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Discomfort-related changes in pup ultrasonic calls of fat-tailed gerbils *Pachyuromys duprasi*

Alexandra S. Zaytseva[a,b], Ilya A. Volodin[a,b], Olga G. Ilchenko[b] and Elena V. Volodina[b]

[a]Faculty of Biology, Department of Vertebrate Zoology, Lomonosov Moscow State University, Moscow, Russia; [b]Scientific Research Department, Moscow Zoo, Moscow, Russia

ABSTRACT

Rodent pups vocalize when placed in social isolation. We apply a method of "joint calls" for examining discomfort in rodent pup ultrasonic (>20 kHz) calls. Previously, this method has been developed for audible calls of fur farm mammals. Using a repeated measures design to exclude effects of individual identity and age on the analysed variables, we compared the ultrasonic call variables produced by 8–40-day pups of fat-tailed gerbils *Pachyuromys duprasi* during two subsequent experimental stages, the Isolation Stage and the Handling Stage. We considered that discomfort-related negative emotional arousal increased towards the Handling Stage compared to the Isolation Stage because of cumulative effects of handling and time of pup isolation from the nest. At the Isolation Stage, the call rate (calls/s) was higher from 10 to 18 days of age, whereas both the maximum amplitude frequency and power quartiles of joint calls were lower than at the Handling Stage from 20 to 32 days of age. At the same time, in audible (<20 kHz) vocalizations of a wide range of mammalian species, both the higher call rate and the upward shift of the maximum amplitude frequency and power quartiles indicate the discomfort-related increase of negative emotional arousal. We discuss the advantages of the method of joint calls for express-analyses of power variables for large sequences of ultrasonic vocalizations of complex acoustic structure during experimental trials.

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Introduction

Mammalian vocalizations encode basic information about the caller, as individual identity (Matrosova et al. 2011; Sibiryakova et al. 2015), age (Grimsley et al. 2011; Schneiderová et al. 2015) and sex (Fernández-Vargas & Johnston 2015). Calls also encode current information about behavioural situations, as in sciurid alarm calls (Matrosova et al. 2011; Schneiderová et al. 2015), resting-associated shrew calls (Schneiderová & Zouhar 2014) and advertisement songs of male singing mice (Fernández-Vargas et al. 2011). In addition, vocalizations convey information on the emotional valence and arousal of callers (Gogoleva et al. 2010; Briefer 2012).

CONTACT Ilya A. Volodin volodinsvoc@gmail.com

Studies conducted on audible calls (20–20,000 Hz) revealed that there are common cues for encoding the levels of emotional arousal or discomfort in mammalian calls (reviews: Volodin et al. 2009; Briefer 2012). The most universal acoustic variables to indicate the increase of emotional arousal in mammals are a higher call rate and a shift of call energy towards higher frequencies (Volodin et al. 2009; Gogoleva et al. 2010; Briefer 2012; Lingle et al. 2012). These indicators have been established for many young and adult mammals: piglets *Sus scrofa* (Weary & Frazer 1995; Weary et al. 1997); domestic cattle *Bos taurus* (Watts & Stookey 1999); Guinea pigs *Cavia porcellus* (Monticelli et al. 2004; Groenink et al. 2015); tree shrews *Tupaia belangeri* (Schehka et al. 2007; Schehka & Zimmermann 2009); red fox *Vulpes vulpes* (Gogoleva et al. 2010a, 2010b); fallow deer *Dama dama* (Charlton & Reby 2011); giant pandas *Ailuropoda melanoleuca* (Stoeger et al. 2012); African elephants *Loxodonta africana* (Soltis et al. 2011; Viljoen et al. 2015); horses *Equus caballus* (Briefer, Maigrot et al. 2015) and goats *Capra hircus* (Briefer, Tettamanti et al. 2015). Estimating the degree of discomfort by the acoustic structure of animal vocalizations is an important issue for management of farm, zoo and laboratory animals (Watts & Stookey 2000; Manteuffel et al. 2004; Groenink et al. 2015).

