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Relaying aversive USVs alarm calls depends on the previous experience. Empathy, social buffering or panic?

Wiktoria Karwicka ¹, Marta Wiatrowska ², Kacper Kondrakiewicz ², Ewelina Knapska ², Miron Kurska ³ and Adam Hamed ^{1,*}

¹ Laboratory of Spatial Memory, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Street, Warsaw 02-093, Poland;

² Laboratory of Emotions Neurobiology, BRAINCITY - Centre of Excellence for Neural Plasticity and Brain Disorders, Nencki Institute of Experimental Biology, Polish Academy of Sciences Warsaw, Poland;

³ Interdisciplinary Centre for Mathematical and Computational Modelling, University of Warsaw, Pawinskiego 5A, 02-106 Warsaw, Poland;

* Correspondence: a.hamed@nencki.edu.pl

Abstract: Ultrasonic vocalizations (USVs) are one of the evolutionarily oldest forms of animal communication. In order to study the communication architecture in an aversive social situation we used a behavioral model in which one animal, the observer, is witnessing as his cagemate, the demonstrator, is experiencing a series of mild electrical foot-shocks (aversive stimuli). We studied the effect of foot-shocks experience in the observer and the influence of a warning sound (emitted shortly before the shock is applied) on USVs communication. These experiments revealed that such warning seems to increase the arousal level, which differentiates the responses depending on previous experience. It can be identified by the emission of characteristic, short 22-kHz calls, of a duration below 100 ms. Furthermore, by analyzing temporally overlapping USVs, we found that in 'Warned' pairs with a naive observer, 22-kHz were mixed with 50-kHz calls. This fact, combined with a high fraction of very high-pitched 50-kHz calls (over 75-kHz), suggests the presence of the phenomenon of social buffering. On the other hand, in 'Warned' pairs with an experienced observer, pure 22-kHz overlaps were mostly found, signifying possible fear contagion with distress sharing. Hence the importance of differentiating 22-kHz calls to long and short.

Keywords: ultrasonic vocalization; social buffering; 50-kHz calls; 22-kHz calls; distress; emotional contagion; fear contagion; aversive state; communication;

1. Introduction

Ultrasonic vocalizations (USVs) of animals are one of the evolutionarily oldest forms of communication [1]. Rats emit sounds varying in frequency, inaudible to humans, in a band above 18 kHz, reaching a frequency of up to 125 kHz. Most rodent vocal communication research focuses on appetitive sounds - in the so-called "50-kHz" band - that derive from a positive emotional state [2, 3]. Ultrasonic vocalizations ranging from 30 to 125 kHz can be induced by addictive substances [4–8], positive social interaction [9–15], the anticipation of reward [16, 17], and in response to a context associated with appetitive conditioning [4, 17–20].

However, rodents, just like humans, not only experience and show their positive states but also express negative emotions [21–23]. Jaak Panksepp, an author of the concept of affective neuroscience, classified seven basic emotions as a result of artificial stimulation of the mammalian brain: seeking, rage, fear, lust, care, panic/grief, and play [24].

"22-kHz" USV calls, with a frequency bandwidth of 18 to 28-kHz, serve as indicators of rats' negative emotional states such as distress, fear, or anxiety [25–28]. These aversive

types of emissions are usually observed in stressful or endangering situations such as the presence of a predator [29–32], aggressive behavior of other conspecific(s) [26, 33–35] or when exposed to negative stimulus [36–38]. Experiments using playback techniques have shown that exposure to 22-kHz calls elicits freezing and avoidance responses [39–41]. Several reports [21, 22, 35, 42] state that 22-kHz calls have a communicative function in a group of rats, and not merely an expression of negative emotions. Litvin *et al.* (2007) proposed a subdivision of 22-kHz calls based on the situation and purpose of their emission: “alarm cries” that are meant to warn conspecifics about the danger [39, 43] and “alarm cries” that are supposed to discourage the predator [29, 31, 32, 43]. In both cases, the USVs are risk assessment dependent and appear only if potential benefits outweigh the costs [43]. Interestingly, the documented functions of ultrasound emission in the 22-kHz band include producing alarm signals to protect the social group [44, 45]. Thanks to the modern technology of recording and analyzing USVs, we can determine the animal’s emotional state with increasing precision. Moreover, we can register sounds lasting even for a few milliseconds with greater accuracy and resolution. With such tools, we can determine the course of emotional states during behaviorally modulated social interaction.

The neurobiological foundations of ultrasonic vocalizations and their association with emotional states of rodents are still under investigation. It was demonstrated that stimulation of cholinergic neurons in the laterodorsal tegmental nucleus triggered 22-kHz calls [46]. Moreover, the previous research has shown that the mPFC plays an important role in generation of 22-kHz calls and lesion of this structure considerably or completely reduces or the number of this type of calls [12, 47, 48]. Dupin *et al.* (2019) investigated the relationship between electrophysiological data, respiration, and emission of USVs, suggesting that sequences of USV calls could result in a differential gating of information within the network of structures sustaining fear or anxiety behavior. Emission of 22-kHz ultrasonic vocalization calls converges with decreased in theta power and increased delta and gamma power in BLA, mPFC, and olfactory piriform cortex (PIR) [49].

In our past research, we noticed occasional 22-kHz signal amplification in pairs of rats. Based on that observation, we aimed at verifying the hypothesis that the experience of an electric shock in the past could change the communication architecture between two familiar rats. An in-depth examination of the communication architecture during stressful situations will explain why an observer might behave differently depending on previous experience.

To study the communicative function of 22kHz USVs, we used a behavioral model in which one animal, the observer, is witnessing as his cagemate, the demonstrator, is experiencing a series of mild electrical foot-shocks. Aversive stimuli in this model elicit 22-kHz vocalizations [50]. We focused on the observers' reactions to their familiar demonstrator aversive responses to foot-shocks. Previous works have shown that experience of aversive stimuli may modulate future empathetic responses of animals [51–53]. For instance, the previous studies showed that prior experience with foot-shocks increases the freezing of the observers in the model we used [54]. Thus, we compared vocalizations of the experienced and inexperienced observers. Besides, in half of the groups, we added in the protocol a 19-second 1.75-kHz audio signal, which was the foot-shock introduction (warning signal). This signal was intended to associate the external stimulus with the demonstrator's foot-shock. Simultaneously, this allowed us to investigate how the prediction of the stimulus affects the behavior of the demonstrator and the observer.

