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# Ultrasonic vocalizations as indicators of welfare for laboratory rats (*Rattus norvegicus*)

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**Abstract**

Adult laboratory rats produce two distinct types of ultrasonic vocalization (USV) that appear to reflect the caller's emotional state, either positive (50 kHz) or negative (22 kHz). If these calls can influence the emotional state and related behaviour of group-mates, then such calls may act as useful indicators of welfare for not only the vocalizing rat, but also other non-vocalizing individuals within auditory range. We therefore investigated the effect of playing back these different USVs on the behaviour of rats in an emergence test, a test of anxiety. In an initial experiment, we compared the response of 20 rats to playback of either background noise or to playbacks of 22 kHz vocalizations from conspecifics. Rats that received playback of the 22 kHz USVs were less likely to emerge, showed an increased latency to emerge and spent less total time in the open arena than rats receiving playback of background noise, suggesting a state of increased anxiety. In a second experiment, the same 20 rats received playback of either background noise or 50 kHz vocalizations from conspecifics. Rats receiving 50 kHz USV playback showed no difference in emergence behaviour to those rats receiving background noise. Taken together, these results suggest that 22 kHz USVs can induce a negative emotional state of increased anxiety in rats hearing the vocalization, and could therefore be a useful indicator of welfare for rat groups; including both callers and non-calling group-mates.


*Keywords:* Ultrasonic vocalizations; Laboratory rats; Animal welfare

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## 1. Introduction

The use of vocalizations as a potential measure for animal welfare has become increasingly common in recent years (e.g. pigs: Weary and Fraser, 1995; cattle: Grandin, 1998; rats: Han et al., 2005). However, whilst the initial focus has, understandably, been directed towards assessment of the vocalizing animal itself, if we assume that a particular vocalization has some communicative function, then there are also likely to be effects of hearing that vocalization on the receiving conspecifics (e.g. Manteuffel et al., 2004), an area of research that has received far less attention. By refocusing our attention on the receiver animals, not only can we investigate the possible modulatory effect of the vocalization on receiver emotional state (e.g. Braun and Poeggel, 2001), it may also help us to clarify the meaning of a specific vocalization beyond the information that can be gained by observing when, and in what context, it is emitted (e.g. Puppe et al., 2003). This approach has clear implications for the assessment of animal welfare because, if vocalizations can be demonstrated to influence the emotions of those individuals hearing the calls, then the measurement of vocalizations may inform us about the welfare of not only vocalizing animals, but also non-vocalizing animals within auditory range. An interesting subject animal for this approach, both in terms of the potential welfare benefit for the animal itself and its use as a potential model for other species, is the laboratory rat, which emits ultrasonic vocalizations.

Adult laboratory rats (*Rattus norvegicus*) produce two different varieties of ultrasonic vocalization, each with distinctive properties. One variety, commonly referred to as 50 kHz vocalizations, are short in duration (3–65 ms, e.g. Sales, 1972), show rapid frequency changes (bandwidth 2–50 kHz, e.g. Sales and Pye, 1974) with a sound frequency ranging from 35 to 70 kHz (e.g. Wintink and Brudzynski, 2001), and are generally produced in irregular bursts. They are emitted in a range of contexts, particularly social situations such as during 'play' (e.g. Knutson et al., 1998), and sexual behaviour (e.g. Barfield et al., 1979), as well as during, and in anticipation of, social contact (e.g. Brudzynski and Pniak, 2002). They also appear to be produced in anticipation of non-social rewarding events (e.g. Knutson et al., 1999) and correlated with non-social exploratory behaviour (e.g. Knutson et al., 2002).

The other variety of ultrasonic vocalization, known as 22 kHz vocalizations, are longer calls (300–3400 ms, e.g. Sales, 1972) with little frequency change within each call (bandwidth 1–5 kHz, e.g. Sales and Pye, 1974) characterised by a frequency between 18 and 32 kHz (e.g. Sales, 1972; Sales and Pye, 1974; Francis, 1977; Sales, 1979; Kaltwasser, 1991; Brudzynski et al., 1993). The vocalizations are usually emitted in a regular series, e.g. from 2 to 20 calls (Brudzynski and Ociepa, 1992). Unlike for 50 kHz vocalizations, there is a typical behaviour pattern associated with the production of 22 kHz USVs, with the rat immobile and producing a deep breathing rhythm that appears to correlate with call emission (e.g. Barfield et al., 1979). Social situations in which 22 kHz vocalizations are produced include during aggressive encounters, particularly during defensive/submissive postures (e.g. Sales and Pye, 1974). They are also emitted in non-social situations such as response to potential predation (e.g. cat: Blanchard et al., 1991; man: Brudzynski and Ociepa, 1992), and either following, or in anticipation of, negative events, e.g. footshock (e.g. Cuomo et al., 1992), startle (e.g. Kaltwasser, 1991) and morphine withdrawal (Vivian and Miczek, 1991).