Mammalian ultrasonic calls (>20 kHz) may also be indicators of emotional valence and arousal of callers. In well-studied adult rats *Rattus norvegicus*, the 22-kHz calls indicate the negative emotional state, whereas their 50-kHz calls indicate the positive emotional state (Brudzynski 2013, 2015). In pup rodents, the increase of emotional arousal results in the increase of the call rate in their ultrasound isolation calls, as in rats (Allin & Banks 1971; Hofer 1996; Shair et al. 2003); California mice *Peromyscus californicus* (Smith 1972); Mongolian gerbils *Meriones unguiculatus* (Elwood 1979); house mice *Mus musculus* (Dirks et al. 2002; Hahn & Schanz 2002) and bank voles *Clethrionomys glareolus* (Szentgyörgyi et al. 2008).

Rodent pups are born blind, deaf, incapable of walking, and with imperfect thermoregulation. In the pups' first days of life, they depend completely on the mother. Any negative impact (falling out of the nest, cooling, handling, rotation) evokes vocal emission pup isolation calls, which trigger maternal behaviour for searching for the pup and its return to the nest (Hofer 1996; Ehret 2005; Hahn & Lavooy 2005). The ultrasonic isolation calls of rodent pups represent a convenient model for studying the acoustic variables encoding emotional arousal in response to discomfort. These situations may be easily modelled experimentally (Hahn & Lavooy 2005).

Acoustic structure of rodent pup ultrasonic isolation calls displays variable frequency contours (flat, chevron, wave, upward and downward), complicated with frequency jumps, biphonations and other nonlinear phenomena (Brudzynski et al. 1999; Hashimoto et al. 2004; Scattoni et al. 2008; Wright et al. 2010; Grimsley et al. 2011; Arriaga & Jarvis 2013). In addition, animals may use different call types depending on the context; this also complicates the studying and applying of the structural acoustic variables of pup ultrasonic isolation calls as indicators of emotional arousal and discomfort. Such complex acoustic structure strongly complicates the use of any acoustic variables, for the exclusion of call rate, for estimating the degrees of discomfort.

To overcome such difficulties, a method of joint calls has been earlier proposed, with which all intervals between calls, recorded within test trials, were cut-off, thus allowing to compare the peak frequency and power quartiles of the joint calls among experimental trials (Gogoleva et al. 2010a, 2010b). Vocal traits of joint calls were more sensitive to gradations of discomfort compared to the same vocal traits measured within different call types, as differences in vocal trait values within particular call types were small and did not achieve the significance level (Gogoleva et al. 2010b). Previously, this method has been applied to audible calls (Gogoleva et al. 2010a, 2010b); for ultrasonic vocalizations, this approach has never been applied so far.

The fat-tailed gerbil (*Pachyuromys duprasi*) is a North African species that in the last dozen years has been distributed among laboratory and zoological collections (Felt et al. 2008; Zaytseva et al. 2016). In captivity, fat-tailed gerbils live in pairs; a male is non-aggressive to pups, the appearance of a second litter is possible without separation of the first one (Zaytseva et al. 2016). The fat-tailed gerbil serves as laboratory model for studying parasitological and tropical diseases including leishmaniasis (Felt et al. 2008; Hanafi et al. 2013), for studying ear morphology and hearing (Lay 1972; Müller et al. 1991; Plassmann & Kadel 1991) and thermoregulation (Refinetti 1998, 1999). Adult fat-tailed gerbils produce low-frequency wideband chirrs along with harmonic audible calls and ultrasonic narrowband vocalizations up to 60 kHz when two unfamiliar animals (male–male, female–female or male–female) are placed together in one cage (Bridelance 1989).

The purpose of this study was to examine the variables encoding discomfort in ultrasonic vocalizations of fat-tailed gerbil pups. We examine both the traditionally used acoustic variable (call rate) and the newly introduced power variables of ultrasonic joint calls. Our prediction was that the increased discomfort would be associated with a higher call rate and with the energy shift towards higher frequencies of ultrasonic calls.

Materials and methods

Study site and subjects

Ultrasonic calls were collected from members of a captive colony of fat-tailed gerbils at Moscow Zoo, Moscow, Russia, in May–July 2013 and in June–August 2014. Our subjects were 40 fat-tailed gerbil pups (17 males and 23 females from 11 litters), examined from birth to 40 days of age. Before parturition, females were checked once a day for the appearance of a litter, and birth dates as well as the number of pups were recorded. The age of the females at the moment of parturition was 3.7 to 20.4 months (mean ± SD = 10.2 ± 5.1 months). The 11 study litters, containing in total 40 study pups, originated from 10 different mothers: nine mothers with one litter per female and one female gave birth to two litters. The litter size varied from 2 to 6 pups (mean ± SD = 4.00 ± 1.34), with 44 pups in total being born. Four pups died at 2 days of age. The registered first day post-partum was considered to be the first day of pup life for the chronological splitting of age groups along ontogeny. Study pups were sexed between 12 and 19 days of age, on average at 15.1 ± 2.0 days of age based on the appearance of nipples in females (Volodin et al. 1996). The small size of pups prevented individual chip marking for ethical reasons until 18–20 days of age. Pups were kept together with their parents until the completion of data collection at 40 days of age.