An essential part of the study design was to exclude the animals' aversive conditioning response. The electric shocks, intended to familiarize experienced observers with aversive stimuli, were administered in a different spatial context. The training cage differed in shape, lighting, smell, and sound from the one in which we performed the test.

In our study we aimed at (1) comparing USVs emissions between foot-shock experienced and inexperienced (naive) observer rats, (2) examining whether experienced observers amplify the 22-kHz aversive signal emitted by demonstrators, (3) testing whether the warning sound signaling foot-shocks will change communication between rats.

2. Materials and Methods

2.2 Animals

Experimentally naïve male Wistar rats (250–300 g at the beginning of the experiment) were used in the experiment. The animals were supplied by the Center of Experimental Medicine in Białystok, Poland. Subjects were randomly paired and housed together in standard home cages (43.0 × 25.0 × 18.5 cm). They were kept in standard laboratory conditions under a 12/12 light–dark cycle and with a free access to food and water. All experiments were carried out in accordance with the Polish Act on Animal Welfare, after obtaining permission (126/2016) from the First Warsaw Ethical Committee on Animal Research.

2.3 Experimental procedure

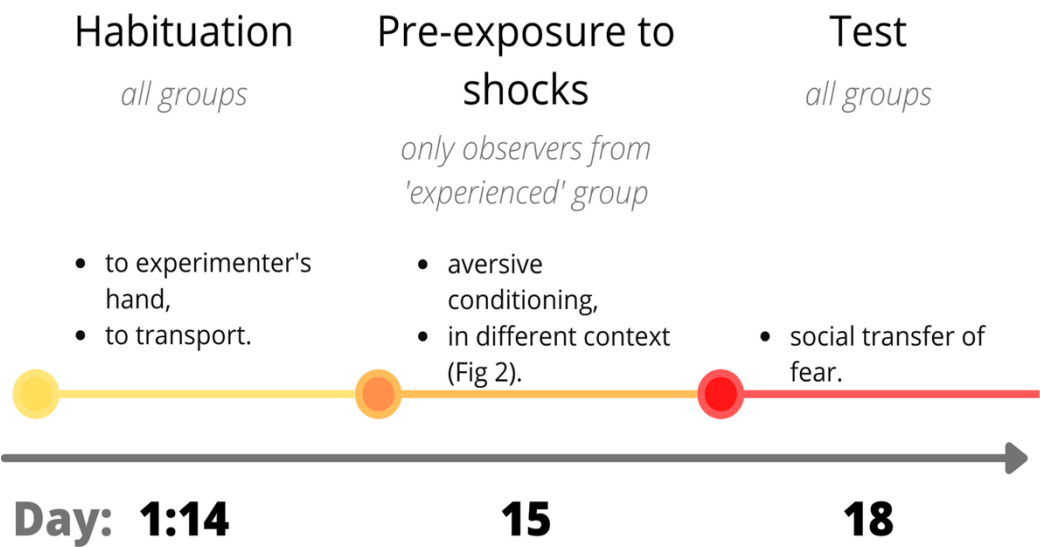


Figure 1. A graphic representation of experiment pipeline.

2.3.1 Habituation

After initial acclimatization to a home cage (4 days) the rats were habituated for the 10 days to the experimenter's hand (4-5 min / pair / day), to the experimental room (20 min for 3 consecutive days in dimmed light) and transport. Pairs of rats were randomly assigned to three experimental groups: control, naïve and experienced. Within each pair, rats were additionally marked either as a demonstrator or observer; this division was not necessary in the control group pairs as both of the rats went under the same procedure. The experiment was carried out in two groups - 'Warned' and 'Unwarned', the 3 subgroups that underwent each procedure consisted of the following number of animals:

1. naïve - 4 demonstrators and 4 observers,
2. experienced - 4 demonstrators and 4 observers,
3. control - 4 animals undivided into demonstrators and observers.

2.3.2 Pre-exposure to shocks

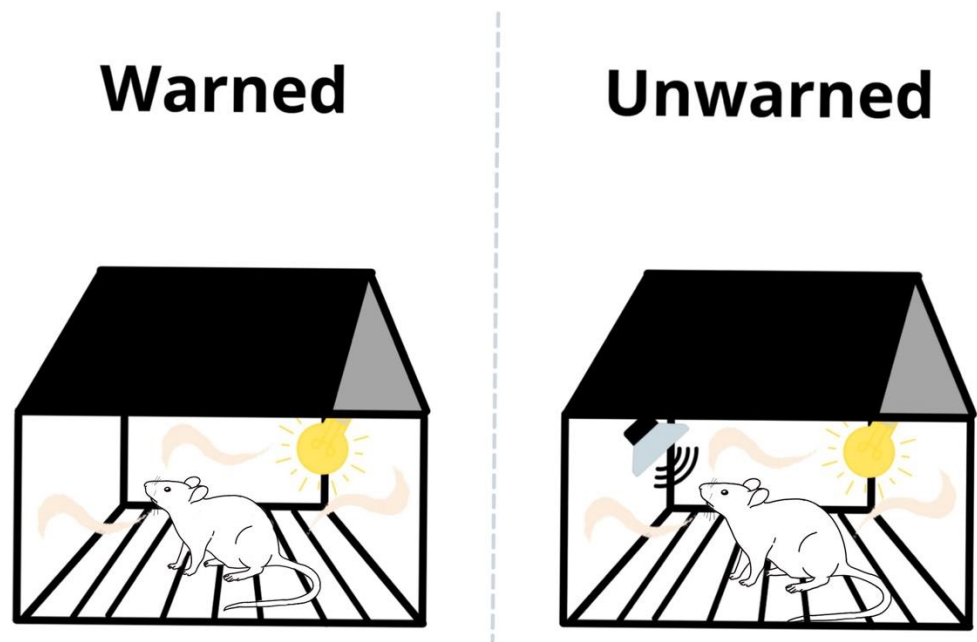


Figure 2. Illustration of the training: pre-exposure to the electric foot-shocks. To exclude the rats' aversive conditioning response the training cage differed from the test cage in terms of smell, light intensity and shape. Depending on the experimental group 'Unwarned' or 'Warned', a 1.75-kHz sound signaling a foot-shock was emitted or not respectively.

