic index were determined. A sound recorder patented by the Zoological Survey of India consisted of a two-walled plastic recording chamber and a microphone attached to the cap. A cell phone was connected to this device via this microphone. Sounds of mosquitoes were captured after introducing the mosquitoes into the chamber at 16-bit and 44.1 kHz.

Fundamental frequency was considered for the discrimination between mosquito species, which provided 95.32% accuracy, which further increased upon analysis of the acoustic entropy index. Two novel matrices were also developed to isolate the mosquito pairs unable to distinguish using fundamental frequency. The diversity of harmonic bands demonstrates an 84.79% success rate compared to 19 species. Abdominal condition, gender, and mixed sound were considered as influencing factors on the acoustic features of mosquitoes. No significant impact of age was found on the buzzing sound of mosquitoes. The average power density was not efficient in the detection of species and provided less than 50% accuracy. This study reports harmonic convergence in *Aedes vittatus* synchronizing the 2nd harmonic of males and the 3rd harmonic of females.

Mosquitoes were confirmed using molecular tools using the COI gene for amplification, and the sequencing of 19 mosquitoes was subjected to Sanger sequencing followed by alignment of sequences in BioEdit and submission to NCBI GenBank. Phylogenetic trees for the COI gene were constructed harnessing the Maximum Likelihood method & Kimura 2-parameter model. These molecular analyses resulted in sequences up to 500bp and reported novel sequences of the five mosquitoes, i.e., *Anopheles roperi, Anopheles umbrosus, Culex alienus,* and *Hulecoetomyia fluviatilis.* Other sequences showed 95-100% while comparing with conspecific sequences submitted to GenBank. The percentage of adenine-thymine content was found in *Mansonia annulifera,* while the lowest guanine-cytosine content was found in *Anopheles elegans.* The phylogenetic tree analysis revealed multiple clusters of species of related genera and demonstrated close evolutionary relationships between different mosquito species. To know the effects of body size, differential body sizes were obtained by rearing mosquito larvae in different densities, providing the same food ration and environmental conditions. The wing lengths were considered as a parameter to denote the effects of this parameter on the acoustics of *Aedes aegypti* and *Aedes albopictus*. The lengths of wings were calculated by harnessing the ImageJ software. The differences in wing lengths were confirmed with the help of one-way ANOVA between the different wing-sized mosquitoes.

The temperature and humidity were considered as environmental factors, and the effects of these factors on the acoustics of *Aedes aegypti* and *Aedes albopictus* mosquitoes were examined. A positive correlation between temperature and fundamental flight tones has been shown in this study. For each degree rise in temperature base frequency increased by 1.29 ± 0.04 Hz and 1.62 ± 0.37 Hz in *Aedes aegypti* and *Aedes albopictus,* respectively, while humidity could not reveal any significant effects. This study also focused on monitoring mosquitoes from remote and resource-constrained areas. The efficacy of machine learning codes to decode and distinguish between different mosquito species using the R language was also investigated. Recordings of wing beat sounds generated by three types of free-flying dengue vectors, *Aedes aegypti, Aedes albopictus,* and *Aedes vittatus,* were undertaken through a low-cost portable device. This study evaluated acoustic indices with the help of machine learning codes using R, using the seewave and soundecology packages to describe the acoustic features of mosquitoes and to compare the mosquitoes based on acoustic signatures. The mean fundamental frequency was evaluated at low and high frequencies. For each 10-second recording, two eco-acoustic indices were calculated: the bioacoustic index and the acoustic entropy for the classification of three mosquito species. The density surveillance study of *Aedes vittatus* was conducted during the study period. Bioacoustics and acoustic entropy index of two populations of *Aedes vittatus* species having different densities demonstrated statistically significant differences. Our study provides detailed acoustic datasets of mosquitoes, addressing acoustic signatures as identification keys that can be further developed for the automated detection of mosquitoes. This study also reveals the efficacy of eco-acoustic indices for indicating mosquito presence and surveillance. The study assessed statistically significant differences in the acoustic characteristics between three mosquito species. *Aedes aegypti* revealed the lowest harmonics, *Aedes vittatus* demonstrated the lowest MAE or median amplitude envelop, and the highest frequency modulation. The acoustic indices, including delta frequency, peak frequency, and delta time, also varied significantly between the dengue-vector mosquito species. Moreover, the study detected correlations between different acoustic indices for each of the individual species of mosquitoes. Both the eco-acoustic and the density of mosquitoes showed a positive correlation as both of the eco-acoustic indices increased with the number of mosquitoes present in the recording device. This, in turn, indicates the possibility of surveillance of mosquitoes from a remote location.

Detailed information on the acoustics-based database, along with morphological, physiological, and environmental effects on the acoustics of mosquito species, have been provided that can be harnessed in the AI model for the analysis and accurate detection of mosquitoes without human supervision.

Table of contents

LIST OF TABLES:

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Vector-borne diseases are now a significant threat. Diptera have contributed to many deadly diseases, causing death and agricultural losses worldwide (Louzeiro *et al*., 2021). Many of these diseases are transmitted by various mosquito species: *Aedes aegypti, Aedes albopictus, Aedes mediavitatus, Aedes sierrensis, Anopheles albimanus, Anopheles arabiensis, Anopheles atroparvus, Anopheles dirus, Anopheles farauti, Anopheles freeboni, Anopheles gamiae, Anopheles merus, Anopheles minimus, Anopheles quadranulatus, Anopheles quadramaculatus, Anopheles stephensi, Culex pipiens, Culex quinquefasciatus, Culex tarsalis, Culiseta incidens,* etc. (NVBDCP, 2020). Vector surveillance, identification, and control are just as important as active case detection and treatment. Despite advances in vector control methods, a high number of mosquito-borne disease cases continue to be reported (NVBDCP, 2020). The indoor residual spray program is scheduled in endemic areas to control mosquitoes, but issues such as insufficient funding, floods, frequent festivals, lack of proper sprayers, and low public acceptance have compromised its effectiveness. Prolonged use of a single insecticide can lead to resistance and pose health risks to humans and animals (Ahmad *et al*., 2024). Mosquito characteristics have also evolved, enabling them to thrive in favorable environmental conditions. There is significant diversity among mosquito species across different regions of India (Sharma *et al*., 2025; De *et al*., 2022). Although many species appear morphologically similar, taxonomic identification traditionally relies on physical features, while molecular analysis provides a more precise way to differentiate species with similar appearances (Batovska *et al*., 2016). Molecular techniques analyze biological markers in the genome and proteome, along with gene expression (Poste, 2001). Molecular markers, such as species-specific mitochondrial DNA and ribosomal DNA, are essential for detection. Efficient molecular identification depends on markers like Cytochrome c oxidase subunit I (COI), internal transcribed spacer-2 (ITS2), and 16S ribosomal DNA. These methods are faster and more accurate (Liu *et al*., 2017). Their importance is growing in pathogen detection and understanding vector biology (Ondrejicka *et al*., 2014). Despite many benefits, molecular methods have some limitations. Proper collection, handling, and storage of specimens are critical for PCR accuracy; contamination can lead to false positives. Additionally, DNA analysis via PCR can be costly and time-consuming. Therefore, accurate vector identification remains a pressing challenge, highlighting the importance of species recognition and understanding their roles in disease transmission and control (Teymouri *et al., 2021*). Recognizing the limitations of classical morphological identification and advanced molecular techniques, researchers have suggested eco-friendly methods to identify and control vector-borne diseases. Bioacoustics, an interdisciplinary field, explores the relationship between living organisms and sound. It covers various aspects, including animal communication, movement, and environmental monitoring. Vocalizations are crucial for communication, helping animals establish territory, reproduce, and navigate (Penar *et al*., 2020). For example, birds use calls to define territory, while whales employ complex vocalizations to socialize and travel vast ocean distances. Bioacoustics research also studies how human-made noise affects wildlife, revealing disruptions in animal acoustic behavior due to urbanization and industrialization. These disturbances alter communication patterns and reduce reproductive success (Lewis *et al*., 2020). While all these aspects involve acoustics in biological organisms, their focus and implications vary. Animal vocalizations mainly help to understand the evolution of communication in different species and ecological contexts. This knowledge supports conservation strategies, emphasizing the need to preserve natural habitats and resources (Turlington *et al*., 2024). Recent advances in research on man-made noises addressed the mal effects of sounds on animals, which can be further subdivided into beneficial and detrimental aspects (Slabbekoorn *et al*., 2018). Although the detrimental effects of sound signals affect biodiversity and conservation, as a benefit, control strategies can be developed, harnessing noise playback for mating reduction and population control of agricultural pests and vector insects like mosquitoes (Mankin, 2012). Bioacoustics serves as the crucial framework from small insects to larger animals for communication, feeding, mating, and other activities, generating sound frequencies ranging from infrasonic to ultrasonic. Several attempts have been made to denote the frequency distributions of different animals along with the sound-associated behaviors (Mcloughlin *et al*., 2019). Bioacoustics has proven to be a low-cost and efficient method reported to be fruitful for the identification as well as the control of insects employing traps and repellents (Lapshin & Vorontsov, 2018; Rohde *et al*., 2019). Animal bio-acoustic covers the aspects of biosonar, communication between animals through sound, neurophysiology, transduction of sound signals, and detection of animals through emitted sound frequencies using hardware and software (Erbe, 2016). The bio-acoustic study is interdisciplinary as it comprises the knowledge and techniques of biology, physics, and mathematics, forming a new aspect of research in biotechnology (Hianik *et al*., 2006; Jung *et al*., 2018; Hult *et al*., 2002; Hiremath *et al*., 2020) and medical sciences through painless drug delivery to visceral organs (Du *et al*., 2018). Mosquitoes produce sounds of different frequencies and varied amplitudes. They possess highly sensitive auditory organs in contrast with other fauna, with the help of Johnston's organ (JO) located at the second segment of the antenna. JO is comprised of numerous ciliated mechanosensory nerve cells conjugated with prongs situated at the basal part of the flagellum and receives acoustic signals. Mosquitoes use sound frequencies to locate conspecific members with the help of Johnston's organ. This feature helps researchers to design acoustic lures for different mosquitoes (Staunton *et al*., 2021).

To distinguish between the bioacoustics of different mosquitoes, proper identification is necessary, while a DNA fingerprinting tool using PCR and Gel electrophoresis can be employed at first to inspect the species of the mosquitoes. Once DNA analysis confirms the species, information related to molecular and genetic structure and polymorphism can be easily combined with sound frequencies. Hence, sound characteristics of mosquitoes can be one of the important candidates for genetic features. Moreover, by utilizing computer programming using deep learning, automated mosquito detection can be done in a way that a trapped mosquito will demonstrate its species, genetic, molecular, morphological, and behavioral characteristics (Bist *et al*., 2021).

CHAPTER 2: REVIEW OF LITERATURE

2.1. Bioacoustics of insects

Bioacoustics is a novel field of study on the sounds generated by living organisms. From the songs of birds to the chirping of crickets, bioacoustics represents the diverse range of sounds of animals for communication, navigation, and even predation. Furthermore, bioacoustics plays a key role in conservation efforts through monitoring populations using wildlife soundscapes, along with analyzing the changes in calls of fauna to track population trends and the health of ecosystems. Bioacoustics study also includes classification of animals through sound frequency through hardware and software, tracing of aquatic animals using biosonar and echo sounders, control of pests and wildlife conflicts using deterrent devices, etc. Bioacoustics adopted by animals is marked as interdisciplinary, as diversified research methods, knowledge, and techniques of biology, mathematics, physics, and zoology are brought together to elucidate animal bioacoustics (Erbe, 2016). Bioacoustics explores the mechanisms of sound production and reception. (Haskell, 2021) described insect sounds along with sound reception and associated behavior. General principles of insect sound were illustrated, focusing on the physical nature of sound, sound capture, and analysis. He emphasized the structure and function of various insects' generating and sound-receiving apparatus. He explained the term 'insect sound' as a mechanical interruption that the insect can strongly prefer to an external source or a source in its vicinity.

2.1.1. Sound production:

A wide variety of structures are engaged in the production and transmission of sounds in insects. The generation of sound in insects can be categorized into five different ways (Alexander, 1957; Ewing, 1989) (Fig. 2.1).

2.1.1.1. Stridulation:

Generation of sound through the collision between two body parts. Some crickets, grasshoppers, bugs, katydids, butterflies, beetles, caterpillars, moths, ants, and beetle larvae are found to adopt stridulation.

2.1.1.2. Percussion:

Generation of sound striking the body parts against the substrate medium. Band-winged grasshoppers strike their feet against the substrate, while cockroaches use the tip of their abdomen, and death-watch beetles involve the head striking against the substrate to produce sound.

2.1.1.3. Vibration:

Production of sound through the vibration of wings and body parts. Mosquitoes, flies, bees, and wasps produce sounds vibrating their wings and body parts.

2.1.1.4. Click mechanism:

Sound generation through the vibration of tymbals or tymbal activities. Cicadas, treehoppers, leafhoppers, and spittlebugs adopt a click mechanism.

2.1.1.5. Air expulsion:

Generation of sound through forcible ejection of air or fluid. Short-horned grasshoppers are found to use this method.

Studies on one cicada insect, *Subpsaltria yangi* revealed the capability of females to produce sounds through the Stridulation mechanism, followed by stimulating phonotactic and acoustic responses from conspecific males. During the production of sound, cicada females were observed striking their bodies through their forewings (Luo and Wei, 2015). There is a great impact of resonators on the sound production of insects. Resonators refer to the apparatus that helps to gain the resonance of a sound. Different types of animals demonstrated different resonators during the production of sound. The tymbal organ is involved as the primary resonator in both typical and bladder cicadas, but the mole cricket demonstrated the harp as the primary resonator. Helmholtz resonator, horn-shaped burrow, and thin-walled bladder were identified as secondary resonators for typical cicada, mole cricket, and bladder cicada, respectively (Bennet-Clark, 1999).

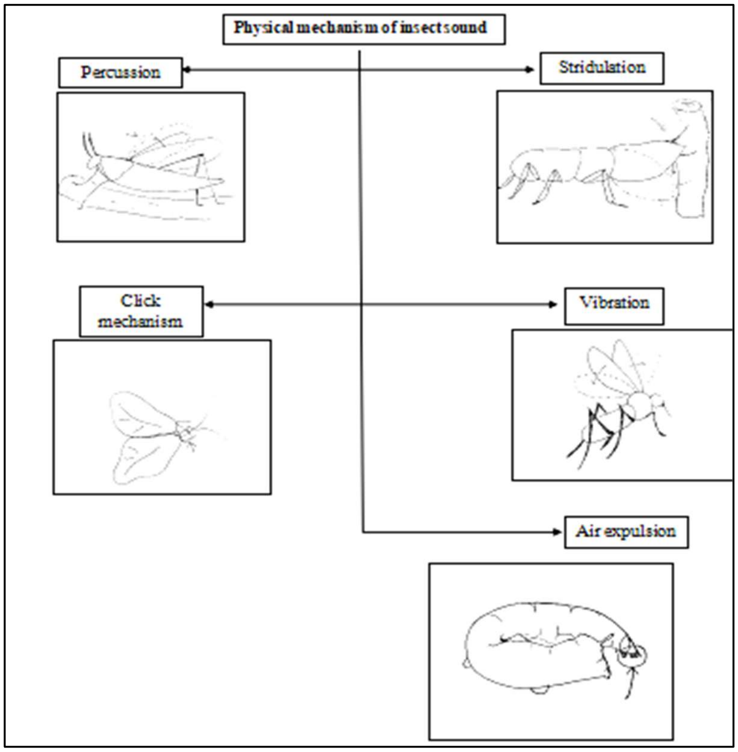


Figure 2.1 Physical Mechanism of insect Sound

2.1.2. Sound reception mechanism with sound receptor organs:

Insects as the smallest unit having ears, along with the chances of being located at almost any of the possible body parts, at legs, wings, mouth parts, abdomen, or thorax. Some insect species were found to locate highly accurate acoustic sources through their directional hearing. Insects adopt hyper-acuity with the help of their ears, which are internally coupled. The following five types of hearing organs are found in insects (Römer & Schmidt, 2016).

2.1.2.1. Hair mechanosensillum

Several arthropods possess cuticular integuments that act as exoskeletons and bear mechanosensilla that serve to detect signals from external sources. Signal transduction is operated by the sensory receptors in three steps: coupling, transduction, followed by encoding. In insects, the mechanoreceptors may carry individual hair or seta projecting from the cuticular surface. This hair mechanosensillum transfers the sound signals to the Central Nervous System through a single neuron. (Capinera, 2008).

2.1.2.2. Chordotonal sensillum

Transduction of sound signals is operated through the chordotonal mechanosensillum, which is connected to sensory neurons in many insects. Those neurons are attached by a comparatively longer axon to the CNS. The scolopale or the sclerotized cap cell is connected to the site of stimulation. Upon receiving stimulation, the adjacent site of scolopale stimulates the dendrites of those sensory neurons, resulting in nerve impulse propagation (Capinera, 2008).

2.1.2.3.Tympanal organs

The paired tympanal organ serves as a potential sound receptor. Air-mediated acoustic stimulation causes oscillation of the tympanum. This results in the transmission of signals through sensory neurons to the CNS (Capinera, 2008).

2.1.2.4. Subgenual organ

Several insects demonstrate subgenual organs as another auditory organ. This organ is comprised of numerous scolopidia forming a complex chordotonal organ that exists adjacent to the joint between the femur and tibia. It serves as a receptor for internal stimuli. It is also capable of identifying the vibrations of the substrate. Crickets and katydids possess well-developed subgenual organs for sound reception. (Capinera, 2008).

2.1.2.5. Johnston's organ:

It is a sensory organ located at the pedicel of antennae of insects and comprises numerous mechanosensitive scolopidia (Fig.2.2). This organ responds to different types of stimuli in various insects, such as an indication of gravity and direction of antennal movement, detection of wing beat frequency in order Diptera, especially sound perception in mosquitoes and other flies. Antennae vibrate while subjected to simultaneous acoustic stimulation. Johnston's organ is strong enough to detect the differences in tones coming from mixed acoustic tones of different frequencies of male and female mosquitoes (Table 2.1). Mosquitoes communicate with the help of a signal received by Johnston's organ, depending on acoustic interactions between male and female mosquito pairs (Capinera, 2008).

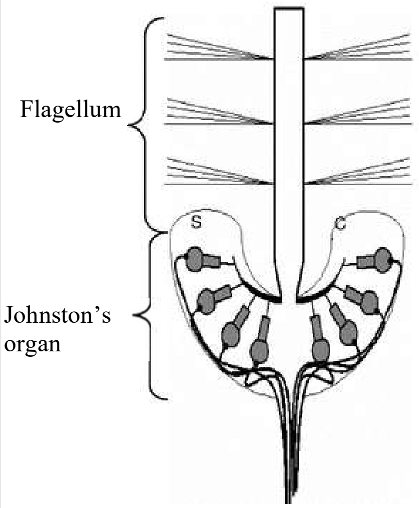


Fig. 2.2. Cross-sectional view of the mosquito's antenna demonstrating Johnston's organ, Gibson *et al.* (2010)

Table. 2.1. Comparative analysis of Johnston's Organ in different mosquitoes

Species	**Sex**	**Flagellar resonant frequency (Hz)**	**Ref.**
Toxorhynchites brevipalpis	Male	420.0 ± 5.0	(Göpfert & Robert, 2000)
Toxorhynchites brevipalpis	Female	244.0 ± 11.0	(Göpfert & Robert, 2000)
Culex pipiens pipiens	Male	85 to 470	(Lapshin & Vorontsov, 2017)
Aedes aegypti	Male	522.69 ± 11.10	(Su *et al.*, 2018)
Aedes aegypti	Female	203.06 ± 2.22	
Culex quinquefasciatus	Male	485.40 ± 7.03	
Culex quinquefasciatus	Female	212.96 ± 2.41	
Anopheles gambiae	Male	506.62 ± 9.03	
Anopheles gambiae	Female	219.70 ± 3.55	

2.1.3. Uses of sound in insects

A variety of insect pests and other pests produce acoustic signals for different functions and behaviors. Orthopteran tettigoniidae insects produce sounds to show aggression and to attract males using percussion and tremulation mechanisms, while cricket uses the same mechanism for call and reception for spermatophores. Blattodea demonstrated percussion and stridulation for mating. Isopteran insect follows percussion through mandibles to generate alarming signals. Snow Flies, needle flies, forest flies, willow flies, salmon flies, and green stone flies attract mates through percussion methods. Heteropterans engaged the tymbal and stridulation method to generate signals for mate attraction. Diptera chloride flies were noted to follow a tremulation mechanism to attract mates. Ants were found to produce signals for attacks and perils through percussion. Most of the insects produce vibrational signals of simple volleys of monophasic nature (Kirchner, 1997). Cicada insects can generate 109 decibels of sound for attracting mates and alarming calls against predators (Heppner *et al*., 2008). Spiders were observed to demonstrate higher persistence while attacking silenced insects than the sound-producing ones. Females of mutillid wasps were experimented with wild-caught mice, *Peromyscus floridanus*, and most of the phonic wasps were observed to be more than the silenced ones (Mitchell, 1979). Sexual selection in the cricket species *Laupala cerasina* is operated through the analysis of acoustic features (Grace & Shaw, 2012). Mosquitoes rely on acoustic signals for the investigation of conspecific mates (Ikeshoji, 1981). Male mosquitoes are found to be attracted by the wingbeat sound of females (Cator *et al*., 2010). Many bark-beetle species demonstrated sexual dimorphism in sound generation; either male or female is observed to produce sound for both sexual and defensive purposes (Low *et al.*, 2021).

2.1.4. Acoustic behavior-based detection of insects

Grace & Shaw (2012) investigated the sexual selection in *Laupala cerasina* through acoustic features. This study demonstrated the discriminating capabilities of females from diverse sounds of nearby populations. The authors revealed that females can detect the acoustic variations between populations and prefer breed true in a usual environment. Mean preferences for each of the populations were found to be similar to the mean pulse rates. Diversity in preference was noted only among populations having different acoustic features. Besides having the efficiency for discriminating against slight differences in sounds, a correlation was observed between evolutionary aspects of sounds and the preference within populations, which in turn suggested the promotion of assortative sexual behavior between populations which resulting in a reduction of genetic flow and speciation. A smaller number of studies have been reported in the case of mosquitoes, although they can make efforts to communicate with each other through the highly efficient Johnston's organ for receiving the species-specific aural cues. In response to acoustic signals, Mosquitoes respond to acoustic signals using their species and sex specific humming song of specific frequency (Simões *et al.*, 2016). Although species-wise mosquito detection using wingbeat sound was proposed several decades ago (Roth, 1948), very few of them demonstrated significant distinguishing parameters between different mosquito species (Alar & Fernandez, 2021; Fernandes *et al.*, 2021; Mukundarajan *et al.*, 2017; Siddiqui & Kayte, 2022). These studies were mainly dependent on wing beat frequencies using fundamental sound frequency for mosquito species identification. Mukundarajan *et al*. (2017) recorded 19 medically important mosquito species using commercially available cell phones and reported fundamental wingbeat frequencies for 19 medically important mosquito species (Table 2.2), which showed overlapping fundamental frequencies among them, and this issue was resolved using location metadata. Moore *et al*. (1986) developed an instrument based on a microcomputer for the recording and analysis of the frequency of wingbeats of mosquitoes, harnessing beams of light, and detected *Aedes aegypti* and *Aedes triseriatus* with an accuracy of 84% with the help of wingbeat frequency measurements. This spectrum-based analysis of recordings of both sexes of two species of *Aedes* suggested the great efficiency of the frequency of wingbeats to distinguish between the four mosquito groups. Identifications were 100% correct in the case of *A. triseriatus* females and males of *Aedes aegypti*, 93% correct for *Aedes triseriatus* males, and 43% correct for *Aedes aegypti* females. Arthur *et al*. (2014) analyzed acoustic tones of tethered *Aedes aegypti* mosquitoes. Flight tones of both male and female mosquitoes of *Aedes aegypti* were recorded using microphones of pressure-gradient type and reported mean frequency as from 571 Hz to 832 Hz in the male mosquitoes, while females showed frequency distribution from 421Hz to 578 Hz. This implied significant sex-based differences. Fundamental frequencies were found to be similar to previously reported studies, but amplitudes were found to be lower. This study reported that female fundamental frequency was lower than that of males, and modulations of frequencies by males were observed to be done over a wide range. Brogdon (1994) recognized fundamental wingbeat frequency of *Aedes aegypti* and *Aedes albopictus* by recording the sounds with a 20 kHz sampling rate and then resampling it to 10 kHz for females and 5 kHz for males, and reported the mean fundamental wingbeat frequency (WBF) of female *Aedes aegypti* and *Aedes albopictus* as 460 Hz and 536 Hz, respectively, while for the males of *Aedes aegypti* and *Aedes albopictus*, with a 5 kHz sampling rate, mean fundamental wingbeat frequencies were visualized as 715 Hz and 724 Hz, respectively. Cator *et al*. (2011) recorded sounds of *Aedes aegypti* mosquitoes using microphones sensitive to pressure placed at the perimeter position of a funnel having a 15cm diameter for the spatially isolated array of microphones. Customized electronic circuit provided power to those microphones along with amplification and modulation of the signal of the output. Data transfer in a computer was conducted using an analog-to-digital converter and measured the mean flight tone frequency of free-flying *Aedes aegypti* in swarms in Thailand and reported flight tones of solo male and females as 982.0 ± 1.0 and 664.3 ± 4.6, respectively, while flight tones of paired male and females were 989.3 ± 7.4 and 609.1 ± 48.5, respectively. Simões *et al*. (2016) recorded the WBF of *Culex quinquefasciatus* using a parabolic microphone while mosquitoes were free flying above a marker inside a huge sound-reduced booth in a dark environment and reported fundamental WBF of *Culex quinquefasciatus* males as 789±10 Hz, and that of females was 474±10 Hz. Aldersley *et al*. (2014) recorded individual flight tones of male and female *Aedes aegypti* mosquitoes and extracted frequency characteristics using Hilbert spectral analysis, which showed peaks of fundamental wingbeat frequency ranging from 492.1 Hz to 880.3 Hz in the different males captured. Mean flight frequency of a single female mosquito of females ranged from 415.9 Hz to 532.6 Hz, along with a group-wide average and SD of 480.6 Hz ± 32.5Hz. Gibson *et al*. (2010) reported a similar range of fundamental frequency distribution in both sexes of *Toxorhynchites brevipalpis*. Irrespective of the fundamental wing-beat frequency of each mosquito, both male and female mosquitoes altered sound frequencies in response to a pure-tone acoustic stimulus for convergence with that pure tone. This frequency matching ranges from 350–500 Hz if the pure tone stimulus is 60 Hz below or 60 Hz higher than the base or fundamental frequency range; otherwise, this alternation can range between 200–345 and 500–800 Hz. Göpfert and Robert (2000) assessed the vibrations of the flagellum of antennae in both male and female *Aedes aegypti,* followed by the comparison and the evaluation of the auditory significance of the hairs of the flagellum. In both sexes, the antennae demonstrated forced damped harmonic motion during acoustic stimulation. The best frequencies of the female and male antennae are around 230 and 380 Hz, respectively, while the lowest or fundamental frequency of female flight sound was found to be equivalent to the best antennal frequency of male. Hairs of male antennae are tuned to frequencies between 2600 Hz and 3100 Hz. Antennal hairs are strongly associated and go along with the flagellar shaft during acoustic evocation at frequencies near 380 Hz. As a result of these coupling arrangements, forces are transmitted to their flagellar shaft and thus to the neural auditory organ at the flagellar base. Dou *et al*. (2021) experimented on the acoustic responses of free-flying *Aedes aegypti* and *Anopheles gambiae* in Baltimore, USA. A high-speed recorder having 125 μm/pixel FPS was employed for extracting the velocity of mosquitoes. This study reported the flight velocity as 0.14-0.18 m/s before being subjected to an acoustic wave. The speed of flight of male *Aedes aegypti* was increased in response to acoustic waves having frequencies ranging from 100 Hz to 800 Hz, while females exhibited a comparatively narrower range between 100 Hz - 300 Hz. Male *Anopheles gambiae* was observed to demonstrate statistically significant responses to acoustic frequencies of about 400Hz, while minimal responses were recorded for female *Anopheles gambiae*. Lapshin & Vorontsov (2017) conducted an elaborate examination of the Johnston organ of male *Culex pipiens.* Focal recordings from antennal axons were made through glass micro-electrodes using three mol l−1 KCl, followed by acoustic and feedback stimulation. This study imparted the presence of a minimum of 8 groups of aural neurons having distinct frequency-tuning features in male mosquitoes. This study addressed the tuning range of neurons from 190 to 270 Hz, which demonstrated equivalent differences between the flight tones of female and male mosquitoes. This study also reported that sensory units of axons of the Johnston organ propagated amplified and graded receptor potential compared to an all-or-none action potential. The authors presented evidence in support of advanced acoustic frequency detection in mosquitoes. They also assessed frequency tuning for individual acoustic receptors of Culicine mosquitoes. The distribution of frequencies in neurons was observed to be dominated by those modulated to the frequency range of 75 to 125 Hz. The authors developed a unique feedback stimulation mechanism for detecting acoustic feature receptors individually. The authors suggested the potential analytic capabilities of mosquitoes to distinguish between different components of frequency. Raman *et al*. (2007) constructed a field-applicable insect flight sound-detecting device involving a noise-reducing microphone combined with a sound recording device having capability of 10 hours. This study generated huge numbers of false positive sounds as ambient sounds, including the sounds of humans, birds, frogs, and vehicles, which were mixed. This study addressed 726 insect humming tunes and 52 cases of false positives. After the estimation with the final algorithm, the clear insect humming tune increased to 784, along with the increase of false positives to 471, which is 28% of the total detected mosquitoes. Acoustic analysis of mosquito wingbeat frequency has been investigated as a tool for species identification, incorporating four mosquito species: *Aedes albopictus, Culex quinquefasciatus, Anopheles crawfordi, and Armigeres subalbatus* (Rajan *et al*., 2025). This study revealed a significant difference in the fundamental frequency among the species, supporting the potential of this method. However, the research revealed a notable limitation: the wingbeat frequency of *Aedes albopictus* and *Culex. quinquefasciatus* is not static but changes throughout its adult life, reaching its peak during the swarming stage. This variation introduces uncertainty into species identification based on a single frequency measurement. Furthermore, the study determined that successful swarming and pairing depend on the convergence of the male's first harmonic (M1) and the female's second harmonic (F2), which is essential for mate selection within the species.