Because of the contrasting contexts in which the two types of USV are produced (see above) it has been proposed that USVs index the affective/emotional state of the vocalizing rat (e.g. Vivian and Miczek, 1991; Knutson et al., 2002), with 50 kHz USVs indicative of a positive emotional state, and 22 kHz USVs indicative of a negative emotional state. This apparent assessment of directly contrasting emotional states makes USVs a potentially valuable tool in the welfare

measurement of a vocalizing laboratory rat, with the additional bonus that, particularly in the case of 22 kHz USVs, they may be more easily automatically recorded than other mammalian calls because of their reduced band width. It makes USVs in laboratory rats eminently suitable for studying the possible modulatory effect of the calls on non-vocalizing receiver emotion because we can make clear predictions about what behavioural response and concurrent emotional state we would predict upon the playback of the two different types of vocalization. There is also still some discrepancy in the contexts in which the USVs are emitted, which suggests some ambiguity of meaning remains. Fifty kilohertz USVs can be emitted during aggression (e.g. Sales, 1972) – although these may be primarily produced during the early explorative stages of an aggressive interaction (e.g. Panksepp and Burgdorf, 2003; Burgdorf and Panksepp, 2006), and 22 kHz USVs during presumably non-aversive sexual behaviour, particularly post-ejaculation (e.g. Barfield et al., 1979). By instigating a 'receiver based approach' investigating the effect of USV playback on receiver behaviour and emotional state, we can therefore not only try to determine whether or not ultrasonic vocalizations influence the emotional state of receiver rats, but also attempt to shed some additional light on the meaning of the vocalizations.

Surprisingly few studies have actually investigated the effect on receiver behaviour of playing back conspecific generated ultrasonic vocalizations. Those studies that have studied this issue have investigated the effect of playing back either 50 kHz (Thomas et al., 1982) or 22 kHz USVs (Sales, 1991; Brudzynski and Chiu, 1995) on conspecific behaviour in situations that did not allow an assessment of receiver emotional state. For instance, in both Sales (1991) and Brudzynski and Chiu (1995), experiments took place in an open test chamber with the emphasis on general changes in behaviour within that chamber. Our aim was therefore to observe the effect on receiver behaviour of playing back conspecific generated 22 and 50 kHz USVs in an emergence test (e.g. Paré et al., 2001), a common test of anxiety in which particular behavioural responses can be attributed to particular emotional states. For example, approach/emergence suggests reduced anxiety, and therefore a positive emotional state, whereas, inhibition of emergence/withdrawal suggests increased anxiety, and therefore a negative emotional state.

## 2. General methods

### 2.1. Animals and housing

Twenty male Wistar rats (Harlan, UK) weighing 200 ± 30 g (ca. 8 weeks of age) at the start of the study, a strain, sex and age previously used in studies of both 22 and 50 kHz USVs (e.g. Brudzynski et al., 1993; Brudzynski and Pniak, 2002), were housed in pairs. The rats were kept on a 12 h light cycle (lights on 06:00–18:00) at 20 ± 1 °C and 46% relative humidity. All rats received ad libitum food (Harlan Teklad Laboratory Diet) and water, were provided with sawdust bedding material, shredded paper nesting material and a plastic tube for shelter. Cages were cleaned using a disinfectant (Virkon–Antec International). All cages (33 cm × 50 cm × 21 cm) were positioned on the same rack such that all the rats would become indirectly familiar with the vocalizations of one another (including non-cage-mates) during the 2-week acclimatisation period prior to the start of the study. Testing took place in a separate 'test' room on the same light cycle, with the experiments carried out between 14:00 and 18:00 h.

### 2.2. Recording of USVs

We selected 10 rats at random to use as a source of ultrasonic vocalizations, half for frequency modulated 50 kHz USVs and half for 22 kHz USVs. All rat vocalizations were recorded in the test room. Twenty-two kilohertz USVs were induced by placing the rat in an empty clean cage – identical to the one in which it was normally housed – and following the methods of Brudzynski and Ociepa (1992), 50–80 g of pressure (as

ascertained using an electrical balance) was applied with a fingertip to the neck of the rat and moved along the spine of the rat in a caudal and cranial direction for 10 s; equivalent to the light pressure applied when gently stroking an animal. This was repeated after a minute if no vocalization was produced, as vocalizations can be produced up to 20 s after induction. The induction was repeated a maximum of three times unless vocalization started, in which case no further induction was necessary (see Section 2.3). Fifty kilohertz vocalizations were induced by placing the rat in an empty clean cage identical to the one in which it was normally housed with the lid open. Vocalizations were emitted during exploration of the cage.

Vocalizations were detected using a QMC S-200 bat detector (QMC Instruments Ltd.) equipped with an ultrasonic microphone model SM1 (frequency response of +6 dB, 10–120 kHz) at a distance of 25 ± 5 cm from the rat. The frequency response of the SM1 microphone falls off rapidly below 20 kHz (unpublished data), though we are aware that signals with high amplitude at frequencies down to 10 kHz or lower may be recorded. Our signals contained little energy in the sonic range, though some low amplitude background noise at sonic frequencies may be represented in recordings. We therefore refer to 'background noise', rather than 'ultrasonic background noise' in our control sequences. The original (undivided) signals obtained from the high frequency output of the detector were stored on a portable ultrasound processor (PUSP Ultra Sound Advice, UK) at a sampling rate of 224 kHz. The PUSP stored 4 s of vocalization that it time-expanded to 40 s. The output from the PUSP was digitised through a standard soundcard and recorded onto a computer as .wav files using BatSound Pro sound analysis v.3.0 software (Pettersson Elektronik AB, Uppsala, Sweden). This software allowed us to assess the acoustic parameters of the vocalizations (see Fig. 1(a) and (b)), and two vocalizations (4 s sequences of calls) were selected for 22 kHz playback and two for 50 kHz playback (all from different animals) based on their similarity to those described in the literature (e.g. Brudzynski et al., 1993; Brudzynski and Pniak, 2002). We also recorded background noise at the same time, by removing all rats, and recording in the same way as for the USVs.