3 days prior to the experiment observers from the experienced subgroup were placed in the aversive conditioning cage (Panlab). After 1-minute habituation the rats received 3 electric shocks (with intensity of 0.7mA and 1 second length) with 1 minute time interval between each. To avoid aversive conditioning to the specific context following measures were taken:

- the exposure was performed in a different behavioral room than the following test,
- the cage was illuminated with a bright, white light,
- the interior was sprayed with 1% acetic acid, which left a strong smell,
- a plastic rooftop was installed to obtain triangular shape for different spatial cues.

In the unwarned group during the conditioning also a 1.75-kHz sound (19-s long) was emitted prior to each shock (Fig. 2).

2.3.2 Test day

On the 18-th day of the experiment the test was carried out in a specially constructed cage (31.2 x 25.4 x 26.7 cm) made out of a transparent plastic (details in [55]). A perforated transparent plexi glass divided the interior into two separate halves - one intended for the observer and the other for the demonstrator. Metal rods installed in the demonstrators' part were connected to the current generator (MedAssociates), which allowed the administration of mild electrical shocks. The rats could see, hear and smell each other throughout the test. During the test the animals were recorded with a digital camera and in case of the ultrasounds with 4 microphones (UltraSoundGate, Avisoft). The test session and the pre-exposure to shocks were conducted in separate rooms. To provide a different context the lights were dimmed and the cage was cleaned with acetone after testing each pair. After 2 minutes from inserting the rats to the appropriate chambers, the demonstrator was given a series of 10 foot-shocks (1-s, 1.0-mA). In the 'Warned' group each electric shock was signaled by a 19-second 1.75-kHz sound giving a total of 80-seconds between 2 shocks whereas in the 'Unwarned' group there was no sound indication and the shocks were administered in a 1-minute interval (Fig. 3).

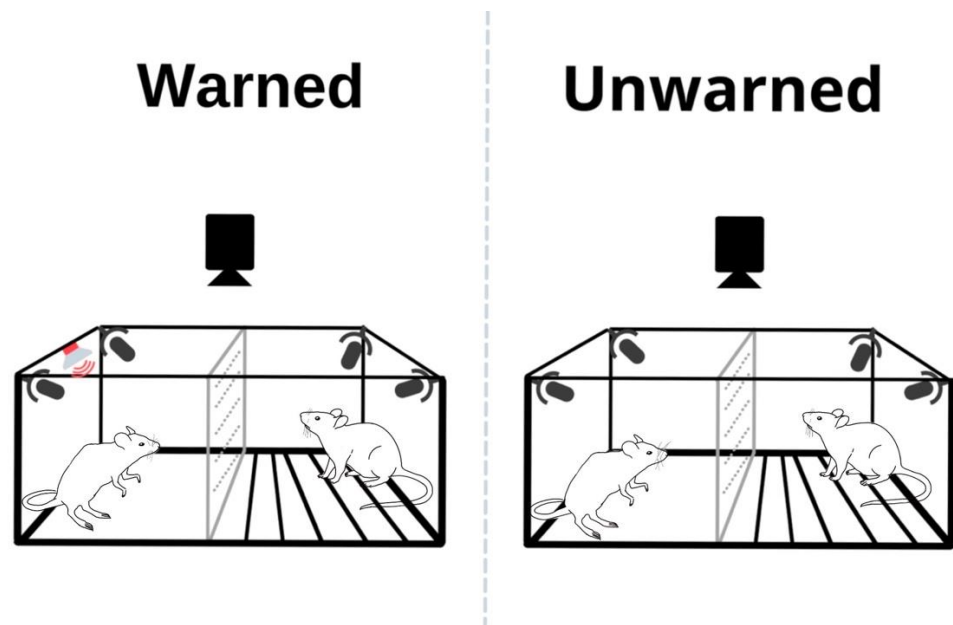


Figure 3. Illustration of the training: pre-exposure to the electric foot-shocks. To exclude the rats' aversive conditioning response the training cage differed from the test cage in terms of smell, light intensity and shape. Depending on the experimental group 'Unwarned' or 'Warned', a 1.75-kHz sound signaling a foot-shock was emitted or not respectively.

2.4 Apparatus and USV recordings

The all subtypes of 50kHz rat calls were recorded using an UltraSoundGate Condenser Microphone CM16 (Avisoft Bioacoustics, Berlin, Germany) that was positioned 25–30cm above the floor of the cage. Microphones were sensitive to frequencies of 15–180 kHz with a flat frequency response (± 6 dB) of between 25 and 140 kHz. It was connected to an amplifier (custom-made, Warsaw) that had the following parameters: a voltage gain of 16 V/V (12 dB), a frequency response of ± 0.1 dB, a range of 30 Hz to 120 kHz, and an input impedance of 600 Ω . The signal was then transferred through a 120 kHz anti-aliasing filter (custom-made, Warsaw). The filtered sounds were sent to a PCI-703-16A data acquisition board (Eagle Technology, USA). This board was a 14-bit 400-kHz analogue input and analogue output board for PCI-based systems. The recorded data were processed using the RAT-REC PRO 7.3 software (custom-made, Warsaw). The signals were processed through a fast Fourier-transformation (1024 or 512, Hamming or Hann window) and displayed as color spectrograms. Each signal was manually marked (Fig. *x*) with the section label included in the automated parameter measurement. Various parameters were determined automatically, including the number of USV calls, the total calling time (s), the mean call length (s), the frequency bandwidth (kHz), the number of gaps, the mean gap length (s), and the mean peak frequency (kHz). The signal from the microphone was sent to another room where the computer and observer were situated (more in [4, 10, 11]).

2.5 Statistics

For the assessment of the relationships between continuous and categorical variables, we have used the Kruskal-Wallis test, followed by the Conover-Iman post-hoc test for identification of precise pairwise differences. Significance level of 0.05 and two-sided testing was employed. All statistical analyses were performed in R, version 4.0.5, using conover.test package version 1.1.5.

