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Species specificity of acoustic signals of malarial mosquitoes of Anopheles maculipennis complex

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Abstract
Acoustic characteristics of three representatives of malaria mosquitoes of Palearctic complex *Anopheles maculipennis*: *An. messeae*, *An. maculipennis s.s.* and *An. atroparvus* were studied. It was found that the spectra of fundamental frequencies for males of all species is significantly higher than for females. *An. atroparvus* and *An. maculipennis* have close acoustic characteristics, which explains the cases of interspecific hybridization of these two species discovered earlier in nature by means of cytogenetic analysis. It was proven, that acoustic spectrum of *An. messeae* differs from the ones of other species. At the same time, the acoustic spectra of intraspecific groups of *An. messeae*, selected on the basis of high frequency of some karyotypes in populations have specific differences. Obtained results correlate with analogous research in groups other *Culicidae*. This makes it possible to consider acoustic communication of malaria mosquitoes as a basic mechanism of specific and intraspecific identification of a mating partner.

Keywords: bloodsucking mosquitoes, acoustics, sound frequency, harmonics, sexual behavior, sibling species, karyotypes, chromosomal inversions

1. Introduction
Acoustic signaling is widespread among insects primarily among flying forms. Acoustic features of insects play a crucial role in reproduction making it possible for species of different sexes to meet as well as providing reproductive isolation of closely related sympatric species [1]. A growing interest to acoustic communication of dipterous insects can be explained by the will to understand the most complex forms of their living activities as well as the necessity to control their behavior and population of vectors of serious transmissible diseases.
Malaria mosquitoes of the *Anopheles* (Diptera, Culicidae) genus are a subject of particular interest to biologists and medical services due to epidemiological significance of these insects. At the same time, this genus represents a perfect model for genetic and ecological analyses [2, 3]. Cytogenetic research of Palearctic malaria mosquitoes revealed several sibling species combined into *Anopheles maculipennis* complex. Some of these species are characterized by intraspecific inversion-chromosome polymorphism, which has an adaptive significance. Especially prominent in this case is widespread *An. messeae* Fall. [4], whose range covers nearly all Palearctics, encompassing diverse natural climatic zones.
Fluctuating inversions of *An. messeae* have wedge-shaped distribution and replace each other naturally in the area of species [4]. Evolutionarily initial versions of chromosomes - XL_0 (sex chromosome), $2R_0$ (right chromosome arm of the second autosomes), $3R_0$, $3L_0$ (right and left chromosome arms of the third autosomes) mainly take place in the southwestern regions of the Palearctic; inversion XL_1, XL_2, $2R_1$, $3R_1$, $3L_1$ - in the northeast; a high level of polymorphism is showed in the center of the area. Moreover, certain chromosomal combinations in populations are registered significantly more often than it was expected in accordance with frequencies of certain chromosome rearrangements [5, 6]. Conformity with this research we made an assumption that assortative mating (preferential mating of genetically similar mosquitoes) takes place in polymorphous hemipopulations of adult *An. messeae*. The five most widespread variants of inversion associations were selected in populations of the central part [5]:

1) $XL_{11}2R_{11}3R_{11(01)}3L_{00(01,11)}$;   2) $XL_{22(12)}2R_{11(01)}3R_{11(01)}3L_{00(01,11)}$;

3) $XL_{11}2R_{01}3R_{00(01,11)}3L_{00(01,11)}$;   4) $XL_{11}2R_{00}3R_{00(01,11)}3L_{00(01,11)}$;

5) $XL_{00(01)}2R_{00}3R_{00(01)}3L_{00(01)}$.

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These variants are conveniently divided into the following two groups according to areas in which they are predominant: "northern" (1), (2) and "southern" (4), (5). Karyotype (3) is produced as a result of hybridization of mosquitoes with "northern" and "southern" chromosomal inversions. Chromosomal polymorphism in *An. messeae* populations is maintained due to different fitness of mosquitoes with different chromosomal combinations during the annual cycle [3]. Mosquitoes with alternative karyotypes have different physiology, behavior, developmental rate, fertility and other characteristics [7, 8, 9, 10].

In spite of the fact that malaria mosquitoes are of specific interest to scientists the mechanisms of acoustic communication of this group are still understudied.

The objective of this work was to research acoustic mechanisms of identification and selection of a sexual partner among sympatric species of malaria mosquitoes of *Anopheles maculipennis* complex.