The use of insect bioacoustics, specifically wingbeat frequency, was explored as a method to build local databases of carrion insect succession for post-mortem interval (PMI) estimation (Gorgeva *et al*., 2023). This approach was intended to overcome the limitations of traditional insect trapping, which was time-consuming, environmentally dependent, and susceptible to sampling bias. While this technique held promise for guiding forensic entomologists toward more accurate developmental studies on dominant local species, it presented several challenges. Wingbeat frequency was influenced by a range of factors, including temperature, humidity, age, size, and sex, necessitating customized recording and post-processing for different species. Additionally, detecting specific flight sounds against a backdrop of environmental noise and a multitude of species in the field posed a significant challenge. Despite these challenges, previous studies have successfully used wingbeat sounds for fly species identification, suggesting that integrating bioacoustics with machine learning could provide a powerful new tool for forensic entomology.

Kohlberg *et al*. (2024) systematically reviewed the effectiveness of automated bioacoustics models for insect monitoring over the past four decades. The review, which analyzed 176 studies, sought to address the gap in understanding the status of these emerging technologies. The findings showed that automated bioacoustics models had been developed for 302 insect species across nine different orders. These models used various types of sounds for identification, including intentional calls, wingbeats from flight, and indirect sounds like the movement of grain. Most of the studies focused on pests, such as weevils and borers, with all disease vector studies concentrating on mosquitoes. The research revealed that machine learning, especially deep learning, was becoming the gold standard for these automated approaches, with some models achieving over 90% accuracy in classifying hundreds of insect species. The review highlighted several advantages of automated bioacoustics models, such as their usefulness in reducing the need for lethal sampling, monitoring insect patterns, and working in challenging locations where traditional methods are less effective. However, it was also noted that not all insect species produce easily detectable sounds and that sound pollution could interfere with recordings in some environments (Ow and Ghosh, 2017). The study concluded that automated bioacoustics methods could be a valuable tool for monitoring insects and addressing important ecological and societal questions. Successful applications included assessing insect biodiversity, distribution, and behaviour, as well as evaluating the effectiveness of restoration and pest control efforts. The authors recommended increased collaboration between ecologists and machine learning experts to expand the use of these models among researchers and practitioners.

Table. 2.2. Frequency distribution by insects

Sr No.	Insects	Species	Sex	Frequency Range (Hz)	Location	Ref.
1	Mosquito	*Aedes aegypti*	male	571-832	Tapachula,Mexico	Arthur *lbu*(2014)
			female	421-578	Tapachula,Mexico	
			male	100-800	Baltimore, Maryland	Dou *et al.* (2021)
			female	100-300	Baltimore, Maryland	
			female	450-650	CDC Atlanta	Mukundarajan *et al.* (2017)
2	Mosquito	*Aedes albopictus*	female	500-700	CDC Atlanta	
3	Mosquito	*Aedes mediavitatus*	female	390-480	Coffee lab, U.C. Davis	
4	Mosquito	*Aedes sierrensis*	female	340-530	Big Basin Redwoods State Park, CA, USA	
5	Mosquito	*Anopheles albimanus*	female	360-500	CDC Atlanta	
6	Mosquito	*Anopheles arabiensis*	female	360-590	CDC Atlanta	
7	Mosquito	*Anopheles atroparvus*	female	380-510	CDC Atlanta	
8	Mosquito	*Anopheles dirus*	female	400-490	CDC Atlanta	
9	Mosquito	*Anopheles farauti*	female	500-700	CDC Atlanta	
10	Mosquito	*Anopheles freeboni*	female	340-450	CDC Atlanta	
11	Mosquito	*Anopheles gamiae*	male	100-500	Baltimore, Maryland	Dou *et al.* (2021)
	Mosquito		female	200	Baltimore, Maryland	
	Mosquito		female	460-650	CDC Atlanta	Mukundarajan *et al.* (2017)
12	Mosquito	*Anopheles merus*	female	400-600	CDC Atlanta	
13	Mosquito	*Anopheles minimus*	female	550-700	CDC Atlanta	
14	Mosquito	*Anopheles quadranulatus*	female	330-520	CDC Atlanta	
15	Mosquito	*Anopheles quadramaculatus*	female	250-450	Mathius Lab, Auburn University	
16	Mosquito	*Anopheles stephensi*	female	500-650	CDC Atlanta	
17	Mosquito	*Culex pipiens*	female	290-400	Santa Clara Vector Unit	

18	Mosquito	*Culex quinquifasciatus*	female	340-410	Santa Clara Vector Unit	
19	Mosquito	*Culex tarsalis*	female	210-370	Stanford University	
20	Mosquito	*Culiseta incidens*	female	200-360	Stanford University	
21	Sandfly	*Phlebotomus argentipes*	male	246.8	Southeast Asia	Araki *et al.* (2020)
22	Sandfly	*Lutzomyia longipalpis*	male	285.9	Brazil	Souza *et al.* (2004)
23	Crickets	*Nisitrus vittatus*	male	7300	Singapore	Robillard *et al.* (2013)
24	Crickets	*Agnotecous robustus*	male	11200	New Caledonia	
25	Crickets	*Gryllus bimaculatus*	male	4800	University of Bristol	(Montealegre-Z *et al.*, 2011)

2.1.5. Acoustic behavior and communication in insects

Cocroft and Rodríguez (2005) conducted experiments based on the ecological aspects of the acoustic behavior of insect communication. This study demonstrated that 92% of insect species adopt substrate vibration mechanisms for transmitting sound signals. Signals through vibratory mechanisms demonstrate extensively different features than air-borne signals. Even substrate-mediated signals having low frequency were found to show pure tone, while plants were observed to mostly use substrate. This study suggested the occurrence of insects' vibratory communication in complex environmental circumstances, including noises emitted from rainfall, the surrounding atmosphere, and signals of other individual animals. Kirchner (1997) described insects' communication through vibration. Experiments on the mechanism of signal functions and signaling methods were explained. This study described orthopteran tettigoniidae insects to show aggression and to attract males using percussion and tremulation mechanisms, while cricket uses the same mechanism for call and reception for spermatophores. Blattodea demonstrated percussion and stridulation for mating. Isopteran insect follows percussion through mandibles to generate alarming signals. Snow flies, needle flies, forest flies, willow flies, salmon flies, and green stone flies attract mates through percussion methods. Heteroptera engaged tymbal and stridulation methods to generate signals for mate attraction. Dipteran chloropid flies were noted to follow a tremulation mechanism to attract mates. Ants were found to produce signals for attacks and perils through percussion. Most of the insects produce vibrational signals of simple volleys of monophasic nature. This study revealed the evolutionary advantages of changes in various insects, which suggested the evolution and development of vibrational communication. (Villarreal and Gilbert, 2013) described the complicated aspects of replying to diversified male sounds by the female katydid *Scuderia pistillata*. This response of females was found to be comprised of a bout of raising the length of multi-syllabic phrases. The authors narrated the responsiveness of females to each of the phrases in a bout with a varying number of ticks. Female forms adapt their tick response, which ranges between 1-8, along with the counts of syllables presented by the male, replying to a maximum number of 7 to 9 syllables for each phrase. Tick response of females was reported to be decreased in subsequent phases while presenting with different influences of a bout of males. Authors reported the occurrence potential provision of information by females to the singing male on their predilection for specific acoustic features through the adaptation of ticks. The authors also suggested that temporal latency has a strong correlation with both counts and the number of ticks produced by her. (Tautz, 2000) developed a precise method of analyzing behavioral studies in insects, along with an analysis of the vibration of a comb at the place of wobble dancing of honeybees. With the help of laser-doppler-vibrometry and a digital video recorder waggle dance of *Drosophila* was recorded. This study revealed that the amplitude of the waggle phase was significantly higher than the return phase. This study also demonstrated no statistically significant differences in regions of flanking frequencies between 100 Hz to 200 Hz and 300 Hz to 400 Hz. This study evaluated the highest peak vibrations ranging from 206 Hz to 292 Hz. Virant-Doberlet and Cokl (2004) provided overviews of vibrational signals in insects and associated behavior. These signals were found to be associated with alarming calls, sexual behavior, and other interactions. Small insects produce far-reaching sounds with low frequency, which are unrecognizable to predators. This study explained the production of signals using vibratory methods in different insects through moving the tergal plate of the abdomen and vibration of the dorso-ventral regions of the whole abdomen. Schilcher (1976) conducted acoustic experiments on courtship dances of *Drosophila*. During this study, simulated courtship sounds of male *Drosophila* species were played. This study resulted in increasing locomotory activities in males, while decreasing trends of locomotion were noted in females. Wingless males were observed to have larger locomotory activities than winged ones. Solo males exhibited higher locomotory and sexual activities than grouped males. Percy and Day (2005) investigated abnormal acoustic behavior in two atypical leafhoppers, namely *Stenocotis depressa* (Walker) & *Austrolopa brunensis* (Evans) of Australia. Authors reported unusual interaction between females and the emanation of an intricate male call mediated by direct bodily contact with females. The authors discussed the evolutionary aspects of the acoustic behaviors based on this study and reported efficient impacts of competition between the same sex and the role of the carrying capacity of plant-substrate in the evolution. Couldridge and van Staaden (2006) examined the acoustic responses to previously captured sounds of males in the bladder grasshopper species *Bullacris membracioides*. Females were subjected to sounds having conspecific frequencies of different kinds of individuals, degraded sounds with conspecific frequencies, and the sounds of two different specific frequencies. This study addressed a statistically significant correlation between the responses of females and seven among eight rhythmic acoustic characteristics in *Bullacris membracioides.* This study also reported the response of females to the conspecific sounds with the levels of degradation corresponding to a sound-emitting male situated 150 meters away, but the intensity was observed to be similar to the male supposed to emit calls from a distance of 25 meters. This study revealed that the sounds of sister taxon *Bullacris intermedia* were observed to be equally attractive to females of *Bullacris membracioides,* while *Bullacris membracioides* demonstrated less preference for the more perceptible calls of *Bullacris serrata* than the other above-mentioned species. This study suggested a lack of distinguishing capability against an equivalent call from a separate species.

2.1.6. Harmonic frequency and harmonic convergence in mosquitoes

Aldersley *et al.* (2014) separated higher harmonic frequency in the male form of *Aedes aegypti* mosquitoes, harnessing a bandpass filter, which is defined by a center frequency and bandwidth, by applying an automated process to the specified filter parameters with the help of Fourier spectrum transformations, and found 9 harmonic bands including one weaker band having low power and frequency above 6 kHz. Mukundarajan *et al.* (2017) demonstrated fundamental frequencies and harmonics using a short-time Fourier transform and described that the harmonics correspond to the subtle differences in wing kinematics, such as deformation of wings during clap and fling. They have excluded the harmonics as potential parameters for mosquito detection as they used different mobiles for having differential frequency response; moreover, the mosquitoes were kept in containers of variable sizes and shapes, which can alter the characteristics of harmonics. This study also explained that the first overtone gets amplified more than the fundamental frequency if the frequency of the mosquito's first overtone is closer to the resonant frequency of the container. Although Brogdon (1994) considered the fundamental frequency observed seven harmonics in both *Aedes aegypti* and *Aedes albopictus* in the recordings sampled at 20 kHz, while five harmonics for *Aedes aegypti* and four harmonics for *Aedes albopictus* were observed in files resampled at 5 kHz, sound files sampled at 1 kHz demonstrated up to the 2nd and 1st harmonics, respectively. Simões *et al*. (2016) visualized lower harmonics and rapid modulation of harmonics in male *Culex quinquefasciatus* in the presence of conspecific female mosquitoes, which resulted in harmonic convergence characterized by two types of harmonic convergence ratios dividing stimulus frequency of females by the male mosquito's frequency just before the onset of rapid frequency modulations influenced by the stimulus. If harmonic convergence ratios = 1/2, the frequency of the 2nd harmonics of female sound is more or less equal to the male's fundamental frequency. Cator *et al*. (2011) assessed the acoustic behavior of swarming *Aedes aegypti* mosquitoes. The sound of free-flying and swarming *Aedes aegypti* mosquitoes was recorded in a natural environmental circumstance in Thailand. The authors described copulatory behaviors, and no statistically significant acoustic difference was observed between solo males and the males who were paired with females. However, males subjected to pairing with males demonstrated a little bit lower sound frequencies than the males reported to fly in pairs with females. This study revealed that the spectrograms of a male mosquito, which was found to be betrothed in pursuit of a female mosquito during flight. This study also demonstrated that the second harmonics of males and the third harmonics of females were closer to the frequencies of convergence. Authors explained that the male mosquitoes orient to the fundamental wing beat frequency of females, and both sexes actively modulate their flight tone before mating to converge at harmonic frequencies and observed a male and female appeared to be in the process of convergence in a paired flight while female's third harmonic and male's second harmonic were at frequencies near to convergence. Harmonic convergence could not occur between two closely related molecular forms of *Anopheles gambiae* (Pennetier *et al*., 2010). It is also evident that studies on mosquito communication using acoustic signals also contribute facts on reproductive isolation. Warren *et al*. (2009) investigated frequency matching in *Culex quinquefasciatus* in the UK, where the WBFs of male and female mosquitoes were found to be different. Instead of convergence on the fundamental, WBFs were observed to converge on the shared harmonic in the vicinity. This demonstrated sexual detection by finding similar wing beat-tone harmonics in the *Culex* mosquitoes. Simões *et al*. (2016) conducted an acoustic behavioral experiment on free-flying *Culex quinquefasciatus* male mosquitoes. They developed two different sets up for this purpose; in one setup, a sound recording of single free-flying male and virgin female pairs under the semi-natural environmental circumstances in a large flight arena in a double-skin sound-attenuated booth and in another setup, the behavior of free-flying mosquitoes was recorded inside a wire-framed arena and were subjected to artificially generated tone stimuli from a sound source which were delivered to the cage. They concluded that a novel stereotypical acoustic characteristic was adopted by male mosquitoes while exposed to the treatment with a fundamental frequency of female flight tones during mating. This male-specific free-flight activity demonstrated phonotactic flight, starting with a steep increase in the wing-beat frequency (WBF), and showed the rapid frequency modulation (RFM) of WBF in the lead-up to the formation of the copula. This study suggested RFM characteristics are truly consistent, even in response to an artificial tone that does not consist of the multi-harmonic components of natural female flight frequencies. RFM behavior of males showed an extremely fast change in WBF and can be adopted without acoustic feedback or the actual presence of female mosquitoes. It has been revealed after comparing audiograms of the robust RFM behavior and the electrical responses of the auditory system that the male Johnston's organ is linked to the distinction between the male and female WBFs and not to female WBFs. According to this study, male mosquitoes rely on their self-made flight tones to locate female mosquitoes through distortion products. Gibson *et al.* (2010) described on acoustic behavior of mosquitoes and revealed that both sexes of *Toxorhynchites brevipalpis* mosquitoes are capable of responding to the acoustic signals of flight tones by changing their WBF. It was observed that mosquitoes of different sexes attract each other due to acoustic treatment, and mosquitoes of the same sex were noticed to be repelled. This study demonstrated that Johnston's organs of mosquitoes are strong enough to detect the differences in tones coming from mixed acoustic tones of male and female mosquitoes. Mosquitoes were found to use distortion products as a sensory signal to communicate with the help of a signal that depends on acoustic interactions between male and female mosquito pairs. Frequency matching is also a tool for the identification of species. It was rarely observed that morphologically similar but progenitively isolated molecular forms of *Anopheles gambiae* hybridize but flutter in the same flock. This study also suggested that acoustic behavior is related to assortative mating, as the acoustic frequency was proven to be similar between both sexes of the same molecular form.

Pantoja-Sánchez *et al*. (2019) conducted studies on acoustic interactions in malarial vector mosquitoes before copulation to determine the acoustic features of *Anopheles* *albimanus*. The authors detected the characteristics of the flight tone of both sexes, acoustic features before the copulatory activities under both free and tethered circumstances, and acoustic interactions between free-flying males. This study reported a statistically significant increase in WBFs of free-flying males and females compared to the tethered mosquitoes. Harmonic convergences were observed between 79% of the paired and tethered mosquitoes. The authors demonstrated major differences in acoustic features between tethered and free-flying forms. This study demonstrated a distinguished pattern of flight along with unique acoustic and visual features after being rejected for copulation. This study suggested the efficiency of acoustic signal transduction for swarming activities. Pantoja-Sánchez *et al*. (2019) conducted experiments on acoustic traps designed for *Aedes aegypti.* Acoustic stimulus produced through the previously recorded sounds of wingbeat signals adopted by *Aedes aegypti* and pure flight tones were determined as attractants to the male mosquitoes in several sentinel sites, including semi-field circumstances and indoors. This study reported statistically significant differences between acoustic traps installed in two different types of sentinel sites. Acoustic traps emitting pure tones were observed to capture more or less 69% of male mosquitoes two hours after the onset of traps, while more or less 78% of male mosquitoes were collected through generating a wingbeat signal. Less than 1.7% of released male mosquitoes were noted to be trapped in semi-field circumstances. Traps failed to improve the capture rate under the semi-field circumstances upon intensifying the signal up to 90 decibels. Feugère *et al*. (2021) assessed an acoustic behavioral study on the African malarial vector *Anopheles coluzzii*. Free-flying and swarming females of *Anopheles coluzzii* were subjected to the prerecorded sounds of male *Anopheles coluzzii and Anopheles gambiae*. This study demonstrated no significant responses of females to the swarm sound level up to 48 decibels SPL. This study concluded that inter-mosquito acoustic communication is limited to the pair interactions in the vicinity. This study also revealed higher sound sensitivity of free-flying male mosquitoes than that of tethered mosquitoes.

2.1.7. Communication and mating in other vector insects

Two different acoustic songs called primary and secondary songs are generated by male *Lutzomyia longipalpis* sandflies during mating. The primary song is emitted in each case of Brazilian specimens of said sand fly and demonstrates different acoustic features, while the secondary song is not mandatory and does not possess any dissimilarity in acoustic characteristics among all populations. *Lutzomyia longipalpis* of Brazilian populations possesses primary songs of three different types: pulse, burst, and mix type. Pulse-type shows variable acoustic parameters in different cryptic species of *Lu. longipalpis* (Araki *et al*., 2020; Souza *et al*., 2004). Male forms of *Lutzomyia cruzi* and *Lutzomyia migonei* sandflies use mating calls for copulation (Vigoder *et al*., 2011), while pre-copulatory song was found to be generated by *Lutzomyia intermedia. Lu. Intermedia* demonstrates a different pre-copulation call than other sand fly species. Three Brazilian populations of *Lu. Intermedia* could not demonstrate mating success despite having similar acoustic parameters. Females of triatomine bugs reject other males after successful mating through stridulatory acoustic signals (Vigoder *et al*., 2013). A vector of sleeping sickness, *Glossina fuscipes fuscipes* tsetse fly, demonstrates prolonged mating song from the pre-copulatory period up to the start of the ejaculatory period (Rudrauf, 1977). Males of another tsetse fly species, *G. m. morsitans,* generate calls after the start of copulation, and there is a chance of second copulation if both male and female produce mating signals. The second copulation will not be successful if females produce different signals for mating rejection.

2.2. Molecular identification of mosquitoes

Hernández-Triana *et al*. (2021) developed an integrated DNA analysis tool to detect the interaction between the host, vector, and the pathogen. Residual DNA extracts of stranded RNA were investigated. Analysis of residual DNA in the extracts of standard RNA was done by the DNA barcoding method. Next-generation sequencing and Sanger were adopted to detect the blood meal source of 291 specimens were found to obtain sequences of DNA barcodes for more than 300 bp of the COI gene, while 130 bp sequences were found to be recovered from 179 specimens. This study reported a higher level of interspecific divergence, which in turn suggested the presence of cryptic species complexes. Blood meal analysis revealed diversified blood meal sources for different mosquito species. *Aedes angustivitatus* was noted to feed on duck and chicken while humans were reported to be the blood meal source for *Psorophora albipes.* Diverse host feeding habits were observed in the case of *Culex quinquefasciatus*. Chicken, human, Turkey, and Mexican grackles were identified as the blood source of *Culex quinquefasciatus.* Genomic DNA was extracted, followed by the amplification of the 735 bp region of the COI gene of mitochondria. The COI gene was edited, and DNA barcodes were compared for identification of the species (Soni *et al*., 2018). *Aedes aegypti* was found to be the most prevalent species. This study revealed 99.77 -100 % similarity of the COI gene in the case of the sequence of the *Aedes aegypti,* while the sequence of *Aedes albopictus* demonstrated 100%

similarity of the COI gene. This study reported that the intra-species diversity within two taxa, *Aedes aegypti* and *Aedes albopictus,* was 0-2.6% and 0.5% respectively. At the nucleotide level, both dengue vectors showed sequence homogeneity of 88.2%-88.7%. Paskewitz & Collins (1990) developed a molecular method to distinguish between two closely related afrotropical malaria vectors: *Anopheles gambiae* and *Anopheles arabiensis*. DNA fragments of two target species were subjected to amplification through PCR from a small amount of unknown DNA along with PCR primers of three different types based on rDNA sequences. A universal primer of plus-strand type was used, which was derived from the 3' end of a conserved DNA sequence that codes for 28s rDNA, while minus-strand primers were subjected to be derived from intergenic spacer sequences. This study revealed the production of 1.3kb fragments using the DNA of *Anopheles gambiae* as a template. In contrast, the DNA template of *Anopheles arabiensis* generated a 0.5 kb fragment. Singh *et al*. (2018) described molecular studies of mosquito fauna in India. According to the molecular studies conducted in different parts of India and worldwide, it was found that mainly the mitochondrial genome and nuclear region were focused on for the molecular analysis. This study concluded that molecular evaluation was mostly performed on COI, ITS1, and ITS2 regions, while other molecular markers were ignored. Most of the research was conducted in southern and north-eastern parts of India, while fewer numbers of studies were noted in northern and north-western regions. Alam *et al.* (2007) conducted a molecular analysis of two sibling species of *Anopheles annularis* detected as A & B through PCR-RFLP. Specimens were captured from Assam, MP, Jharkhand, and UP. The study was conducted with domain 3 of each specimen from each of the locations. The specimens of UP demonstrated two different sequences that correspond to the two sibling species of *Anopheles annularis.* Sibling species A demonstrated uncommon restriction sites for Mval and Eco24l, while B showed Nrul and Hinfl sites. Domain 3 sequence of A & B showed a unique type of restriction site for Alw26l and Kpnl sites, respectively. Bhattacharyya *et al*. (2010) assessed molecular experiments on the *Anopheles philippinensis-nivipes* complex to differentiate between these two morphologically similar species of north-eastern India. A molecular evaluation was conducted by adopting the allele-specific method of PCR using rDNA ITS2 sequences. Investigations for malarial parasites were done using nested PCR along with the 18s rDNA of that parasite. Among 337 specimens of that species complex, 275 specimens and 62 specimens were detected as *Anopheles nivipes* and *Anopheles philippinensis,* respectively. This study revealed the presence of a malarial parasite in the thorax and head of two specimens. Batovska *et al*. (2016) conducted molecular studies on 29 mosquito species using COI gene sequences. An 840-base pair COI gene fragment was amplified with the help of primer pairs LCO1490 and R-COI650. A 648 base pair COI fragment was amplified for two dry-pinned specimens, harnessing universal primer pair LCO1490 and HCO2198. This study obtained 17 species of mosquitoes that were not barcoded previously. *Macleaya macmillani, Culex palpalis,* and *Tripteroides atripes* were previously misidentified as a single species with morphological characters and were distinguished through DNA barcoding. This study reported average conspecific and congeneric p-distance as 0.8% and 7.6%, respectively. This study also identified an egg of *Stegomyia aegypti.*

Torres-Gutierrez *et al*. (2016) investigated the use of the mitochondrial COI gene as an essential tool in the taxonomy of *Melanoconion*. A data set containing 120 COI gene sequences of *Culex* specimens collected from Brazil was harnessed to address the utility of the COI barcodes for species detection. Genetic divergences were evaluated between specimens and clustering patterns of species in the topologies acquired with Maximum Likelihood, Neighbour Joining, and Bayesian phylogenetic inferences. All specimens were previously identified with morphological characters. Most of the taxonomic identifications were confirmed through COI barcodes. This study produced COI gene sequences belonging to 48 species of *Melanoconion*. This study reported a 3% mean intraspecific genetic divergence. This study addressed higher values of all interspecific divergence than intraspecific divergence. This study is the first report of subgenus *Melanoconion,* indicating COI as a utilizable and convenient DNA barcode.