The animals were kept under a natural light regime at room temperature (24–26 °C), in family groups consisting of two parents and littermates. The animals were housed in wire-and-glass cages of 51x42.5x41.5 cm, with a bedding of sawdust and hay, various shelters, cardboard boxes and tree branches as enrichment. They received custom-made small desert rodent chow with insect and mineral supplements and fruits and vegetables *ad libitum* as a source of water. All study animals were descendants of 8 animals (5 males and 3 females), collected in December 2007 in a natural population in Egypt.

Experimental procedure and call recording

All acoustic recordings were conducted in a separate room where no other animals were present, at room temperature 23–28 °C (mean ± SD = 25.1 ± 2.4) during daytime, at the same level of background noise. For sound recordings (sampling rate 384 kHz, 16 bit resolution), we used a Pettersson D1000X recorder with built-in microphone (Pettersson Electronik AB, Uppsala, Sweden). The microphone was positioned stationary at a distance of 5–15 cm from the animals, which guaranteed a high signal/noise ratio of recordings. Pup ultrasonic calls were recorded from each littermate separately. In total, each pup participated in 15 experimental trials (one trial per pup per age), at ages of 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, 15–16, 17–18, 19–20, 21–24, 25–28, 29–32, 33–36 and 37–40 days after birth.

Immediately before an experimental trial, the focal pup was taken from the nest and transferred in a small clean plastic hutch to the experimental room within the same floor of the building. Time from removal of the focal pup from the nest to the start of an experimental trial did not exceed 60 s. The experimental trial started when the focal pup was placed in the experimental set-up. Duration of each experimental trial was 420 s. Each trial took place in two stages, both in the context of moderate discomfort: the Isolation Stage (210 s) followed by the Handling Stage (210 s). The duration of tests in this study was within range typical for those used in medical tests with rodent pups (2–15 min, Groenink et al. 2015).

For the duration of the Isolation Stage, a pup was located either in a clean plastic hutch (190x130x70 mm, for 1–18-day pups) or in a plastic cylinder without bottom (diameter 193 mm, high 170 mm, for over 18-day pups), standing on a plastic table surface. For the duration of the Handling Stage, the experimenter (ASZ) took the pup in hands and rotated it on its back until the end of the trial, which was uncomfortable for pups of any age. Although the experimenter hand surface temperature (28–30 °C, Ceron et al. 1995) was slightly higher than the temperature in the experimental room, a pup was held by the fingers, avoiding the possibility of additional warming of the pup by the hand. We considered that discomfort increased towards the Handling Stage compared to the Isolation Stage because of cumulative effects of handling and time of pup isolation from the nest. Earlier, the isolation (separation) context was considered as providing lower discomfort compared to the handling context in studies of discomfort-related changes in variables of audible calls (Lingle et al. 2012; Scheumann et al. 2012).

After the end of a trial, the focal pup was placed in a heating hutch with a bedding of cotton fabric, standing in the neighbouring room. Experimental trials with all littermates were done consequently in the same manner. Then, all the litter in total was returned to their home cage with their parents; the time of pup stay out of the nest did not exceed 40 min. Although pups were not individually identified, the sequential trials with littermates allowed controlling that each pup participated in experiments only once per age.

Each trial was recorded as a wav-file, the transition between the Isolation and Handling Stages was labelled by voice of the experimenter. For some litters at some ages, recording trials were missing due to non-functioning equipment or for other reasons. As a result, 589 recording trials were made from the potential 600 trials (40 pups in 15 ages).