The problems of the research included: determination of acoustic characteristics of mosquitoes of different sexes within three model species: *An. messeae* Falleroni, *An. maculipennis* Meigen and *An. atroparvus* van Thiel; comparative analysis of sound frequencies of intraspecific karyotypic forms of *An. messeae* as well as investigation of possible relationship between acoustic characteristics and mating preferences.

2. Materials and methods

The material for this work were the adults of the three types of malarial mosquitoes. Two species – *An. messeae* and *An. maculipennis* – were represented by the progeny of females captured in natural biotopes; *An. atroparvus* mosquitoes were taken from a laboratory strain (the age of the line at the moment of the experiment ≈15 years). Reproductively active *An. messeae* females were collected in a cowshed in the village of Teguldet (Tomsk oblast') on 04.08.2010 (52°15' N 25°56' E). Insects were transported to Tomsk and on the next day each female was placed in a separate vial, quarter-filled with water for oviposition. After eclosion first-instar larvae were placed in plastic cups with 200 ml of tap water.

An. maculipennis mosquitoes were obtained from females specimens collected in a cowshed in the village of Merchitsy (Pinsk region, Brest oblast', Belarus, 52°15' N 25°56' E) on 15.09.2012. It should be noted that sympatric *An. messeae* and *An. maculipennis* are sibling species and morphologically indistinguishable in Europe, but they are well identified by their characteristic color eggs for each species, and cytogenetically on stage larvae and adults. Each female mosquito caught in village Merchitsy was planted in a separate bottle with water, and when she laid eggs, determined their species. Progeny of *An. maculipennis* was taken to Tomsk and was brought the imago.

Before the advent of pupae, larvae of all three species *Anopheles* were fed with a mixture of ground dried nettle leaves and oat flakes in a ratio of 1:1. The development is at air temperature 20-24 °C.

It should be clarified that only *An. messeae* is inversionly polymorphic from the three used species, and *An. maculipennis* and *An. atroparvus* are cytogenetically monomorphic. In this connection, five specimens of fourth-instar larvae *An. messeae* were selected from each progeny during the experiment; these larvae were fixed in alcohol-acetic mixture 3:1 for cytogenetic analysis. Temporary preparations of polytene chromosomes using laktoatsetorseinovoy methods were prepared from the salivary glands of larvae and karyotypes siblings were determined [11]. The rest of the larvae of the progeny developed to adult stage.

Acoustic characteristics were obtained for each mosquito 24 hours after reaching the adult stage. Before recording mosquitoes were placed in glass vials with a cotton plug and were anesthetized with ether. Then, the dorsal surface of mosquito's thorax was glued to the tip of the preparation needle with a medical adhesive (Russian brand "BF-6"), in order to keep the wings free. After 5-8 minutes mosquitoes, completely recovered from anesthesia, started to flap their wings while staying fixed on the tip on the needle (Fig. 1). Preparation needle with a mosquito was placed into a box with inner surfaces covered with foam rubber to provide sound insulation (internal parameters of box: 28×26×28 cm; the thickness of the foam rubber – 1.5 cm). Dynamic microphone (50-15000 Hz, 1 mV/Pa) (Fig. 2) was at a distance of 2 cm from the imago; microphone signal fed to the sound card Sound Blaster 1095. Free software Audacity® (registered trademark of Dominic Mazzoni) is used for determination the frequency characteristics of sounds and its graphic display.

Digital audio recording was performed for each mosquito for 20 to 60 seconds.

Acoustic characteristics were determined for 240 females and 76 males of *An. messeae*, 72 females and 29 males of *An. atroparvus*, 11 females and 16 males of *An. maculipennis*. All statistics processing was carried out at the significance level of 0.05.

Amplitudes of information signals and low-frequency noise in the spectrum are very often comparable particularly due to nonlinearity of amplifying equipment, therefore the values of fundamental frequency were identified and verified using frame-by-frame breakdowns of the video recordings of flapping *An. atroparvus* males and females recorded with Centurio C100 high-speed video camera (1700-2000 fps). This approach made it possible to match wing flapping frequency with the values of fundamental frequency.