2.3. Effect of environmental condition and body size on bioacoustics

Bertram and Fitzsimmons (2011) investigated the impacts of aging on the acoustic behavior of *Gryllus veletis,* commonly known as the spring field cricket. The authors recorded the sounds of males simultaneously throughout their life span. This study revealed that the songs of male forms of spring field crickets were found to be altered with age. Younger males were found to be more acoustically active than the older ones. This study also discovered that the life span of acoustically active males was longer than that of males who produced less sound, which in turn suggested the correlation between longevity and acoustic behavior. Zhang *et al*. (2015) conducted an acoustic study to investigate the impact of humid content of wheat on the propagation of acoustic waves. The authors developed a novel detection device to evaluate the propagation constant of the acoustic wave. These propagation constants of twelve specimens of wheat were measured along with the evaluation of the range of moisture contents. This study revealed a decreasing tendency of the velocity of acoustic waves with increasing moisture, which was found to range from 1.1% to 7.8%. At the same time, the attenuation coefficient was found to rise slightly. On increasing humidity from the 7.8% - 21.2% range, the velocity of acoustic velocity was observed to increase moderately. Insects demonstrate morphometrically tiny body size and closely located ears, leading to low-intensity differences and interaural time, which in turn result in directional hearing (Römer, 2020). Villarreal *et al*. (2017) established a study on different free-flying female *Aedes aegypti* mosquitoes with different morphometric parameters collected from several geographical locations having multiple environmental conditions. They revealed that female fundamental frequency greatly relies on the environmental temperature. They analyzed that for each degree rise in temperature resulted in the gain of 8-13Hz female fundamental frequency. It was also concluded that there is no statistically significant relationship between the body size of mosquitoes and fundamental frequency. According to this study, the range of female fundamental flight tones is not influenced by tethering effects. Bennet-Clark (1999) narrated the impacts of size and scale effects on the acoustic features of insects. The author described the emission of higher acoustic frequencies in smaller insects due to having lower muscular strength and tiny acoustic sources. During the production of precise loud pulses from a prolonged contraction of muscles, frequency-multiplier methods and a power amplification mechanism were assumed to be adopted by the insects. The acoustic frequency of air-borne insects was reported to range from 1kHz to 5kHz, along with consistent pure tone properties and a definite pulse pattern. Higher acoustic frequency resulted in briefer pulses. The author suggested the probability of association of scale effects with sound-generating methods and sound reception systems. The authors explained that small insects communicate through the vibratory method with small energy. Tiny insects assess long-distance communication with a decreasing signaling property. Costello (1974) described the emission of higher acoustic frequency in smaller insects due to having lower muscular strength and tiny acoustic sources. During the production of precise loud pulses from a prolonged contraction of muscles, frequency-multiplier methods and a power amplification mechanism were assumed to be adopted by the insects. Acoustic frequencies of air-borne insects were reported to range from 1kHz to 5kHz, along with consistent pure tone properties and definite pulse-pattern. Higher acoustic frequency resulted in briefer pulses. The author suggested the probability of association of scale effects with sound-generating methods and sound reception systems. The authors explained that small insects communicate through the vibratory method with small energy. Tiny insects assess long-distance communication with decreasing signaling properties. The author investigated the effect of environmental and physiological factors on the acoustic behavior of *Aedes aegypti*. Temperature, humidity, pressure, and light were considered as environmental factors, while age, mating, oviposition, and feeding were marked as physiological factors. The effects of these factors were examined on acoustic behavior in both larval and adult forms. The acoustic behavior of larvae was found to be affected by rearing temperature in both male and female forms. An increase in rearing temperature and humidity produces mosquitoes with higher wing beat frequencies. Other environmental factors could not demonstrate a significant impact of light and pressure on acoustic features. Newly emerged mosquitoes showed higher wingbeat frequency than aged ones. Mated males showed increased wingbeat frequency and less acoustic responsiveness but could not affect the acoustic behavior of females. Blood-fed females demonstrated increased wing-beat frequencies, but a sooner decrease was noted after oviposition.

2.4. Application of bioacoustics in surveillance and control of mosquitoes and other insects:

Initially, mating disruption for the control of different pests was carried out using pheromone-based mating control strategies. With the advancements in scientific techniques, bio-acoustic technology contributed to potential control strategies preventing mating successes. Disruption of the mating behavior of insects is achieved through the interruption of substrate-mediated vibrational communication. The implication of vibrational mating disruptions was found to be fruitful in controlling the population of different pests (Eriksson *et al*., 2012; Mazzoni *et al*., 2019; Polajnar *et al*., 2016). The prerecorded female sound of species *B. cockerelli* prevents successful mating events. Most of the males of *B. cockerelli* were found to be attracted to the speaker emitting female sound instead of calling the female species in an experimental setup, resulting in lower mating success (Avosani *et al*., 2020). Disturbing noise was found to be effective for the prevention of mating success in different agricultural pests (Mazzoni *et al*., 2019). Disturbance noise was formed by artificially broadcasting small devices attached to the plant, which is capable of injecting non-specific vibrational signals into the substrate. It masked the natural mating signals by overlapping in frequency with the signal produced by the opposite sex. The continuous noise saturates the vibrational "channel," making it hard to detect the courtship signals. Similarly, the population of mosquitoes can also be reduced by preventing copulatory success upon playing disturbing noise. The song "Scary Monsters and Nice Sprites" resulted in less copulatory activities in *Aedes aegypti* mosquitoes (Dieng *et al*., 2019). The song's aggressive mix of very low and high frequencies overwhelmed the mosquito's Johnston's organ. The intense low-frequency bass also created physical vibrations. Together, this auditory and vibrational chaos created a powerful sensory distraction that interfered with the mosquito's ability to perceive its environment and detect mates.

Most of the attempts at detection of communication and mating behavior in mosquitoes were conducted using solo mosquitoes with acoustic playbacks. Ikeshoji (1981) reported the insemination of 15.3% - 100% females of *Aedes albopictus, Culex pipiens, Aedes aegypti, and Anopheles stephensi* during acoustic treatment with male wing beat sound. Males of *Aedes albopictus* and *Aedes aegypti* were found to respond better than other mosquitoes during bio-acoustic treatments. *Culex pipiens* were observed to be inseminated more quickly than others. The response of female forms of *Anopheles stephensi* and *Aedes aegypti* to their conspecific sound frequencies was statistically insignificant.

Pantoja-Sánchez *et al*. (2019) conducted studies on acoustic interactions in malarial vector mosquitoes before copulation to determine the acoustic features of *Anopheles albimanus*. The authors detected the characteristics of the flight tone of both sexes, acoustic features before the copulatory activities under both free and tethered circumstances, and acoustic interactions between free-flying males. This study reported a statistically significant increase in WBFs of free-flying males and females compared to the tethered mosquitoes. Harmonic convergences were observed between 79% of the paired and tethered mosquitoes. Authors demonstrated major differences in acoustic features in tethered than that of free-flying forms. This study demonstrated a distinguished pattern of flight along with unique acoustic and visual features after being rejected for copulation. This study suggested the efficiency of acoustic signal transduction for swarming activities.

Lapshin & Vorontsov (2018) concentrated the swarming mosquitoes of the *Aedes diataeus* species with sound having conspecific female frequencies. After the onset of the sound signal, most of the mosquitoes were found to leave the adjacent area of the sound source within one second. Results demonstrated that the sounds having frequencies ranging from 140Hz to 200Hz were capable of repelling male mosquitoes of *Aedes diataeus.*

Pantoja-Sánchez *et al*. (2019) conducted experiments on acoustic traps designed for *Aedes aegypti*. Acoustic stimulus produced through the previously recorded sounds of wingbeat signals adopted by *Aedes aegypti* and pure flight tones were determined as attractants to the male mosquitoes in several sentinel sites, including semi-field circumstances and indoors. This study reported statistically significant differences between acoustic traps installed in two different types of sentinel sites. Acoustic traps emitting pure tones were observed to capture more or less 69% of male mosquitoes two hours after the onset of traps, while more or less 78% of male mosquitoes were collected through generating a wingbeat signal. Less than 1.7% of released male mosquitoes were noted to be trapped in semi-field circumstances. Traps failed to improve the capture rate under the semi-field circumstances upon intensifying the signal up to 90 decibels.

Staunton *et al*. (2021) assessed male *Aedes* sound traps tuned to either 550Hz, 500Hz, 450Hz, or 600Hz for capturing *Aedes aegypti* and *Aedes albopictu*s, followed by the comparison of those frequency-dependent sound traps with BG sentinel traps in Pacific and Latin American regions. Traps set to 450-500Hz demonstrated the highest number of male *Aedes aegypti*, while traps emitting 550Hz showed a great number of both *Aedes* males collection which is higher than the collection of the BG sentinel trap. In Molas of Mexico, the abundance of *Culex quiquefasciatus* and *Culex restanus* was great in traps tuned to 450-500Hz. In all cases, traps set to 600Hz showed a lower abundance of mosquitoes than any other traps.

Johnson and Ritchie (2016) conducted acoustic studies on *Aedes aegypti* mosquitoes, aiming at male collection for the sterile insect technique along with modification of the population agenda. A passive collection method was adopted using a gravid *Aedes* trap. In those traps, three types of frequencies were set up using two female tones of 484 Hz and 560 Hz, along with one male tone of 715 Hz. For this experiment, three sound devices were designed to generate tones at 484 Hz, 560 Hz, and 715 Hz. One control device was also set up using no audio. The highest male density was found in the case of a trap tuned to 484Hz. The collection rate of male mosquitoes was moderate in the case of the device designed with 560 Hz, while the rest showed a very low collection rate of male mosquitoes. This study suggested the effectiveness of a sound-baited gravid *Aedes* trap in collecting male mosquitoes.

Feugère *et al*. (2021) assessed an acoustic behavioral study on the African malarial vector *Anopheles coluzzii.* Free-flying and swarming females of *Anopheles coluzzii* were subjected to the pre-recorded sounds of male *Anopheles coluzzii* and *Anopheles gambiae.* This study demonstrated no significant responses of females to the swarm sound level up to 48 decibels SPL. This study concluded that inter-mosquito acoustic communication is limited to the pair interactions in the vicinity. The study also revealed higher sound sensitivity of free-flying male mosquitoes than the tethered mosquitoes, i.e., immobilized by gluing a tiny pin or a thin wire to the thorax.

Leemingswat *et al*. (1988) conducted acoustic experiments on a laboratory-reared colony of *Culex tritaeniorhynchus* mosquitoes. Wingbeat frequency of male and female mosquitoes was evaluated, followed by acoustic treatments on mosquitoes. A sound-emitting speaker, connected to a tape recorder capable of playing different sounds with variable frequencies, was positioned in a caged hamster with a covering of a polyvinyl bag having adhesive sprayed on the outer surface to capture mosquitoes. Sounds having 500-800Hz frequencies captured a high number of female mosquitoes. The highest density of females was found at a sound frequency of 600 Hz. In contrast, traps emitting 300-400Hz frequencies demonstrated a very low density of female mosquitoes. This result suggested the repulsion of female mosquitoes while subjected to sounds of female wingbeat frequencies.

Kerdpibule *et al*. (1989) assessed an acoustic experiment on male *Culex tritaeniorhynchus* mosquitoes through a sound trap to control the density of these mosquitoes. Wingbeat frequencies of female *Culex tritaeniorhynchus* mosquitoes were used for trapping the males. A black cloth was placed inside the trap to induce swarming activities. An improved version of this trap was designed with dry ice and a hamster as attractants for males. A trap having a cylindrical structure emitting 350 Hz sound frequencies was developed for the male *Culex tritaeniorhynchus* collection, while another trap having a cage-like structure was designed with the emission of sound with 530 Hz frequencies to capture the females. Both of the traps were installed every alternate day after the setting of the sun for three weeks in a rice farming field. No decrease in the population densities of mosquitoes was evident. Balestrino *et al*. (2016) investigated the responses of *Aedes albopictus* to acoustic and visual stimuli through acoustic traps. The authors analyzed the responses of male mosquitoes to different acoustic stimuli generated with separate frequencies and volumes, along with optical cues for optimizing male captures. The generation of modified frequencies, along with simultaneously changing features, but having a typical range of acoustic emission of females, demonstrated acoustic pressure within 75 decibels and 79 decibels. This study revealed the black color of the acoustic trap as the attracting factor for males staying in the vicinity of a sound source. The authors observed the increase in males' response to auditory stimulation at four days of age and then demonstrated a sharp decline in acoustic responses. A plastic-made acoustic trap was found to be capable of generating the most impacted stimuli while tested in laboratory circumstances.

A new approach was proposed to overcome the limitations of existing sensor-based mosquito detection systems, which suffered from issues with noisy environments, a lack of adaptability, and data scarcity (Seervi *et al*., 2025). This study developed an adaptable pipeline to create environment-specific deep-learning models for mosquito detection. The research addressed the challenge of data scarcity and evaluated various feature extraction strategies, including log-mel and per-channel energy normalization (PCEN), to improve model robustness. The proposed solution successfully generated models with accuracy exceeding 90% for any given environment. Experimental results, which involved testing CNN and TCN models in different settings, confirmed the effectiveness of this approach. PCEN preprocessing was found to outperform log-mel, with the CNN model achieving the highest accuracy of 93.25% in an open environment.

Cross-testing results further validated the use of environment-specific models. The findings demonstrated improved adaptability, which could support public health efforts to control vector-borne diseases.

2.5. Involving AI in acoustic biology for insect detection

The developing field of artificial intelligence represents the indices-based detection of acoustic characteristics and dynamics of aural cues, along with focusing on the ecological aspects of soundscapes (Sánchez-Giraldo *et al.*, 2021). The role of eco-acoustic technology extends from species-wise detection of the animals (Aide *et al.*, 2013) to estimating the ecological properties of soundscapes (Pijanowski *et al.*, 2011). In these intricate acoustic circumstances, animals experience challenges due to the limitation of the acoustic space. They split the soundscapes using the time duration and frequency to generate an audible signal. This type of partitioning enables them to communicate efficiently in a bustling acoustic environment (Erbe *et al.*, 2018; Marín-Gómez *et al.*, 2020). Sound recordings can efficiently represent the diversity, density, and other conditions of an ecosystem using acoustic parameters (Gasc *et al.*, 2013). Harnessing advanced technology in recording, storing, and data processing acoustic monitoring has become an easily accessible and low-cost tool (Gibb *et al.*, 2019) compared to conventional monitoring techniques. Eco-acoustic monitoring based on Artificial Intelligence revealed the successful detection of animals along with their density and differential behaviors through some eco-acoustic indices in a semi-supervised way. While mosquito detection and monitoring through AI are lagging, very few efforts are being made to decode mosquito fauna with their density and behavioral aspects using machine learning codes. Several R packages contain tools designed for efficient management of acoustic-based monitoring programs. These encompass the surveillance and management of animal survey data, the generation and manipulation of different templates, automation in detection, and the management of results. They are developed to be measurable, accommodating multi-directional approaches in long and short-term projects covering extensive and spatial aspects. Specialized workflow accompanied by the utilization of these tools within different packages for sound analysis also consists of sequential functions through binary point matching and using spectrum-based detectors. Acoustic signals are specific to species and show low intraspecific differences while exhibiting high interspecific variation. This characteristic enables them to be suitable for the detection of inconsistencies in taxonomic identifications in different species (Köhler *et al.*, 2017). The biological component of soundscapes captured at specific regions and times can be anticipated to measure diversity in animals along with the facilitation of the development of acoustic-mediated detection strategies (Farina *et al.*, 2021). Moreover, socio-ecological interactions among the same and different species can be managed using aural signals (Laurenceau-Cornec *et al.*, 2015; Magrath *et al.*, 2015). Acoustic monitoring strategies are not only capable of species confirmation but also provide meaningful insights into the socio-economic and ecological conditions and ecological dynamics (Laiolo, 2010). The measurement of the acoustic features of mosquitoes indicated the technological feasibility of constructing an acoustic-based control strategy to detect individual mosquitoes and their populations. Previous studies predicted the mosquito species using fundamental wingbeat frequency as a metric and misidentified the mosquitoes as 26 out of 29 species demonstrated overlapped frequency (Kim *et al.*, 2021). Mankin *et al.* (2021) explained the acoustic detection, investigation, and management of insects. Different types of acoustic devices were noted to be successful in the detection of insects from stored products, soils, and trees. Rice weevils were reported to be detected in the grains through an acoustic device. Some of the devices involved the processing of digital signals along with statistical analysis using machine learning and neural networks to differentiate the targeted pests from other individual organisms and background noise.

Bist *et al*. (2021) proposed a deep learning mechanism for the investigation of mosquitoes. The authors integrated hardware along with software technologies. They demonstrated the possibilities for improving the pipelined process with the help of the latest designed technique using a gated neural network. Their designed architecture included input as audio files, whose features will be extracted and analyzed through a deep learning model along with NVIDIA Nano Jetson and a flying machine to integrate the designed architecture.

CHAPTER 3: HYPOTHESIS

HYPOTHESIS:

Morphologically based detection of mosquitoes is not fruitful because of having closely related morphological features. Different species of mosquitoes were identified as the same species, while molecular detection revealed the differences in the DNA sequences and detected them as separate. **These lead to molecular detection as an efficient method for species identification.** Mosquitoes generate species and sex-specific aural cues for different functions. Studies reported the efficiency of acoustic signatures of mosquitoes in species detection, as the range of frequency distribution of wingbeats of mosquitoes varies from species to species. **Mosquitoes of different species can be isolated using acoustic features.** Mosquitoes under different physiological and environmental conditions may show differences in acoustic features; hence, the **effects of physiological and environmental conditions on acoustics are required to be monitored.** In response to contiguous acoustic signals antenna vibrates, which helps to measure the highly amplified intermodulation distortion products along with the electrical responses of Johnston's Organ (JO). JO enables mosquitoes to navigate other mosquitoes using sound frequencies. **These features facilitate the chances for acoustic-based capture and control of mosquitoes.**

CHAPTER 4: OBJECTIVES

OBJECTIVES:

1. Identification of different species of mosquitoes through acoustic signals.
2. Confirmation of mosquito species using molecular tools.
3. Monitoring the effects of morphometric parameters on the bioacoustics of mosquitoes.
4. Evaluation of the climatic influences on bioacoustics of mosquitoes.
5. Development of data metrics for the surveillance of mosquitoes using acoustic signals.

CHAPTER 5: MATERIALS AND METHODS

5.1. General materials and methods

5.1.1. Mosquito collection, rearing, and species identification:

The field surveys were conducted to collect 19 species of mosquitoes (Table 5.1) from various regions in different Indian states. During these surveys, mosquitoes of the genera *Anopheles, Aedes, Armigeres, Culex,* and *Mansonia* were captured using different traps (CDC-LT trap and BG-Sentinel trap), and larvae were collected from various water bodies using ladles. For *Aedes, eggs* were collected by harnessing ovitraps and reared to the adult form under 26 ± 1 °C, 12:12 (L:D), and 65 ± 5% relative humidity following the method of (Sharma *et al*., 2022; De *et al*., 2022). The adult mosquitoes were fed a 10% sucrose solution in cotton located on aluminum foil *ad libitum*. These adult mosquitoes were confirmed using morphological taxonomic keys (Reuben *et al*., 1994; Tyagi *et al*., 2015; Christophers, 1933).

Table 5.1. Mosquito species under study

Serial no.	Mosquito species
1	*Anopheles subpictus*
2	*Aedes aegypti*
3	*Aedes albopictus*
4	*Anopheles stephensi*
5	*Mansonia annulifera*
6	*Culex quinquefasciatus*
7	*Culex gelidus*
8	*Armigeres subalbatus*
9	*Culex tritaeniorhynchus*
10	*Aedes vittatus*
11	*Anopheles culiciformis*
12	*Anopheles elegans*
13	*Mansonia uniformis*
14	*Anopheles sinensis*
15	*Culex mimuloides*
16	*Culex alienus*
17	*Anopheles roperi*
18	*Anopheles umbrosus*
19	*Hulecoetomyia fluviatilis*

5.1.2. Sound recording device and procedure for recording mosquito sound tones:

The sound-capturing instrument developed by Suman (2021) (Automated surveillance system for mosquito and other insects. Patent Application (India): 202031011565A, March 2021) was used. It comprises two components: one sound-capturing chamber and one data collection apparatus. The sound-capturing chamber is manufactured by transforming the conical-shaped bottom part of a 15 ml (100 mm in length, 29 mm in diameter) and another 50 ml (100 mm in length, 16 mm in diameter) centrifuge tubes made up of polypropylene plastic. Caps of these two centrifuge tubes adhered with the help of hot glue, followed by punching to create a hole. A plug of cotton was also accommodated on the opening of the 15ml tube to prevent the exit of mosquitoes and allowance of acoustic aeration and transmission. A collar microphone of omnidirectional type was then fixed to the cap of the tube, secured with hot glue. This microphone is placed 2 mm below a mesh and attached to an acoustic capturing device as a mobile phone to capture the sound in WAV format, along with 16-bit resolution and 44100 Hz sampling rate (Samsung Voice Recorder Android Application, version 5). Settings on the voice recorder are configured to acquire audio files in WAV format, employing 16-bit resolution at a sampling rate of 44.1 kHz. An individual male or female mosquito was transferred to the recording chamber of the device for the capture of sound. After an acclimatization time of 2 to 3 minutes, the sound of the mosquito was recorded under standard laboratory conditions (26 ± 1 °C temperature, 12:12 light and dark cycle, & 65 ± 5% RH or relative humidity) using the voice recorder of the mobile device. 5-10 sound files can be generated from each mosquito, with a time duration of 5-20 seconds in each file.

Harnessing a 50 ml centrifuge tube as a recording chamber of the recording device by Suman (2021), the sounds of multiple mosquitoes can be recorded for sex-based, convergence-based, and ecological studies. Sound files were stored using specific code for each for subsequent acoustic analysis.

5.1.3. Mosquito sound characterization and analysis:

Using the Audacity ver. Noise reduction was performed for each audio file generated for individual or grouped mosquitoes at -12 dB. 2.4.2 package (http://www.audacityteam.org). Audacity is a free, open-source, and cross-platform software package. We did not adjust the amplitude or frequency modulation during the noise reduction process in Audacity. The audio files recorded from individual mosquitoes of 19 species, including females and males, to characterize the acoustic signatures, were used to generate spectrograms with amplitude and frequency in the time domain with the Raven Pro 2.0. (Bioacoustics Research Program, Laboratory of Ornithology, Cornell University, Ithaca, NY). Using a similar method, the audio files of the grouped mosquitoes were processed. The baseband's fundamental frequency (*F0*), higher harmonics, and their amplitude (*dB*) were visualized and estimated. For the estimation of the frequency of each band, an average of the upper and lower limits of frequencies was considered. These parameters were used for a variety of purposes, including identifying species, sex, density, and combinations of sex or species.

5.2. MATERIALS AND METHODS FOR OBJECTIVE 1: IDENTIFICATION OF DIFFERENT SPECIES OF MOSQUITOES THROUGH ACOUSTIC SIGNALS.

5.2.1. Species differentiation using different parameters of sound:

The identification of mosquito species was conducted based on *F0*, the width of the *F0* band, *dB,* and harmonic bands. We also developed suitable matrices using these attributes as ratios and multiplication factors to differentiate a maximum number of species.

5.2.1.1. Species identification using a single parameter matrix:

5.2.1.1.1. The fundamental frequency (F0):

The *F0* was estimated as an average of higher and lower frequencies of the baseband, which represent the baseband sound wave oscillations. Using the Raven Pro package's spectrogram, these frequencies for each species were manually measured following the methods of Aflitto and Hofstetter (2014). A robust data set for fundamental frequency was generated by drawing a total of 305 samples from 19 species of female mosquitoes.

5.2.1.1.2. The dispersion of baseband frequency (bandwidth):

The base bandwidth dispersion represents the area between lower and higher frequencies. It was estimated for each sample (n = 305) of each species, taking into account a unique feature of the species. The data set was subjected to a One-way ANOVA to assess differences among species.

5.2.1.1.3. Harmonic band diversity:

The frequency spectrogram of mosquito sounds was manually assessed to see if other higher frequency bands were present relative to the fundamental frequency band. The number of harmonic frequency bands, visually distinguishable from the background noise, for all species and their replicates was counted. This characteristic is considered by observing its consistency and pattern in preliminary studies.

5.2.1.1.4. Amplitude (dB) of the fundamental frequency band:

The amplitude of the fundamental frequency band (*dB*) on a spectrogram of a mosquito sound file was manually estimated by covering the higher and lower edges of the frequency band. For all species and their replicates, the *fdB* was estimated following the same method.

5.2.1.1.5. Acoustic entropy (H):

The acoustic entropy was evaluated for species showing an overlapped fundamental frequency.

5.2.1.2. Using a matrix to identify species based on the combination of multiple sound parameters:

- **The combination of fundamental frequency (F0) and the total number of harmonic bands (hbn):**

These features contributed significantly to the sound and were used in combination to generate new matrices to find an alternative method with better resolution for identification.

The ratio matrix for fundamental frequency and harmonic bands:

Fundamental frequency (F0) of the species / total number of harmonic bands (bn) in the species

The multiplication matrix for fundamental frequency and harmonic bands:

Fundamental frequency (F0) of the species x total number of harmonic bands (hbn) in the species

In this estimation, a total of 305 sound files of 19 species were analyzed to generate data on *F0* and *hb*.

- **Combination of amplitude of top harmonic band (hbdB) and the total number of harmonic bands (hbn):**

The top harmonic band amplitude represents the lowest energy level visible in the highest frequency band of the species, as it covers the entire frequency spectrum of the individual species. In the current study, the matrices were estimated as a ratio using *hbdB* and *hbn*, then the results were multiplied to identify the species:

The ratio matrix for the amplitude of the top harmonic band and the total of harmonic bands:

Amplitude of the top harmonic band (hbdB) of the species / total number of harmonic bands (hbn) in the species

The multiplication matrix for the amplitude of the top harmonic band and the total of harmonic bands is:

Amplitude of the top harmonic band (hbdB) of the species x, total number of harmonic bands (hbn) in the species

5.2.2. Differentiation of male-female and the impact of abdominal fed vs unfed condition on fundamental frequency

The *F0* parameter was used to distinguish between males and females of the same species, as well as between fed and unfed (starved) females. For the fed-unfed experiment, 10 ± 2 days old, 10 fed mosquitoes from every species were taken. The starved females were prepared by not feeding them for 24 hrs. For sex detection, 134 samples from nine species of male mosquitoes were drawn to generate a robust data set for fundamental frequency.