### 2.3. Ethical note

During the 22 kHz induction, the rats continued to move around the cage in an apparently normal fashion without attempting to escape unless they started to vocalize, in which case they remained stationary. Rats that vocalized following 22 kHz induction stopped vocalizing after about 1 min. They then resumed normal exploratory behaviour. Upon return to their home cage all the rats, those that had vocalized and those that had not, appeared to show no subsequent ill effects of the induction procedure, exhibiting normal behaviour and food/water intake. Similarly, no apparent ill effects of the testing procedure were observed. We gave rewards (sugar-coated cereal) to all of the rats once they had been returned to the home room, and these were immediately, and enthusiastically, eaten.

### 2.4. Playback of USVs

To ascertain at what intensity the recorded USVs should be played back in order to replicate the same intensity as a rat would naturally emit, we stimulated several rats to produce USVs a specific distance (10 cm) from a calibrated Bruel and Kjaer $4235\frac{1}{4}''$ microphone (preamplifier and amplifier frequency response 1–100 kHz ± 2 dB) connected to a cathode ray oscilloscope (Gould 500 Digital Storage oscilloscope, sample rate 200 MHz). We could then calculate the intensity at which the rats were vocalizing from a distance of 10 cm. This intensity was then adjusted further to take into account the distance of the emergence box (60 cm) from the speaker position, as the further away the speaker is from the receiver rat, the higher the attenuation by spherical spreading (atmospheric attenuation was negligible). Assuming an attenuation of 6 dB per doubling of distance, we played back the 22 kHz USVs at 72 dB ($2 \times 10^{-5}$ Pa RMS) and the 50 kHz USVs at 95 dB. Time-expanded recordings (40 s) were replayed from the computer into the PUSP, recompressed 10 times to recreate the original timebase (4 s), and broadcast through an amplifier (Ultra Sound Advice) and, initially, an oscilloscope to ensure standardization of playback intensity, before being played through the ultrasound speaker (Ultra Sound Advice Ultrasound Speaker, frequency response 20–100 kHz ± 6 dB) during the test. Background noise was played at the same intensity as the USV for the particular experiment, i.e. 72 dB for experiment 1 and 95 dB for experiment 2.

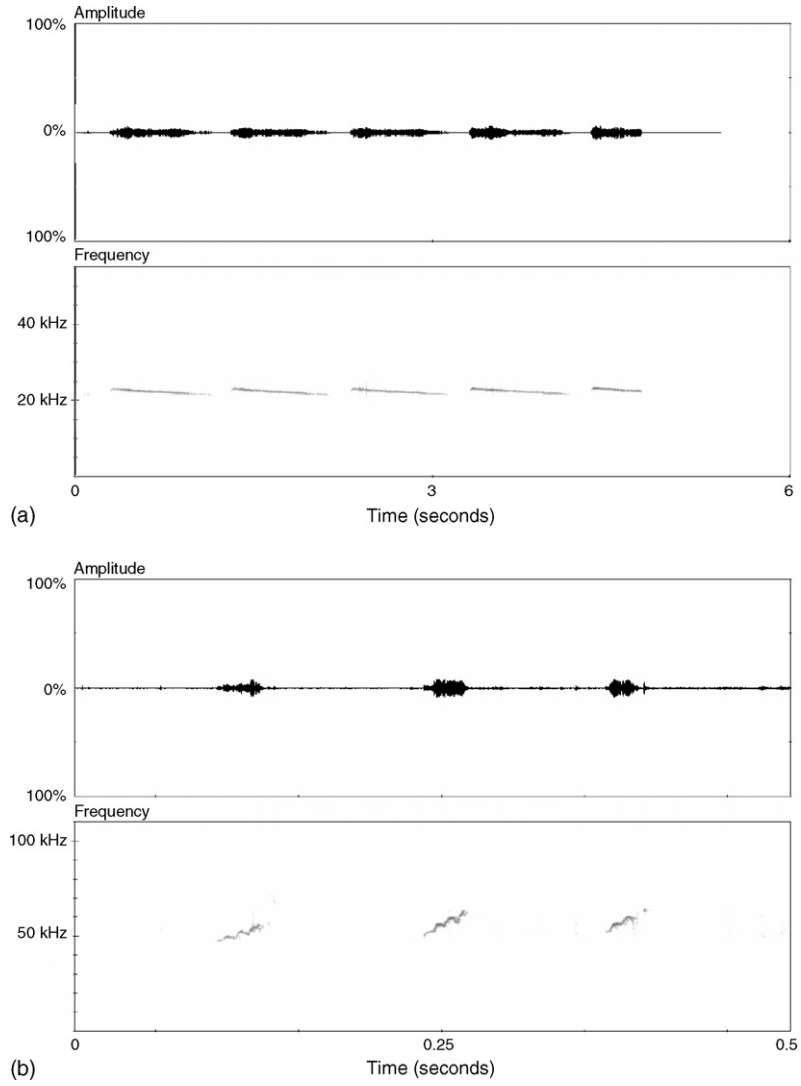


Fig. 1. (a) An example of a spectrogram and waveform (FFT size 512, Hanning window) for a 22 kHz ultrasonic vocalization. (b) An example of a spectrogram and waveform (FFT size 512, Hanning window) for a 50 kHz ultrasonic vocalization.

