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Distinct classes of low frequency ultrasonic vocalizations in rats during sexual interactions relate to different emotional states

Michal Bialy[1]*, Martyna Podobinska[1], Jaroslaw Barski[2], Wiktor Bogacki-Rychlik[1] and Elzbieta M. Sajdel-Sulkowska[1,3]

[1] Department of Experimental and Clinical Physiology, Laboratory of Centre for Preclinical Research, Medical University of Warsaw, Poland,
[2] Department for Experimental Medicine, Medical University of Silesia, Katowice, Poland,
[3] Department of Psychiatry, Harvard Medical School, Boston MA, USA,
** Email: michalbialy@yahoo.com*

This study examined low-frequency ultrasonic vocalizations (lUSVs) in rats during two types of sexual interactions: postejaculatory interval (PEI) and barrier – noncontact (NC) test. We report distinct classes of lUSVs that can be assigned to different emotional states; relaxation *vs.* frustration. Totally flat, 22-kHz calls (Class A), were observed during the relaxation state following ejaculation; characterized by immobilization or grooming during the PEI. On the other hand, two-three component lUSVs (Class B) that start at a higher frequency (45-kHz: flat, upward or short signal) and then shift to 35–23-kHz (mostly to 28-23-kHz), correspond as we assume, to arousal and frustration – active states associated with sniffing a hole or exploration during the NC test. We suggest that momentary, abrupt decreases of arousal during the frustration state correspond to Class B lUSVs. The detailed spectral analysis of the high-frequency component of two-component lUSVs is crucial for establishing the relationship between such lUSVs and the corresponding behavior and emotional states. Our studies indicate that while the two-component Class B 22-kHz lUSVs may relate to the frustration state, a single component, flat, Class A lUSV relates to the relaxation state. The results of these studies support a notion that rats emit distinct vocalization patterns, reflecting their emotional states.

Key words: 22-kHz, ultrasonic vocalizations, arousal, ejaculation, frustration, rats

INTRODUCTION

Ultrasonic vocalizations (USVs) in rodents are elements of both innate and learned behaviors (Bialy et al., 2000; 2010; Arriaga and Jarvis, 2013) that have been shown to attract attention, express emotions, and signal changes of arousal during social interactions (Sales and Pye, 1974; Barfield and Geyer, 1975; Holy and Guo, 2005; Wöhr and Schwarting, 2007; Burgdorf et al., 2008; Asaba et al., 2014; Brudzynski, 2015; Burke et al., 2017; Wöhr, 2018). On the other hand, some authors have expressed skepticism regarding the communication function of USVs in rats (Blumberg and Alberts, 1991; Ågmo and Snoeren, 2015).

In adult male rats, two main types of ultrasonic calls (USVs) can be detected. Firstly, so-called "50-kHz" (35-kHz to 70-kHz) USVs, which include short calls (10–150 ms). Secondly, low frequency ultrasounds (lUSVs) below 35-kHz and conventionally referred to as "22-kHz", which include flat, long-lasting (sometimes with a duration of 1–3 seconds) loud sounds; although some 22-kHz calls are shorter than 300 ms (Brudzynski et al., 1993; Brudzynski 2007; 2015). Adult female rats also produce multiple brief 50-kHz calls with structural characteristics and frequencies similar

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to those produced by male rats (Thomas and Barfield, 1985; White and Barfield, 1987; Snoeren and Ågmo, 2013; Inagaki and Mori, 2015). From mostly pharmacological research, Brudzynski (Brudzynski 2007; 2015) proposed a simple and very attractive model explaining the function of these calls in the expression of emotions, with the 50-kHz USVs reflecting a positive emotional state of the animals, and the 22-kHz USVs reflecting a negative one. Recently, Burgdorf et al. (2018) proposed that lUSVs reflect a transition from approach to avoidance during the refractory period for motivated behavior.

Long-lasting 22-kHz ultrasound vocalizations (lUSVs) occurring during an aversive situation are well documented. They serve as alarm cries altering the behavior in a colony of rats (Blanchard et al., 1991), and reflect an enhanced level of anxiety, but not fear (Jelen et al., 2003).

Low-frequency 22-kHz lUSVs observed during agonistic behavior are not of constant frequency, but show different types of modulation (Vivian and Miczek, 1993a); some of them show a high-frequency prefix and/or a high-frequency suffix emitted during the threat of the attack (Vivian and Miczek, 1993b).

The modulation of the 22-kHz frequency calls has been observed under different conditions including; conditioning training (Bang et al., 2008), the emotionally enhanced state in alcohol-preferring rats (Reno et al., 2015; Thakore et al., 2016), chronic pain (Calvino et al., 1996; Naito et al., 2006), and aggressive or agonistic interactions (Takeuchi and Kawashima, 1986; Vivian and Miczek, 1993a; b), as well as during sexual interactions (Burgdorf et al., 2008). Moreover, different patterns of low-frequency vocalizations have been observed during the postejaculatory period and during defeat or aversive stimulation (van der Poel and Miczek, 1991).

These observations suggest that frequency modulation reflects different emotional states and further investigation during appetitive behavior is warranted to establish the significance of low-frequency vocalizations during emotional expression in rats.

Bell (1974) proposed that 22-kHz lUSVs correlate with a state of abrupt decreases of arousal, a suggestion that raised the possibility that 22-kHz vocalizations in addition to the aversive situation can also be associated with a positive emotional state after ejaculation. Male rats, after ejaculation, emit long-lasting 22-kHz calls (Barfield and Geyer, 1972; Sachs and Bialy, 2000). Recently, we proposed that postejaculatory 22-kHz lUSVs reflect a positive emotional state – a relaxation that occurs as the result of an abrupt decrease of high arousal associated with ejaculation. We have demonstrated that postejaculatory 22-kHz vocalizations occurred only in a familiar place, corresponding to conditioning to a new place, and were inhibited by anxiety-related odor-cues from unfamiliar males (Bialy et al., 2016). In a sexual context, the 22-kHz vocalizations can also be observed before ejaculation, during repeated copulation and ejaculations (Brown, 1979).