5.2.3. Harmonic convergence in the presence of both sexes

A pair of 3–4-day-old male and female *Ae. vittatus* was placed in a large sound recording chamber to study the convergence in harmonic band frequency. The audio was recorded as described above and visualized in the Raven Pro software package to generate harmonic band profiles for both male and female mosquitoes (n = 26 sound samples from 10 pairs). The harmonic band profiles of both sexes were analyzed to determine the convergence of harmonic band frequencies.

5.2.4. Statistical analysis

Data from different replicates of each species were pooled to conduct the descriptive analysis and to assess the normality of the distribution using Skewness and Kurtosis. Data on male and female *F0,* dispersion of baseband, combination of *F0* and *hb,* and amplitudes, *i.e.,* ratio and multiplication matrix, were subjected to One-way analysis of variance (ANOVA) to compare and differentiate the species, sex (male and females), abdominal condition (fed and unfed), and mixed individual of different species using multiple range test with least significant difference (LSD value) at p <0.05. In One-way ANOVA, *df*, *f*-ratio, and *p-value* were also estimated. The success rate of each parameter for species identification was estimated as a percentage of species pairs that showed significant differences in multiple-range tests of One-Way ANOVA using the following equation:

% success rate = (No. of species pairs with significant difference / total pairs used in multiple range test) x 100

All the '±' values refer to standard deviation.

All statistical analyses were performed using the STATGRAPHICS Plus 5.0 (Stat-Graphics Technologies, Inc., Virginia, U.S.A.) software package.

5.3. MATERIALS AND METHODS FOR Objective.2: Confirmation of mosquito species using molecular tools.

5.3.1.DNA isolation: A leg was removed from each frozen mosquito for DNA isolation. At first, working buffer solutions were prepared for DNA isolation.

Buffer B3: Buffer B3 has been made from chaotropic salt and detergents.

Wash Buffer B5: Wash Buffer B5 was made by adding 24ml of 96–100 % ethanol to the 6ml Buffer B5 Concentrate. It was stored at room temperature, 18–25 °C.

Proteinase K solution: For preparing Proteinase K solution, 260 µl Proteinase Buffer PB was dissolved in 6mg lyophilized Proteinase K.

Preparation of sample: Each leg of the mosquito was placed into a 1.5 ml microcentrifuge tube.

Lysis of sample: 100 µl Buffer T1 and 10 µl Proteinase K solution have been added and mixed through vortexing. Incubation was done at 56 °C for 1-4 hours. After that Thermal heating block was set to 70 °C for the next process. 80 µl Buffer B3 has been added to the solution and has been incubated at 70 °C for 5 minutes. The lysate has been allowed to cool down to ambient temperature.

DNA binding: 80 µl 96–100 % ethanol has been added to the lysate and mixed with the help of a vortex.

One NucleoSpin® Tissue XS column has been placed into a 2ml collection Tube for individual samples. A sample was applied to the column. Samples were subjected to be centrifuged for one minute at 11,000 x g. The flow-through has been discarded, and the column has been placed into a new sterilized collection tube.

Washing: At first, 50 µL Buffer B5 has been added to the NucleoSpin® Tissue XS column. Centrifugation has been done for 1 minute at 11,000 x g. Again, the addition of 50 µL Buffer B5 to the NucleoSpin® Tissue XS column was done, followed by centrifugation for 2 minutes at 11,000 x g. Collection tubes have been discarded with flow-through.

Elution of DNA: NucleoSpin® Tissue XS column has been placed in a new 1.5 ml microcentrifuge tube, and 20 µL Buffer BE has been added directly to the middle of the silica membrane of the column. Centrifugation was done for 1 minute at 11,000 x g.

Removal of residual ethanol: Residual ethanol was removed by the incubation of the elution fraction without closing the lid for 8 minutes at 90 °C.

5.3.2. PCR amplification**:** Cytochrome C Oxidase Subunit 1 appears to be the most conserved protein-coding gene in the mitochondrial genome of animals (Brown,1985). No other genetic region can be found in taxonomically verified databases with sequences covering so many taxa. After a literature search, found following primers were found to be effective in the amplification of the COI gene in the DNA barcoding of different mosquito species (Suman et al., 2022):

F-LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3')

R-COI650 (5'-TAG CAG AAG TAA AAT AAG CTC G- 3')

The amplification with the COI gene was performed using an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, 45°C for 45 s, and 72°C for 45 s, and final extension at 72°C for 5min.

5.3.3. Agarose gel electrophoresis and sequencing: The PCR product was confirmed for bands in a 1.5% agarose gel.

Reagents:

1X TAE buffer: To make 1X TAE buffer, first, a 1-liter 50X stock of TAE was prepared by using 242 g Tris-base, 57.1 mL 100% acetic acid, 100 mL of 0.5 M sodium EDTA, and adding dH2O up to 1 liter. To make 1x TAE from 50X TAE stock, the stock was diluted to 20 in 980 ml of DI water. 1.5g agarose powder was dissolved in 100ml 1X TAE buffer, followed by keeping in the microwave for 1min and then resting for 15min. After cooling down, 2µl ETBR (ethidium bromide). The gel tray was blocked at two sides by transparent adhesive tapes, and of mixture in the gel tray with the two well combs in place and rested for 30 minutes. Once solidified, agarose gel was placed into the gel box (electrophoresis unit) and the gel box was filled with 1xTAE until the gel was completely covered. 2µl DNA ladder and 1µl loading dye were loaded in the first well of each comb, 2µl sample DNA + 1µl loading dye was loaded in the other wells. Electrophoresis runs for 1.5 hours at 80-150 V until the movement of dye lines.

Sanger sequencing was conducted, and after getting the results, the sequences were aligned using Bioedit and submitted to GeneBank. Thereafter, the sequences were compared with other mosquito species sequences obtained from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and also further analyzed for the phylogenetic tree generation, Nucleotide composition using Bioedit and Mega X software. The analyzed sequences have been submitted to GenBank. The details of Accession numbers and nucleotide composition have been described below (Table 9). Phylogenetic trees for COI were constructed using the Maximum Likelihood method and the Kimura 2-parameter model.

5.4. MATERIALS AND METHODS FOR Objective 3. Monitoring the effects of morphometric parameters on the bioacoustics of mosquitoes.

5.4.1. Mosquito rearing in different densities:

Aedes aegypti and *Aedes albopictus* were considered for this study. The differences in adult size were obtained by rearing mosquito larvae in different densities, adopting procedures described by Ochola *et al*. (2022) and De *et al*. (2022), with slight modifications. Rearing of larvae was performed in water pans of 200 ml. 20, 50, and 100 first instar larvae were placed in different water pans of the same volume, presenting 1 larvae/ml density in the water pan having 20 larvae, while 2.5 larvae/ml and 5 larvae/ml in water pans having 50 and 100 larvae, respectively. Temperature 26 ± 1 °C and 65 ± 5% relative humidity were maintained throughout the experiment.

5.4.2. Morphological identification and sound recording:

After the emergence of adults from different pans, female mosquitoes were separated and transferred into separate cages, followed by the demarcation of rearing densities and sizes. Sounds of mosquitoes were recorded following the methodology described in the general methodology part.

5.4.3. Evaluation of morphometrics:

Wing size was considered for analyzing the effect of morphometrics on bioacoustics. Wing size was evaluated by ImageJ 1.54 g (Wayne Rasband and contributors, National Institutes of Health, USA) following the methodology narrated by Rueden *et al*. (2017). ImageJ is a free, open-source image processing program that can display, edit, analyze, process, save, and print various image types.

5.4.4. Statistical analysis:

Data from different replicates of each species were pooled to conduct the descriptive analysis and to assess the normality of the distribution using Skewness and Kurtosis. Data on wing size and fundamental frequency of female *Aedes aegypti* and *Aedes albopictus* were subjected to One-way analysis of variance (ANOVA) to compare the fundamental frequencies of mosquitoes using multiple range tests with the least significant difference (LSD value) at $p < 0.05$.

5.5. MATERIALS AND METHODS FOR Objective.4: Evaluation of the climatic influences on the bioacoustics of mosquitoes.

5.5.1. Mosquito rearing in different temperatures and humidity

Aedes aegypti and *Aedes albopictus* mosquitoes were reared following the methodology described by Costello (1974). 1st instar larvae of *Aedes aegypti* and *Aedes albopictus* were reared at different temperatures (24℃, 28℃, and 31℃) and different humidity (65%, 70%, and 75%), having 1.5 larvae/ml in each.

5.5.2. Morphological identification and sound recording

After the emergence of adults from different pans, female mosquitoes were separated and transferred into separate cages, followed by demarcation of rearing temperature and humidity. Sounds of mosquitoes were recorded following the methodology described in the general methodology part.

5.5.3. Statistical analysis

Data from different replicates of each species were pooled to conduct the descriptive analysis and to assess the normality of the distribution using Skewness and Kurtosis. Data on temperature, humidity, and fundamental frequency of female *Aedes aegypti* and *Aedes albopictus* were subjected to One-way analysis of variance (ANOVA) to know if there are any differences in fundamental frequencies of mosquitoes using multiple range tests with the least significant difference (LSD value) at $p < 0.05$.

5.6. MATERIALS AND METHODS FOR Objective.5: Development of data metrics for the surveillance of mosquitoes using acoustic signals.

5.6.1. Acoustics-based identification

Eleven acoustic indices of tunes of *Aedes aegypti, Aedes albopictus,* and *Aedes vittatus* were evaluated, comprising 9 acoustic parameters: low and high frequency of fundamental frequency, delta time, delta frequency peak frequency, number of peaks, median amplitude envelope, frequency and amplitude modulation, and 2 eco-acoustic indices using R (Ver 4.2.2; R Core Team, 2022) using the see wave (Ver 2.2.0; Sueur *et al*., 2008) and sound ecology (Ver 1.3.3; Villanueva Rivera and Pijanowski, 2018) packages. The mean fundamental frequency was evaluated at low and high frequencies. For each 10-second recording, two eco-acoustic indices were calculated: the bioacoustic index (Boelman *et al*., 2007) and the acoustic entropy (Sueur *et al*., 2008) for the classification of three mosquito species.

5.6.2. Comparing mosquito density based on acoustics

To investigate the change in eco-acoustic indices, multiple free-flying *Aedes vittatus* mosquitoes were transferred into the sound recording device for the recording of the sound of multiple mosquitoes together. Standard laboratory conditions for mosquitoes were maintained at 26 ±2ºC temperature and 65±5 relative humidity.

5.6.3. Monitoring the effect of acoustic treatment on mosquito movements

To know the effect of different frequencies on male and female mosquitoes were subjected to prerecorded sounds of *Aedes aegypti* (458.95 Hz to 467.75 Hz) and *Aedes albopictus* (488.70 Hz to 530.25 Hz).

5.6.4. Statistical analysis

Fundamental frequencies from low and high frequency, peak frequency, delta frequency, delta time, bioacoustic index, and acoustic entropy of each flight tone of individual species were subjected to a one-way ANOVA for statistically significant differences at $p < 0.05$, followed by comparison of eco-acoustic indices between individual and multiple mosquitoes in the same way, along with two-tailed Spearman's rank correlations to test relationships between mosquito counts and eco-acoustic indices. The Spearman rank correlation test was also conducted between sound frequency and the activity of mosquitoes to determine if there was any significant correlation. All statistical analyses were assessed using the STATGRAPHICS Plus 5.0 (Stat-Graphics Technologies, Inc., Virginia, U.S.A.) software package. All the '±' values refer to standard deviation.

5.6.5. Database generation for surveillance

A database on acoustic indices of mosquitoes was generated through machine learning codes in R and incorporated with other acoustic parameters evaluated in this study for automated surveillance of mosquitoes.

CHAPTER 6. RESULTS AND DISCUSSION

6.1. OBJECTIVE 1. Identification of different species of mosquitoes through acoustic signals.

6.1.1. Fundamental Frequency:

The fundamental frequencies of 19 mosquito species females ranged between 269.09 ± 2.96 Hz to 567.51 ± 3.82 Hz, being the lowest in *Anopheles culiciformis* and the highest in *Aedes vittatus* followed by *Culex alienus* (539.44 ±0.629 Hz), *Aedes albopictus* (510.90 ± 11.76 Hz), *Hulecoetomyia fluviatilis* (473.16 ± 1.60 Hz), *Aedes aegypti* (463.07 ± 3.22 Hz), *Anopheles elegans* (461.872 ± 3.31 Hz), *Culex tritaeniorhynchus* (449.48 ± 13.49 Hz), *Anopheles subpictus* (445.46 ± 2.01 Hz), *Culex gelidus* (426.56 ± 10.45 Hz), *Anopheles roperi* (410.62 ± 1.75 Hz), *Culex quinquefasciatus* (408.90 ± 17.74 Hz), *Armigeres subalbatus* (399.6 ± 16.74 Hz), *Mansonia annulifera* (397.65 ± 5.78 Hz), *Anopheles umbrosus* (390 ± 0.63 Hz), *Anopheles sinensis* (371.75 ± 4.07 Hz), *Mansonia uniformis* (356.89 ± 15.18 Hz) and *Culex mimuloides* (308.485 ± 1.87 Hz) (ANOVA: F ratio= 757.89, df= 304, P=0.00001). Overall, the average fundamental frequency produced 95.32% success in differentiating 19 species in multiple comparison tests (171 combinations), i.e., about 95.32% of all the 171 possible pairwise comparisons, the average fundamental frequency compared was statistically different. For approximately 163 among 171 pairs (171×0.9532≈163), the fundamental frequency was a reliable way for pairwise comparison. The statistical analysis of fundamental frequencies of these species shows significant differences among them except for the following pairs of mosquitoes: *Aedes aegypti - Anopheles subpictus; Anopheles elegans - Anopheles stephensi; Anopheles roperi - Culex quinquefasciatus; Anopheles subpictus*

- *Culex tritaeniorhynchus; Anopheles umbrosus - Mansonia anulifera; Armigeres subalbatus - Mansonia anulifera* (F ratio= 757.89, df= 304, P=0.0001) (Fig.6.1).

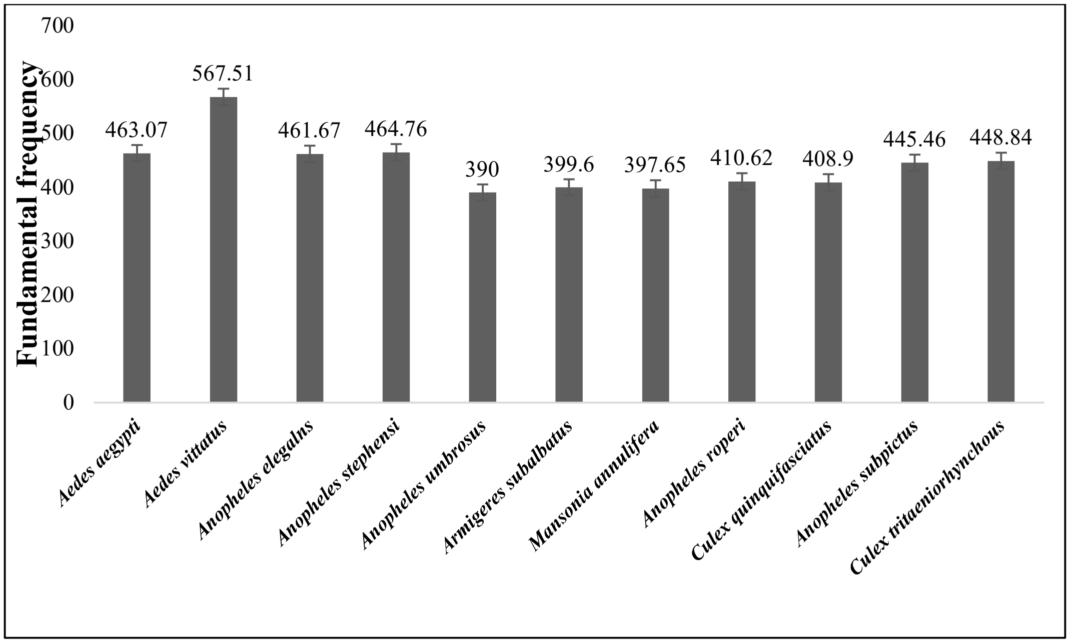


Fig. 6.1. Mosquito species could not be isolated using fundamental frequency

6.1.2. Width of fundamental frequency band:

The width of the fundamental frequency band (bandwidth) ranges from 171.875 to 382.26 Hz, being the highest and lowest in *Culex quinquefasciatus* and *Anopheles culiciformis,* respectively. This parameter can produce 84.79% differentiation statistically significant between 171 pair-wise comparisons among 19 species (ANOVA: df, 18, 286, f = 125.5, p = 0.0001). The bandwidth has differentiated species pairs: *Aedes aegypti - Anopheles elegans; Anopheles elegans - Anopheles stephensi; Anopheles roperi - Culex quinquefasciatus;* and *Armigeres subalbatus - Mansonia anulifera.*

6.1.3. Harmonic band diversity:

The sound profile showed a consistent number of harmonic bands for each species. The harmonic band number ranges from 5 to 14, being lowest in *Anopheles culiciformis* and highest in *Aedes albopictus,* indicating significant differences among them. This harmonic band diversity showed an 84.79% success rate among the species, comparing 171 pairs (ANOVA: df, 18, p = 0.0001).

6.1.4. Amplitude (dB) of fundamental frequency band:

The amplitude of the fundamental frequency band showed an inconsiderable success rate for the identification of species (49.7%) with 84 pairs significantly different from 171 (ANOVA: df, 18, 286, f = 9.86, p = 0.0001).

6.1.5. Development of matrices, amplitude, and frequency or their combinations for unresolved species identification:

There are two matrices for the detection of mosquitoes having similar fundamental frequencies.

- **Combination of the amplitude of fundamental and highest harmonic frequency bands:**

The ratio and multiplication of these parameters were estimated for species differentiation. The ratio ranged between 0.484 (*Culex tritaeniorhynchus*) and 0.889 (*Anopheles culiciformis*). Out of 171 pairs, a comparison of 19 species, 104 pairs showed significant differences among them, indicating 60.82% capability of species differentiation (ANOVA: df, 18, 286, f = 25.38, p = 0.0001). Interestingly, the multiplication of the same parameters improved the ability of species identification to 58.39% (ANOVA: df, 18, 286, f = 17.93, p = 0.0001).

- **Combination of fundamental frequencies and harmonic bands:**

Both of these parameters were used to estimate the ratio and multiplication for species differentiation. Among 19 species of mosquitoes subjected to fundamental frequencies, four groups of (3-3 & 2-2 species) from 10 species that shared similar fundamental frequencies were not separable for species identification (Fig.6.1). Using the ratio between fundamental frequencies – numbers of total harmonic bands, 96.50% success rate for the species differentiation among 171 pairs except *Aedes albopictus – Armigeres subalbatus, Anopheles roperi - Hulecoetomyia fluviatilis, Anopheles subpictus – Culex. tritaeniorhynchus,* and *Armigeres subalbatus - Mansonia annulifera* (ANOVA: df, 18, 286, f = 3033.81, p = 0.0001). The multiplication of these parameters further improved the success rate of species differentiation to 97.66% with 167 pairs confirmed out of 177, except *Anopheles stephensi - Hulecoetomyia fluviatilis, Anopheles subpictus – Culex. tritaeniorhynchus, Anopheles. sinensis – Culex. mimuloides complex* and *Armigeres subalbatus - Mansonia annulifera* (ANOVA: df, 18, 286, f = 3315.17, p = 0.0001).

6.1.6. Acoustic indices-based differentiation of mosquitoes:

The acoustic entropy index of the 10 mosquitoes ranged from 0.04101± 0.001 to 0.32005 ± 0.0001, being highest in *Culex tritaeniorhynchus* and lowest in *Anopheles roperi*. The acoustic entropy index demonstrated statistically significant differences among themselves (F ratio=114336.44, df=99, P value=0.00001) (Table 6.1 and Fig.6.2).

Table 6.1. Acoustic entropy index of 10 mosquito species

Species	Acoustic entropy Index
Aedes aegypti	**0.0600155 ± 0.000014**
Anopheles elegalns	**0.1412 ± 0.000014**
Anopheles stephensi	**0.1299 ± 0.0003**
Anopheles umbrosus	**0.290063 ± 0.00005**
Armigeres subalbatus	**0.2307± 0.0007**
Mansonia annulifera	**0.050092 ± 0.0001**
Anopheles roperi	**0.04101± 0.001**
Culex quinquefasciatus	**0.1141 ± 0.002**
Anopheles subpictus	**0.17011 ± 0.0002**
Culex tritaeniorhynchus	**0.32005 ± 0.0001**
ONE-WAY ANOVA	**F ratio= 114336.44, df= 99, P value=0.00001**

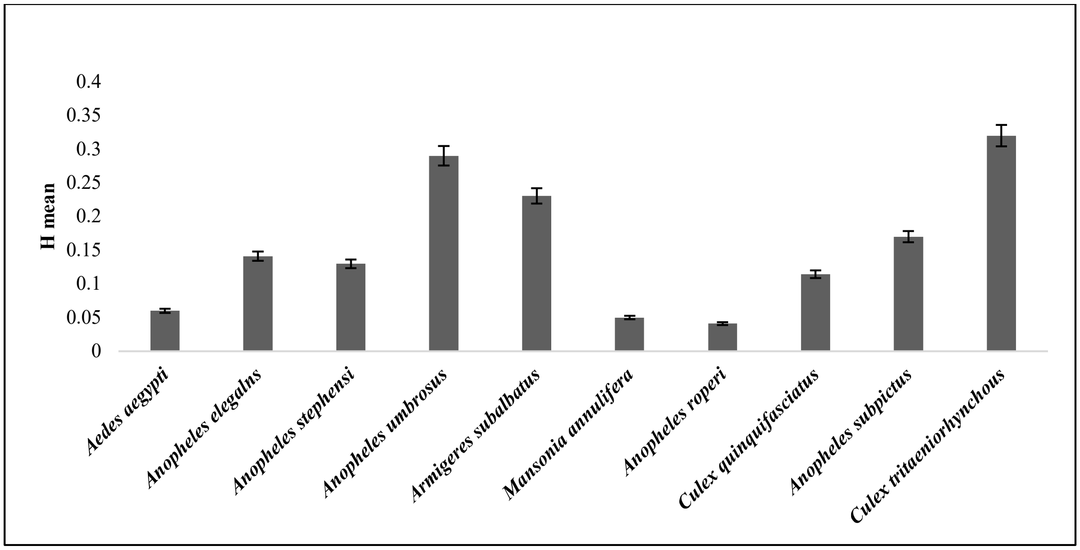


Fig. 6.2. Acoustic entropy Index of 10 mosquito species

6.1.7. Sexual differences

The fundamental frequency of male mosquitoes was higher than that of female mosquitoes. Mean frequencies of males ranged from 587.68 ± 4.28 Hz to 960.85 ± 2.42 Hz, being highest in *Aedes vitatus,* followed by *Aedes albopictus*, *Culex* *tritaeniorhynchus*, *Anopheles subpictus, Culex quinquefasciatus, Hulecoetomyia fluviatilis, Armigeres subalbatus,* and *Aedes aegypti,* while con-specific females ranged from 399.60 ±2.54 to 567.51±0.71 Hz (Table 6.2). Male and female mosquitoes under the study of sexual differences differed significantly in fundamental frequencies. All male mosquitoes can be differentiated based on fundamental frequencies (ANOVA: df=109, F=9771.26, P=0.00001).

Table 6.2. Effects of sexual differences on the Fundamental Frequency of mosquitoes

Species	**Male**	**Female**	**One-Way ANOVA**
Aedes albopictus	799.38±5.09	510.90±11.76	F=9884.23, df=56, P= 0.00001
Aedes vitatus	960.85±2.42	567.51±3.82	F=150380.97, df=39, P=0.00001
Culex tritaeniorhynchus	722 ± 5.65	449.48±13.49	F=6762.47, df=54, P=0.00001
Culex quinquefasciatus	687.32±1.49	408.90±17.74	F=2427.84, df=21, P=0.00001
Armigeres subalbatus	587.68±4.28	399.60±16.74	F=1578.52, df=44, P=0.00001
Hulecoetomyia fluviatilis	627.24±4.98	473.16±1.60	F=8667.80, df=19, P=0.00001
Anopheles subpictus	696.81±6.01	445.46±2.01	F=39126.24, df=38, P=0.00001
Anopheles stephensi	645.81±7.2	464.76±0.439	F=6260.89, df=19, P=0.00001
One-Way ANOVA	df=109, F=9771.26, P=0.00001		

6.1.8. Impact of abdominal fed vs unfed condition on fundamental frequency:

The results showed that the blood-fed females had a significant upsurge in the fundamental frequency to unfed females being 458.24±2.75 Hz in comparison to 445±2.01 Hz for *Anopheles subpictus* (ANOVA: df=37, F=243.08, P=0.001)*,* 482.61±5.12 Hz than 399.60±16.74 Hz in *Armigeres subalbatus* (ANOVA: df=43, F=281.38, P=0.001), and 418.64±3.41 Hz than 408.90±17.74 Hz in *Culex quinquefasciatus* (ANOVA: df=27, F=4.65, P=0.040 (Table.6.3.).

Table 6.3. Impact of abdominal fed vs unfed condition on fundamental frequency

Species	**Fed**	**unfed**	**ANOVA**
Anopheles subpictus	458.24±2.75	445±2.01	F=243.08, df=37, P=0.001
Armigeres subalbatus	482.61±5.12	399.60±16.74	F=281.38, df=43, P=0.001
Culex quinquefasciatus	418.64±3.41	408.90±17.74	F=4.65, df=27, P=0.040

6.1.9. Mixed sound of both sexes and harmonic convergence:

The tone of *Aedes vittatus* was characterized by higher modulations of the sound frequencies in the presence of the opposite sex. Paired males and females showed a decrease in fundamental frequency than that of solo mosquitoes (Fig. 6.3). Our studies on the acoustic behavior of *Aedes vittatus* revealed evidence of harmonic convergence for mating success. No significant difference (ANOVA: f=0.001, df=24, P=0.9719) was found between 2nd harmonic of males (1340.49 ± 6.97) and 3rd harmonic of females (1340.4 ± 5.74). Harmonic convergence was observed between 2nd male harmonic and 3rd female harmonic frequency (Fig. 6.3).