Selection of test trials for acoustic analysis

Visual inspection of spectrograms of acoustic files with Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany) revealed that ultrasonic calls occurred from the 6th day of pup life. From the total of 589 trials, only 256 trials contained ultrasonic calls, whereas 333 did not contain the US calls. Trials from 2-day and 4-day pups (80 trials) did not contain the ultrasonic calls; in 6-day pups, the ultrasonic calls were only presented in 4 of 40 trials. From the total of 256 trials containing ultrasonic calls, the 91 trials contained ultrasonic calls emitted only at Isolation Stages; the 92 trials contained ultrasonic calls only at Handling Stages, and the 73 trials contained ultrasonic calls in both Isolation and Handling Stages. For analysis, we took the 73 trials that contained ultrasonic calls in both Isolation and Handling Stages. These 73 trials were recorded from at least 35 pups of the total of 40 study pups, and distributed across ages in the following way: 3 trials from 8-day pups; 6 trials from 10-day pups; 8 trials from 12-day pups; 12 trials from 14-day pups; 10 trials from 16-day pups; 7 trials from 18-day pups; 8 trials from 20-day pups; 4 trials from 24-day pups; 6 trials from 28-day pups; 3 trials from 32-day pups; 2 trials from 36-day pups; and 4 trials from 40-day pups.

Acoustic analysis

Acoustic measurements were conducted with Avisoft SASLab Pro software and exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Before measurements, all wav-files were subjected to 20 kHz-high-pass filtering to remove low-frequency noise. Spectrograms for analysis were created using sampling frequency 384 kHz, Hamming window, FFT 1024 points, frame 50%, overlap 0%, providing a frequency resolution of 375 Hz and 2.7-ms time resolution.

Each of the 73 analysed acoustic files, corresponding to 73 experimental trials, was split into Isolation Stage and Handling Stage based on the voice label. By spectrogram, we measured in each trial the duration of the Isolation Stage and Handling Stage and calculated the number of ultrasonic calls at each stage. In total, 3958 ultrasonic calls were examined. To calculate the call rate (calls/sec), we divided the number of calls by the duration of the trial stage (in seconds).

In addition, we prepared "joint calls", by manually cutting out the silent spaces between the ultrasonic calls produced within a trial stage (Figure 1). The method of preparing the "joint calls" mainly followed that applied in the study of audible red fox calls (Gogoleva et al. 2010b). In total, we prepared 146 joint calls for the 73 trials (one joint call per trial stage per trial). Using the automatic parameter measurement option of Avisoft, we measured the maximum amplitude frequency (fpeak) and three quartiles (q25, q50 and q75), covering respectively 25, 50 and 75% of call energy (hereafter the lower, medium and upper quartiles) from the mean power spectrum of each joint call (Figure 1). These call variables describe the relative distribution of energy over a call spectrum. The low values of the maximum amplitude frequency and of the quartiles reflect the shift of energy towards the lower frequencies, while the high values of these variables reflect the energy shift towards the higher frequencies.