Playback of either 22, 50 kHz or background noise occurred continuously for only the first 3 min of the 5 min test (see Section 2.6). As such, the 4 s vocalizations were replayed a total of 45 times in each test. The 4 s 22 kHz vocalizations consisted of a sequence of 5 separate calls (sequences of 2–20 calls are commonly emitted by rats), resulting in a playback rate of 1.25 calls/s. The 4 s 50 kHz vocalizations consisted of eight separate calls, resulting in a playback rate of 2 calls/s. There have been reports of defeated rats emitting 22 kHz USVs continuously for 5–10 min (Sales and Pye, 1974), however, it is possible that the playback of recorded 22 and 50 kHz USVs in this study resulted in either greater repetition and/or duration of vocalization than rats would receive in a natural situation—potential implications for interpretation of the results are therefore included in Section 6.

### 2.5. The emergence test

The test arena (see Fig. 2) consisted of a wooden 'emergence box' (200 mm × 400 mm × 270 mm) with a removable lid and a trap door at the front connecting it to a circular open arena (diameter: 1200 mm). The lid of the box could be removed to allow the subject rat to be placed inside, and the trapdoor could be raised when required to allow the rat the opportunity to emerge into the open arena (see Section 2.6). The open arena itself was divided into three 'zones' of concentric circles (e.g. Mechan et al., 2002) delineated with black tape, with some further subdivision of the zones into sectors (see Fig. 2) to allow assessment of locomotion. A wooden surrounding wall (300 mm high) enclosed the entire open arena. We wiped the floor of the test arena with dilute (10%) Virkon and allowed it to dry before the start of each trial. The test arena was situated in the test room away from where the rats were housed, and the animals were tested in the same phase of the light cycle in which they were housed (i.e. the light phase). Observations were recorded using a video camera positioned at 90° to the emergence box, and the ultrasound speaker for playback was positioned opposite the video camera, 60 cm away from the emergence box.

### 2.6. The experimental procedure

Each cage containing a pair of rats was transferred to the test room 10 min prior to testing, with both rats of a pair tested individually before moving onto the next pair of rats. The cage was placed in a side room adjoining the test room with the door closed during testing to prevent the waiting rat from hearing playback of the USV/background noise. At the start of a test, each individual rat was carefully placed inside the emergence box by the same familiar experimenter with the trap door to the open arena closed for 1 min before the trap door was opened for 5 min. Fifteen seconds prior to the opening of the trap door we started the playback loop of either 22, 50 kHz USVs or background noise, and this playback continued for 3 min from after the trapdoor was opened. For the final 2 min that the trapdoor was open there was no playback of any kind. Five minutes after the trapdoor had first been opened, the rat was carefully removed from the arena and returned to its home cage. Throughout testing, all equipment with the exception of the video camera and the PUSP was turned off. Apart from placing the rat in the arena at the start of each test and removing it at the end, the experimenter remained stationary and out of the line of sight during testing, 2 m away from the arena. Testing took 3 days, with either four or eight rats tested each day.

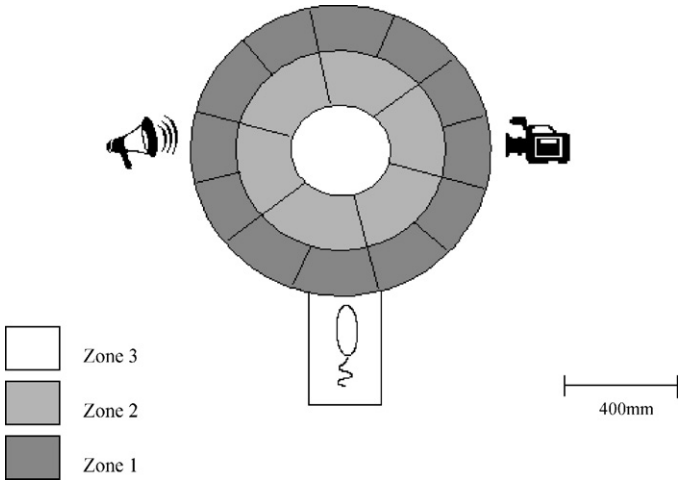


Fig. 2. The test arena showing the emergence box, the division of the arena into zones and sectors and the position of the loudspeaker and video camera in relation to the emergence box.

### 2.7. Behavioural observations

At the end of the study, videotapes of the tests were analysed using Observer software (Noldus Information Technology, Wageningen, Holland). The experienced observer was 'blind' to the experimental design. We recorded the following behaviours: (1) the latency for the nose to emerge (when the tip of the nose to level of eyes emerged into the open arena from the emergence box); (2) the total time spent with at least the nose in the open arena (out of the emergence box) as a percentage of available time once emerged; (3) the total number of other behaviours, including rearing, defecation, freezing and grooming; (4) the total number of rats entering (all four limbs) into zones 1, 2 or 3. In addition to studying behaviours over the whole 5 min during which the trap door was open and the rats were able to emerge into the arena, we were also able to compare behaviour within the first 3 min (during which the playback occurred) and within the last 2 min (during which the playback had stopped). For the latency data, rats that failed to emerge at all during the 5 min test were ascribed values of 300 s. We analysed the data using SPSS version 11.5 (SPSS Inc., Chicago, USA), specific statistical tests are referred to in the text.