Many authors assume that 22-kHz ultrasonic vocalizations are associated with aversive behavior. On the other hand, the association of the 22-kHz with "frustration" has only been suggested by Engelhardt et al. (2017) and Wöhr and Schwarting (2009), who found that rats exposed to playback 50-kHz ultrasonic vocalizations, tried to reach sound source and in some cases emitted 22-kHz vocalizations as an effect of frustration state. Frustration, a negative emotional response as defined by Amsel (Amsel, 1958; 1992; Dudley and Papini, 1997), has been experimentally tested in a sexual context in male rats (Freidin and Mustaca, 2004). According to Amsel's theory of frustration, the absence of expected appetitive reinforcement elicits a negative emotional response, defined as frustration, with both behavioral and physiological correlates (Amsel, 1958; Dudley and Papini, 1997). During the sexual separation test, frustration (as we assume) occurs when a sexually aroused male (familiar with the copulatory chamber where previously male had free access to a female) is prevented from contact with a receptive female. Such frustration is especially visible during the first noncontact test; under these conditions, lUSVs were characterized by an abrupt shift from 50-kHz to 22-kHz (Geyer et al., 1978). While Barfield mentions the 22-kHz lUSVs observed during increased frustration in his later work, he did not identify it as such in his original report. During noncontact tests, male rats, familiar with the noncontact procedure, display enhanced sexual arousal manifested by penis erections (Sachs et al., 1994). This type of erection is related to the odor cues from a receptive female (Sachs, 1997), which requires medial amygdala activation (Kondo and Sachs, 2002) via androgen receptor activation in the posterior-dorsal medial amygdala (Bialy and Sachs, 2002; Bialy et al., 2011).

The present study aimed to examine the hypothesis that rats emit distinct vocalization patterns reflecting their emotional states. Specifically, we aimed to characterize and compare lUSVs as well as behavior of male and female rats during two types of sexual interactions: (1) the postejaculatory interval (PEI) characterized by relaxation, and (2) the first noncontact encounter during the noncontact (NC) test characterized by enhanced level of arousal and assumed frustration, evoked by unexpected problem with access to receptive female.

METHODS

Animals

Long-Evans male (N=9) and female (N=9) rats, 4.5 months old at the start of the experiment, were the subjects in this study. The males and females were housed in separate rooms, two or three animals per standard (42×29×16 cm) laboratory cage, with chow and water freely available. Rats were maintained on a 12 hour light-dark cycle (lights switched off at 10:00 h), and temperature maintained at 21±1°C. The ovariectomized females were brought into estrus with a single injection of estradiol benzoate (50 μg/rat s.c., Sigma-Aldrich) and progesterone (500 μg/rat s.c., Sigma-Aldrich). Hormonal injections were given between 48–72 h for estradiol and between 4–8 h for progesterone respectively before the test.

These protocols were approved by the Animal Care and Use Committee of the Medical University of Warsaw (2014).

Apparatus

A transparent Plexiglas test chamber (50×25×30 cm) was used for postejaculatory (PEI) and barrier noncontact (NC) tests. For NC tests, the long axis of the chamber was bisected by a set of three partitions placed 1 cm apart. The two outer partitions had four equally spaced rectangular holes (2.5×7.5 cm) at floor level; the center partition had four holes (2.5×9.0 cm) aligned above the holes in the other partitions. These holes allowed reciprocal olfactory, visual, and auditory stimulation, but their size and offset positions prevented direct contact between animals. For the PEI tests, the barriers were removed (Fig. 1).

Ultrasounds and behavioral analysis

Behavior was recorded using the Noldus Ethovision system, simultaneously on the same computer, with ultrasounds recorded using the Metris Sonotrack system and spectral analyses of the ultrasounds were performed using the Sonotrack software.

The microphone was placed 50 cm above the floor, in the middle of the chamber, ultrasounds were recorded from both the male and the female. The frequencies of ultrasound were analyzed: (1) at the beginning of the ultrasound; and (2a) at the beginning, (2b) in the middle, and (2c) at the end of the low-frequency fragment of the ultrasound. All ultrasounds were analyzed manually using the Sonotrack cursor allowing to measure accurate time, frequency and intensity of the ultrasound. Visualization of ultrasounds in a computer program is based on Fourier analysis, with a set level of sensitivity, which caused problem with determining the continuity of the ultrasound. Additionally, ultrasounds were listened in slow motion and, based on continuity of sound, referred to as one ultrasound.

The activity of the male and the female was scored during the low-frequency ultrasounds. Male behavior was analyzed in terms of immobilization, hole sniffing, exploration of the chamber, rearing and grooming. The females were additionally analyzed for sex soliciting behavior (hopping, darting followed by immobilization, ear wiggling).