Fig 1: Fixation of a mosquito in front of the microphone
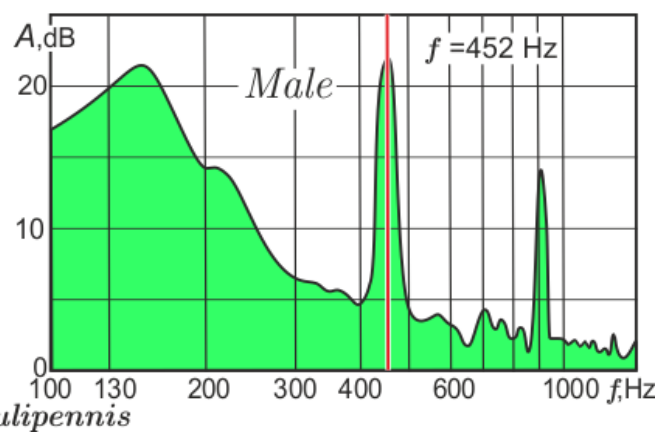
Fig 2: Sound chamber with a microphone

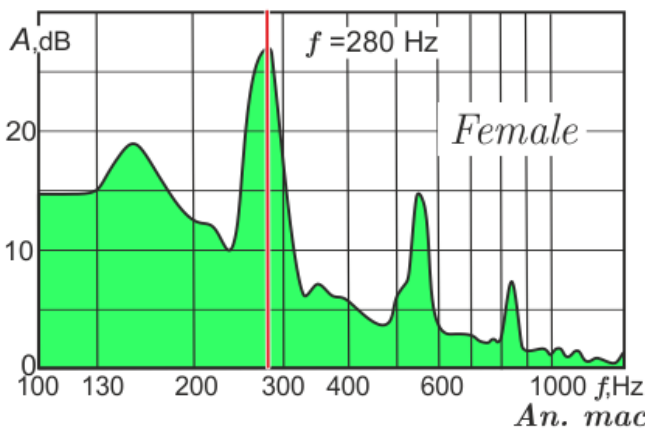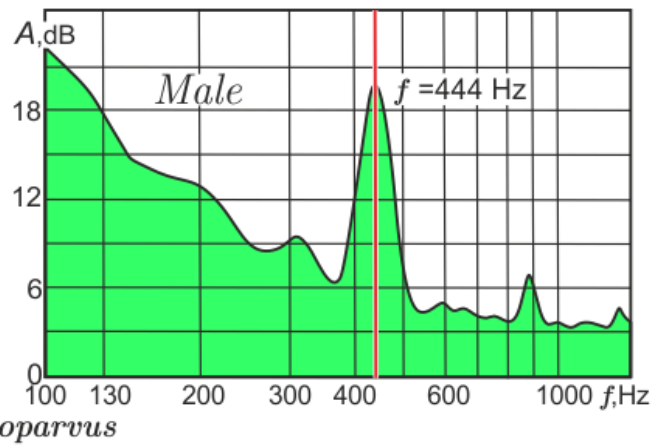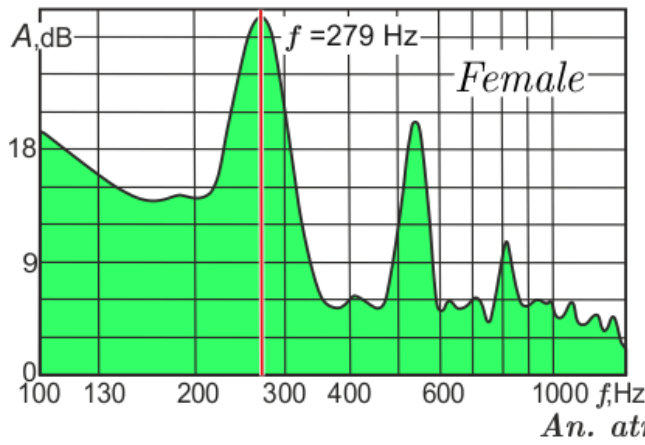## 3. Results and discussion

Sympatry of malaria mosquitoes *Anopheles maculipennis* complex in various combinations was observed in different Palearctic regions [3, 12]. Hence, reliable biological pre-copulative mechanisms providing meeting and identification of mating partners of mosquitoes of the same species must have been developed during their evolution. Since mosquitoes mate while flying and very often in the darkness hours [2], the acoustic signals of flapping wings are of important consequence for species identification in mixed swarms with multiple forms and (or) similar species (shown for the *Anopheles gambiae* complex [13]).