## 3. Experiment 1: playback of 22 kHz USVs

The 20 rats were randomly allocated into three groups: 'background' ($n = 10$), '22 kHz a' ($n = 5$) and '22 kHz b' ($n = 5$), with the rats in the '22 kHz a' group hearing the USV obtained from one individual and the rats in the '22 kHz b' group hearing the USV obtained from another (see Section 2.2). This was intended to identify any difference in response to the vocalizations recorded from particular individuals. The rats from each group were tested on two separate occasions (tests 1 and 2), $120 \pm 15$ min apart and on the same day. To counter any potential order/day effects, we tested the same number of rats from each group on each day, and the order of testing was balanced between the groups. For test 1, all three groups received playback of background noise (see Section 2.2). However, in test 2, whilst one of the groups of rats ('background') received a further playback of background noise, the other groups ('22 kHz a' and '22 kHz b') received playback of 22 kHz USVs.

Our prediction would therefore be that playback of 22 kHz USV, if it signifies a negative emotional state in the caller and is able to modulate the emotional state of a conspecific that hears it, should inhibit receiver rat emergence.

## 4. Results

We first investigated whether or not the three groups of rats differed in the number of times that they emerged into the open arena, using a likelihood ratio $\chi^2$-test. When we compared the total number of rats to emerge into zones 1 and 2 (no rats entered into zone 3) during test 1, we found no significant difference between the groups in the number of rats emerging into zone 1 ('background' 4/10, '22 kHz a' 1/5, '22 kHz b' 3/5) or zone 2 ('background' 1/10, '22 kHz a' 0/5, '22 kHz b' 1/5). However, in test 2 we found that significantly fewer rats exposed to the 22 kHz USVs ('22 kHz a' 0/5, '22 kHz b' 1/5) emerged into zone 1 of the open arena compared to those exposed to background noise ('background' 7/10) (likelihood ratio $\chi^2$: $N = 20$, $P < 0.01$), but no such difference was found for emergence into zone 2 ('22 kHz a' 0/5, '22 kHz b' 0/5 'background' 3/10).

We then examined behaviour in the arena using a general linear model (GLM), with behaviour in test 1 acting as a covariate in the model for the analysis of behaviour in test 2 (dependent variable) across the three groups ('group', independent variable). Thus we were able to ask, how does behaviour in test 2 vary between the groups once the initial differences in behaviour (in test

1) have been taken into account? All of the assumptions upon which the GLM is based were met by the data, although the latency data required log transformation. There were no significant interactions for either behaviour, and these were therefore eliminated from the analysis to give a more appropriate model. Once the variation explained by behaviour in test 1 ($F_{1,19} = 0.77$, NS) had been taken into account, we found a significant group effect on latency for the nose to emerge ($F_{2,38} = 4.39$, $P < 0.05$), with rats receiving playback of '22 kHz a' showing a significantly greater latency to emerge than rats receiving background noise (post hoc Bonferroni pairwise comparison) (see Fig. 3(a)). Similarly, once the variation explained by behaviour in test 1 ($F_{1,19} = 1.19$, NS) had been taken into account, there was also a significant group effect on the % total time spent with at least the nose in the open arena ($F_{2,38} = 8.25$, $P < 0.01$), with rats receiving playback of '22 kHz a' showing less % total time emerged than rats receiving background noise (post hoc Bonferroni pairwise comparison) (see Fig. 3(b)). The post hoc tests also revealed that there was no significant difference, for either behaviour, between the '22 kHz a' and '22 kHz b' groups, nor was there a significant difference between the '22 kHz b' and the 'background' groups (see Fig. 3(a) and (b)).

We investigated in more detail the difference in % time spent with at least the nose emerged by comparing this behaviour between the groups within the first 3 min of the test (0–3 min) when the playback occurred, and within the final 2 min of the test (3–5 min) during which the playback had