Based on the previous articles and observations of long-lasting breathing pattern, we can assign all the 22-kHz ultrasounds following ejaculation to the male (Barfield and Geyer, 1972; Blumberg and Moltz, 1987; Bialy et al., 2016). During the NC test, the number of ultrasounds with a shift from 50-kHz to 22-kHz strongly depends on the male arousal state, and therefore these ultrasounds have been assigned primarily to the males

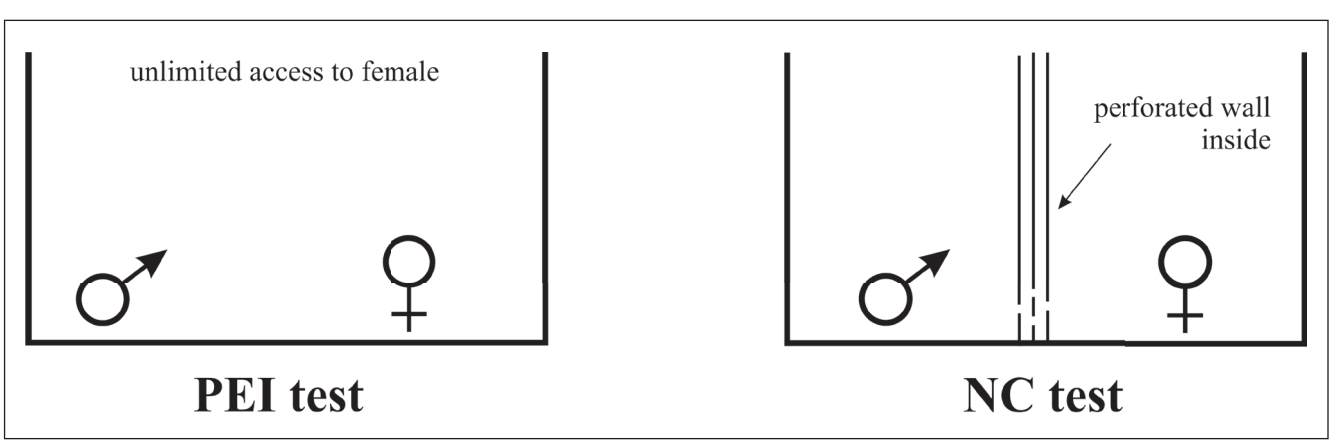

Fig. 1. Schematic representation of the apparatus used for postejaculatory (PEI) and barrier noncontact (NC) tests.

(Geyer et al., 1978). However, since it has been reported that female vocalize at around 22-kHz, but the total time of such vocalizations is much lower compared to males (Inagaki and Mori, 2015), some of the 22-kHz could be emitted by the females.

Behavioral procedures

All behavioral tests were conducted between 12:00 and 17:00 h, during the dark phase of the light-dark cycle, with one week between individual tests. The males were acclimated 3–4 times to handling and being in the Plexiglas testing chamber, for 10 minutes in the first session, then 5 minutes in subsequent sessions.

Postejaculatory test

Males acquired sexual experience during three copulation sessions in the Plexiglas testing chamber without barriers. Each male was placed into the chamber, and a stimulus estrous female was introduced 5 minutes later. Sessions continued until the first intromission after ejaculation; if a male did not achieve intromission within 15 minutes or did not ejaculate within 30 minutes after the first intromission, then the session was terminated. We analyzed postejaculatory vocalizations in sexually experienced males during the third session. Two males did not copulate during all three tests (one achieved ejaculation only during the first session and the other male only during the second session); these two males were excluded from the analysis of lUSVs in the postejaculatory test.

Noncontact test

All males, regardless of whether or not they copulated during all three sessions, were used in the barrier noncontact (NC) test. We did not exclude the two non-copulating males during the third copulatory session from the NC test. Social contact has some rewarding value, and a male can be aroused in the presence of a female even in the absence of copulation as noncontact erections with enhanced sexual arousal, and erections during copulation are regulated by different mechanisms, related to autonomic neural networks and different hormonal profiles (Sachs, 2000).

The male was placed in the left side of the chamber, and 5 minutes later an estrous female was introduced on the right side. The test was terminated 20 minutes after the introduction of the female. Noncontact erection was scored when, in conjunction with genital grooming by the male, any of the following was observed: penile erection, or hip flexion, hip constriction, "tiptoe posture" (elevation of heels of the hind paws) – behaviors which correlate with penile erections in male rats. Such behavior related to penile erection despite NC situation can be observed during copulatory behavior after intromission or ejaculation.

Statistics

The *chi*-squared test was used to analyze the frequency of Class A and B lUSVs during the postejaculatory period and the NC test. The time duration of ultrasounds during PEI test and NC test was compared by *t*-student test with previous analysis of normal distribution and equal standard deviation of this parameter in subsequent tests.

RESULTS

This study examined the low-frequency vocalizations (lUSVs) during two types of sexual interactions: PEI interval (described in the Postejaculatory ultrasonic vocalizations subsection below) and the NC test, in which the male and female rats were separated by a Plexiglas wall.

Seven out of nine males copulated during all three copulatory sessions and all of these seven males vocalized during the postejaculatory period. Five out of nine males during the NC session emitted lUSVs (in this group, one male excluded from PEI analysis emitted 22-kHz vocalizations during the NC test). Only one male displayed noncontact erection and this male emitted only a few lUSVs.

Distinct classes of lUSVs

Two major classes of lUSVs were observed during both the postejaculatory and noncontact paradigms (Fig. 2).

Class A lUSVs, consisting of flat 22-kHz lUSVs, were observed mostly during PEI (Fig. 2, Class A). Additionally, we observed Class A lUSVs with some frequency fluctuation – Class A FM with a frequency below 35-kHz (Class A FM, Fig. 2), which occurred mostly during NC (65% total Class A lUSVs during NC test).