3. Results

3.1. The effects of the warning signal. Fractions of the ultrasonic vocalizations during social communication in the social transfer of fear paradigm.

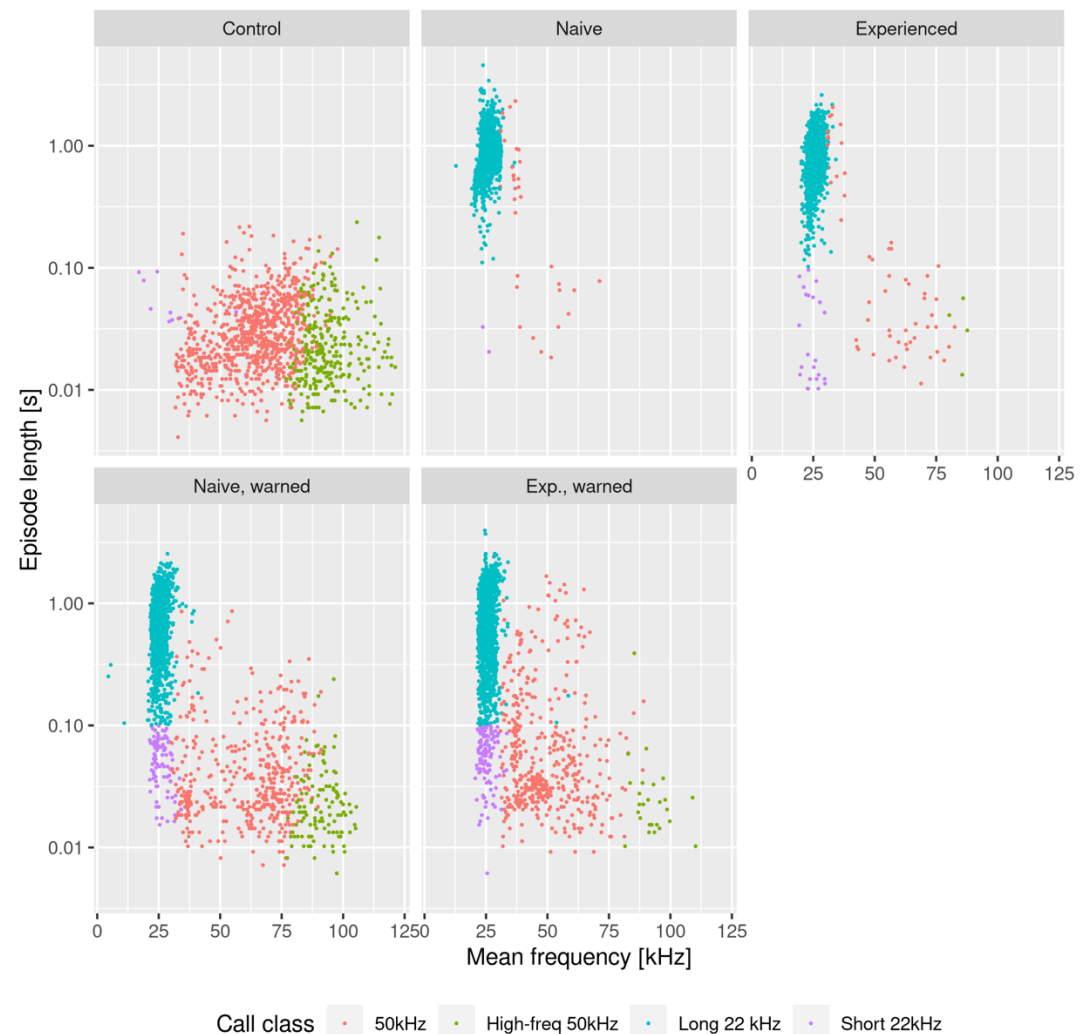


Figure 4. Scatterplot of basic properties of the recorded USV calls, mean frequency and length. Each panel collects all vocalizations recorded for pairs of a certain class. Color denotes the call fraction. Note that the length is shown on a logarithmic scale.

Figure 4 presents the joint distribution of length and frequency of every USV call considered in the paper, split into experimental groups. The various fractions of calls are clearly visible, so are quantitative and qualitative differences between them over the groups. The 22-kHz fraction is strongly present in all groups except control. Though, in 'Unwarned' groups, its length span is substantially limited, which corresponds to a lack of episodes to which we will later refer to as "short 22-kHz" calls.