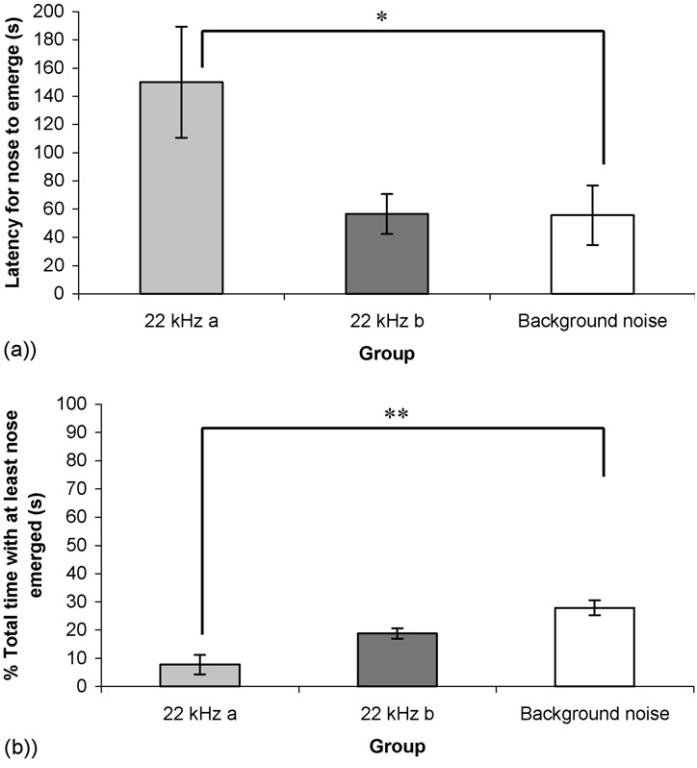


Fig. 3. (a) Latency for the nose to emerge (s) for the '22 kHz a', '22 kHz b' and 'background' groups in test 2, experiment 1. Data shown are the untransformed means ± standard error. $^{*}P < 0.05$. (b) % Total time with at least nose emerged (s) for the '22 kHz a', '22 kHz b' and 'background' groups in test 2, experiment 1. Data shown are the untransformed means ± standard error. $^{**}P < 0.01$.

stopped. This might reveal whether the 22 kHz vocalizations were influencing behaviour directly, as they were played, or subsequently, once they had stopped, i.e. a latent effect (e.g. Brudzynski and Chiu, 1995). As before, we used a GLM with behaviour in test 1 as a covariate in the model for the analysis of behaviour in test 2 for the three groups. Once variation in behaviour in test 1 had been taken into account (0–3 min: $F_{1,19} = 3.13$, NS; 3–5 min: $F_{1,19} = 0.10$, NS), we found that % total time emerged (means ± S.E.: 'background' 17.1 ± 3.81, '22 kHz a' 2.99 ± 1.94, '22 kHz b' 14.79 ± 1.17) showed no significant group effect from 0 to 3 min ($F_{2,38} = 1.86$, NS), but achieved significance during 3–5 min ('background' 20.34 ± 3.68, '22 kHz a' 7.18 ± 2.95, '22 kHz b' 8.29 ± 2.41) ($F_{2,38} = 4.27$, $P < 0.05$). There were no significant interactions at either time interval. Thus it seems that, whilst there does appear to be an increasing difference between the groups after playback has ceased (3–5 min), because this effect is not as strong as that observed over the whole 5 min test (see above), then this small latent effect does not fully explain the overall difference observed in the current study.

Insufficient numbers of rats emerged fully into the open arena to enable us to compare any other behaviours between the two groups, e.g. rearing or locomotion between sectors.

### *4.1. Discussion*

These results suggest that the behaviour of rats in an emergence test can be affected by the playback of a 22 kHz USV, and that the consequence of this playback is to reduce both the likelihood of emergence, the latency to emerge and the % total time spent emerged. Such behaviour in an emergence test suggests a state of increased anxiety in the receiver rat upon hearing playback of conspecific generated 22 kHz USVs. This extends the findings of previous studies investigating the effect of playing back conspecific-generated 22 kHz USVs on receiver behaviour, tested in open arenas without the opportunity to escape/withdraw, which found that playback reduced activity in adult rats (Sales, 1991; Brudzynski and Chiu, 1995).

We also observed that, although there was no significant difference in behavioural response to playback of the 22 kHz USVs recorded from different individuals ('22 kHz a' and '22 kHz b'), the significant differences observed in receiver behaviour only occurred between rats in the 'background' group and those in the '22 kHz a' group. This suggests that the '22 kHz b' USV provoked an intermediate effect less, but not significantly so, than the '22 kHz a' USV, and more, but again not significantly so, than playback of background noise. There are several possible explanations for this apparent differential effect of 22 kHz USVs emitted by different individuals, and these are addressed in Section 6.

Despite the results of experiment 1, we cannot yet draw any conclusions about what information the playback of the 22 kHz USV might contain, because the response of the receivers might have been the same for the playback of any conspecific-generated USV, or even an artificially generated USV (e.g. Sales, 1991). In order to address this issue, in a second experiment we repeated the first experiment, but this time using a different conspecific generated USV, the 50 kHz USV (see Section 1), for which we might predict a different behavioural response.

## 5. Experiment 2: playback of 50 kHz USVs

One month after experiment 1, the same 20 rats were reused as subjects. In order to limit any possible effects of previous experience on subject performance (such as whether rats had previously received playback of background noise and a 22 kHz USV, or background noise only),

rather than randomly allocate the 20 rats into the three new groups, the new groups were made up of roughly equal numbers of rats from the 'background' and 22 kHz groups in experiment 1. The new groups were 'background' ($n = 10$), '50 kHz a' ($n = 5$) and '50 kHz b' ($n = 5$), with the rats in the '50 kHz a' group hearing the USV obtained from one individual and the rats in the '50 kHz b' group hearing the USV obtained from another (see Section 2.2). As in experiment 1, the rats from each group were tested on two separate occasions (see Section 3 for further details). For test 1, each group of rats received playback of background noise. However, in test 2 the 'background' group received further playback of background noise, whereas, the '50 kHz a' and '50 kHz b' groups received playback of 50 kHz USVs.