Class B lUSVs, consisting of lUSVs starting at a frequency around 45-kHz and rapidly shifting to a low frequency below 35-kHz, were detected mostly during NC (Fig. 2, Class B). This high frequency part was flat,

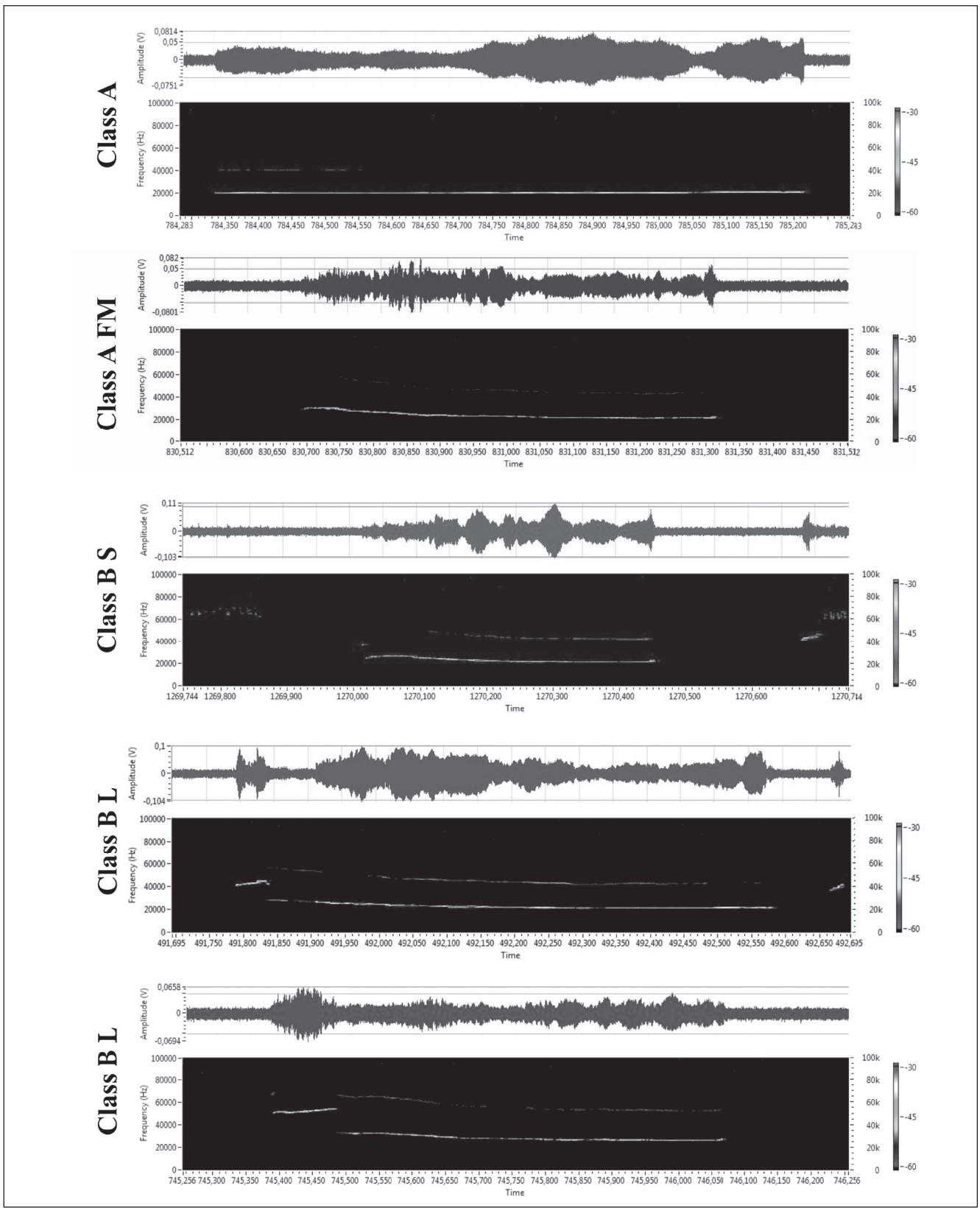

Fig. 2. Different classes and subclasses of 22-kHz lUSVs. Class A: extremely flat 22-kHz lUSVs, Class A FM: Class A with some frequency fluctuation, Class B S: lUSVs starting with a short element (at the frequency around 45-kHz) and rapidly shifting to a low frequency below 35-kHz, Class B L: prefix consist of flat or upward element.

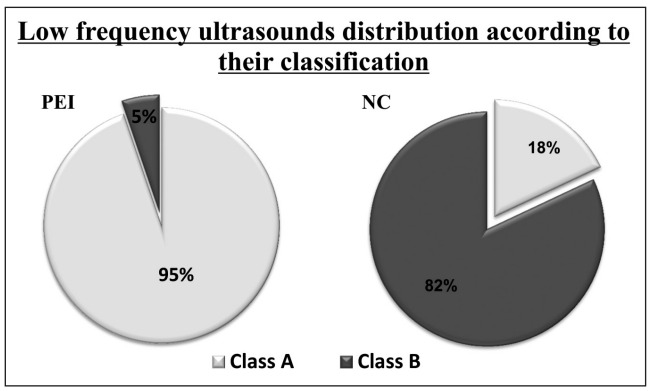

Fig. 3. Percentage of different lUSVs classes emitted during the PEI and NC tests.

upward or short. When the 45-kHz prefix was short, we assigned it to Class B short (Class B S), when it was longer than 10 ms we assigned to Class B long (Class B L).

The duration of 22-kHz vocalizations had normal distribution during PEI as well as during NC tests respectively, with no significant clusters of time duration. Mean duration of 22-kHz ultrasounds was significantly longer during PEI than during the NC test ($P<0.0001$, $t=9.242$ with 608 degrees of freedom, means PEI; 1118 sec. (SD=655, SE=36.2) and means NC; 682 sec. (SD=517.7, SE=30.1), respectively.

PEI vocalizations

A total of 327 lUSVs were detected during PEI from all seven males; 310 lUSVs were of class A, and 17 lUSVs were of Class B (Fig. 3).

The majority (81%) of Class A lUVSs were characterized by a constant frequency with the oscillation in frequency not exceeding 1-kHz. Few lUVSs (19%) displayed oscillation at a frequency greater than 1-kHz. The mean frequencies were very close to 22-kHz: Accompanying the 22-kHz lUSVs were harmonic elements around 45-kHz (Fig. 2, Class A). Class B was observed rarely and mostly at the end of the postejaculatory vocalizations.