It was shown for the mosquitoes *Anopheles gambiae* complex [13], *Aedes aegypti* [14] and *Culex quinquefasciatus* [15] that both the fundamental frequency and harmonics having frequencies multiple of it in their acoustic spectrum play a crucial role in identification and selection of mating partner. Fundamental frequency and harmonics determine the individual tone of each insect. The research shown that the timbre is well over for males than for females of this species. Revealed regularities were right for the researching species of mosquitoes of the *Anopheles maculipennis* complex: the female buzz well "lower" than the males - the average frequency ratio of sexes is virtually identical for all three representatives ($\male/\female \approx 1.51 \div 1.65$; $\chi^2 = 0.73$; $df = 2$; $\chi^2_{crit} = 5.99$). Probably, these differences in flapping frequencies are determined by different size and weight of the body as well as geometry and surface area of wings.

Interspecific comparative analysis of acoustic characteristics of three representatives of the *Anopheles* genus regarding genders has indicated ambiguous differences in their frequencies.

Three peaks of acoustic spectrum were observed for *An. atroparvus* females (Fig. 3, *Female*): the first one with the highest amplitude – fundamental frequency (average value for a sample group $f = 293 \pm 5$ Hz, standard deviation $\sigma = 21$ Hz, number of specimens $n = 72$); the second and the third peaks – harmonics or overtones multiple of the value of the main peak (584±10 Hz, $\sigma = 41$ Hz and 880±15 Hz, $\sigma = 65$ Hz, respectively).
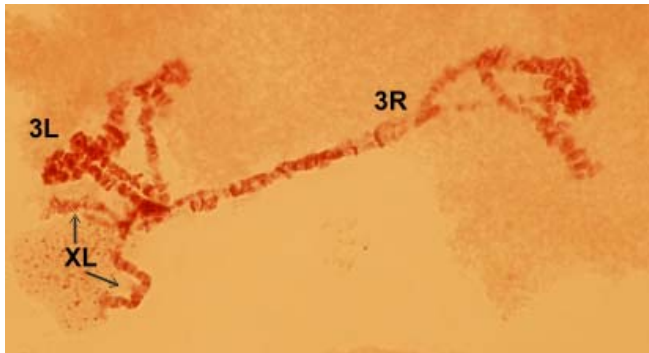

Fig 3: Typical acoustic spectra of sound frequencies of *An. atroparvus* and *An. maculipennis* (males and females). The red lines on the diagrams indicate fundamental frequency peaks

An. atroparvus males have two pronounced peaks (the third one is hardly distinguishable from background oscillations) (Fig. 3, *Male*): the first one (fundamental frequency), average value for a sample group $f = 444\pm6$ Hz, $\sigma = 15$ Hz; the second one – harmonic – average frequency 887 ± 12 Hz ($n = 29$). Thus, the average fundamental frequency for the male mosquito is ≈1.52 higher than for the female, harmonics have the corresponding difference.

The acoustic spectra of *An. maculipennis* females also have three peaks (Fig. 3, *Female*): the first one (fundamental frequency), average value for a sample group $f = 272\pm7$ Hz, $\sigma = 11$ Hz; the second and the third peaks (harmonics) – 548 ± 22 Hz, $\sigma = 32$ Hz and 820 ± 30 Hz, $\sigma = 45$ Hz, respectively ($n = 11$). *An. maculipennis* males as well as *An. atroparvus* males have two pronounced peaks in acoustic spectra (Fig. 3, *Male*): the first one (fundamental frequency), average value for a sample group $f = 448\pm11$ Hz, $\sigma = 20$ Hz; the second one (harmonic) – average frequency 890 ± 23 Hz, $\sigma = 43$ Hz ($n = 15$). The average fundamental frequency for the male mosquito is ≈1.65 higher than for the female, harmonics have the corresponding difference.

Thus, *An. atroparvus* and *An. maculipennis* have close acoustic characteristics (Tukey HSD Test showed nonsignificant difference for males), which explains the cases of interspecific hybridization of these two species. Individual interspecific hybrids at larval stage, captured earlier in natural biotopes of Moldavia (Balty) [16] and Kalmykia (Elista) [12], were discovered by means of cytogenetic analysis (Fig. 4).