Our prediction would therefore be that playback of 50 kHz USV, if it signifies a positive emotional state in the caller and is able to modulate the emotional state of a conspecific that hears it, should encourage receiver rat emergence.

### 5.1. Results

Although we had counterbalanced the rats between treatments, we decided to identify whether there was any evidence of a carry-over effect on subject behaviour from experiment 1. We compared those rats in the 'background' treatment that had previously received playback of background noise ($n = 5$) in experiment 1, with those that had previously received playback of 22 kHz USVs ($n = 5$). Mann–Whitney tests revealed no significant differences in behaviour between the rats, either in test 1 (latency: $U = 32$, $N_1 = N_2 = 5$, NS; % total time: $U = 22$, $N_1 = N_2 = 5$, NS) or test 2 (latency: $U = 31$, $N_1 = N_2 = 5$, NS; % total time: $U = 32$, $N_1 = N_2 = 5$, NS).

As for experiment 1, we then investigated whether or not the three groups of rats differed in the number of times that they emerged into the open arena, using a likelihood ratio $\chi^2$-test. When we compared the total number of rats to emerge into zones 1 and 2 (no rats entered into zone 3) during test 1, we found no difference between the groups in the number of rats emerging into either zone 1 ('background' 2/10, '50 kHz a' 0/5, '50 kHz b' 2/5) or zone 2 ('background' 1/10, '50 kHz a' 0/5, '50 kHz b' 0/5). In test 2, again we found no significant difference between the groups in the number of rats emerging into either zone 1 ('background' 1/10, '50 kHz a' 0/5, '50 kHz b' 2/5) or zone 2 ('background' 0/10, '50 kHz a' 0/5, '50 kHz b' 1/5).

We then examined behaviour in the arena using a general linear model (GLM), with behaviour in test 1 acting as a covariate in the model for the analysis of behaviour in test 2 for the three groups. All of the assumptions upon which the GLM is based were met by the data, although the latency data required log transformation. Once the variation explained by behaviour in test 1 (latency: $F_{1,19} = 0.34$, NS; % total time: $F_{1,19} = 3.54$, NS) had been taken into account, we found that there was no group effect on either latency for the nose to emerge ($F_{2,38} = 0.09$, NS) (see Fig. 4(a)) or the % total time spent with at least the nose in the open arena ($F_{2,38} = 1.74$, NS) (see Fig. 4(b)).

As in experiment 1, insufficient numbers of rats fully emerged into the open arena to enable us to compare any other behaviours between the two groups, e.g. rearing or locomotion between sectors.

### 5.2. Discussion

Unlike the playback of 22 kHz USVs in experiment 1, there appeared to be no effect on behavioural response in an emergence test to the playback of a 50 kHz USV, with rats responding similarly to the playback of a 50 kHz USV and background noise. The only other experiment, as

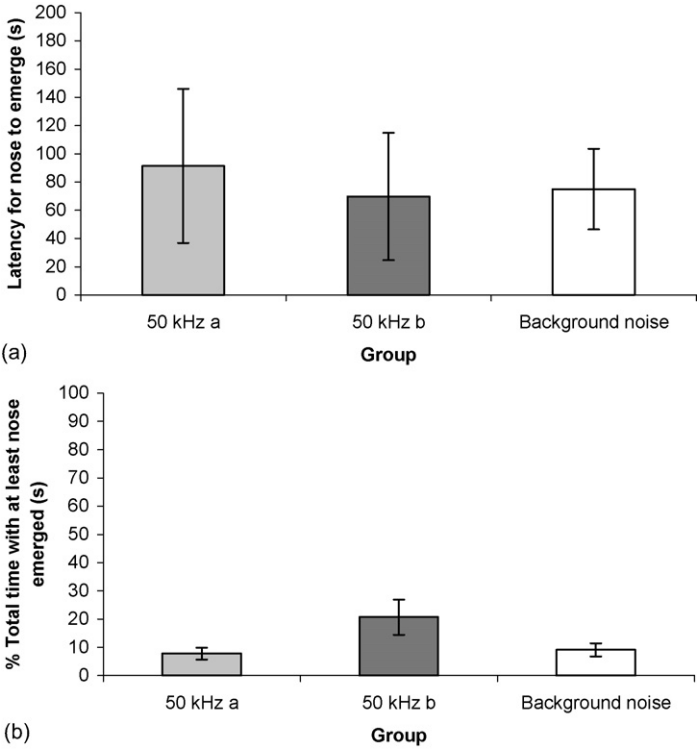


Fig. 4. (a) Latency for the nose to emerge (s) for the '50 kHz a', '50 kHz b' and 'background' groups in test 2, experiment 2. Data shown are the untransformed means ± standard error. (b) % Total time with at least nose emerged (s) for the '50 kHz a', '50 kHz b' and 'background' groups in test 2, experiment 2. Data shown are the untransformed means ± standard error.

far as we are aware, to study the effect of 50 kHz USV playback in rats was carried out by Thomas et al. (1982), who found that playback of 50 kHz USVs increased the receptive behaviour of female rats during mating, although it has been observed that rats prefer to approach rats that emit relatively high numbers of 50 kHz USVs (e.g. Panksepp and Burgdorf, 2003). It is difficult, therefore, to compare the results of that study with those of the present investigation.