Male behavior during Class A and B vocalization (PEI)

During Class A calls in the PEI test, the males displayed mostly immobilization and, less frequently, grooming and exploration, consisting of sniffing, rearing and exploration of the chamber (Fig. 4). Grooming coincided mostly with shorter than 600 ms lUSVs.

Class B lUSVs were very rarely detected during PEI, we found a total of 17 lUVSs emitted by two males. During class B lUSVs, the males displayed mostly ex-

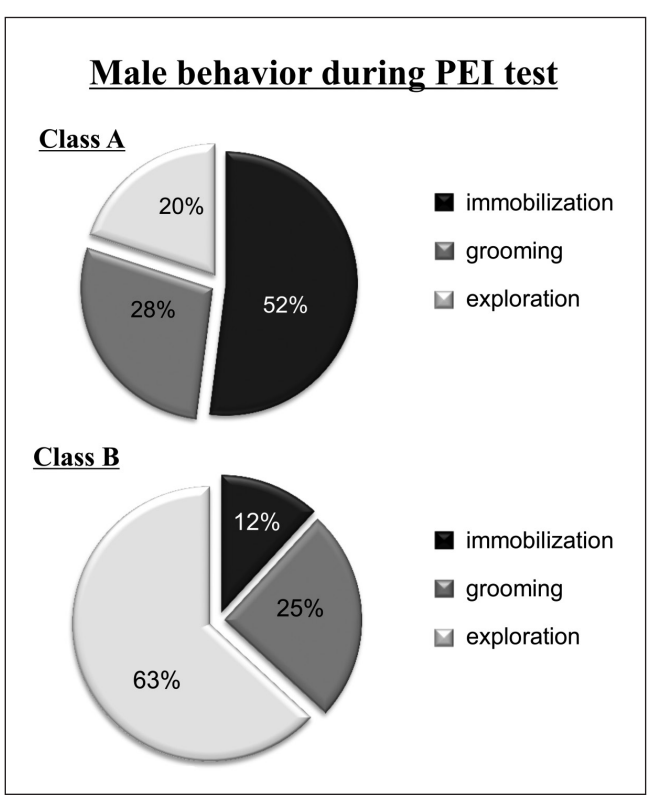

Fig. 4. Male behavior during Class A and B lUSVs in the PEI test.

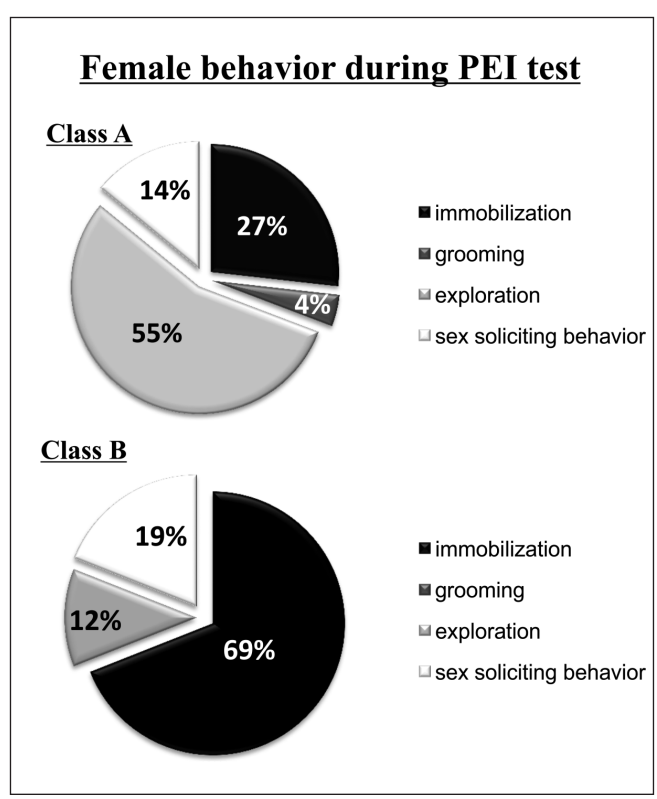

Fig. 5. Female behavior during Class A and B lUSVs in the PEI test.

ploration and less frequently grooming and rarely immobilization (Fig. 4).

Female behavior during Class A and B vocalization (PEI)

Females during Class A lUSVs in the PEI test displayed mostly exploration and, less frequently, immobilization, sex soliciting behavior or grooming (Fig. 5).

On the other hand, females during Class B lUSVs in the PEI test, displayed mostly immobilizations, less frequently sex soliciting behavior and exploration (Fig. 5).

Barrier NC vocalizations

In contrast to PEI, in the NC test, mostly Class B and rarely Class A lUSVs were observed. We detected a total of 296 Class A and Class B lUSVs.

Five males emitted Class B lUSVs and only three out of five males emitted Class A vocalizations (Fig. 3). The proportion of these two classes of ultrasounds were thus significantly different in the PEI and the NC test (*chi*-squared test=376.90, d=1, P<0.0001).

Class B lUSVs in the NC test

Typically, Class B lUSVs started at a high frequency (mean=45-kHz, flat, upward or short signal) and then shifted to a frequency lower than 35-kHz (Fig. 2, Class B). Most of the Class B lUSVs (80%) consisted of the flat or upward elements (Class B L), and were observed mostly as a prefix (99%). Rarely, we detected Class BS lUSVs with a shorter or equal 10 ms high frequency element (18%) and they were observed as prefix (61%), prefix with suffix (18%) and suffix (21%). We observed only a single lUSV with a thrill preceding the 22-kHz (Class B FM) lUSV. The most frequently observed low frequency element of Class B, showed frequency around 28-kHz at the beginning and 23-kHz at the end. Some lUSVs (37) had frequencies between 30-35-kHz, and these calls were shorter than 600 ms. Longer Class B ultrasounds have generally lower frequencies at the central and at the end parts of ultrasound.