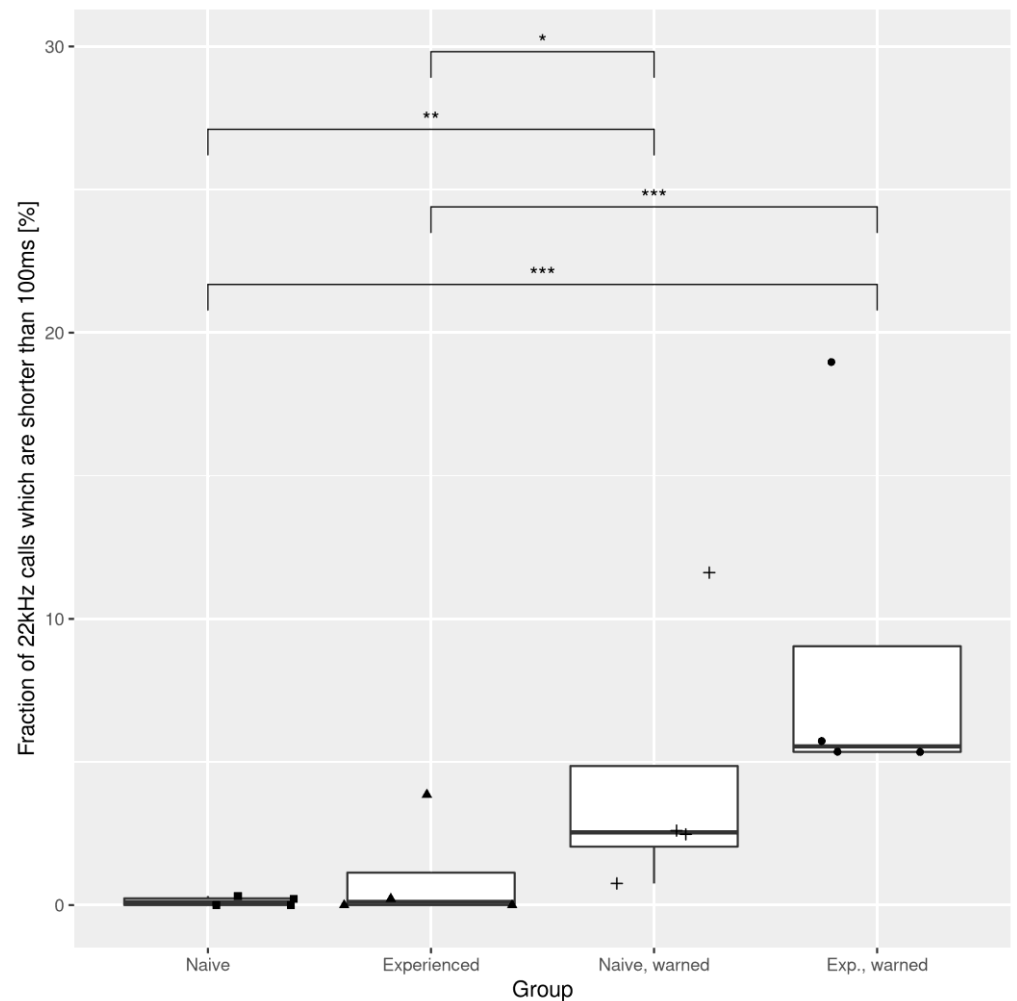


Figure 5. Fraction of short (<100ms) 22-kHz calls in different experimental groups. Brackets mark significant differences identified by the Conover-Iman test.

Figure 5 shows the quantitative difference together with a result of the Conover-Iman test; one can see that short 22-kHz calls are especially abundant in 'Experienced Warned' class, and significantly more numerous than in either of 'Unwarned' groups; similarly, also 'Naive Warned' group emitted significantly more short 22-kHz calls than aforementioned 'Unwarned' groups.

Going back to Figure 4, one can note that the 50-kHz calls build a sparse cluster with a wide span in both parameters. Again, it is less populated in the 'Unwarned' groups. The center of the 50-kHz cluster also varies between groups, which is reflected in the average frequency, as well as in the abundance of 50-kHz calls above the 75-kHz boundary, to which we will later refer to as "high frequency 50-kHz" calls (Figure 6).

Figure 6 shows the comparison of high frequency 50-kHz calls abundance between groups; as with the fraction of short 22-kHz calls, each of the 'Warned' groups exhibited significantly higher abundance than either of the 'Unwarned' groups. The highest fraction of high frequency calls was found in the 'Naive Warned' group, however, up to over 10% of all calls. The same conclusions can be drawn from Figure 8, which presents the mean frequencies of 50-kHz calls in each pair. Here, the 'Naive Warned' group generated the highest-pitched calls of all experimental groups, with median over 75 kHz. This result was similar to the achieved by the control pairs. Other than that, we can also see that among Unwarned groups, 'Experienced' rats produced higher USVs than 'Naive' ones.

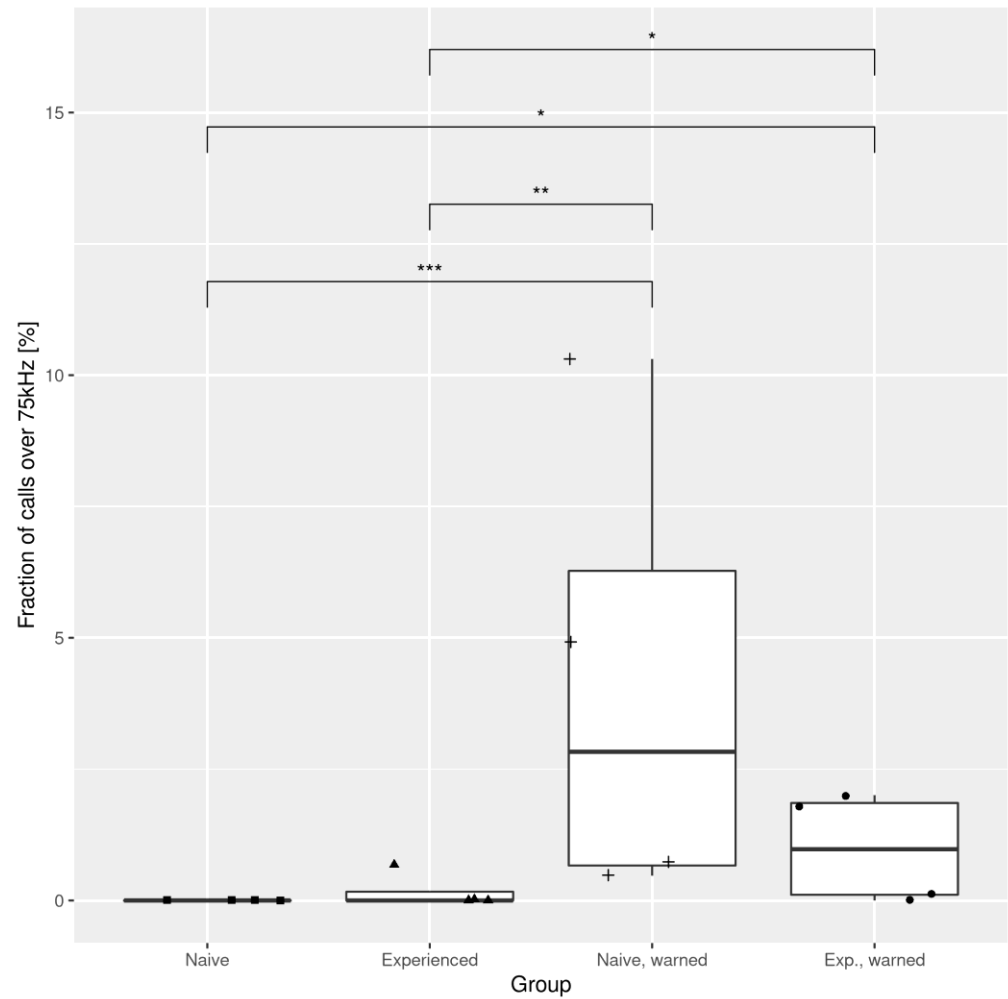


Figure 6. Fraction of 50 kHz calls with frequency over 75 kHz, in different experimental groups. Brackets mark significant differences identified by the Conover-Iman test.