The fact that playback of 50 kHz USVs did not significantly inhibit emergence, as observed for playback of 22 kHz USVs in experiment 1, suggests that the behaviour in experiment 1 was not simply a general response to the perception of *any* conspecific-generated USV, but specific to the perception of the 22 kHz USV. Unlike in experiment 1, we found no suggestion of a difference in behavioural response to 50 kHz USVs emitted by two different individuals.

## 6. General discussion

The results of this study reveal that playback of a conspecific generated 22 kHz USV can inhibit emergence behaviour, and, in the specific context of this test of anxiety, such a behavioural response suggests that playback of 22 kHz USVs induced a negative emotional state (increased anxiety) in the receiver rats. This inhibitory effect on receiver behaviour also provides additional information on which to base interpretation of the 22 kHz USV in laboratory rats. Although

22 kHz USVs are generally associated with the experience and/or anticipation of negative events, they can also occur during what might appear to be non-aversive situations such as during sexual behaviour, including pre and post-ejaculation (e.g. Barfield et al., 1979). The results of our study therefore contribute further evidence that 22 kHz USVs reflect the negative emotional state of the vocalizing rat because playback of these calls appeared to induce a similar negative emotional state in receiver rats. Emission of 22 kHz USVs might therefore not only inform us as to the current affective state of the vocalizing rat itself, but also that of non-vocalizing conspecifics in auditory range.

Interestingly, we did not observe any increase in emergence (therefore suggesting reduced anxiety and a positive emotional state) upon playback of 50 kHz USV, although we did observe no inhibitory effect relative to background noise. This could be because the perception of the 50 kHz USV does not influence receiver rat behaviour, but it is also possible that in an anxiety test a behavioural response is more likely to occur upon perceiving a 'negative', rather than a 'positive', vocalization. In other words, if the rats were already in an anxious state then they may have been more predisposed to the perception of (and response to) negative stimuli than positive stimuli (e.g. Harding et al., 2004). If this were the case, then testing in a lower anxiety situation might increase the chance of observing an approach response, and, if the animals were tested in a positively valenced situation, then they might respond better to the playback of vocalizations associated with positive, rather than negative, events. This is an interesting area for future research, although we cannot entirely rule out the possibility that our failure to observe a predicted effect of 50 kHz playback on listener behaviour might be explained, at least partly, by the experimental design. By testing the rats initially with 22 kHz playback and then, 1 month later, with 50 kHz playback, there could have been either age/strain-related or motivational influences on performance. Our analysis of the data did not find an effect of carry-over from experiment 1, but, because our sample was small, this is an additional factor that would need to be considered in the design of future experiments into this area.

The selected intensity and duration at which the vocalizations were replayed during the tests was a potential influence on the outcome of the experiment. But, any confounding effect on the results, e.g. habituation to repeated playback of the same vocalization during a particular test, would most likely have resulted in the suppression of any differences in behavioural response between the groups. Thus, the fact that we were still able to observe differences in behaviour between the groups, despite the possible behavioural suppression by some elements of the methodology, indirectly strengthens the findings of this study. However, further experiments with reduced intensity/duration of playback would certainly provide an interesting comparison with the results of the current study. A further aspect of the methodology of interest was that we used ultrasonic vocalizations recorded from two different individuals for playback. This was done in order to ascertain any difference in response to vocalizations emitted by different individuals. Although no difference in behavioural response to the two 50 kHz USVs was found, we did observe possible differences in response for some behaviours to the 22 kHz ultrasonic vocalizations emitted by different individuals (see Sections 3 and 4). Possible explanations for this result may include: (1) differences in playback quality between the two vocalizations – despite our attempts to minimise any such differences (see Section 2.4); (2) differences in information carried in the two vocalizations – including information about the severity of the event provoking the vocalization and/or about the motivational state of the signaller; (3) any preferential response to vocalizations from particular individuals – certain individuals (e.g. dominant ones) may be attended to more than others. Distinguishing between these different possible explanations is an area for further enquiry.

## 7. Conclusion

In this study, we have described how receivers were given the opportunity to choose their response to the playback of conspecific generated 22 and 50 kHz vocalizations in an emergence test—either to approach/emerge or avoid/withdraw. We observed that playback of a 22 kHz USV appeared to inhibit rat emergence into an open arena, whereas, playback of a 50 kHz USV did not. These results suggest that, in the same experimental situation, rats were able to alter their behavioural response according to the specific type of USV played back—with the 22 kHz USV apparently conveying information about the signaller's negative emotional state resulting in the inducement of a similar negative emotional state (increased anxiety) in the receiver. Whilst what may be viewed as a transient state of increased anxiety does not necessarily imply poor welfare, the results of this study do suggest that measurement of 22 kHz USVs in laboratory rats may be a useful indication of the current emotional state of both individual rats and groups of rats, including those vocalizing and non-vocalizing individuals that are in auditory contact. The subjective emotional state of an animal is thought by many researchers to be the critical component in the study of animal welfare (e.g. Dawkins, 1990; Mendl and Paul, 2004). If such negative emotional states become more frequent and long lasting (less transient), then this may allow conclusions to be drawn concerning the compromisation of welfare. There are also implications for the use of laboratory rats in behavioural research because, if subject rats are observed in the presence of other rats, then any emission of 22 kHz USVs may significantly alter subject behaviour.

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