Class A in the NC test

Class A lUSVs in the NC test very often displayed fluctuations in the frequencies of more than 1-kHz

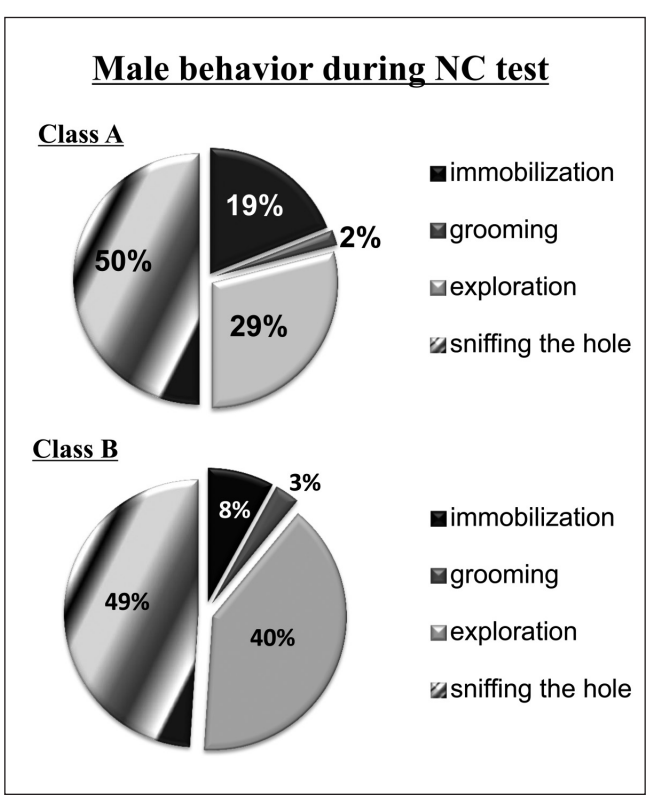

Fig. 6. Male behavior during Class A and B lUSVs in the NC test.

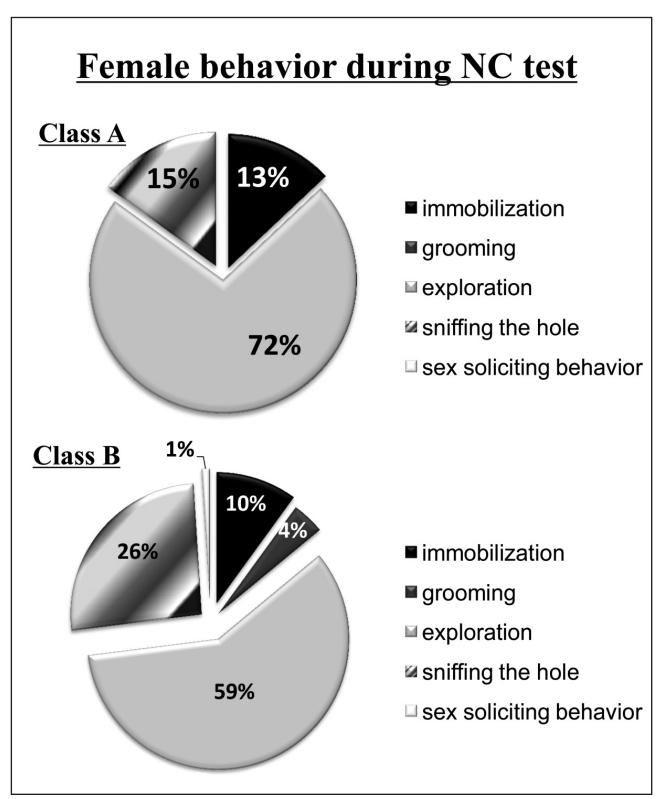

Fig. 7. Female behavior during Class A and B lUSVs in the NC test.

(65% of total Class A lUSVs). These fluctuations in Class A lUSVs were more frequently observed in the NC test compared with the PEI test (*chi*-squared test=47.66, d=1, P<0.0001). Flat Class A lUSVs displayed a frequency around 19–20-kHz, and frequency modulated Class A lUSVs showed an initial mean 28-kHz component that decreased at the end to a mean frequency around 23-kHz.

Male behavior during Class A and B vocalization (NC)

Males during Class A lUSVs displayed either exploration or sniffing a hole or immobilization and less frequently grooming (Fig. 6). Males during Class B vocalizations displayed mostly sniffing a hole, and exploration (exploration of the chamber and rearing), but less frequently immobilization or grooming (Fig. 6).

Female behavior during Class A and B vocalization (NC)

Females during Class A USVs displayed mostly exploration and less frequently immobilization and sniffing a hole. During Class A lUSVs, females did not display any sex soliciting behavior or grooming (Fig. 7).

Females during Class B lUSVs mostly explored the chamber, less frequently sniffed a hole, and rarely displayed immobilization, or grooming. We observed sex soliciting behavior only once (Fig. 7).

DISCUSSION

The results presented here indicate that the low-frequency ultrasounds (lUSVs) are not a homogeneous group, but can be divided into at least two distinct classes; Class A consisting of flat ultrasounds at a frequency around 22-kHz sometimes with some frequency modulation, and Class B with sounds initiated at 45-kHz, shifting below 35-kHz before reaching a lower frequency. Calls containing a component below 35-kHz, by convention, have been referred to as 22-kHz USVs. In our studies, Class A and Class B lUSVs can be observed during specific behaviors and distinct emotional states. Class A lUSVs are most frequently seen during the PEI and are associated with male immobilization or grooming and are accompanied by relaxation. Class B lUSVs are the main calls during the NC test and are associated with an active state consisting of sniffing a hole or exploration and as we assume are accompanied by enhanced arousal and frustration.