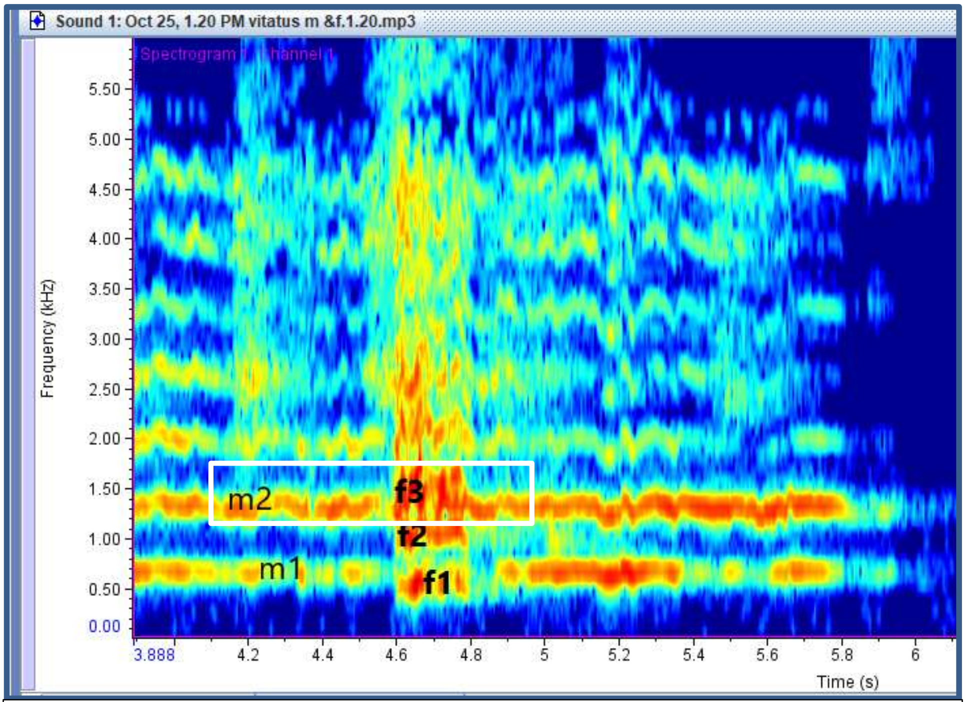


Fig.6.3. Spectrogram showing harmonic convergence in *Aedes vittatus*; m1: 1st harmonic of male, m2: 2nd harmonic of male, f1: 1st harmonic of female, f2: 2nd harmonic of female & f3: 3rd harmonic of female. Synchronization between m2 and f3 has been marked in white.

6.1.10. Effect of age on bioacoustics of mosquitoes:

Sounds of *Aedes albopictus* mosquitoes have been recorded in three age durations: freshly emerged, on the 3rd day, and 7th day of emergence in 26 ± 1 ºC temperature and 65 ± 5% relative humidity. Freshly emerged mosquitoes showed the highest frequency (501.02 ± 5.98) followed by 3rd (499.348 ± 5.41) and 7th day (499.268 ±1.33), but the difference was not statistically significant (F ratio= 0.42, df=28, P=0.66). Fundamental frequency in each age also matches the frequency of field-collected specimens.

6.1.11. Discussion:

Mosquitoes communicate with each other through a highly developed Johnston's organ (JO) by receiving species-specific aural cues. In response to acoustic signals, mosquitoes emit a buzzing sound having a species-specific frequency of vibrating wings (Simões *et al*., 2016). However, species-wise mosquito detection based on sound was proposed several decades ago (Goeldi, 1905). A microcomputer-based instrument with a laser light beam has also been used to detect wing beat frequency to identify *Aedes aegypti* and *Aedes triseriatus* populations; however, only an accuracy of 84% could be achieved (Moore *et al*., 1986). Very few researchers were able to demonstrate significant differences among mosquito species using mainly fundamental sound frequency (Alar & Fernandez, 2021; González-Pérez *et al*., 2022; Mukundarajan *et al*., 2017; Siddiqui & Kayte, 2022). Recently, (Mukundarajan *et al*., 2017) recorded sounds of 19 medically important mosquito species with commercially available cell phones which resulted in great variations in the fundamental frequencies within the species, i*.e., Aedes aegypti* (450-650 Hz), *Aedes albopictus* (500-700 Hz), *Aedes mediavitatus* (390-480 Hz), *Aedes sierrensis* (340-530 Hz), *Anopheles albimanus* (360-500 Hz), *Anopheles arabiensis* (*3*60-590 Hz), *Anopheles atroparvus* (380-510 Hz), *Anopheles dirus* (400-490 Hz), *Anopheles farauti* (500-700 Hz), *Anopheles freeboni* (340-450 Hz), *Anopheles gamiae* (460-650 Hz), *Anopheles merus* (400-600 Hz), *Anopheles minimus* (550-700 Hz), *Anopheles quadranulatus* (330-520 Hz), *Anopheles quadramaculatus* (250-450 Hz), *Anopheles stephensi* (500-650 Hz), *Culex pipiens* (290-400 Hz), *Culex quinquifasciatus* (340-410 Hz), *Culex tarsalis* (210-370 Hz) and *Culiseta incidens* (200-360 Hz). This caused problems in establishing significant differences statistically among the species due to overlapping frequencies. Therefore, Mukundarajan *et al*.

(2017) had to link the species prevalence data with geographical distribution metadata to separate species based on sound frequencies statistically. This suggests that fundamental frequencies are not enough to distinguish mosquito species when present in large numbers. In our study, we have found a 95.32% success rate for differentiating 19 mosquito species using fundamental frequency only. Out of 171 pairs of species, only 7 pairs were not able to distinguish statistically: *Aedes aegypti - Anopheles subpictus, Anopheles elegans - Anopheles stephensi, Anopheles roperi - Culex quinquefasciatus, Anopheles subpictus - Culex tritaeniorhynchus, Anopheles stephensi - Hulecoetomyia fluviatilis, Anopheles umbrosus - Mansonia anulifera, Armigeres subalbatus - Mansonia anulifera.* It concerns areas with high species diversity, which may result in compromising the success rate. Therefore, the involvement of other sound parameters may be helpful for better identification of the species.

In addition to the fundamental frequency, the present study explores the other parameters of the sound that were not considered by previous authors. For example, the width of the fundamental frequency band has shown 84.79% successful differentiation of species. This parameter successfully separated the pairs of *Aedes aegypti* - *Anopheles elegans, Anopheles Stephensi – Anopheles elegans,* and *Armigeres sublabatus – Mansonia annulifera* that could not be differentiated with fundamental frequency. This suggests the utility of bandwidth in species differentiation using the sound of mosquitoes.

Higher harmonics are the prominent features of the sound profile; however, the harmonic frequency bands were not given significance earlier (Alar & Fernandez, 2021; González-Pérez *et al*., 2022; Mukundarajan *et al*., 2017; Siddiqui & Kayte, 2022). Similarly, Mukundarajan *et al*. (2017) also excluded the harmonics as potential parameters for mosquito detection as they used different mobiles for having differential frequency response, probably because of using mosquito containers of variable sizes and shapes, which can alter the characteristics of harmonics. Initially, Brogdon (1994) considered harmonics for *Aedes aegypti* and *Aedes albopictus* identification and observed seven harmonics in both species at 20 kHz, five in *Aedes aegypti* and *Aedes albopictus* at 5 kHz, and two and one harmonics at 1 kHz, respectively. (Aldersley *et al*., 2014) had isolated higher harmonic frequencies in male *Aedes aegypti* mosquitoes using a bandpass filter. The present study has included the standard device development to produce similar results with multiple repetitions and found consistent reproducibility among 19 species of mosquitoes, ranging from 5 (*Anopheles culiciformis*) to 14 (*Aedes albopictus*), which were able to differentiate among species. Efforts were also made in the design to reduce external noise during sound recording, resulting in a double-chamber device (Suman,2021*,* Indian patent publication). The ratio of fundamental frequency and number of harmonic bands showed a 97.7% success rate for species differentiation, showing a promising ability to identify most of the species that resolve a majority of undifferentiated species pairs in fundamental frequency, but produced some new undistinguishable pairs. Interestingly, instead of a ratio, the multiplication factor of these parameters not only further improves the success rate (98.25%) for differentiating the species but also resolves the species pairs where the fundamental frequency failed. This suggests that the multiplication factor provides more power than either the use of fundamental frequency or the ratio between fundamental frequency and harmonic band numbers for species identification.

The sex differentiation based on sound frequency has also been an important component for the surveillance of mosquitoes. For example, Arthur *et al*. (2014)

analyzed acoustic tones of tethered *Aedes aegypti* mosquitoes showing significant differences in males (571 to 832 Hz) and females (421 to 578 Hz); however, the success rate for identifications was 100% in *Aedes triseriatus* females and *Aedes aegypti* males, 93% in *Aedes triseriatus* males, and only 43% for *Aedes aegypti* females (Moore *et al*., 1986). Simões *et al*. (2016) observed the fundamental frequency of *Culex quinquefasciatus* males as 789±10 Hz, and that of females was 474±10 Hz. Aldersley *et al*. (2014) recorded individual flight tones *of Aedes aegypti* mosquitoes ranging from 492.1 to 880.3 Hz for males and 415.9 to 532.6 Hz for females. (Brogdon, 1994) recognized the fundamental frequency of female *Aedes aegypti* and *Aedes albopictus* as 460Hz and 536Hz, respectively, whereas the males of these species showed higher frequencies 715 Hz and 724Hz, respectively. In agreement with these studies, our results also showed that male mosquitoes of all eight species produce significantly higher frequency sounds in comparison to the females. Also, it is interesting to note that the males of all these species were significantly different among these species belonging to the genera *Aedes, Anopheles, Armigeres, Culex, and Hulecoetomyia.* This suggests that the detection ability of species using males alone may support morpho-taxonomy and proactive detection of mosquito populations.

Acoustic communication in con-specific insects is vital and is being used to understand the mating behavior of mosquitoes with the phenomenon of harmonic convergence and modulation of sound frequencies. (Warren *et al*., 2009) demonstrated sexual detection by finding similar wingbeat-tone harmonics in the *Culex quinquefasciatus* mosquitoes in the UK. (Pantoja-Sánchez *et al*., 2019) Determined the acoustic features before copulation of *Anopheles albimanus* and showed a significant increase in wing beat frequency of free-flying males and females compared to the tethered mosquitoes, with harmonic convergences of 79% of the paired and tethered mosquitoes.(Simões *et al*., 2016) Visualized two types of harmonic convergence ratios (HCR) (2:1 and 3:2, female-male harmonic relationship) in male *Culex quinquefasciatus* in the presence of conspecific female mosquitoes, which resulted in harmonic convergence. (Cator *et al*., 2011) Observed that the male mosquitoes orient to the fundamental frequency of females, and both sexes actively modulate their tone to converge at harmonic frequencies before mating; in a paired flight, the female's third harmonic and the male's second harmonic were at frequencies near convergence. However, harmonic convergence could not be detected between two closely related molecular forms of *Anopheles* gambiae (Pennetier *et al*., 2010). In our study, the tones of *Aedes vitatus* paired male and female showed a decrease in fundamental frequency than that of the solo mosquito, and harmonic convergence was found between the 2nd male harmonic and 3rd female harmonic frequency, suggesting evidence of harmonic convergence for mating success in this species. This harmonic convergence may be used for designing sex-specific traps for mosquitoes and disease transmission inhibition.

6.2. Objective2. Confirmation of mosquito species using molecular tools.

6.2.1. DNA sequences and accession numbers:

19 mosquito species used in the objective collected from different locations (Table 6.4) were sequenced. PCR analysis was conducted with the help of primers used by Suman et al. (2022). PCR products were subjected to agarose gel electrophoresis, confirming the segregation of DNA fragments up to 501 bp, followed by Sanger sequencing of the PCR amplicons. DNA sequences of mosquito species were aligned and submitted to the NCBI GenBank repository, and accession numbers have been provided. Based on COI-based DNA analysis, 14 species, i.e., *Aedes aegypti, Aedes albopictus, Aedes vittatus, Anopheles elegans, Anopheles sinensis, Anopheles subpictus, Anopheles stephensi, Armigeres subalbatus, Culex glides, Culex implodes, Culex quinquefasciatus, Culex tritaeniorhynchus, Mansonia annulifera,* and *Mansonia uniformis* were compared with the sequences from the GenBank, which revealed 95-100% similarities (Table 6.4).

Table. 6.4. Mosquito species with Accession numbers submitted to NCBI

Mosquito species	Accession numbers
Anopheles subpictus	PP413386
Aedes aegypti	PP423053
Aedes albopictus	PP423057
Anopheles stephensi	PP423061
Mansonia annulifera	PP423085
Culex quinquefasciatus	PP423087
Culex gelidus	PP436810
Armigeres subalbatus	PP436904
Culex tritaeniorhynchus	PP437077
Aedes vittatus	PP437207
Anopheles culiciformis	PP723313
Anopheles elegans	PP437296
Mansonia uniformis	PP437845
Anopheles sinensis	PP438316
Culex mimuloides	PP449032
Culex alienus	PP455247
Anopheles roperi	PP454704
Anopheles umbrosus	PP468572
Hulecoetomyia fluviatilis	PP468573

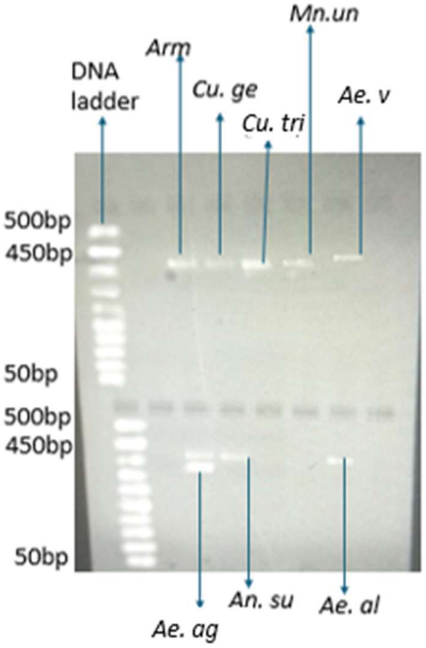


Fig. 6.4. DNA bands on agarose gel electrophoresis.

6.2.2. Nucleotide composition

The percentage of AT content in 19 mosquitoes ranged between 64.34% to 68.75%, being lowest in *Mansonia annulifera* and highest in *Anopheles elegans,* followed by *Aedes albopictus, Aedes aegypti, Culex tritaeniorhynchus, Armigeres subalbatus, Culex quinquefasciatus, Anopheles subpictus, Anopheles culiciformis, Mansonia uniformis, Aedes vittatus, Hulecoetomyia fluviatilis, Anopheles stephensi, Culex alienus, Anopheles roperi, Anopheles sinensis, Culex gelidus, Culex mimuloides, and Anopheles umbrosus.* GC content in 19 mosquitoes exhibited the exact opposite trend (Fig. 6.5 - Fig. 6.7).

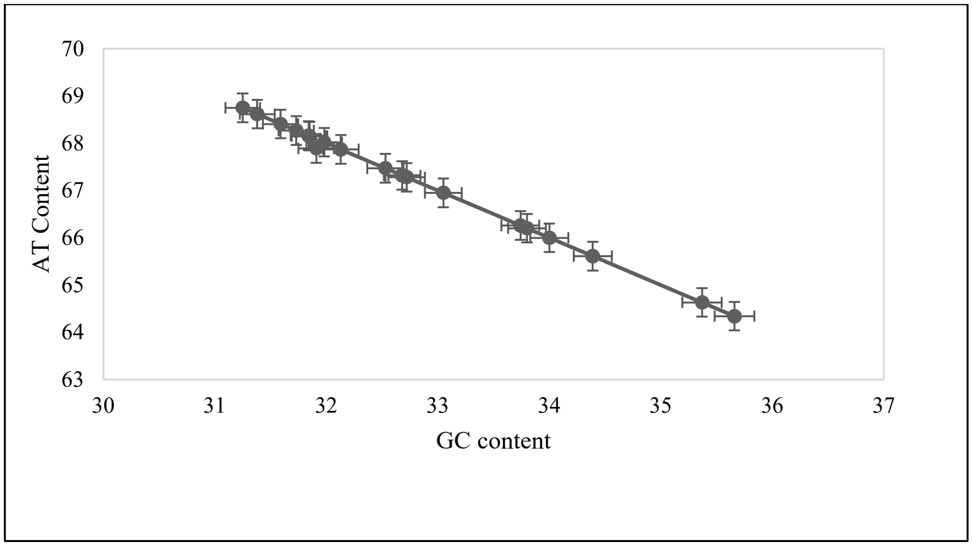


Fig. 6.5. Relationship between AT and GC content

Detailed nucleotide compositions are as follows;

1. DNA molecule: *Aedes aegypti*

Length = 474 bp

MW of DNA = 143089.00 D SS

MW of DNA = 286923.00 D DS

Guanine (G) and Cytosine (C) content = 34.39%

Adenine (A) and Thymine (T) content = 65.61%

Nucleotide	Number	Mol%
A	127	26.79
C	90	18.99
G	73	15.40
T	184	38.82

2. DNA molecule: *Aedes albopictus*

Length = 410 bp

MW of DNA = 124025.00 D SS

MW of DNA = 248255.00 D DS

Guanine (G) and Cytosine (C) content = 35.37%

Adenine (A) and Thymine (T) content = 64.63%

Nucleotide	Number	Mol%
A	110	26.83
C	84	20.49
G	61	14.88
T	155	37.80

3.DNA molecule: *Aedes vittatus*

Length = 501 bp

MW of DNA = 151333.00 D SS

MW of DNA = 303106.00 D DS

Guanine (G) and Cytosine (C) content = 32.53%

Adenine (A) and Thymine (T) content = 67.47%

Nucleotide	Number	Mol%
A	145	28.94
C	91	18.16
G	72	14.37
T	193	38.52

4.DNA molecule: *Anopheles culiciformis*

Length = 382 bp

MW of DNA = 114908.00 D SS

MW of DNA = 231131.00 D DS

Guanine (G) and Cytosine (C) content = 32.72%

Adenine (A) and Thymine (T) content = 67.28%

Nucleotide	Number	Mol%
A	111	29.06
C	57	14.92
G	68	17.80
T	146	38.22

5. DNA molecule: *Anopheles elegans*

Length = 240 bp

MW of DNA = 72806.00 D SS

MW of DNA = 145167.00 D DS

Guanine (G) and Cytosine (C) content = 31.25%

Adenine (A) and Thymine (T) content = 68.75%

Nucleotide	Number	Mol%
A	73	30.42
C	49	20.42
G	26	10.83
T	92	38.33

6.DNA molecule: *Anopheles roperi*

Length = 449 bp

MW of DNA = 135289.00 D SS

MW of DNA = 271598.00 D DS

Guanine (G) and Cytosine (C) content = 31.85%

Adenine (A) and Thymine (T) content = 68.15%

Nucleotide	Number	Mol%
A	135	30.07
C	70	15.59
G	73	16.26
T	171	38.08

7.DNA molecule: *Anopheles sinensis*

Length = 490 bp

MW of DNA = 147482.00 D SS

MW of DNA = 296394.00 D DS

Guanine (G) and Cytosine (C) content = 31.84%

Adenine (A) and Thymine (T) content = 68.16%

Nucleotide	Number	Mol%
A	148	30.20
C	72	14.69
G	84	17.14
T	186	37.96

8.DNA molecule: *Anopheles stephensi*

Length = 492 bp

MW of DNA = 148138.00 D SS

MW of DNA = 297618.00 D DS

Guanine (G) and Cytosine (C) content = 31.91%

Adenine (A) and Thymine (T) content = 67.89%

Nucleotide	Number	Mol%
A	152	30.89
C	71	14.43
G	86	17.48

T	182	36.99

9.DNA molecule: *Anopheles subpictus*

Length = 348 bp

MW of DNA = 105069.00 D SS

MW of DNA = 210583.00 D DS

Guanine (G) and Cytosine (C) content = 33.05%

Adenine (A) and Thymine (T) content = 66.95%

Nucleotide	Number	Mol%
A	102	29.31
C	61	17.53
G	54	15.52
T	131	37.64

10. DNA molecule: *Anopheles umbrosus*

Length = 494 bp

MW of DNA = 149140.00 D SS

MW of DNA = 298775.00 D DS

Guanine (G) and Cytosine (C) content = 31.38%

Adenine (A) and Thymine (T) content = 68.62%

Nucleotide	Number	Mol%
A	154	31.17
C	81	16.40

G	74	14.98
T	185	37.45

11. DNA molecule: *Armigeres subalbatus*

Length = 426 bp

MW of DNA = 128409.00 D SS

MW of DNA = 257828.00 D DS

Guanine (G) and Cytosine (C) content = 33.80%

Adenine (A) and Thymine (T) content = 66.20%

Nucleotide	Number	Mol%
A	108	25.35
C	80	18.78
G	64	15.02
T	174	40.85

12.DNA molecule: *Culex alienus*

Length = 394 bp

MW of DNA = 118856.00 D SS

MW of DNA = 238342.00 D DS

Guanine (G) and Cytosine (C) content = 31.98%

Adenine (A) and Thymine (T) content = 68.02%

Nucleotide	Number	Mol%
A	115	29.19

C	67	17.01
G	59	14.97
T	153	38.83

13. DNA molecule: *Culex gelidus*

Length = 394 bp

MW of DNA = 118640.00 D SS

MW of DNA = 238325.00 D DS

Guanine (G) and Cytosine (C) content = 31.73%

Adenine (A) and Thymine (T) content = 68.27%

Nucleotide	Number	Mol%
A	108	27.41
C	66	16.75
G	59	14.97
T	161	40.86

14. DNA molecule: *Culex mimuloides*

Length = 459 bp

MW of DNA = 138463.00 D SS

MW of DNA = 277626.00 D DS

Guanine (G) and Cytosine (C) content = 31.59%

Adenine (A) and Thymine (T) content = 68.41%

Nucleotide	Number	Mol%

A	139	30.28
C	75	16.34
G	70	15.25
T	175	38.13

15.DNA molecule: *Culex quinquefasciatus*

Length = 489 bp

MW of DNA = 147509.00 D SS

MW of DNA = 295948.00 D DS

Guanine (G) and Cytosine (C) content = 33.74%

Adenine (A) and Thymine (T) content = 66.26%

Nucleotide	Number	Mol%
A	141	28.83
C	84	17.18
G	81	16.56
T	183	37.42

16. DNA molecule: *Culex tritaeniorhynchus*

Length = 447 bp

MW of DNA = 135088.00 D SS

MW of DNA = 270551.00 D DS

Guanine (G) and Cytosine (C) content = 34.00%

Adenine (A) and Thymine (T) content = 66.00%

Nucleotide	Number	Mol%
A	132	29.53
C	81	18.12
G	71	15.88
T	163	36.47

17.DNA molecule: *Hulecoetomyia fluviatilis*

Length = 442 bp

MW of DNA = 133482.00 D SS

MW of DNA = 267384.00 D DS

Guanine (G) and Cytosine (C) content = 32.13%

Adenine (A) and Thymine (T) content = 67.87%

Nucleotide	Number	Mol%
A	140	31.67
C	72	16.29
G	70	15.84
T	160	36.20

18. DNA molecule: *Mansonia annulifera*

Length = 488 bp

MW of DNA = 147711.00 D SS

MW of DNA = 295502.00 D DS

Guanine (G) and Cytosine (C) content = 35.66%

Adenine (A) and Thymine (T) content = 64.34%

Nucleotide	Number	Mol%
A	133	27.25
C	101	20.70
G	73	14.96
T	181	37.09

19. DNA molecule: *Mansonia uniformis*

Length = 407 bp

MW of DNA = 122876.00 D SS

MW of DNA = 246252.00 D DS

Guanine (G) and Cytosine (C) content = 32.68%

Adenine (A) and Thymine (T) content = 67.32%

Nucleotide	Number	Mol%
A	115	28.26
C	74	18.18
G	59	14.50
T	159	39.07

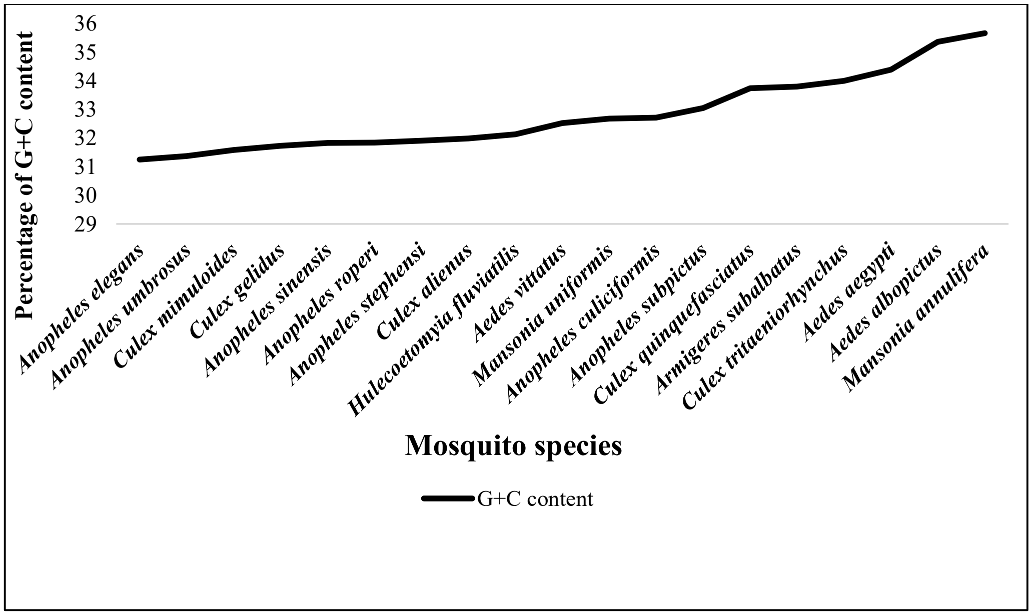


Fig. 6.6. G+C content in 19 mosquitoes

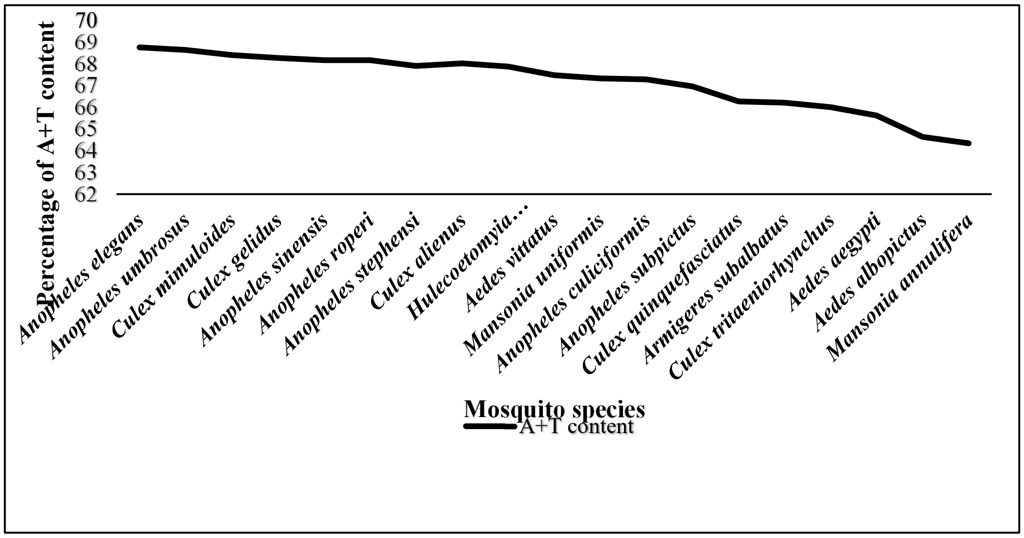


Fig. 6.7. A+T content in 19 mosquitoes

6.2.3. Phylogenetic analysis

The tree with the highest log likelihood (-1458.53) is shown (Fig. 6.8). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.4456)). This analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). The phylogenetic tree analysis based on COI gene sequences demonstrated multiple clusters of different species. The tree shows a deep evolutionary split dividing the mosquito species into two primary super-clades. One major lineage contains the genera *Aedes*, *Hulecoetomyia*, and *Anopheles*. The other major lineage contains the genera *Mansonia*, *Culex*, and *Armigeres*. The genus *Aedes* is shown to be a sister group to the combined *Hulecoetomyia* + *Anopheles* clade. Within the other major lineage, the genus *Mansonia* branches off first, indicating it's a sister group to the combined *Culex* + *Armigeres* clade. *Aedes aegypti, Aedes albopictus & Aedes vittatus* formed a clade, while *Aedes aegypti & Aedes albopictus* showed a close relationship as they formed a different clade and seem to be sister taxa. *Anopheles elegans & Anopheles culiciformis* formed a separate clade showing a close relationship. There were multiple clusters of *Culex* species; among them, *Culex mimuloides* showed a clade with *Culex quinquefasciatus*, *Culex alienus* with *Culex tritaeniorhynchus.* Two species of *the Mansonia* genus formed a clade together. The species *Armigeres subalbatus* occupies a basal position relative to the *Culex* species shown. This means it diverged earlier and is a sister taxon to the entire *Culex* clade presented in this analysis. Some species show a greater degree of genetic change, indicated by their longer branch lengths. For instance, *Anopheles roperi* and *Anopheles umbrosus* have notably long branches compared to other *Anopheles* species, suggesting a higher rate of nucleotide substitution in their COI gene since diverging from their common ancestors.