3.2 *The effects of the warning signal. Overlapping of the ultrasonic vocalizations during social communication in the social transfer of fear paradigm.*

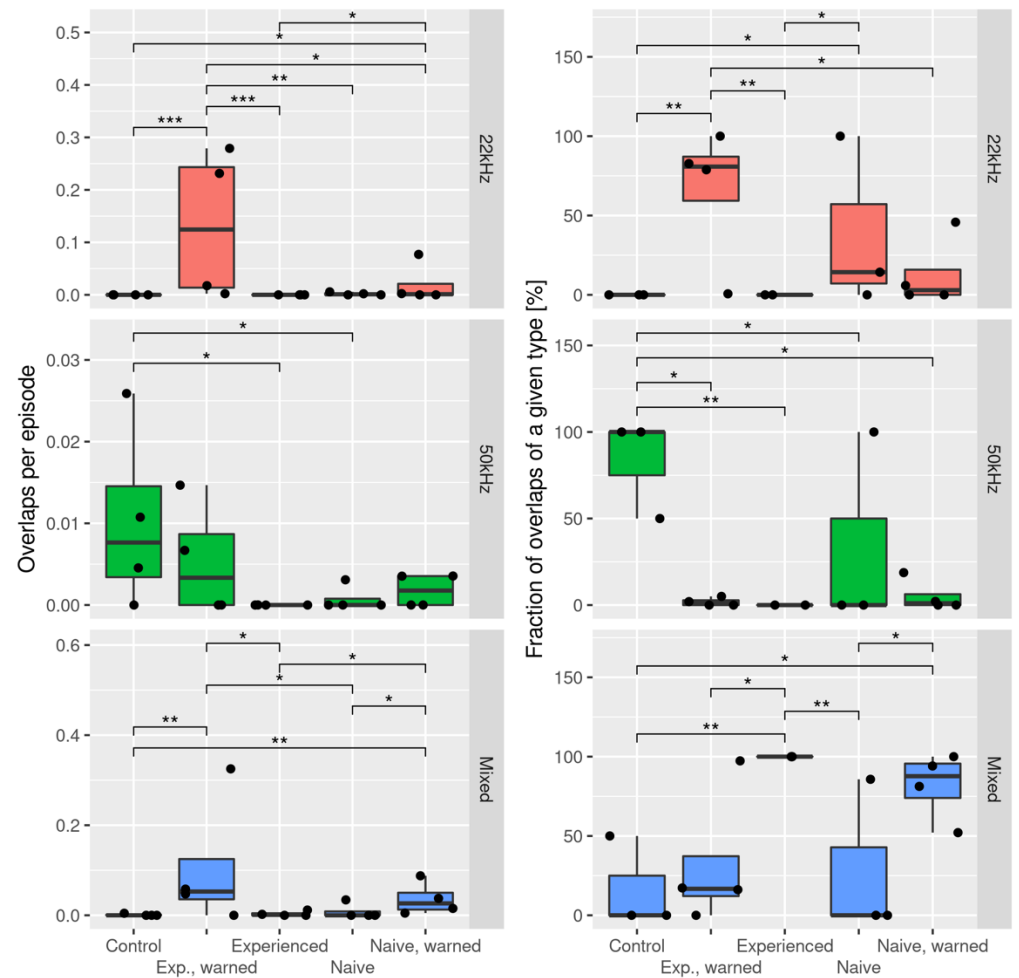


Figure 7. Fractions of temporal USV overlaps which belong to a certain class: 22kHz -- two 22 kHz calls overlapping, 50 kHz -- two 50 kHz calls overlapping, Mixed -- 22kHz and 50kHz call overlapping, in different experimental groups. Left panels show the count normalized by the episode count, while the right panels show the count normalized by the total count of temporally overlapping USV. Brackets mark significant differences identified by the Conover-Iman test.

Some of the reported USV calls were temporally overlapping, that is one coherent signal could be seen superimposed on another one, as can be seen on Figure 9. We have extracted all of such events and denoted the frequencies of each of two involved vocalizations. Using this key, overlaps can be divided into three types: a pair of 22 kHz calls, a pair of two 50kHz calls, and an overlap of 22 and 50 kHz calls, which we will later refer to as a mixed overlap. Figure 7 reports the distribution of said overlap types, both in relation to all recorded calls, and to the overall USV count.

One can see that 'Experienced Warned' rats have a substantial number of pure 22 kHz overlaps, up to almost 3 per 10 episodes in case of one pair. This is significantly higher than in any other experimental group.

In the 'Naive Warned' rats, the overlap rate was smaller than in 'Experienced Warned', by larger than in either of 'Unwarned' experimental groups. On the relative terms, the mixed overlaps constituted from about 50% up to 100% of all overlaps identified in this group.

Finally, in the control group, the most substantial type of overlaps were pure 50 kHz.