After ejaculation, males vocalize at the 22-kHz band (Barfield and Geyer, 1972), and these ultrasounds coexist with slow-wave, spindling, sleep-like EEG activity (Barfield and Geyer, 1975). Postejaculatory 22-kHz vocalizations occur with a latency of 30–40 seconds after the first ejaculation and usually terminate 2–4 minutes later. Postejaculatory vocalization latencies increase progressively after repeated ejaculation (Sachs and Barfield, 1976). During the time from ejaculation to the onset of vocalization, male rats have a penile erection, groom their genitals and move to a preferred place in the chamber (Sachs and Barfield, 1976; Sachs and Bialy, 2000). Recently, we proposed that postejaculatory vocalizations reflect a relaxation state that occurs as a result of the shift from a high arousal state preceding ejaculation to a very low level of arousal during the first minutes of the postejaculatory period (Bialy et al., 2016). Bell (1974) proposed that 22-kHz lUSVs reflect a state of abrupt decrease in arousal. In our opinion, such a hypothesis offers the most appropriate explanation for postejaculatory 22-kHz vocalizations. These lUSVs can be observed, only when males are relaxed and exposed to familiar cues, while entirely new environmental cues or an enhanced level of anxiety evoked by the odor cues of an unfamiliar male inhibited postejaculatory vocalizations (Bialy et al., 2016). In the aversive situation, lUSVs are inhibited by conditioning danger signal, but rats start to emitting lUSVs when exposed to subsequent conditioning safety stimuli (Jelen et al., 2003). Such results can additionally support the hypothesis that the 22-kHz lUSVs reflect relaxation or lower arousal state after high arousal evoked by exposition to aversive conditioning stimuli.

The results of the present study show that postejaculatory 22 kHz ultrasounds emitted during low male activity are nearly totally flat; only 19 % of Class A calls have frequency fluctuations of more than 1 kHz. These 22-kHz lUSVs, with a very long duration, require a stable, long-lasting breathing pattern, which in addition to lUSV emission can be involved in the cooling of the brain after intense sexual interactions (Blumberg and Moltz, 1987). The dominance of long-lasting, extremely flat 22-kHz ultrasounds during the PEI suggests that such ultrasounds reflect a stable relaxation state during immobilization or a low activity state. Grooming or exploration changes the activity level and leads to the shortening of lUSVs during PEI. Usually, following the first ejaculation, and about one minute before the last 22-kHz vocalizations, males display noncontact erections. Presence of erections at this time suggests fluctuation of sexual arousal even before the end of postejaculatory lUSVs (Sachs and Bialy, 2000). We can assume that the tendency to a less stable frequency in the Class A lUSVs and the presence of Class B lUSVs at this time

probably reflects changes in sexual arousal at the end of postejaculatory vocalizations.

In our experiments, females exposed to Class A vocalizations during the postejaculatory period explored the test chamber. Previously, it has been reported that females were immobilized during 22-kHz postejaculatory vocalizations (Barfield and Geyer, 1972). A later report found no correlation between 22-kHz male ultrasonic vocalizations and female behavior, which suggests that such vocalizations have no significant effect on female behavior (Thomas et al., 1982). Recently, it has been reported that natural and artificial 22-kHz ultrasounds had an influence on the emotional state and response of the autonomic nervous system (decreased heart rate) in recipients rats, but the effect could only be observed for a relatively short time period (about 20–30 seconds) after the beginning of ultrasound transmission (Filipkowski et al., 2017). A relatively short response time may explain why female's immobilization is not a typical behavior observed during exposition to all long lasting (usually from 2 to 4 minutes) postejaculatory vocalizations, and usually is seen at the beginning of such calls.

Both, the characteristics of Class A lUSVs and the behavior of males are different in the PEI and the NC test. During the postejaculatory period, extremely flat frequency lUSVs predominated, and males mostly displayed immobilization. However, during the NC test the lUSVs with a frequency fluctuation (Class A FM) around 20–28-kHz were the most frequently observed, and males mostly explored the chamber or sniffed a hole, but rarely displayed immobilization.

In our experiment, males acquired sexual experience in the chamber used later in the NC test. This may explain why males in the first NC test were not only aroused by exposition to odor cues from receptive female (Sachs, 1997), but were also frustrated. During the first NC test, the animals were presented with an unexpected situation of being unable to achieve direct sexual interaction - the familiar, expected goal in this situation. This led to frustration, influencing their behavior and vocalizations. Geyer et al. (1978) reported that the low frequency vocalizations occur during the first barrier test and only occasionally in rats familiar with the situation. Moreover, a shift from 50-kHz to 22-kHz positively correlated with the level of male arousal; a lower number of such calls were observed in sexually fatigued males and a higher number in sexually aroused males after three intromissions without ejaculation. This suggests that Class B lUSVs (the shift from 50-kHz to 22-kHz) are generally emitted by males in the NC test and correspond to the male arousal state. However, we cannot exclude the possibility that some of the ultrasounds are also emitted by the females (Thomas and Barfield, 1985; White and Barfield, 1987; Snoeren and Ågmo, 2013), as it has been reported that the females vocalize at around 22-kHz, but the total time of such vocalizations is much lower compared to males (Inagaki and Mori, 2015).