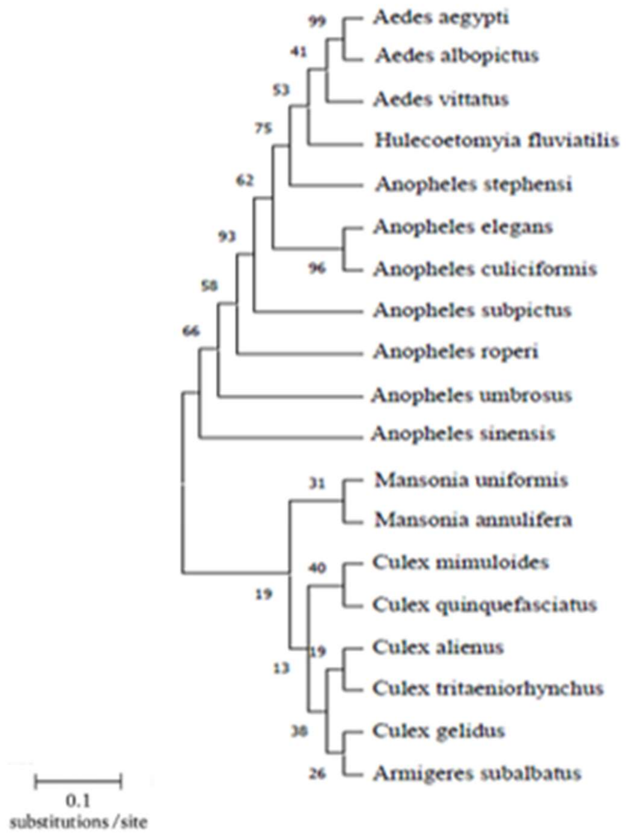


Fig.6.8. Phylogeny tree of 19 mosquito species

6.2.4. Discussion:

This study provides a robust molecular characterization of 19 mosquito species from different vector-borne disease-affected areas of India through phylogenetic analysis of the mitochondrial Cytochrome c oxidase I (COI) gene. The use of COI as the standard DNA barcode for animals is predicated on its ideal evolutionary rate, which is effective for distinguishing species (Hebert et al., 2003). The high AT-content (64-69%) observed in the sequences is a hallmark feature of insect mitochondrial genomes, serving as an internal validation of the data's quality and consistency with broader findings in insect molecular evolution (Cameron, 2014).

The Maximum Likelihood phylogenetic analysis successfully recovered the monophyly of the principal genera, including *Aedes*, *Anopheles*, and *Culex*. This result reinforces the efficacy of COI-based DNA barcoding for the rapid and accurate identification of medically important mosquito vectors, a finding that aligns with numerous regional and global barcoding initiatives (Kumar, 2007). Such accurate identification is the foundational step for implementing targeted vector control strategies against the distinct diseases each genus transmits, such as malaria (*Anopheles*), dengue (*Aedes*), and Japanese Encephalitis (*Culex*).

At a deeper evolutionary level, phylogeny supports a sister-group relationship between the *Aedes* and *Anopheles* lineages, setting them apart from a clade containing *Mansonia*, *Culex*, and *Armigeres*. This topology is largely congruent with findings from multi-gene molecular studies that have significantly reshaped our understanding of the Culicidae family evolution, often challenging traditional morphology-based classifications (Reidenbach et al., 2009). However, the analysis also highlights the limitations of using a single marker, as shown by low bootstrap support for some nodes, particularly within the species-rich *Culex* genus. This indicates that while COI is excellent for genus-level identification, resolving more recent speciation events may require a more data-rich phylogenomic approach. A significant outcome of this research is its contribution of sequences to the NCBI GenBank public database, establishing a permanent and accessible resource for the global scientific community. This work serves as a vital biodiversity baseline for mosquitoes in different areas of India. The generated reference sequences are foundational for future research, including broader phylogenomic studies to resolve taxonomic uncertainties and population genetic analyses to investigate gene flow, population structure, and the spread of insecticide resistance within these vector populations.

6.3.Objective3. Monitoring the effects of morphometric parameters on the bioacoustics of mosquitoes

6.3.1. Estimation of wing size:

ImageJ software was used to measure the wing lengths of female *Aedes aegypti* and *Aedes albopictus* mosquitoes. An image of known size was selected, and a line was drawn using a line ruler, then the measurement data was typed manually, harnessing the “SET SCALE” tool & “SET MEASUREMENT” window. The images of the wing size of mosquitoes under study were selected, and lines were drawn to evaluate the wing length through the “MEASURE” option from “ANALYZE”.

6.3.2. Confirmation of different-sized mosquitoes:

To demonstrate the size differences, one-way ANOVA was conducted between three different-sized mosquito groups. The wing size of mosquitoes revealed differences that are statistically significant in *Aedes aegypti* (F ratio = 731.89, df= 30, P=0.0001) and *Aedes albopictus* (F ratio = 407.93, df=30, P=0.0001)

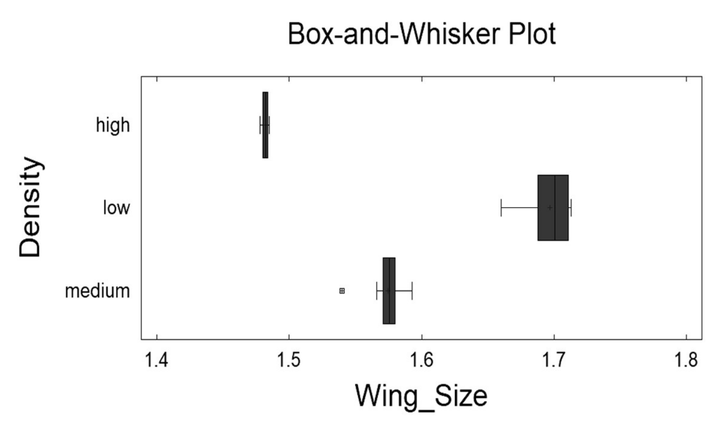


Fig. 6.9. Box and whisker plot to show differences in wing size of adults of *Aedes aegypti* reared in different densities

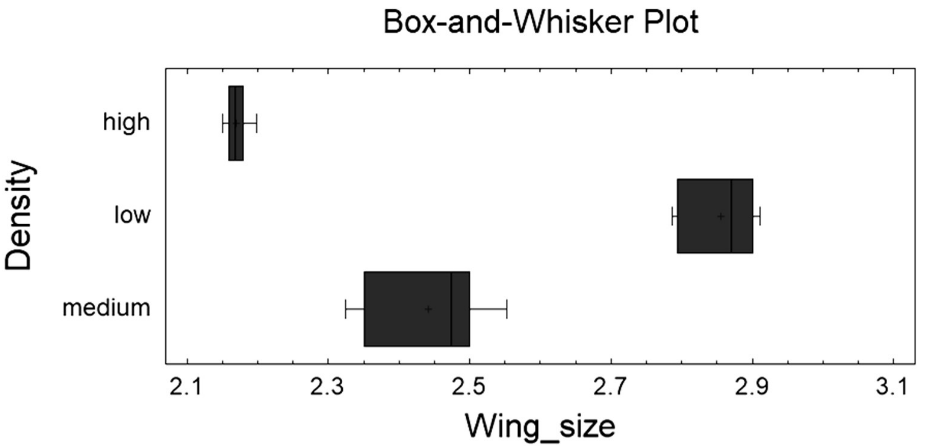


Fig. 6.10. Box and whisker plot to show differences in wing size of adults of *Aedes albopictus* reared in different densities

Flight tone frequencies of female *Aedes aegypti* and *Aedes albopictus* mosquitoes were not affected by wing size. Wing sizes of *Aedes aegypti* ranged from 1.478 mm to 1.709 mm in length could not show any significant impact on sound frequencies (F ratio= 1.58, df= 29, P= 0.2245), While wing sizes of *Aedes albopictus* ranged from 2.15mm to 2.91mm in length and sound frequencies found to be similar (F ratio= 0.08, df=29, P= 0.9255) (Table.6.5, Fig. 6.9, Fig. 6.10)

Table. 6.5. Flight tones by different wing sizes

Wing size	**Flight tone frequency (Hz)**	
	Aedes aegypti	***Aedes albopictus***
Small	**464.771 ± 4.72**	**499.068 ± 2.69**
Medium	**461.97 ± 2.38**	**498.28 ± 5.55**
Large	**463.65 ± 3.10**	**498.862 ± 5.12**

6.3.3. Correlation between wing size and sound frequency:

Pearson and Spearman correlation between wing size and flight tone revealed statistically non-significant value in both of the *Aedes aegypti* (r =0.2076, N= 30, P=0.2710; r=0.1623, N= 30, P=0.3822) and *Aedes albopictus* (r =-0.0398, N= 30, P=0.8346; r=0.0615, N= 30, P=0.7405) (Fig. 6.11).

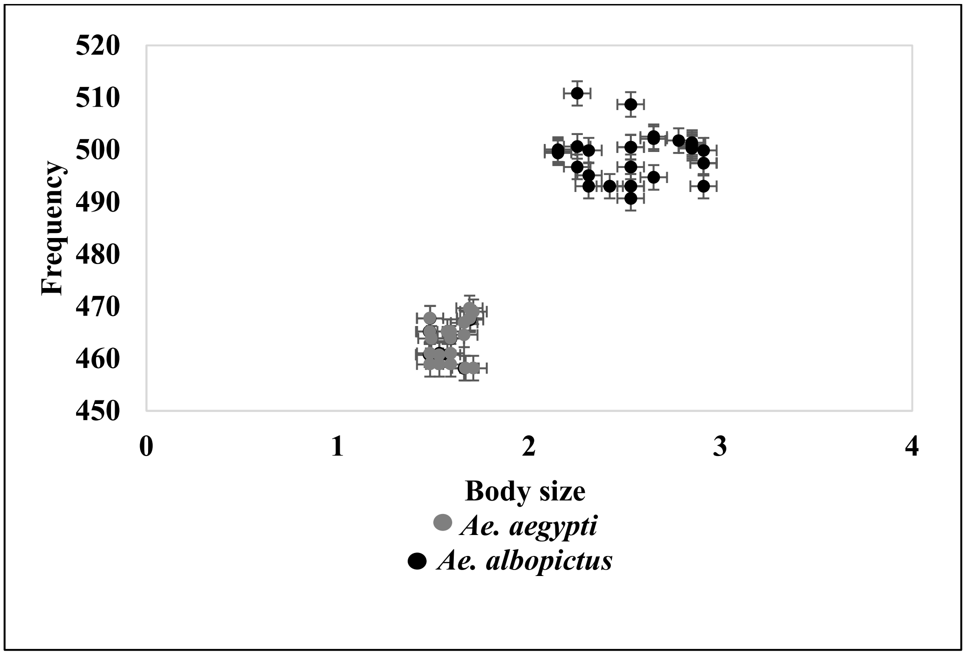
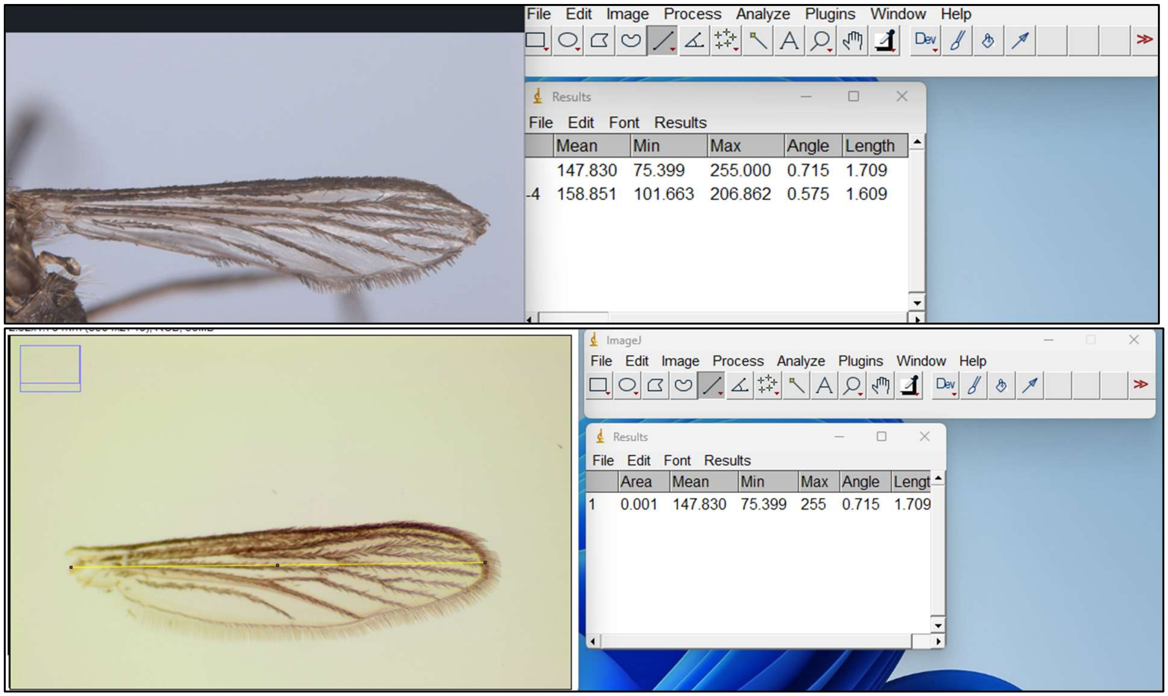


Fig. 6.11. Effects of wing size on flight tone



	Mean	Min	Max	Angle	Length
	147.830	75.399	255.000	0.715	1.709
-4	158.851	101.663	206.862	0.575	1.609

	Area	Mean	Min	Max	Angle	Lengt
1	0.001	147.830	75.399	255	0.715	1.709

Fig. 6.12. Wing size measurement through ImageJ

6.3.4. Discussion:

In this objective, the effects of morphometric parameters on the sound attributes of mosquitoes were addressed. In previous studies, it was estimated that sound signals can be changed with changes in body size (Cator *et al*., 2016). Large-bodied male and female mosquitoes showed quicker responses to the sound signal from the opposite sex and during the alternation of harmonic frequencies than the smaller mosquitoes. Studies also revealed that sound frequency and body size are positively and statistically correlated in *Anopheles gambiae*. There is a correlation between body or wing size and acoustic features in several insects and animals. Copulatory success is highly correlated with the body size of insects and mosquitoes. Females of katydids also demonstrated higher and quicker responses to larger mates than the smaller insects, which also suggests the efficiency of body size on the differentiation of acoustic signals (Erregger et al., 2018). There is a correlation between insemination and body size in the aspects of the amount of ejaculation and duration in mosquitoes (Helinski & Harrington, 2011). Okanda *et al*. (2002) revealed that higher and quicker reproductive success in larger female mosquitoes of *A. gambiae* demonstrated greater mating success than the smaller mosquitoes. It is also evident that the larger mosquitoes will emit different acoustic signals than the smaller ones, and the modulation of harmonic frequencies is also different. In our study, different-sized mosquitoes were obtained from rearing mosquito larvae in three densities, while most of the studies produced different-sized mosquitoes, providing different diets and environmental conditions. Most of the studies enlighten the control measure-oriented approach while discussing the impacts of body size on the acoustics of mosquitoes. Most of the studies emphasized the changes in harmonics due to the change in body size. However, Villarreal *et al*. (2017) revealed no significant impact of body size on the fundamental frequency of *Aedes aegypti* mosquitoes. In this study, we have found similar results, which signify no change in fundamental flight frequencies with the changes of wing size from 1.478 mm to 1.709 mm in *Aedes aegypti* and from 2.15mm to 2.91mm in *Aedes albopictus*. Automated measurements were performed using ImageJ, which enhances accuracy (Fig. 6.12). Cator *et al*. (2016) evaluated 266 copulatory incidences in which the morphometrics of mosquitoes showed impacts on the harmonic convergence of females only. More than 48% of larger mosquitoes succeeded in forming a copula through harmonic matching than female mosquitoes of small sizes. The rate of frequency modulation was correlated with the body sizes of females. These changes in frequency ranged between 22.65 Hz to 31.69 Hz in larger females, while smaller females exhibited frequency modulation from 13.38 Hz to 17.46 Hz during harmonic convergence. Both types of females, either it is small or large, significantly responded only to the larger females.

6.4. Objective 4. Evaluation of the climatic influences on the bioacoustics of mosquitoes.

6.4.1. Effect of Temperature on the fundamental frequency of mosquitoes:

Fundamental sound frequencies varied significantly with the change of temperature from 24ºC to 28 ºC and 31 ºC in both *Aedes aegypti* (F-Ratio= 46.19, df=29, P=0.001) and *Aedes albopictus* (F ratio= 43.91, df=29, P=0.001) (Table 6.6). 24ºC demonstrated lower frequencies, while 31ºC exhibited higher sound frequencies. Moreover, Pearson correlation and Spearman's rank correlation between temperature and fundamental sound frequencies in *Aedes aegypti* (r = 0.8781, N= 30, P= 0.001; r_s=0.8925, N= 30, P= 0.001) and *Aedes albopictus* (r = 0.8627, N= 30, P= 0.001; r_s=0.9035, N= 30, P= 0.001) were statistically significant (Fig. 6.13).

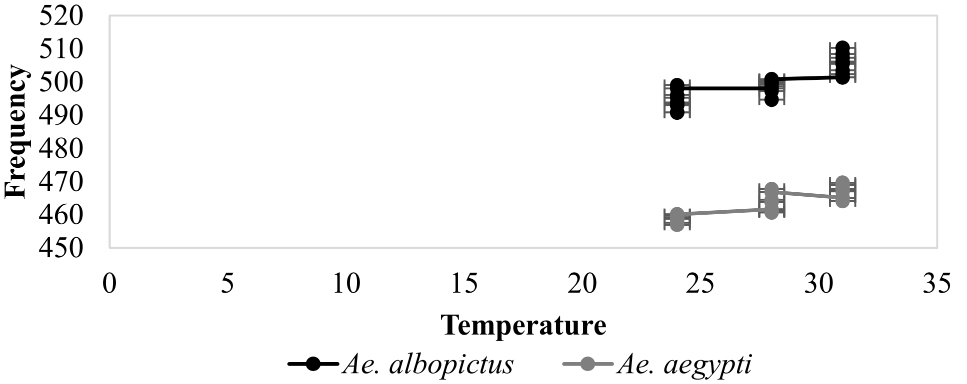


Fig. 6.13. Change of fundamental frequencies with temperature

6.4.2. Effects of humidity on the fundamental frequency of mosquitoes:

Relative humidity between 65 to 75% had no demonstrable influence on flight tone frequencies of *Aedes aegypti* (F-Ratio= 0.04, df=29, P= 0.9582) and *Aedes albopictus* (F ratio= 1.89, df= 29, P=0.1704) (Table.6.6). Moreover, Pearson correlation and spearman rank correlation between humidity and fundamental sound frequencies in *Aedes aegypti* (r =0.0540, N= 30, P= 0.7768; r_s=0.0836, N= 30, P= 0.6525) and *Aedes albopictus* (r =-0.2099, N= 30, P=0.2656; r_s=-0.0425, N= 30, P= 0.8190) were statistically insignificant.

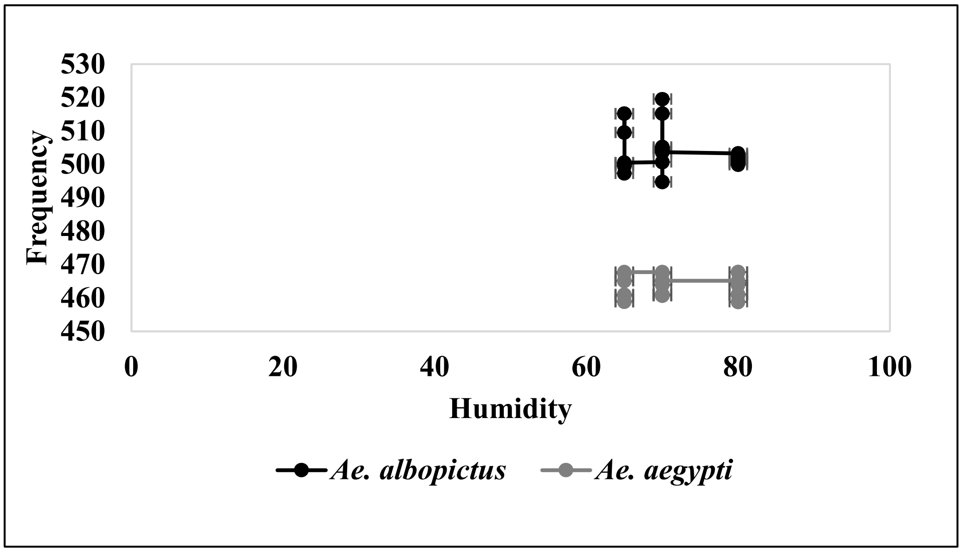


Fig. 6.14. Effects of humidity on sound frequency

Table. 6.6. Effects of Temperature and Humidity on Flight Tones

Factors	**Flight tone frequency (Hz)**	
	Aedes aegypti	***Aedes albopictus***
Temperature		
24ºC (Low)	**458.992 ± 1.01**	**494.37 ± 2.76**
28ºC (Medium)	**463.27 ± 2.57**	**499.02 ± 1.90**
31ºC (High)	**467.236 ± 1.8**	**505.22 ± 2.99**
Humidity		
65% (Low)	**463.225 ± 3.39**	**504.7 ± 6.9**
70% (Medium)	**463.415 ± 2.84**	**506.73 ± 7.55**
75% (High)	**463.512 ± 2.97**	**501.6 ± 1.13**

6.4.3. Discussion:

Several studies have reported the effects of environmental factors on the acoustic features of mosquitoes; most of them focused on evaluating the impacts of temperature and humidity on flight tones. Villarreal *et al*. (2017) established a study on different free-flying female *Aedes aegypti* mosquitoes with different morphometric parameters collected from differentiated geographical locations having multiple environmental conditions. They revealed that female fundamental frequency greatly relies on the environmental temperature. They analyzed the gain of 8-13Hz female fundamental frequency for each degree rise in temperature. Gerhardt and Huber (2002) summarized communication in insects and anurans through bioacoustics and the significant impact of temperature on acoustic signal transduction, which in turn influences communication between ectothermic animals. Zhang *et al*. (2015) conducted an acoustic study to investigate the impact of humid content of wheat on the propagation of acoustic waves.

The authors developed a novel detection device to evaluate the propagation constant of the acoustic wave. These propagation constants of twelve specimens of wheat were measured along with the range of moisture contents. This study revealed a decreasing tendency in the velocity of acoustic waves with increasing moisture, which was found to range from 1.1% - 7.8%. At the same time, the attenuation coefficient was found to rise slightly. On increasing humidity from the 7.8% - 21.2% range, the sound velocity was observed to increase moderately. Insects demonstrate morphometrically tiny body size and closely located ears, leading to low-intensity differences and interaural time, which in turn result in directional hearing (Römer, 2020). Costello (1974) investigated the effect of environmental and physiological factors on the acoustic behavior of *Aedes aegypti*. Temperature, humidity, pressure, and light were considered as influencing environmental factors, while age, mating, oviposition, and feeding were marked as physiological factors. The effects of these factors were examined on the acoustic behavior in both larval and adult forms. The acoustic behavior of larvae was highly affected by rearing temperature in both male and female forms. An increase in rearing temperature and humidity produces mosquitoes with higher wing beat frequencies. Our study reports a direct positive relationship between temperature and fundamental flight tones of two dengue vector mosquitoes of the *Aedes* genus, i.e., *Aedes aegypti* and *Aedes albopictus. Aedes aegypti* exhibited a 1.29 ± 0.04 Hz increase in fundamental frequency for each degree rise in temperature, while a 1.62 ± 0.37 Hz increase in fundamental frequency for each degree rise in temperature was noted in *Aedes albopictus.* Humidity could not show any statistical significance on the flight tone frequency of *Aedes aegypti*. Although *Aedes albopictus* demonstrated a rise in fundamental frequency with the increase of humidity, the difference was not statistically significant, and the correlation study revealed insignificant effects of humidity on fundamental sound frequency.

6.5. Objective 5. Development of data metrics for the surveillance of mosquitoes using acoustic signals.

6.5.1. Description of acoustic signatures of mosquitoes:

We have visualized diverse acoustic characteristics of three *Aedes* mosquitoes. Spectrograms of three mosquitoes showed a different number of harmonic patterns addressing the highest harmonics in *Aedes albopictus* (Fig. 6.15). Median amplitude envelop is significantly different between the three mosquitoes at $p < 0.05$ and noticeably lower in *Aedes vittatus* (0.0000809465 ± 0.0000000779008) (Fig. 6.16). The frequency modulation graph (Fig. 6.17) showed higher frequency modulation in *Aedes vittatus*, while *Aedes aegypti* showed higher amplitude changes. *Aedes aegypti* and *Aedes vittatus* had 47 frequency peaks; however, *Aedes albopictus* had 37 peaks assessed.

6.5.2. Machine learning-based evaluation of fundamental frequency and other related parameters of the sound of mosquitoes:

The fundamental frequencies of dengue vector mosquitoes range between 463.21± 2.93 Hz to 568.61 ±3.87 Hz, being highest in *Aedes vittatus,* followed by *Aedes albopictus* (498.065 ± 6.99 Hz) and lowest in *Aedes aegypti*. These vectors can be isolated based on fundamental frequency (F ratio=1189.40, df=29, P value=0.001). Other acoustic features, including peak frequency, delta frequency, and delta time, showed significant differences between *Aedes aegypti*, *Aedes albopictus*, and *Aedes vittatus* (Table 6.7). *Aedes vittatus* mosquitoes showed higher peaks and delta frequency than others, while *Aedes aegypti* showed higher delta time, which addresses a noticeably longer buzzing period. The bioacoustic index of these three *Aedes* mosquitoes ranged from 3.27 ± 0.005 to 5.63 ± 0.002, being highest in *Aedes albopictus* and lowest in *Aedes aegypti*. *Aedes albopictus* showed the highest acoustic entropy, followed by *Aedes aegypti*, and the lowest in *Aedes vittatus*. These two eco-acoustic indices demonstrated statistically significant differences at P<0.05 among themselves (Table 6.8).