Fig 4: Interspecific hybrid karyotype *An. atroparvus* × *An. maculipennis* (♀). The preparation is made from the salivary glands of the larvae of IV age who caught in the biotope Elista, Rep. Kalmykia. 3R and 3L – right and left arms of the third chromosome respectively; asynaptic homologues of XL sex chromosome are indicated with arrows.
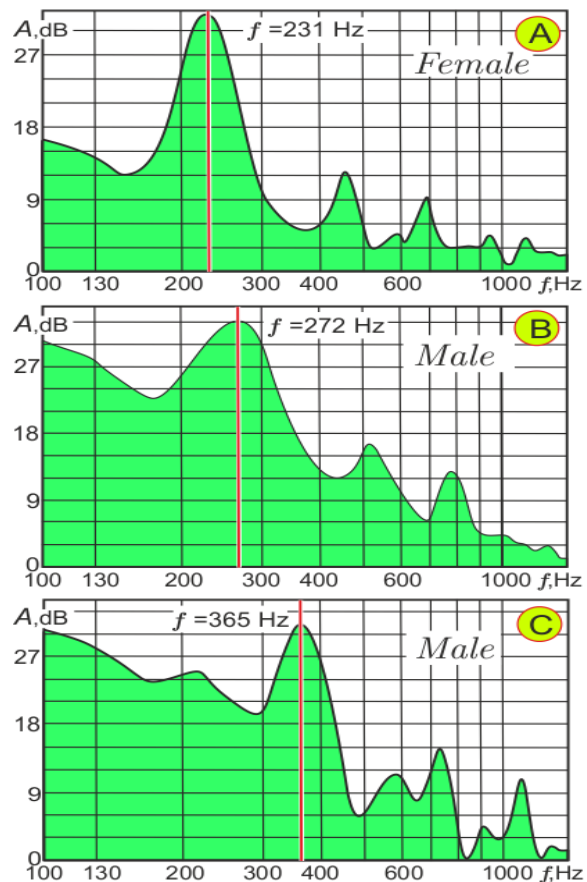
The discovery of natural hybrids *An. atroparvus* × *An. maculipennis* is a truly unique event for all species of *Anopheles maculipennis* complex. This phenomenon is also confirmed by the results of crossing of species in laboratory conditions by means of free copulation in holding cages as well as forced copulation [17, 18]. Laboratory hybridization of *An. atroparvus* × *An. maculipennis* have shown that F_1 males are completely sterile and F_1 females have shown some fertility signs. The females although they were capable of mating with *An. atroparvus* males, did not develop eggs. Obviously, the reproductive isolation between *An. atroparvus* and *An. maculipennis* which is manifested in sterility of hybrids is not supported by behavioral isolation. So we can draw a conclusion that there are no complete ethological isolation barriers for crossing of these species, which correlates with obtained sound frequency data.

Acoustic characteristics of the third of the species considered – *An. messeae* – significantly different from the ones for the two described above. Firstly, average frequency peaks for *An. messeae* by sex are well lower than the ones for *An. atroparvus* and *An. maculipennis* (Tukey HSD Test; Table 1; Fig. 5). Secondly, two typical variants of acoustic spectrum peaks were observed for *An. messeae* males: 1) three single pronounced peaks (Fig. 5 B); 2) the males of certain karyotypes have demonstrated typical double ("batch") peaks of overtones (Fig. 5 C). Thirdly, the range of fundamental frequencies for *An. messeae* males is significantly more varied than for representatives of other studied species. Interspecies comparison of their dispersions of the fundamental frequency by the Fisher's criterion shown in Table 1.

Table 1: Comparison of the values of the fundamental frequencies Tukey HSD test and their dispersions by the Fisher's criterion (F) from three species of *Anopheles*.

Compared pairs of species	Males		Females	
	HSD	F/Fcrit	HSD	F/Fcrit
An. messeae /*An. atroparvus*	P<0.01	6.43/1.75	P<0.01	3.07/1.35
An. messeae / *An. maculipennis*	P<0.01	3.56/2.21	P<0.01	1.25/2.56
An. atroparvus / *An. maculipennis*	nonsignificant	1.80/2.06	P<0.01	3.84/2.61

At the same time, dispersion of the fundamental frequency of the females *An. messeae* doesn't differ from the dispersion of *An. maculipennis* and significantly smaller than *An. atroparvus* (Table. 1). Probably a wide range of sound frequencies of male *An. messeae*, associated with intraspecific inversion polymorphism of this species.