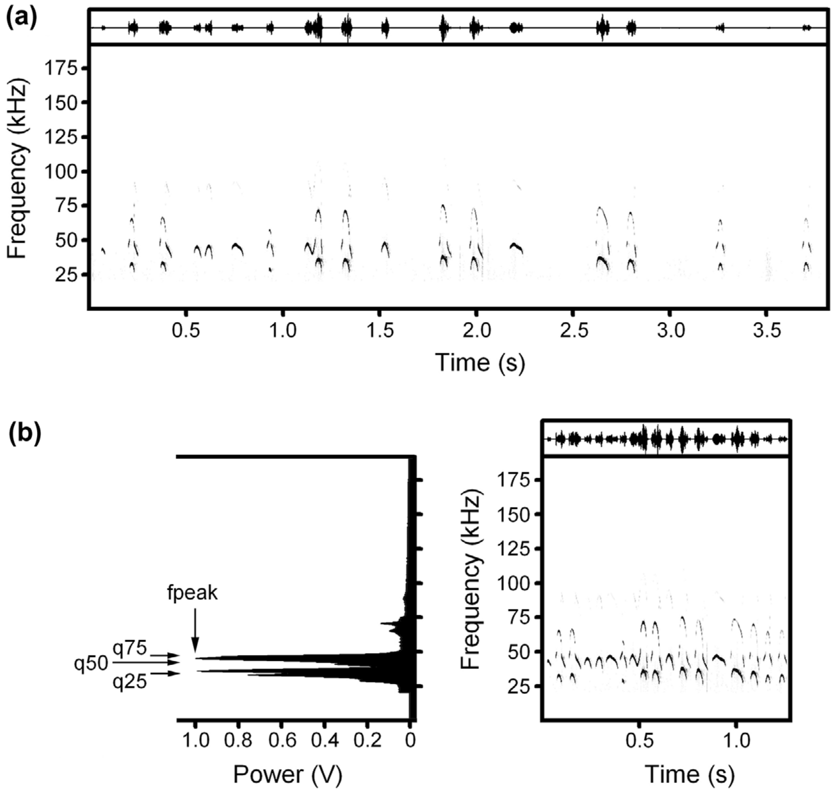

Figure 1. Procedure for preparation of a "joint call" and the maximum amplitude frequency and three power quartiles measure.
Notes: (a) Spectrogram and wave-form of an intact natural sequence of ultrasonic calls, separated with silent spaces, produced by 18-day pup of the fat-tailed gerbil. (b) Spectrogram, wave-form and power spectrum (left) of a part of the future joint call, made from the call sequence shown above. Measured acoustic variables: fpeak – the maximum amplitude frequency; q25 – the lower quartile; q50 – the medium quartile; q75 – the upper quartile.

Statistics

All statistical analyses were carried out with STATISTICA (StatSoft, Inc., Tulsa, OK, USA). All tests were two-tailed and differences were considered significant where $p < 0.05$. We used GLM for repeated measures (with trial stage as the repeated factor and pup age as the fixed factor) with Tukey *post hoc* test to compare the vocal traits between the trial stages. The repeated measures GLM was used because two sequential measurements of each acoustic variable were taken on the same experimental animal unit at two trial stages. This statistical analysis allows excluding effects of individual identity and litter on the analysed variables. Since the values of calling rate did not satisfy the criteria of normality with the Kolmogorov–Smirnov test, we root-square transformed the data to introduce them into GLM.

Results

GLM for repeated measures showed significant effects for both trial stage and pup age on the call rate, the maximum amplitude frequency and three quartiles of joint calls (Table 1).

Table 1. Results of GLM for repeated measures for separate and conjoint effects of trial stage and pup age, for the call rate, the maximum amplitude frequency (fpeak), the lower (q25), medium (q50) and upper (q75) quartiles of joint calls.

Variable	Trial stage	Pup age	Trial stage & Pup age
Call rate	$F_{1,61} = 24.0; p < 0.001$	$F_{11,61} = 1.99; p = 0.04$	$F_{11,61} = 4.08; p < 0.001$
fpeak	$F_{1,61} = 20.6; p < 0.001$	$F_{11,61} = 3.01; p = 0.003$	$F_{11,61} = 4.68; p < 0.001$
q25	$F_{1,61} = 21.8; p < 0.001$	$F_{11,61} = 3.69; p < 0.001$	$F_{11,61} = 3.28; p = 0.001$
q50	$F_{1,61} = 38.1; p < 0.001$	$F_{11,61} = 5.07; p < 0.001$	$F_{11,61} = 3.81; p < 0.001$
q75	$F_{1,61} = 53.1; p < 0.001$	$F_{11,61} = 5.85; p < 0.001$	$F_{11,61} = 4.27; p < 0.001$

Conjoint effects of trial stage and pup age also were found on the call rate and all variables of joint calls (Table 1).

The call rate was higher at the Isolation Stage compared with the Handling Stage from 10 to 18 days of age, showing significant differences at days 14, 16 and 18 (Figure 2). From 20 days of age the call rate did not differ between Isolation and Handling Stages, being similarly low. In contrast, the maximum amplitude frequency, lower and medium quartiles did not differ between Stages from 8 to 18 days of age and were higher at Handling Stage compared with Isolation Stage from 20 to 32 days of age, showing significant differences at day 28 (Figure 2). Similar to other variables of joint calls, the upper quartile did not differ between Stages up to day 14 and was higher at Handling Stage compared with Isolation Stage from 16 to 32 days of age, showing significant differences at days 16, 20, 24 and 28 (Figure 2). All variables of joint calls were similar at ages 36 and 40 days.

Discussion

In this study of ultrasonic vocalizations of fat-tailed gerbil pups, we found that in the lower-discomfort Isolation Stage the call rate was higher, whereas both the maximum amplitude frequency and power quartiles of joint calls were lower than during the higher discomfort Handling Stage. The difference in values of acoustic variables between these contexts was found to be noticeably age-dependent.