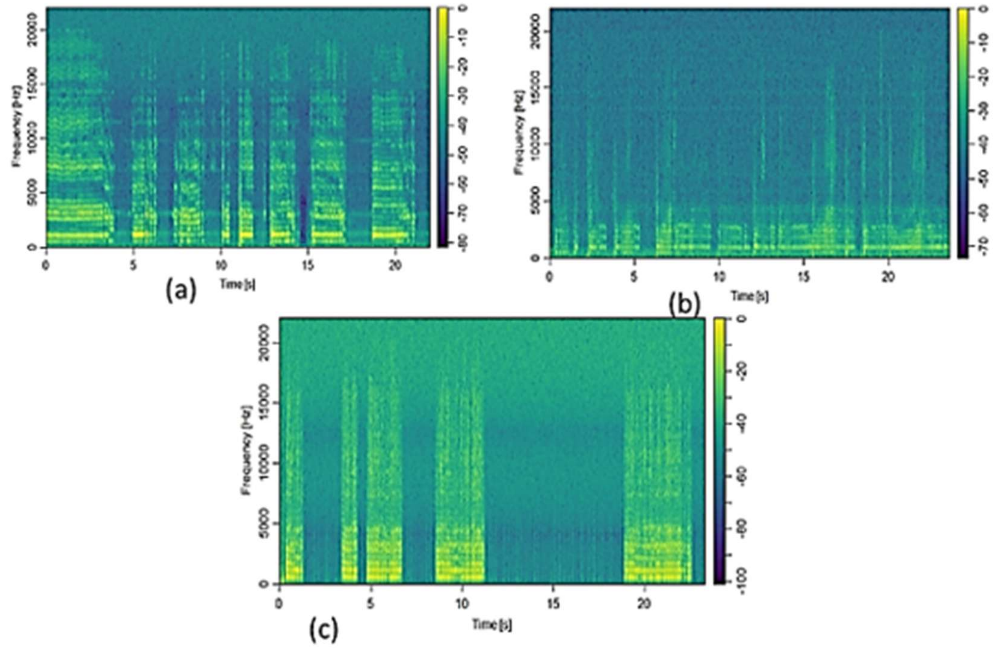


Fig. 6.15. Spectrograms of three mosquitoes; *a= Aedes albopictus, b=Aedes aegyti, c=Aedes vittatus*

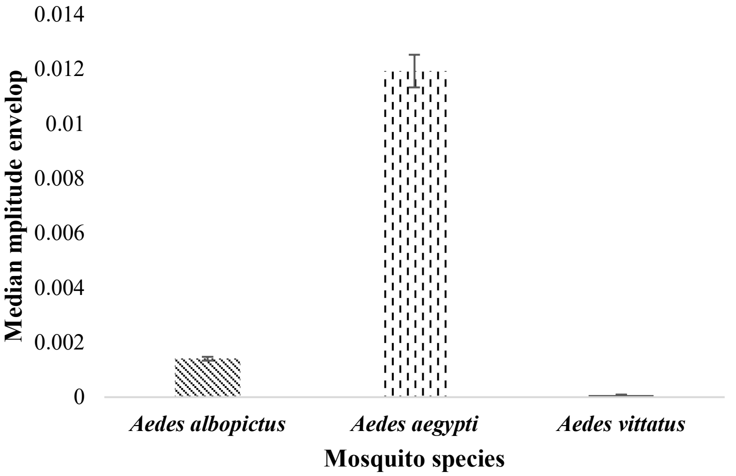


Fig. 6.16. Median amplitude envelop representation of wingbeat sounds of *Aedes albopictus, Aedes aegypti* and *Aedes vittatus*

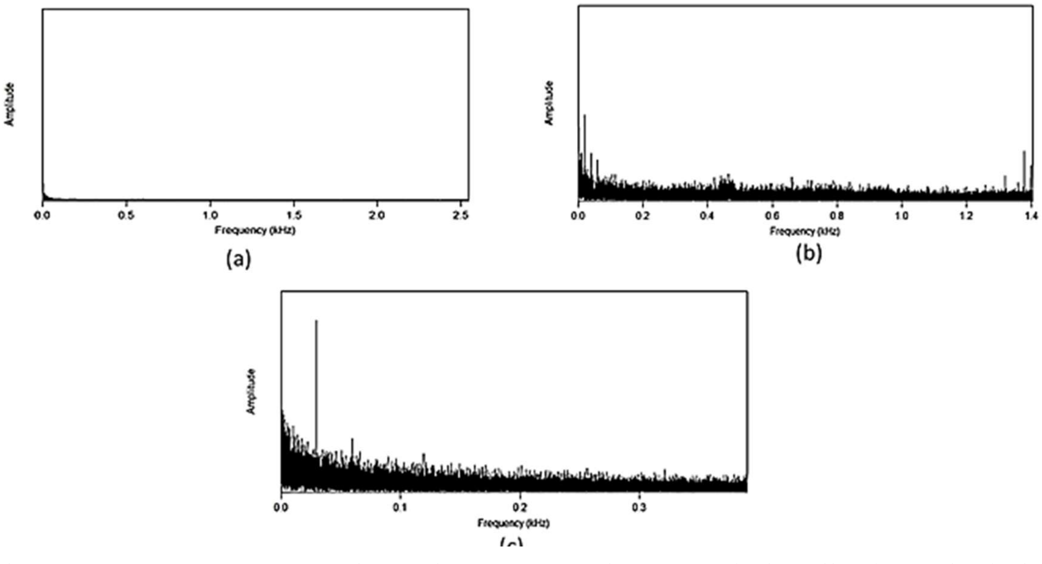


Fig.6.17. Frequency modulations of three mosquitoes; *a= Aedes albopictus, b=Aedes aegyti, c=Aedes vittatus*

Table. 6.7. Acoustic indices in 3 *Aedes* mosquito species

Species	**Acoustic indices**				
	Peak frequency	**Delta frequency**	**Delta time**	**Bioacoustic index (BI)**	**Acoustic entropy(H)**
Aedes albopictus	**564.289 ± 2.58**	**240.93 ± 9.71**	**2.19 ± 0.302**	**5.63 ± 0.002**	**0.748 ± 0.00065**
Aedes aegypti	**488.53 ± 2.31**	**201.42 ± 4.88**	**4.63 ± 0.062**	**3.27 ± 0.005**	**0.7644 ± 0.001**
Aedes vittatus	**566.52 ± 2.15**	**277.17 ± 6.91**	**2.0182 ± 0.14**	**4.48808 ± 0.048**	**0.6462 ± 0.0033**
One-way ANOVA	**F ratio 3539.66,** **df=29,** **P value=0.001**	**F ratio 259.37,** **df=29,** **P value=0.001**	**F ratio 554.12,** **df=29** **, P value=0.001**	**F ratio 17507.12,** **df=29,** **P value=0.001**	**F ratio=8311.98,** **df=29,** **P value=0.001**

6.5.3. Correlations between acoustic indices:

In *Aedes albopictus*, frequency peak is negatively correlated with fundamental mean frequency (*r(10)* = -0.6687, p=0.0448) and high frequency (*r(10)*= -0.7693, p=0.0210) while delta frequency is negatively correlated with low frequency, *r(10)* = -0.7285, p=0.0289. In *Aedes aegypti,* delta time is negatively correlated with delta frequency (*r(10)* = -0.6375, p=0.0474) and peak frequency, *r(10)* = -0.6332, p=0.0494 but positively correlated with low frequency, *r(10)* = 0.6791, p=0.0308, while delta frequency showed strong positive correlation with high frequency, *r(10)* = 0.7821, p=0.0075. The results of Spearman rank correlations also revealed that acoustic entropy is negatively correlated with delta time *r(10)*= -0.8309, p=0.012 in *Aedes albopictus* and with low frequency, *r(10)* = -0.6440, p=0.0445 in *Aedes aegypti.* In *Aedes vittatus,* delta time is negatively correlated with high frequency *(r(10)* = -0.6332, p=0.0494) (Fig.6.18).

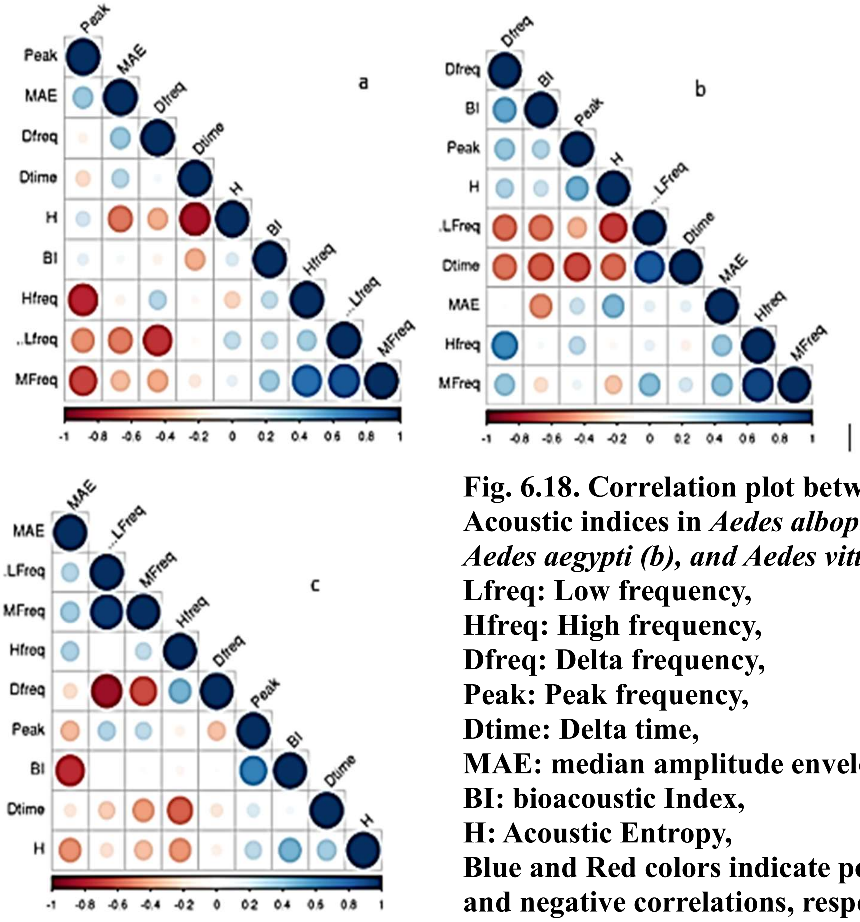


Fig. 6.18. Correlation plot between 7 Acoustic indices in *Aedes albopictus (a), Aedes aegypti (b), and Aedes vittatus (c).*
Lfreq: Low frequency,
Hfreq: High frequency,
Dfreq: Delta frequency,
Peak: Peak frequency,
Dtime: Delta time,
MAE: median amplitude envelop,
BI: bioacoustic Index,
H: Acoustic Entropy,
Blue and Red colors indicate positive and negative correlations, respectively.

6.5.4. Comparison of eco-acoustics between two different mosquito counts:

We constructed a comparison and correlation study of two acoustic indices to determine distinct changes in indices with the changes in mosquito counts. Comparing the Bioacoustic index (BI) and acoustic entropy (H) between sounds of solo and multiple *Aedes vittatus* mosquitoes with two-sample t-tests (Table 6.8) and found significant differences in eco-acoustics between them. The Spearman rank correlation test revealed a significant positive correlation between mosquito count and both eco-acoustic indices (Table 6.9).

Table. 6.8. Comparison of eco-acoustics between solo and multiple *Aedes vittatus*

Aedes vittatus count	Bioacoustic Index	Acoustic Entropy Index
Single	4.48808 ± 0.048	0.6462 ± 0.0033
Multiple	10.19 ± 0.017	1.14261404795 ± 0.0017
T test	t= -352.17, p=0.001	t=-413.634, p=0.001

Table. 6.9. Correlation between eco-acoustic indices and mosquito count *

Variables	Mosquito count	Acoustic Entropy (H)	Bioacoustic Index (BI)
Mosquito count		0.8681 (20) 0.0002	0.8674 (20) 0.0002
Acoustic Entropy(H)	0.8681 (20) 0.0002		0.8373 (20) 0.0003
Bioacoustic Index (BI)	0.8674 (20) 0.0002	0.8373 (20) 0.0003	
***The first value indicates the Correlation coefficient, the value in brackets represents the sample size, followed by the P-value.**			

6.5.5. Frequency-based surveillance:

To know the effect of different frequencies on male and female mosquitoes were subjected to prerecorded sounds of *Aedes aegypti* (458.95 Hz to 467.75 Hz) and *Aedes albopictus* (488.70 Hz to 530.25 Hz). The Spearman rank correlation test could not reveal any significant correlation between sound frequency and the activity of mosquitoes (Table 6.10).

Table. 6.10. Correlation between Attraction, Repulsion, and Frequency *

Variables	**Attraction of male count**	**Frequency (Hz)**	**Repulsion of the female count**
Attraction of male count		-0.0377 (20) 0.8693	-0.4098 (20) 0.0740
Frequency	-0.0377 (20) 0.8693		-0.0327 (20) 0.8868
Repulsion of the female count	-0.4098 (20) 0.0740	-0.0327 (20) 0.8868	
***The first value indicates the Correlation coefficient, the value in brackets represents the sample size, followed by the P-value.**			

6.5.6. Discussion

Dengue and Zika virus incidents are increasing worldwide. Current control methods have not stopped the spread of *Aedes* mosquitoes and diseases worldwide. This condition has led to a worldwide resurgence of dengue, Zika, and other mosquito-borne diseases, which in turn highlighted the urgent need for novel and sustainable control measures. The soundscape that is captured at a specific time and location is capable of reflecting the diversity in animals (Farina *et al*., 2021). Moreover, socio-ecological interactions can also be regulated using bioacoustic principles (Magrath *et al*., 2015). Acoustic-based monitoring also reveals behavioral aspects and ecological conditions in addition to species identification (Laiolo, 2010). The measurement of the acoustic features of mosquitoes indicated the technological feasibility of constructing an acoustic-based control strategy to detect individual mosquitoes and their populations. Previous studies predicted the mosquito species using fundamental wingbeat frequency as a metric and misidentified the mosquitoes as 26 out of 29 species demonstrated overlapped frequency (Kim *et al*., 2017). Hence, there is a need to incorporate other acoustic parameters for accurate classification of different mosquito species. Potential acoustic indices can help to develop proper and easy mosquito detection tools to mitigate the errors associated with similar WBF distributions. The present study addresses the utility of 11 acoustic indices to reflect a meaningful facet of the classification of mosquitoes and ecological conditions. Mean fundamental wing beat frequency distributions showed a narrower range in all three mosquitoes under this study than the recent attempts at wing beat frequency-based classification of mosquitoes by Kim *et al*. (2021). This variability may have resulted from capturing sounds through different sound recording equipment. Moreover, wingbeat sounds were recorded at 23.0 ± 0.5 °C in the studies of Kim *et al*. (2021), while we captured the mosquito sounds at 26 ±1ºC, hence there may be an impact of temperature on the acoustic characteristics of mosquitoes, which caused this variability.

In our study, the soundscape composition by 3 different mosquito species and their frequency distributions were found to be different and strong enough to distinguish based on those acoustic features. Three mosquitoes, viz, *Aedes albopictus, Aedes aegypti, and Aedes vittatus,* can also be differentiated using eco-acoustic indices. Although no efforts were made for noise cancellation, clear waveforms and spectrograms were visualized, which confirms that the device can minimize the noise. Spectrograms represented a data matrix of frequency, estimating the amplitude and frequency components within a specific time duration. Harmonic distributions showed obvious dynamic patterns, being highest in *Aedes albopictus* and the same in both *Aedes aegypti* and *Aedes vittatus*. Aldersley *et al*. (2014) recorded wingbeat tones of female *Aedes aegypti* mosquitoes and evaluated a similar distribution of the mean frequency of 480.6

± 32.5Hz. *Aedes aegypti* and *Aedes albopictus* represented similar patterns of frequency modulations. Delta time or the buzzing period of mosquito flight tone is a noticeable factor that has diverse influences on other acoustic indices. We also detected several indices that were insensitive to the changes in other acoustic parameters. We demonstrate the effect of mosquito count on the eco-acoustic indices of flight tones of *Aedes vittatus*. Both eco-acoustic indices increase with the increase in mosquito counts; hence, mosquito density can be assumed to estimate the bioacoustic index and acoustic entropy of their buzzing tone. The use of acoustic indices has been proven to be suitable for detecting changes in ecological conditions. A correlation between acoustic indices and ecological state is well demonstrated for the avian community (Sueur *et al*., 2008). There is an utter paucity of eco-acoustic studies on other animals and insects. At present, there lies a gap in incorporating similar acoustic sensor-based techniques in the surveillance and control of mosquitoes and the generation of ecological databases on mosquitoes (Ferguson *et al*., 2010). This study represents the eco-acoustic indices of mosquitoes, along with addressing major implications for ecological studies. Our analytical framework demonstrated a positive correlation between bioacoustic index and acoustic entropy. Previous studies mostly focused on establishing the relationship between acoustic indices with species richness (He *et al*., 2022; Sueur *et al*., 2008). This is the first attempt to systematically investigate the effect of mosquito count or abundance on the acoustic indices of buzzing sound in mosquitoes and matches the result of a previously performed acoustic-based biodiversity assessment by Aclocer *et al*. (2022). Moreover, we also addressed the relationship between eco-acoustic indices and acoustic parameters of wingbeats of mosquitoes. With the increase in the buzzing period of *Aedes albopictus*, acoustic entropy decreased, but this phenomenon was not observed in *Aedes aegypti* and *Aedes vittatus.* Acoustic indices show an overall positive correlation with insect richness and acoustic entropy was found to be negatively correlated with it while our studies demonstrated positive correlations between acoustic entropy and mosquito count. Studies indicated acoustic entropy as one of the best detectors of the ecological state of a soundscape. Detailed information on density, habitat associations, and geographic distributions of different mosquito species could be harnessed in machine learning algorithms for automated and semi-automated classification of a particular mosquito species. Along with ecological and geographic data, climatic influences on the acoustic features of mosquitoes should be addressed and incorporated while developing the codes for the most accurate prediction of mosquitoes.

CHAPTER 7. SUMMARY AND CONCLUSIONS

This research enlightens many parameters, such as the fundamental frequency, bandwidth of the fundamental frequency or 1st harmonic, diversity in harmonics, and amplitude of the fundamental frequency, along with the development of novel matrices for mosquitoes having a similar range of frequencies.

The fundamental sound frequencies of female mosquitoes of 19 species were evaluated. The fundamental frequencies exhibited a wide range from 269.09 ± 2.96 Hz - 567.51 ± 3.82 Hz. This study explored statistically significant differences in the fundamental frequencies between the mosquito species. Although base or fundamental frequency-based detection contributed to a more than 95% success rate for the differentiation among 19 mosquito species using multiple comparison tests, several species pairs could not be isolated using the base frequency features. The bandwidth or the delta frequency of the base frequency also acted as a crucial signature in differentiating the mosquito species. This feature also exhibited high accuracy in the detection of mosquito species, demonstrating statistically significant differences between 171 pairs of differentiation in 19 mosquito species. Furthermore, this also delved into the diversity in higher harmonic bands of harmonic frequencies. Higher harmonic frequencies showcased the band numbers consistently for each of the 19 species, which also indicated more than 84% success in the comparison. The amplitude was considered an efficient parameter to denote the differences between the mosquitoes in previous studies; however, it could not show a considerable success rate and isolated only 84 pairs.

The novel matrices were found to increase the success rate a bit higher than the previous detection harnessing base frequency. One metric combines the amplitude of the 1st harmonic and the highest or top frequency, while another incorporates the fundamental or 1st harmonic frequencies and the number of total harmonic bands. These matrices provided meaningful insights into the improvements in the capability of detecting species. The sounds generated by *Aedes albopictus* and *Aedes aegypti* were captured at the interval of three age periods: newly emerged, on the third day of emergence, and on the seventh day under standard laboratory conditions. Age-based studies revealed that newly emerged individuals exhibited a higher frequency than others, which also signified the negative effects of aging on acoustic features. However, these differences were not statistically significant. This study also provides evidence of the similarities in the frequency distributions between laboratory-reared and field-collected specimens. Acoustic entropy-based detection of mosquitoes has made the identification of mosquitoes easier, as it isolates the mosquitoes showing overlapped frequencies. Notably, the males showed higher fundamental frequencies than the female mosquitoes. Mosquitoes of both sexes exhibited statistically significant differences in base or fundamental frequencies. Additionally, the female blood-fed mosquitoes exhibited a significant increase in fundamental frequency in comparison with unfed females. *Aedes vittatus* exhibited higher modulations of sound frequencies upon reaching the vicinity of the opposite sex. A decrease in base frequency was noted in paired male-female mosquitoes. Furthermore, harmonic convergence was also reported in *Aedes vittatus* mosquitoes. Notably, the second harmonic of males and the third harmonic of females showed no statistically significant differences, indicating modulation of harmonic frequencies followed by frequency matching and copulatory success.

The molecular study of 19 mosquitoes delved into a variety of aspects of vector-borne disease research. This encompassed the meticulous procedures of Polymerase Chain Reaction analysis, along with the comprehensive steps of agarose gel electrophoresis, followed by the methodical approaches of Sanger sequencing for the sequencing of DNA. Five sequences of rare mosquito species among a total of 19 sequences were novel and the first report of COI DNA sequences. The remaining 14 sequences, along with 5 novel sequences, were submitted to the NCBI GenBank repository. Additionally, these molecular studies explored the differentiation of mosquito species based on DNA analysis. Correlation and description of purine and pyrimidine contents were also addressed and denoting the in-depth differentiation between AT and GC contents. The phylogenetic tree revealed the close relationship of the newly sequenced species *Hulecoetomyia fluviatilis* with mosquitoes of the *Aedes* genus. The phylogenetic tree also denoted the clusters of different species *Culex* genus. Mosquitoes of the *Anopheles hyrcanus* group also clustered together, indicating a close evolutionary relationship. This study offers a detailed evaluation of molecular information, addressing a wide array of biotechnological and molecular techniques and aiming to provide an in-depth understanding of the genetic and evolutionary relationships and significant variations between 19 mosquito species.

The effects of environmental and morphometrical factors were also well illustrated. Although body size and humidity did not show any impacts of bioacoustics, fundamental frequency varied significantly with a single-degree rise in temperature.

Lastly, in the 5th objective, the main goal of my thesis was obtained. The data matrices for acoustic-based mosquito identification tools were developed. Machine learning codes were modified for the analysis of sound parameters without the help of sound-analyzing software or applications. This study addressed the efficient tools for real-time large-scale analysis of acoustic indices of mosquitoes. A total of 11 acoustic parameters, including eco-acoustic indices, were evaluated and provided a database for the development of an AI model for further automated detection of mosquitoes. Previous studies attempted automated detection harnessing limited numbers of acoustic parameters, while this study provided detailed acoustic features along with a description and way of execution. Moreover, harmonics were not considered an important factor in earlier studies; however, this study has proven the efficiency of higher harmonics in the detection of species and understanding of the eco-acoustics of mosquitoes. This study meticulously explored the low-cost and real-time technology for mosquito surveillance without the help of any commercially available acoustic analyzer. This is the first report of detailed and comprehensive bioacoustics studies on a large number of mosquito species found in India, along with addressing novel acoustic parameters. This study is also the first evidence of harmonic tuning and copulatory success in the *Aedes vittatus* mosquito, which gives insights into mating control of these dengue vectors in an eco-friendly way.