Fig 5: Typical acoustic spectra of *An. messeae* female and males

Statistical analysis using Tukey HSD Test revealed significant differences for main frequency peaks of males with different karyotypes the mostly meeting in the population center of the area. At the same time males have shown significant differences between all variants, especially between adult mosquitoes with karyotypes widespread or predominant in different parts of the area of species (Table. 2, karyotypes (1-2) and (4-5)). Females with different variants of the karyotype compared in pairs (Tukey HSD Test) and significant differences emerged between the last center frequency values (Table. 2): between the karyotypes (2) and (4), (5). At the same time, none of the consecutive pairs of karyotypes – from (1) to (5) – have shown significant differences. It should be noted that effects of associative interaction of chromosomal inversions are significantly more pronounced for males [5].

Table 2: Average frequencies of main peaks of acoustic spectrum of adult *An. messeae* with inversion combinations for XL- and 2R-arms

No. of karyotype	Inversion combinations	1st peak, Fundamental frequency	2nd peak, 1st harmonic	3rd peak, 2nd harmonic	n
Males					
1	XL_12R_{11}	363±5	726±7	1095±14	38
2	XL_22R_{11}	380±6	758±6	1140±15	10
	XL_22R_{01}	326±6	652±10	970±17	8
4	XL_12R_{00}	301±1	600±2	888±5	12
5	XL_02R_{00}	261±7	513±4	780±17	8
Females					
1	$XL_{11}2R_{11}$	227±2	447±4	672±6	173
2	$XL_{22(12)}2R_{11}$	216±2	427±6	641±8	46
3	$XL_{11}2R_{01}$	217±4	439±12	657±27	6
4	$XL_{11}2R_{00}$	205±4	409±4	594±5	6
5	$XL_{01}2R_{00}$	208±3	405±7	607±5	9

Note: variants of inversions for two homologues in a zygote are marked in karyotypes with double subscripts for each arm of the corresponding chromosome. Male sex chromosome XL consists of only one homologue and therefore is marked with a single subscript. The frequencies of the first peaks in "batches", typical for certain karyotypes are not given. The third chromosome is not represented because all variants of zygotes for both arms of this autosome were found among the sibs of studied progenies.

It should also be noted that in pairs of karyotypes predominant in different regions of the area of species only one of cytogenetic forms of males have distinct "batches" of overtones: mosquitoes with karyotypes $XL_12R_{11}3R_{01(00,11)}3L_{00(01,11)}$ – for the pair of "northern" variants (1) and (2) and mosquitoes with karyotypes $XL_12R_{00}3R_{00}3L_{00}$ – for the pair of "southern" variants (4) and (5). Probably, these differences in frequency spectra were fixed during evolution and determine additional features of sound signals of males in coexisting subpopulation groups.

Considering the fact that it is males with different inversion combinations have different acoustic characteristics it is obvious that females of *An. messeae* play the main role in mating partner selection. It is known that *Anopheles* males gather in swarms in the nighttime to amplify the sound in order to attract females [2]. It is still an open question how females identify their mating partners, for karyotypic structure of swarms for representatives of complex *Anopheles maculipennis* is still not studied. Two options are possible: either the swarm predominantly consists of males with certain karyotypes and the female identifies it remotely; or the swarm is a polymorphous hemipopulation of males, in this case the female selects its male partner while in the swarm. Some African mosquito species of complex *Anopheles gambiae* shown different variants of swarm composition in different biotopes [13].

Acoustic characteristics of adult *An. messeae* indicate that mosquitoes with higher frequency are predominant in northern parts of the area of species, while "southern" forms are characterized by lower frequencies. So we can make an assumption that adult mosquitoes predominant in the northern parts with relatively short summer period have larger size which makes it possible for females to lay more eggs and provide rapid boost of population (r-strategy) [10]. "Southern" mosquitoes are characterized with smaller size and lower fertility (K-strategy). In this case larger "northern" mosquitoes must flap their wings more rapidly (other parameters being equal) and therefore have higher values of sound frequency.

Another interesting fact is that adult mosquitoes of *An. messeae* with the sets of inversions typical for western part of the area of species have the most significant differences in acoustic characteristics from other studied species – *An. atroparvus* and *An. Maculipennis*. This can be explained by sympatry of *An. messeae* with these species in European part of Palearctic regions. It should be added, that hybrids of *An. messeae* and *An. atroparvus* as well as hybrids of *An. messeae* and *An. maculipennis* are not found in nature.

Thus, obtained results for acoustic characteristics of closely related species of malaria mosquitoes make acoustic communication to be considered as a basic mechanism of specific and intraspecific identification of a mating partner.

4. Acknowledgments

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