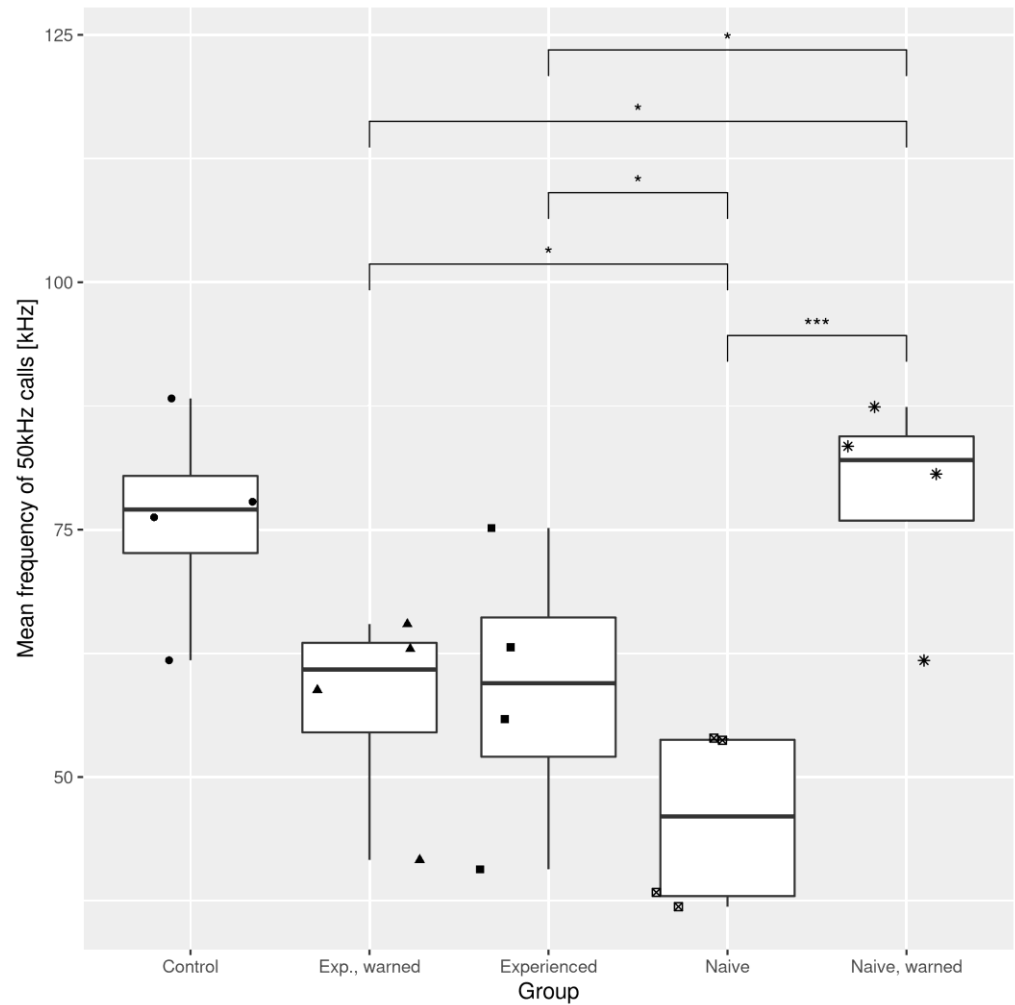


Figure 8. Mean frequency of 50 kHz calls, in different experimental groups. Brackets mark significant differences identified by the Conover-Iman test.

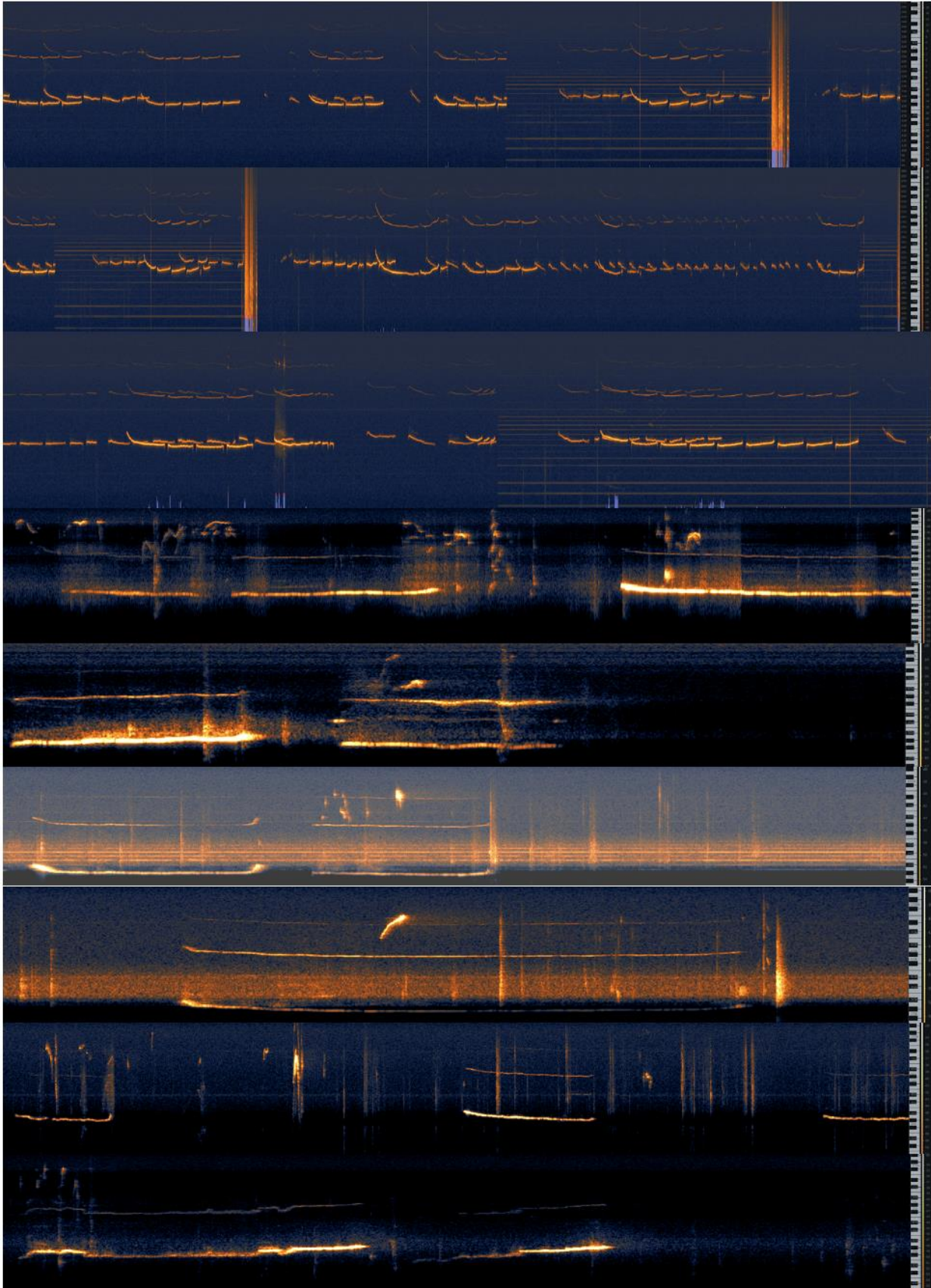


Figure 9. An example of overlapping ultrasonic vocalization presented on a spectrogram.