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Appendices:

NVBDCP	National Vector Borne Disease Control Programme
COI	Cytochrome c oxidase subunit I
ITS	Internal transcribed spacer
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
HZ	Hertz
CDC	Centre for Disease Control
WBF	Wing Beat Frequency
RFM	Rapid Frequency Modulation
SPL	Sound Pressure Level
Mval	Mevalonic Acid Lactone
BG	Biogents
LT	Light Trap
L:D	Light: Dark
F0	Fundamental Frequency
ANOVA	Analysis of Variance
fdB	Amplitude of fundamenta frequency band
hbn	Harmonic band numbers
hbdB	Combination of amplitude of top harmonic band
LSD	Least significant difference
NCBI	National Center for Biotechnology Information
Lfreq	Low frequency
Hfreq	High frequency
Dfreq	Delta frequency
Peak	Peak frequency
Dtime	Delta time
MAE	Median amplitude envelop
BI	Bioacoustic Index
H	Acoustic Entropy
MW	Molecular weight
D	Dalton
SS	Single stranded
DS	Double stranded

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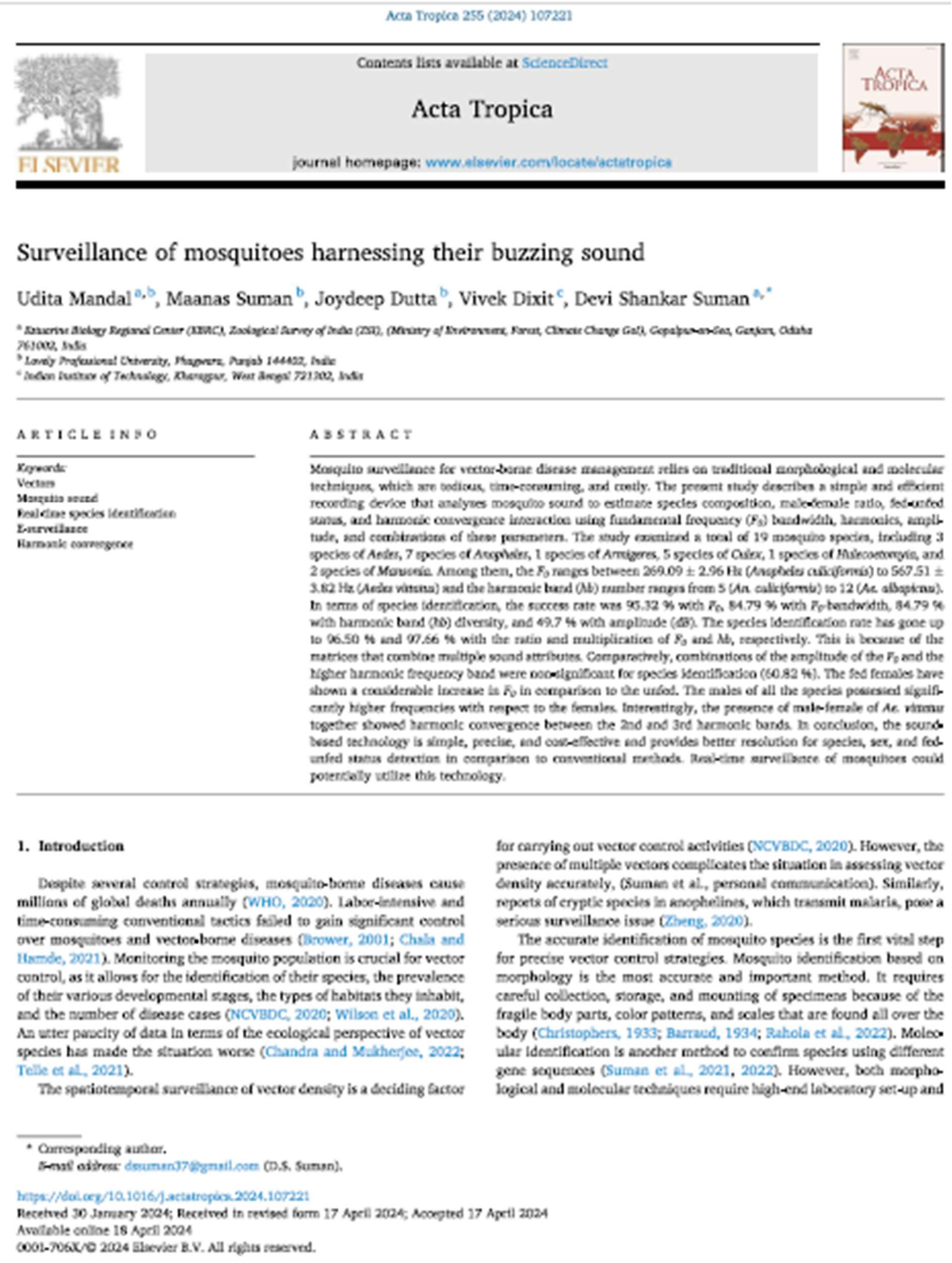

Acta Tropica 255 (2024) 107221

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Surveillance of mosquitoes harnessing their buzzing sound

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A R T I C L E I N F O

Keywords:
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Mosquito sound
Real-time species identification
E-surveillance
Harmonic convergence

A B S T R A C T

Mosquito surveillance for vector-borne disease management relies on traditional morphological and molecular techniques, which are tedious, time-consuming, and costly. The present study describes a simple and efficient recording device that analyses mosquito sound to estimate species composition, male-female ratio, fed-unfed status, and harmonic convergence interaction using fundamental frequency (F_0) bandwidth, harmonics, amplitude, and combinations of these parameters. The study examined a total of 19 mosquito species, including 3 species of *Aedes*, 7 species of *Anopheles*, 1 species of *Armigeres*, 5 species of *Culex*, 1 species of *Hulecoeteomyia*, and 2 species of *Mansonia*. Among them, the F_0 ranges between 269.09 ± 2.96 Hz (*Anopheles culiciformis*) to 567.51 ± 3.82 Hz (*Aedes vittatus*) and the harmonic band (*hb*) number ranges from 5 (*An. culiciformis*) to 12 (*Ae. albopictus*). In terms of species identification, the success rate was 95.32 % with F_0, 84.79 % with F_0-bandwidth, 84.79 % with harmonic band (*hb*) diversity, and 49.7 % with amplitude (*dB*). The species identification rate has gone up to 96.50 % and 97.66 % with the ratio and multiplication of F_0 and *hb*, respectively. This is because of the matrices that combine multiple sound attributes. Comparatively, combinations of the amplitude of the F_0 and the higher harmonic frequency band were non-significant for species identification (60.82 %). The fed females have shown a considerable increase in F_0 in comparison to the unfed. The males of all the species possessed significantly higher frequencies with respect to the females. Interestingly, the presence of male-female of *Ae. vittatus* together showed harmonic convergence between the 2nd and 3rd harmonic bands. In conclusion, the sound-based technology is simple, precise, and cost-effective and provides better resolution for species, sex, and fed-unfed status detection in comparison to conventional methods. Real-time surveillance of mosquitoes could potentially utilize this technology.

1. Introduction

Despite several control strategies, mosquito-borne diseases cause millions of global deaths annually (WHO, 2020). Labor-intensive and time-consuming conventional tactics failed to gain significant control over mosquitoes and vector-borne diseases (Brower, 2001; Chala and Hamde, 2021). Monitoring the mosquito population is crucial for vector control, as it allows for the identification of their species, the prevalence of their various developmental stages, the types of habitats they inhabit, and the number of disease cases (NCVBDC, 2020; Wilson et al., 2020). An utter paucity of data in terms of the ecological perspective of vector species has made the situation worse (Chandra and Mukherjee, 2022; Telle et al., 2021).

The spatiotemporal surveillance of vector density is a deciding factor for carrying out vector control activities (NCVBDC, 2020). However, the presence of multiple vectors complicates the situation in assessing vector density accurately, (Suman et al., personal communication). Similarly, reports of cryptic species in anophelines, which transmit malaria, pose a serious surveillance issue (Zheng, 2020).

The accurate identification of mosquito species is the first vital step for precise vector control strategies. Mosquito identification based on morphology is the most accurate and important method. It requires careful collection, storage, and mounting of specimens because of the fragile body parts, color patterns, and scales that are found all over the body (Christophers, 1933; Barraud, 1934; Rahola et al., 2022). Molecular identification is another method to confirm species using different gene sequences (Suman et al., 2021, 2022). However, both morphological and molecular techniques require high-end laboratory set-up and

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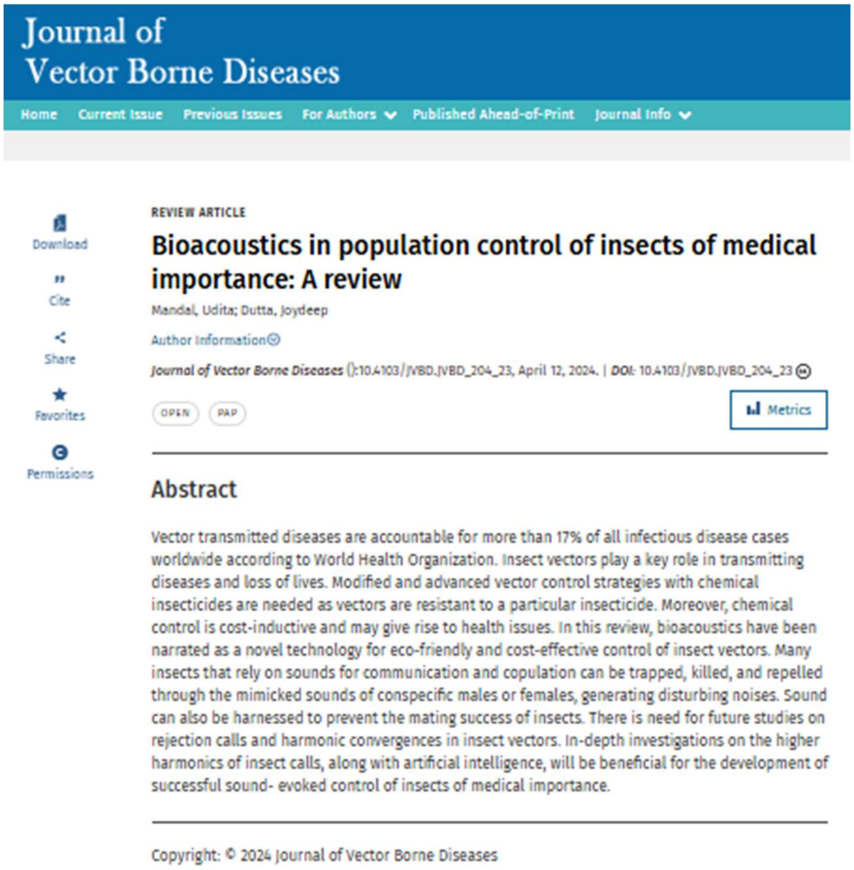

Journal of
Vector Borne Diseases

REVIEW ARTICLE

Bioacoustics in population control of insects of medical importance: A review

Mandal, Udita; Dutta, Joydeep

Journal of Vector Borne Diseases ():10.4103/JVBD.JVBD_204_23, April 12, 2024. | **DOI:** 10.4103/JVBD.JVBD_204_23

Abstract

Vector transmitted diseases are accountable for more than 17% of all infectious disease cases worldwide according to World Health Organization. Insect vectors play a key role in transmitting diseases and loss of lives. Modified and advanced vector control strategies with chemical insecticides are needed as vectors are resistant to a particular insecticide. Moreover, chemical control is cost-inductive and may give rise to health issues. In this review, bioacoustics have been narrated as a novel technology for eco-friendly and cost-effective control of insect vectors. Many insects that rely on sounds for communication and copulation can be trapped, killed, and repelled through the mimicked sounds of conspecific males or females, generating disturbing noises. Sound can also be harnessed to prevent the mating success of insects. There is need for future studies on rejection calls and harmonic convergences in insect vectors. In-depth investigations on the higher harmonics of insect calls, along with artificial intelligence, will be beneficial for the development of successful sound- evoked control of insects of medical importance.

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SHORT COMMUNICATION

Medical and Veterinary Entomology

Ecological variations in adult life table attributes of *Aedes aegypti* (L.) from the desert and coastal regions of India

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Abstract

The ecological variation in biological and adult life-table attributes of two populations of *Aedes aegypti* (Diptera: Culicidae) from the desert (Jodhpur) and coastal (Kolkata) regions of India are assessed to understand the reproductive and survival strategies. The results showed that females lived longer than males in both strains. The desert strain was more r-strategist because of its higher intrinsic rate of increase ($r_m = 0.23$), finite rate of increase ($\lambda = 1.25$), lower life expectancy of males (7.9 days) and females (14.4 days), mean generation time ($T = 19.2$ days) and doubling time (DT = 3.0 days). However, there was no difference in net reproductive rate (R_0) between the desert and coastal strains. The coastal strain showed a longer female life expectancy (22.0 days) than the desert strain (14.4 days). However, the fecundity (eggs/female/day) was lower in the coastal strain (11.4) than in the desert strain (15.1). Conclusively, the desert (Jodhpur) strain is adapted to a better r-strategy than the coastal (Kolkata) strain of *Ae. aegypti*, which might be helpful to flourish in harsh environmental conditions. This study may provide accurate predictions of *Ae. aegypti* population dynamics for vector management.

KEYWORDS
biological variation, ecology, mosquitoes, population dynamics, reproductive potential, survival

INTRODUCTION

Aedes aegypti (Linnaeus) is the primary vector responsible for various arboviral epidemics, such as dengue, chikungunya, Zika and yellow fever. Globally, 5.2 million cases of dengue have been reported in 2019, which was an 8-fold increase in the last two decades (World Health Organization [WHO], 2021). It is believed that *Ae. aegypti* has invaded other continents from Central Africa through human movements and global trade (Tabachnick, 1991). This mosquito occupied geographical areas between latitudes 35° N and 35° S, including all the ecological regions of India (Kalra et al., 1997), suggesting their great ability to thrive in a variety of climates.

Ecological parameters impact the population size and distribution due to the poikilothermic nature of mosquitoes (Paupy et al., 2003). Variations in life-history traits have shown the evolutionary adaptation in mosquitoes due to natural selection in response to native conditions (Reisen et al., 1979; Suman et al., 2011). MacArthur and Wilson (1967) mentioned that the *r*-strategist species mature quickly, have a shorter life span, a larger number of offspring, and are smaller in size. The Japanese strains of *Culex tritaeniorhynchus* were less r-selected in comparison to other Asian strains (Reisen et al., 1979). Suman et al. (2011) found that desert and semi-desert strains of *Culex quinquefasciatus* were more r-selected than the coastal strain. The altitudinal variations in life-history traits have also been reported in *Anopheles sacharovi* from Turkey (Yurttas & Alten, 2006).

There are fewer data reported on the biological parameters of *Aedes* mosquitoes. For example, three ecological populations of *Ae. aegypti* from Argentina were different in larval developmental time, larval-pupal survival and net reproductive rate (Grech et al., 2010). Crovello and Hacker (1972) have observed significant variations in life expectancy and reproductive potential among 13 strains of *Ae. aegypti* from feral and urban areas. These pieces of evidence call for further

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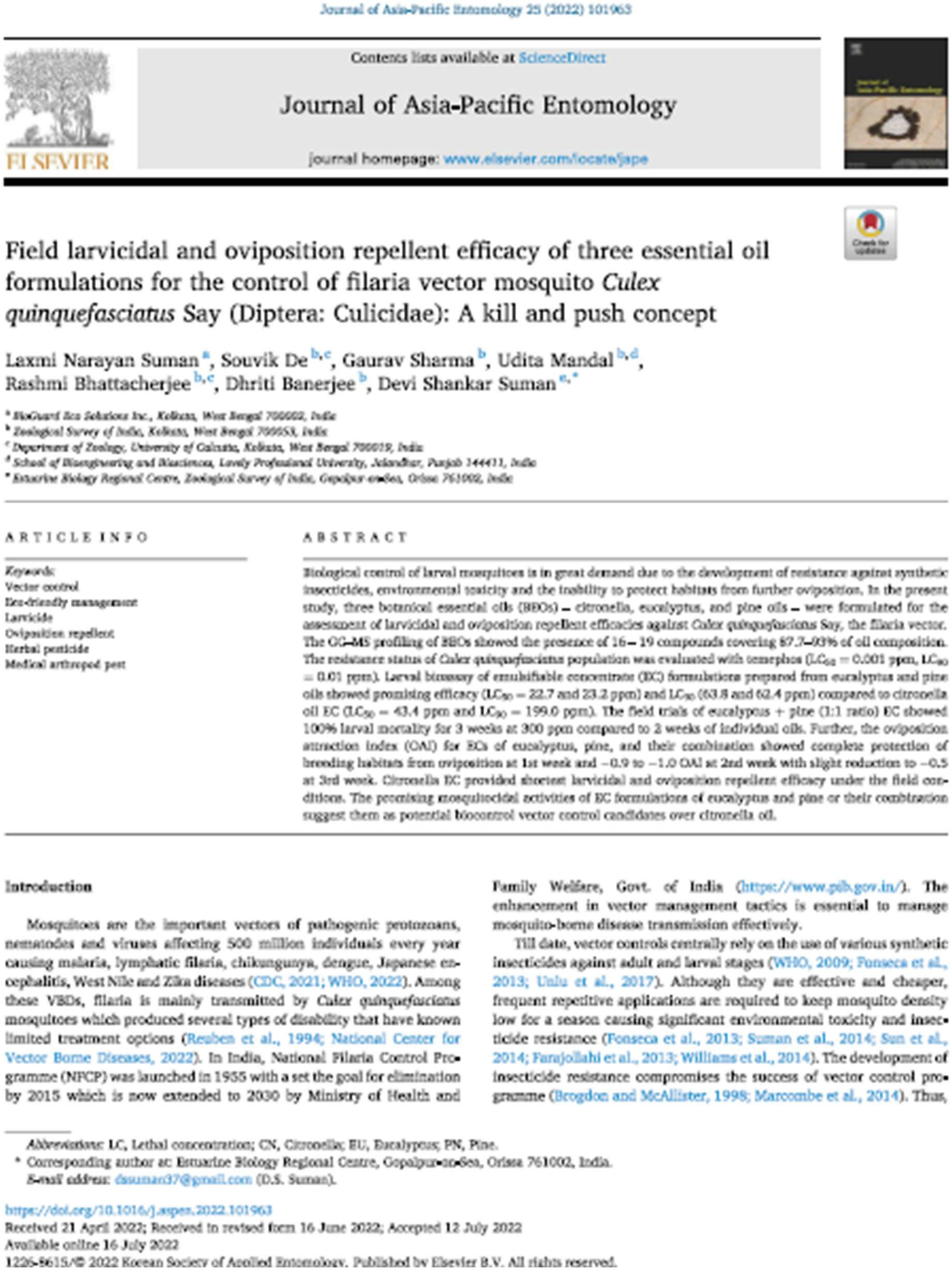

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Field larvicidal and oviposition repellent efficacy of three essential oil formulations for the control of filaria vector mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae): A kill and push concept

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ARTICLE INFO

Keywords:
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Eco-friendly management
Larvicide
Oviposition repellent
Herbal pesticide
Medical arthropod pest

ABSTRACT

Biological control of larval mosquitoes is in great demand due to the development of resistance against synthetic insecticides, environmental toxicity and the inability to protect habitats from further oviposition. In the present study, three botanical essential oils (BEOs) – citronella, eucalyptus, and pine oils – were formulated for the assessment of larvicidal and oviposition repellent efficacies against *Culex quinquefasciatus* Say, the filaria vector. The GC–MS profiling of BEOs showed the presence of 16–19 compounds covering 87.7–93% of oil composition. The resistance status of *Culex quinquefasciatus* population was evaluated with temephos (LC_{50} = 0.001 ppm, LC_{90} = 0.01 ppm). Larval bioassay of emulsifiable concentrate (EC) formulations prepared from eucalyptus and pine oils showed promising efficacy (LC_{50} = 22.7 and 23.2 ppm) and LC_{90} (63.8 and 62.4 ppm) compared to citronella oil EC (LC_{50} = 43.4 ppm and LC_{90} = 199.0 ppm). The field trials of eucalyptus + pine (1:1 ratio) EC showed 100% larval mortality for 3 weeks at 300 ppm compared to 2 weeks of individual oils. Further, the oviposition attraction index (OAI) for ECs of eucalyptus, pine, and their combination showed complete protection of breeding habitats from oviposition at 1st week and −0.9 to −1.0 OAI at 2nd week with slight reduction to −0.5 at 3rd week. Citronella EC provided shortest larvicidal and oviposition repellent efficacy under the field conditions. The promising mosquitocidal activities of EC formulations of eucalyptus and pine or their combination suggest them as potential biocontrol vector control candidates over citronella oil.

Introduction

Mosquitoes are the important vectors of pathogenic protozoans, nematodes and viruses affecting 500 million individuals every year causing malaria, lymphatic filaria, chikungunya, dengue, Japanese encephalitis, West Nile and Zika diseases (CDC, 2021; WHO, 2022). Among these VBDs, filaria is mainly transmitted by *Culex quinquefasciatus* mosquitoes which produced several types of disability that have known limited treatment options (Reuben et al., 1994; National Center for Vector Borne Diseases, 2022). In India, National Filaria Control Programme (NFCP) was launched in 1955 with a set the goal for elimination by 2015 which is now extended to 2030 by Ministry of Health and Family Welfare, Govt. of India (https://www.pib.gov.in/). The enhancement in vector management tactics is essential to manage mosquito-borne disease transmission effectively.

Till date, vector controls centrally rely on the use of various synthetic insecticides against adult and larval stages (WHO, 2009; Fonseca et al., 2013; Unlu et al., 2017). Although they are effective and cheaper, frequent repetitive applications are required to keep mosquito density low for a season causing significant environmental toxicity and insecticide resistance (Fonseca et al., 2013; Suman et al., 2014; Sun et al., 2014; Farajollahi et al., 2013; Williams et al., 2014). The development of insecticide resistance compromises the success of vector control programme (Brogdon and McAllister, 1998; Marcombe et al., 2014). Thus,

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Abbreviations: LC, Lethal concentration; CN, Citronella; EU, Eucalyptus; PN, Pine.

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Life table, survival and fecundity parameters of *Aedes albopictus* (Diptera: Culicidae) strains from desert and coastal regions of India

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A R T I C L E I N F O

Keywords:
Mosquitoes
Population dynamics
Survival
Fecundity
Ecological variations

A B S T R A C T

The Asian tiger mosquito, *Aedes albopictus* (Skuse, 1895) is the anthropophilic and day-biter mosquito, residing in peridomestic habitats and is responsible for vector-borne diseases, i.e., chikungunya and dengue. The knowledge of mosquito life demographics is very important to provide a foundation for a successful vector control program. In the present study, a comparative adult life table, survival, and fecundity parameters were evaluated on desert (Jodhpur, Rajasthan) and coastal (Kolkata, West Bengal) strains of *Aedes albopictus* (Diptera: Culicidae) from India under the standard laboratory conditions. The results showed significant differences between these strains for the life expectancy of males, fecundity (total eggs/cohort and eggs/female life span), oviposition days, intrinsic rate of increase (r_m), finite rate of increase (λ), and doubling time (*DT*). Female mosquitoes lived longer than the males in both strains. Significant differences were observed in the net reproductive rate (R_0) between Kolkata and Jodhpur strains. The coastal (Kolkata) strain showed a longer male life expectancy (5.6 days) than the desert strain (4.0 days). However, the fecundity (eggs/female lifespan) was higher in Kolkata (360.2) than the Jodhpur strain (229.7). The Kolkata strain showed higher R_0, r_m, λ, and the lower T, DT than the Jodhpur strain. Conclusively, the results indicate that the coastal strain of *Ae. albopictus* is more r-strategist than the desert strain. This study may help in the precise prediction of *Ae. albopictus* population dynamics surviving in these geographical areas for vector management.

1. Introduction

The Asian tiger mosquito, *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) are highly invasive, anthropophilic, and day-biter mosquitoes that reside in peridomestic habitats, serving as an important vector of arboviruses throughout the world (Hawley, 1988; Reinert et al., 2004; Gratz, 2004; Benedict et al., 2007). *Aedes albopictus* originated from South East Asia and now has spread in most of the world (Kraemer et al., 2015). *Aedes albopictus*, originally considered a secondary vector of dengue virus, has recently been suggested to also play a role in the transmission of chikungunya virus in several countries bordering the Indian Ocean, Europe, and Central Africa (Black et al., 2002; Gubler, 1998). In 2019, globally 5.2 million cases of dengue have been reported which was an 8-fold increase in the last two decades (WHO, 2021).

Many behavioural and physiological changes in mosquitoes have been reported previously under various conditions such as temperature exposure, food limitation, predator exposure, microbial infection, and breeding site disturbances (Day et al., 1994; Costero et al., 1998; Maharaj, 2003; Mohammed and Chadee, 2011; Grech et al., 2015). Ecological parameters also have a contribution to the population size and vector competence of mosquitoes (Paupy et al., 2003).

Over time, adaptation and ecological isolation to local conditions may lead to the development of geographically isolated strains that differ in various biological traits in fecundity, survivorship, and morphology (Reisen et al., 1979; Suman et al., 2009, 2011). Among different populations of *An. Sacharovi* (Favre, 1903) from Turkey, the altitudinal variations in life-history traits have also been reported (Yurttas and Alten, 2006). Different egg morphology and morphometrics of coastal *Cx. quinquefasciatus* (Say, 1823) strains have been reported from the desert and semi-desert strains in India (Suman et al.,

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Theme
Animals of Medical Importance: Mosquitoes

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From the Director's Desk

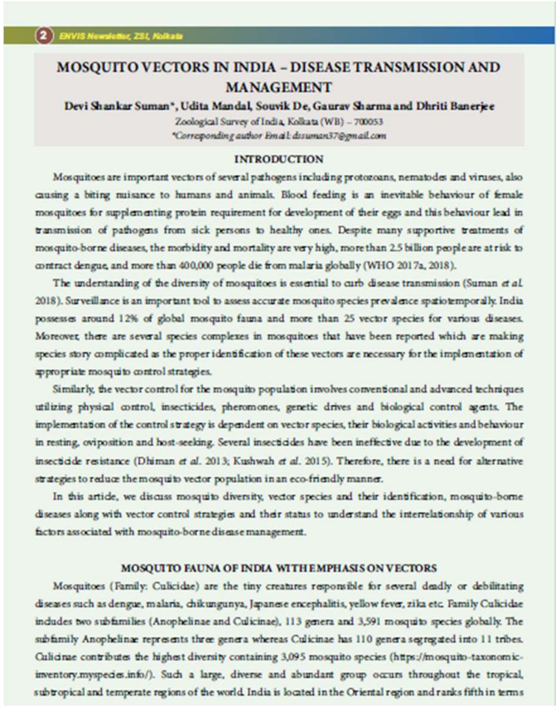
Mosquitoes are often listed as one of the most dangerous creatures, since they act as vectors of several deadliest diseases like dengue, malaria, filaria, japanese encephalitis and Zika. As per some reports by the WHO, almost 50 percent of the global population is at risk due to mosquito-borne diseases.

The mosquito control programs, often focus on reducing the population of mosquitoes in order to minimize the risk of disease spread. Use of insecticides like DDT though much popular during middle of the twentieth century is no more advocated, because of the adverse effects on the environment and as well as development of resistance in mosquitoes. To achieve an acceptable level of control, an integrated control strategy is often employed, comprising of several tactics including habitat management to reduce mosquito breeding, use of structural barriers like mosquito nests, and controlling both larvae and adult mosquitoes using moderate chemical applications.

The current issue of the ENVIS Newsletter has discussed in depth about the Mosquito vectors in India, along with their disease transmission and management.

Dr. Dhriti Banerjee
Director, ZSI

In this issue ....



2 *ENVIS Newsletter, ZSI, Kolkata*

MOSQUITO VECTORS IN INDIA – DISEASE TRANSMISSION AND MANAGEMENT

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INTRODUCTION

Mosquitoes are important vectors of several pathogens including protozoans, nematodes and viruses, also causing a biting nuisance to humans and animals. Blood feeding is an inevitable behaviour of female mosquitoes for supplementing protein requirement for development of their eggs and this behaviour lead in transmission of pathogens from sick persons to healthy ones. Despite many supportive treatments of mosquito-borne diseases, the morbidity and mortality are very high, more than 2.5 billion people are at risk to contract dengue, and more than 400,000 people die from malaria globally (WHO 2017a, 2018).

The understanding of the diversity of mosquitoes is essential to curb disease transmission (Suman *et al.* 2018). Surveillance is an important tool to assess accurate mosquito species prevalence spatiotemporally. India possesses around 12% of global mosquito fauna and more than 25 vector species for various diseases. Moreover, there are several species complexes in mosquitoes that have been reported which are making species story complicated as the proper identification of these vectors are necessary for the implementation of appropriate mosquito control strategies.

Similarly, the vector control for the mosquito population involves conventional and advanced techniques utilizing physical control, insecticides, pheromones, genetic drives and biological control agents. The implementation of the control strategy is dependent on vector species, their biological activities and behaviour in resting, oviposition and host-seeking. Several insecticides have been ineffective due to the development of insecticide resistance (Dhiman *et al.* 2013; Kushwah *et al.* 2015). Therefore, there is a need for alternative strategies to reduce the mosquito vector population in an eco-friendly manner.

In this article, we discuss mosquito diversity, vector species and their identification, mosquito-borne diseases along with vector control strategies and their status to understand the interrelationship of various factors associated with mosquito-borne disease management.

MOSQUITO FAUNA OF INDIA WITH EMPHASIS ON VECTORS

Mosquitoes (Family: Culicidae) are the tiny creatures responsible for several deadly or debilitating diseases such as dengue, malaria, chikungunya, Japanese encephalitis, yellow fever, zika etc. Family Culicidae includes two subfamilies (Anophelinae and Culicinae), 113 genera and 3,591 mosquito species globally. The subfamily Anophelinae represents three genera whereas Culicinae has 110 genera segregated into 11 tribes. Culicinae contributes the highest diversity containing 3,095 mosquito species (https://mosquito-taxonomic-inventory.myspecies.info/). Such a large, diverse and abundant group occurs throughout the tropical, subtropical and temperate regions of the world. India is located in the Oriental region and ranks fifth in terms