Unlike other rodent pups, the fat-tailed gerbil pups displayed an unusual pattern of delayed emission of isolation ultrasonic calls during development. We detected the first ultrasonic calls of this species only from the 6th day of pup life, with a maximum of ultrasound emission in 12–15-day pups. At the same time, other rodents produce their isolation-induced ultrasonic calls earlier in their life: Mongolian gerbils from the 1st day of pup life, with a maximum of ultrasound emission in 2–6-day pups (de Ghett 1974; Broom et al. 1977); California mice from the 1st day of pup life, with a maximum of ultrasound emission in 3-day pups (Vieira & Brown 2002); voles *Microtus pinetorum* and *M. pennsylvanicus* from the 1st day of life with a maximum of ultrasound emission in 8–14-day and 8-day pups, respectively (Blake 2012); Norway rats from the 1st day of life with a maximum of ultrasound emission in 4–9-day pups (Noirot 1968; Allin & Banks 1971); house mice from the 2nd day of life with a maximum of ultrasound emission in 4–8-day pups (Noirot 1966; Hahn et al. 1998); mandarin voles *Microtus mandarinus* from the 2nd day of life with a maximum of ultrasound emission in 11-day pups (Yu et al. 2011); and golden hamsters *Mesocricetus auratus* from the 3rd day of life (Okon 1971; Schneider & Fritzsche 2011). As in other species of rodents, in fat-tailed gerbil pups in this study, the isolation-induced

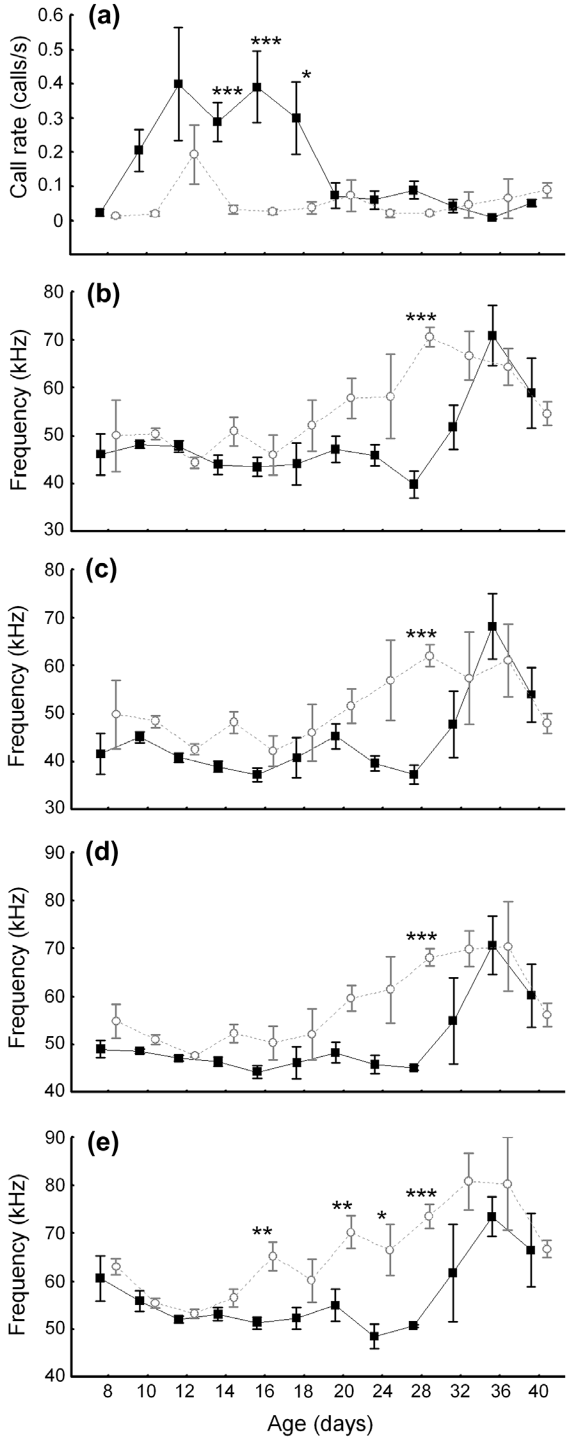

Figure 2. Values of acoustic variables of joint calls across ages from 8 to 40 days.
Notes: (a) the call rate; (b) the maximum amplitude frequency; (c) the lower quartile; (d) the medium quartile; (e) the upper quartile. Black squares indicate Isolation Stage and empty circles indicate Handling Stage of experimental trials. Central points show means, whiskers show SE. Significant differences between trial stages: * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; Tukey *post hoc* test.