4. Discussion

In this study, we conducted a detailed analysis of the ultrasonic vocalizations emitted by the pairs of rats during two slightly different aversive situations. In one of the groups, the electrical shocks were signaled by the emission of a 19 second audible (warning) sound ('Warned' group), while the other experimental group did not receive any external acoustic signals before the electrical stimulus ('Unwarned' group). The tested animals either had previous experience with foot-shocks ('Experienced' group) or did not have such experience ('Naive' group). We were interested in whether there was a difference in communication architecture between 'Warned' and 'Unwarned' animals and whether the past experience of the electrical stimulation would change the animals' behavior in an aversive situation. The warning sound emitted before the electrical stimulus seems to increase arousal level, which differentiates the responses depending on previous experience.

In the animals from the 'Warned' group, we have identified a fraction of short calls in the 22-kHz band, which was practically absent in the 'Unwarned' animals. It was suggested earlier [26, 56], that such calls are a sign of distress (internal negative emotional state), rather than a response to external, aversive stimuli triggering the common, longer 22-kHz calls. Our observations provide additional, substantial evidence in favor of this theory. This could mean that the anticipation of electric shock, induced by the warning signal, leads to a higher stress level in contrast to unpredictable shocks, which stays in line with the results of fear conditioning studies [57].

Many studies showed that USVs playback or synthetic sound emission in the 22-kHz band activates the perirhinal cortex, periaqueductal grey matter, amygdala or hypothalamus; the structures involved in defensive behavior [41, 58, 59]. The ultrasonic vocalizations produced by rats in a stressful or threatening situation may be crucial for their conspecifics' survival or wellbeing [43]. Both 22-kHz calls [56] and freezing behavior [60] can be interpreted as a warning and used by the observers to learn about danger. Importantly, the reception of the 22-kHz calls changes the function of the brain fear circuit, which probably helps the recipients quickly adapt to the threatening situation. For example, Dupin *et al.* (2019), who investigated the relationship between emission of USVs, respiration and electrophysiological activity of brain structures, showed that, 22-kHz calls result in a decrease of theta power and increased of delta and gamma power in the BLA, mPFC, and the piriform olfactory cortex (PIR), the structures involved in fear responses [49]. Intense fear observed as a panic attack is associated with hyperventilation and breathlessness [61], which can be reduced by paroxetine, a selective serotonin reuptake inhibitor [62]. Interestingly, Willadsen *et al.* (2020) documented that animals lacking the serotonin transporter emitted a lower number of 22-kHz USVs [63]. These results, together with the correlational studies demonstrated by Dupin *et al.* (2019), indicate that the animals warned by the audible sound in our research (the ones emitting short 22-kHz signals) were in intense distress. This indicates the crucial role of 22-kHz USVs in the emotional contagion within a social group.

Further, we detected the difference between the 'Experienced' and 'Naive' groups in the distribution of frequency of the emitted ultrasonic vocalizations.

The simplest form of empathy, which can be observed also in animals, is defined as the ability to understand or share another individual's emotional state [64]. It plays an essential role in regulating social behavior and can be modulated by prior experience [65]. An interesting phenomenon observed in our study is that short 22-kHz episodes which reflect distress are emitted both by the demonstrator and the observer. Additional intensification of the ultrasound signal in the 22-kHz band appears shortly after, thus indicating emotional contagion; the observer is being "infected" with the demonstrator's emotional state [50]. Presence of the short 22-kHz USVs indicates accelerated breathing (tachypnea) typically connected to hyperventilation, which is characteristic to panic states. Henceforth, the occurrence of the same 22-kHz calls in the demonstrator and observer indicates that distress may be shared by the pair of rats.

On the other hand, we have noticed an interesting fraction of very high-frequency 50-kHz calls (over 75-kHz) present exclusively in the Naive Warned experimental group. We believe this indicates that naive rats are more inclined to emit soothing calls to reduce

the distress of their partners, following the social buffering phenomenon [64, 66]. It is known that in parallel to fear transfer from the demonstrators to the observers, the observers may provide social support and moderate stress response of demonstrators, the phenomenon is known as social buffering [64]. It has been shown that social buffering is more effective among familiar animals [67]. Further, naive animals are more effective in social buffering than animals subjected to fear conditioning [66, 67].

While we were not able to unambiguously attribute each call to an individual rat, we have relied on a phenomenon of temporally overlapping calls, which we assume to come from either rat at a particular time. This allows us to elaborate on their interactions. In particular, we observed all of the possible overlap classes: two 22-kHz calls, two 50-kHz calls, and a mixture of both. Analysis of the architecture of USVs communication reveals that in the presence of an experienced observer most of the overlapping ultrasonic vocalization covered a 22-kHz band (Fig. 7). In the case of the 'Naive Warned' observer, we noticed that USVs overlaps are mixed signals, including 22-kHz and 50-kHz calls (Fig 7). Moreover, the ultrasounds from the 50-kHz band in the 'Naive' 'Warned' group are mostly constituted of USV calls over 75-kHz (Fig. 4, 6, 8).

In conclusion, our detailed analysis of the USV communication architecture has shown that it is critical to divide the 22-kHz calls into long and short classes, in order to precisely quantify the emotional processing in rats. In addition, for the future studies, we also suggest to consider extracting the 50-kHz fraction and dividing it into low and high-frequency '50-kHz' using 75-kHz threshold, or analysing their dominant frequency distribution. Nowadays, the typical 50-kHz analysis approach distinguishes a whole range of subtypes based on their spectrogram shape, but without simultaneously considering their frequency. We strongly believe that the ultrasonic vocalizations have a prosodic character, which carries most essential information in social communication and the division into specific sub-episodes distinguished by a different shape reflected in the FFT should be taken into account as a second factor.

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Institutional Review Board Statement: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experimental procedures using animal subjects were approved by the 1st Local Committee for Animal Care in Warsaw as compliant with Polish Law (21 of January 2005). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Data Availability Statement: Data available upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

National Science Centre, Poland had no role in study design; in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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