In the present study, male behavior during Class B vocalizations in the NC test differed dramatically when compared with Class A lUSVs during PEI. During Class B lUSVs in the NC test, the males actively sniffed a hole or explored the chamber, but rarely showed immobilization or grooming, which was characteristic for postejaculatory Class A vocalizations during the PEI test. Recently, Burke et al. (2017) found that emission of the trill-22-kHz ultrasounds during playing behavior inhibited escalation from playing to aggression in adult male rats. Hernandez et al. (2017) observed the trill-22-kHz lUSVs in relatively young, sexually naïve, but not experienced males during the NC test. In this experiment, males acquired sexual experience when co-housed with a female for 12 hours and acquisition of the sexual experience was not associated with cues present in the noncontact test chamber. In this case, sexually experienced males were aroused, but probably not frustrated, and did not emit lUSVs observed by us in the sexually experienced males during the first NC test. On the other hand, sexually naïve males were not frustrated, because they were not familiar with the sexual interaction and contextual reward, and had no expectations associated with such behavioral situation. We can assume that lUSVs consisting of the trill component correspond to play or social interactions. Authors also show that males produced two-component (trill and 22-kHz) ultrasounds during one breathing cycle that further supports the concept of two-component functionally one ultrasound (Hernandez et al., 2017).

Thus, we assume the high-frequency component of lUSVs appears to be different and specific in different behavioral situations – flat, upward during frustration and arousal, *vs.* trill in rats during playing interactions inhibiting possible escalation to aggression. We propose that the trill-22-kHz lUSVs should be distinguished as an additional and distinct class of lUSVs; we also propose an additional Class B FM (B, frequency modulated). In our opinion, distinct classes of lUSVs, including flat frequency Class A, frequency modulated Class A FM, Class B with a shift from high to low frequency (below 35-kHz), represent a continuum, corresponding to different emotional states. These may include a stable, long-lasting relaxation state after ejaculation, relaxation with some fluctuation of arousal influencing frequency modulation lUSV, high level of arousal during frustration when the rat initiates vocalization at a higher frequency and proceed to a lower frequency to a relatively short-lasting, quick, abrupt arousal shift. We further propose that pre-

fix/suffix can be specific for different behavioral situations, such as; short during agonistic behavior (Vivian and Miczek, 1993a; b), flat and upward during frustration (our present results) and trill during social playing interaction with the inhibition of aggressive behavior (Burke et al., 2017).

On the other hand, differences in the spectral analysis of the 22-kHz vocalization may also reflect differences in neural networks and neurotransmitter activations. It has been proposed that 22-kHz vocalizations reflecting a negative emotional state depend on muscarinic stimulation (Brudzynski, 2015). Our suggestion that postejaculatory vocalizations reflect relaxation state implies that pharmacologically elevated level of sexual arousal should diminish lUSVs. Dopamine has an excitatory and serotonin an inhibitory effect on sexual activity and arousal (Hull and Rodriguez-Manzo, 2017). After ejaculation, an elevated level of serotonin in lateral hypothalamus can be observed, and contributes to the suppression of copulation during the postejaculatory interval (Lorrain et al., 1999). Administration of the serotonin 5HT-1A receptor agonist 8-OH-DPAT, which decreases the level of free serotonin in the brain, suppresses postejaculatory vocalizations (Mos et al., 1991; Bialy et al., 2018) at the dose which enhances sexual arousal in sexually satiety (Rodriguez-Manzo and Fernández-Guasti, 1994) and old inactive males (Bialy et al., 2018).

Additionally, dopaminergic D2 agonists and, less effectively D1 receptor agonists, decrease postejaculatory vocalizations (Cagiano et al., 1989; Beck et al., 2002). These data suggest that pharmacologically-induced elevated level of arousal probably blocks the abrupt decrease of arousal typical for postejaculatory period simultaneously with blocking the lUSVs after ejaculation. Opioids have ambiguous effects on postejaculatory vocalizations that are strain-dependent in rat. In the WAG/Rij rats, morphine decreased the duration of postejaculatory 22-kHz vocalization, in Han Crl naltrexone also decreased the 22-kHz vocalization, while there was no significant effect in Sprague-Dawley rats on length 22-kHz vocalizations after ejaculation (Bialy et al., 2014). During the agonistic behavior, 5HT-1A agonists and the opioid agonist morphine decreased lUSVs, but less efficiently than an anxiolytic drug like diazepam (Vivian and Miczek, 1993a; b; 1999). These data suggest that several different neurotransmitters and neural networks can be involved in the regulation of different classes of 22-kHz vocalization, depending on different behavioral and emotional context.

In conclusion, we suggest that flat 22-kHz vocalizations reflect an emotional relaxation state associated with the transition from a high arousal level to a low arousal level during the first minutes of the postejaculatory period. Frequency fluctuations and two-component calls reflect a level of gradual disturbance of the relaxation state. We hypothesize that even during a high level of arousal, combined as we assume with frustration, the momentary decrease in arousal can take place and is reflected in a shift from high to low-frequency calls.

CONCLUSIONS

We report the presence of distinct classes of low-frequency vocalizations (lUSVs) in male rats during sexual interactions that are associated with different emotional states and behavior. The single component, totally flat 22-kHz Class A lUSVs occur during the PEI test and accompany the relaxation state following ejaculation when male rats display mostly immobilization. Class B accompanies arousal, combined as we assume with frustration, in the NC test, with lUSVs starting at 45-kHz (flat, upward or short signal) followed by the 22–28-kHz (occasionally 30-35 kHz) calls. Based on our data we propose that the modulation of the 22-kHz USVs, and specifically the shift from high, flat, upward or short signal to the 22-kHz calls, reflects a momentary decrease of arousal during the arousal - frustration state. Thus, the results of our studies support a hypothesis that rats emit distinct lUSVs classes reflecting their emotional states and arousal fluctuation. Further detailed analysis of both the high and low-frequency components of lUSVs is crucial for establishing the relationship between the lUSVs and the corresponding behavior and animal emotional states.

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