ultrasound emission practically ceased by the 20th day of life (Noirot 1966, 1968; Allin & Banks 1971; Okon 1971; de Ghett 1974; Motomura et al. 2002; Vieira & Brown 2002; Schneider & Fritzsche 2011; Blake 2012). At the age of 20 days, rodent pups develop their own thermoregulation, as has been reported for rats, mice, Golden hamsters and Mongolian gerbils (Okon 1970, Okon 1971; Schneider et al. 1995). Available data do not allow explaining the reason of this ontogenetic delay for emission of isolation-induced ultrasonic calls in fat-tailed gerbils compared to other rodents. This ontogenetic delay is only related to ultrasonic calls, whereas the audible calls could be recorded from pups of this species from the 1st day of life at both Isolation and Handling experimental trials (our unpublished data).

In pups of fat-tailed gerbils, the call rate of ultrasonic calls was higher during isolation than during handling. This result contradicts the data on raised call rate with increase of negative emotional arousal reported for audible calls of many mammals (Volodin et al. 2009; Briefer 2012). Also, call rate for ultrasonic vocalizations by California mice pups is reported higher for the handling context than for the isolation context (Smith 1972). However, for mice pups, the results are inconsistent. Whereas the study by Hahn and Schanz (2002) showed that call rates for ultrasonic calls are higher during rotation (handling) than during isolation from the 2nd to the 8th day of life, the study by D'Amato et al. (2005) showed that call rates are lower during handling than during isolation on the 8th day of life but indistinguishable on the 4th day of life.

In pups of fat-tailed gerbils, the values of the peak frequency and power quartiles of ultrasonic joint calls increased with an increase of the negative emotional arousal. These results are consistent with data on audible joint calls of red fox (Gogoleva et al. 2010a, 2010b) and with data on audible calls of many other mammalian species (reviews: Volodin et al. 2009; Briefer 2012; Lingle et al. 2012). However, in fat-tailed gerbil pups, significant differences between the handling and isolation contexts has appeared only in 20-day pups and older (for the upper quartile in 16-day pups and older), that is, when ultrasonic calls have nearly ceased. Thus, the shift of call energy towards higher frequencies has been observed in time period when the call rate values are becoming both low and indistinguishable between the contexts.

This study suggests universality of the method of joint calls for different species and call types, either audible or ultrasonic. The acoustic variables of joint calls are easy both for manual and for automated measuring. For comparison, the changes in the degree of discomfort reflected in proportions of different call types are not universal across species; their measuring depends on researcher personality and cannot be automated (Weary & Frazer 1995; Gogoleva et al. 2010a, 2010b). In addition, in both pup and adult rodents, the acoustic structure of ultrasonic calls displays variable frequency contours: flat, chevron, wave, upward and downward (Brudzynski et al. 1999; Scattoni et al. 2008; Wright et al. 2010; Grimsley et al. 2011; Arriaga & Jarvis 2013). The fundamental frequency contours of ultrasonic calls are often broken with frequency jumps of 10 and more kHz, and up to 40–50% ultrasonic calls consist of a few syllables (Scattoni et al. 2008; Grimsley et al. 2011). Such structure makes difficult both the classifying of ultrasonic calls to types and the comparison of acoustic structures between call types. Using the joint calls method resolves this problem, as this approach is appropriate for any calls independently on their acoustic structure. Applying the joint calls also simplifies the acoustic analysis.

In conclusion, this study represents the first successful application of the method of joint calls for analysis of discomfort-related vocal cues in power variables of ultrasonic vocalizations. The method of joint calls is promising for developing fast analyses of power variables in ultrasonic calls of other rodents, mainly for analysis of long ultrasonic sequences during experimental trials. This study confirmed the prediction that increased discomfort would be associated with an energy shift towards higher frequencies in ultrasonic calls and revealed the age-related relations of vocal ultrasonic responses to discomfort. However, the ultrasonic calls cannot be considered as indicators of discomfort for pups of fat-tailed gerbils across ages, because of the species-specific delay in producing any ultrasound up to the age of 6 days and very rare ultrasonic emission up to the age of 10 days. Further research should establish whether this delay in ultrasonic vocalizing is unique for this species and how the ontogeny of ultrasonic emission is related to the species biology.

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Disclosure statement

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ORCID

Ilya A. Volodin http://orcid.org/0000-0001-6278-0354
Elena V. Volodina http://orcid.org/0000-0001-9755-4576

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