Feel-good songs: application of a novel playback paradigm to induce a positive affective state in juvenile male Wistar rats

Hammond, Tayla J.; Brown, Sarah M.; Meddle, Simone L.; Nielsen, Birte L.; Lawrence, Alistair B.; Bombail, Vincent

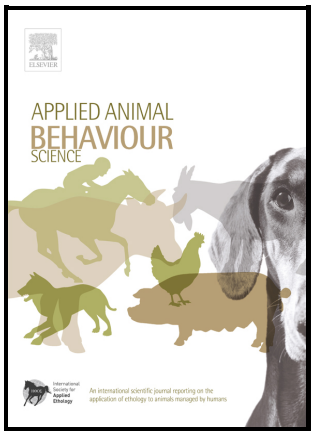
Published in:
Applied Animal Behaviour Science

DOI:
10.1016/j.applanim.2024.106296

First published: 27/05/2024

Document Version
Version created as part of publication process; publisher's layout; not normally made publicly available

Feel-good songs: application of a novel playback paradigm to induce a positive affective state in juvenile male Wistar rats



Tayla J. Hammond, Sarah M. Brown, Simone L. Meddle, Birte L. Nielsen, Alistair B. Lawrence, Vincent Bombail

PII: S0168-1591(24)00144-8

DOI: https://doi.org/10.1016/j.applanim.2024.106296

Reference: APPLAN106296

To appear in: *Applied Animal Behaviour Science*

Received date: 2 February 2024
Revised date: 15 May 2024
Accepted date: 20 May 2024

Please cite this article as: Tayla J. Hammond, Sarah M. Brown, Simone L. Meddle, Birte L. Nielsen, Alistair B. Lawrence and Vincent Bombail, Feel-good songs: application of a novel playback paradigm to induce a positive affective state in juvenile male Wistar rats, *Applied Animal Behaviour Science,* (2024) doi:https://doi.org/10.1016/j.applanim.2024.106296

Feel-good songs: application of a novel playback paradigm to induce a positive affective state in juvenile male Wistar rats

Tayla J. Hammond[1,2], Sarah M. Brown[2], Simone L. Meddle[2], Birte L. Nielsen[3], Alistair B. Lawrence[1,2] and Vincent Bombail[1]

[1] Animal & Veterinary Sciences, Scotland's Rural College (SRUC), Roslin Institute, Easter Bush, UK

[2] The Roslin Institute, The Royal (Dick) School of Veterinary Studies, The University of Edinburgh, United Kingdom, UK

[3] Universities Federation for Animal Welfare (UFAW), Wheathampstead, UK

Corresponding author: tayla.j.hammond@gmail.com, (+44) 07548401323

Keywords: rats, play, positive animal welfare, playback of ultrasonic vocalisations, 50-kHz ultrasonic vocalisations

Abstract

Across animal welfare science there is a lack of validated models of positive affective states. Previous work has shown that presentation of contrastingly valenced ultrasonic vocalisations (USVs) to rats alters their behaviour. However, the potential of using playback of USVs to induce a positive affective state and promote positive animal welfare has yet to be explored. We used three cohorts of juvenile male Wistar rats (37 days old) in three independent experiments to develop a novel home cage playback paradigm to induce a positive affective state in rats. The intention behind this paradigm was to create a low-stress environment, given the heightened susceptibility of positive affective states to stress. Rats were presented in pairs with a playback track consisting of positively valenced 50-kHz USVs, White Noise (within the 30 – 100 kHz range), or Background Noise in their home cage. In Experiments 1 (N = 7 cages) and 2 (N = 14 cages), rats received a single presentation of each playback track in a Latin square experimental design. In Experiment 3 (N = 20 cages), rats received repeated presentations of the same playback track over five consecutive days. Changes in affective state were measured through USV production, approach to the stimulus, and play behaviour. Across all three experiments, the presentation of 50-kHz stimuli USVs increased subject-produced positively valenced 50 kHz USVs compared to presentation of Background Noise (e.g. Experiment 2; $F_{2,239} = 6.05$, $p < 0.05$). Similarly, rats also expressed an increase in approach behaviour towards the speaker in response to 50 kHz stimuli USVs compared to White Noise and Background Noise (Experiment 3 duration of approach behaviour; $F2,479 = 10.55$, $p < 0.001$). Whilst there was complexity in the relationship between the presentation of different acoustic stimuli and play behaviour, rats presented with the 50 kHz stimuli showed increased social play in the ten minutes during presentation under some of our test conditions. The impact of acoustic stimuli on measures of affective state across cohorts provides evidence that the home cage playback paradigm holds promise as a method for inducing a positive affective state in rats.

Introduction

A conservative estimate reported that over 1.9 million rats were used for research purposes in the UK, USA and EU per annum as of 2018 (Carbone, 2021, UK Home Office, 2016). Constant revision and improvement to the welfare standards for these animals are vital from both ethical and scientific viewpoints; we have a moral responsibility to give animals a 'life worth living' at a minimum (Hubrecht, 2014) and there is evidence that scientific validity is compromised when subject animals are experiencing poor welfare (Neville et al., 2022; Bayne and Würbel, 2014). However, the current rat welfare standards are limited by focusing solely on mitigating unnecessary harm, pain, or distress (Cait *et al*., 2022; Makowska and Weary, 2020). In recognition of this discrepancy, there has been a much-needed drive to promote positive experiences for laboratory rats, such that providing a 'good life' should be a precondition for their use (Makowska and Weary, 2020). To do this, it is necessary to develop practical and validated methods of inducing and assessing positive affective states.

A promising method to induce positive affective states in animals, such as rodents, is the playback of vocalisations. The use of acoustic playback is based on the evidence that the production of vocalisations, and their acoustic structure, is sensitive to the affective state of the producer (Briefer, 2012). Considering that the primary role of vocalisations is communication, playback of these calls can be reasonably assumed to result in valence-matched effects on conspecifics (e.g., Manteuffel *et al*., 2004).

Playback of vocalisations to manipulate affective state is particularly promising for rats, considering that the ultrasonic vocalisations (USVs) produced by juvenile and adult rats are widely accepted as signals of affective state (Wöhr and Schwarting, 2009). Broadly, adult rats produce two classes of USVs which perform different communicative functions and are associated with contrasting affective states; calls in the 30 – 90 kHz range, known as 50 kHz USVs, and calls in the 18 - 30 kHz range, known as 22 kHz USVs. As the role of 50 kHz USVs is to facilitate social interaction, perception of these calls is likely to induce a positive

affective state in the recipient (Burgdorf *et al*., 2008). In contrast, as 22 kHz USVs serve as alarm calls for conspecifics in aversive or threatening situations, such as in response to a predator, perception of these calls is likely to induce a negative affective state in the recipient (Litvin *et al*., 2007). As these types of USVs do not overlap in their acoustic structure (frequency, amplitude, or duration), the recipient of the USVs receives an unambiguous signal to the affective state of the producer without the need to visually perceive the producer or the USV-inducing situation (Brudzynski, 2013, 2007). The differential perception of these USVs by rats is further evidenced when tested using cognitive bias: Saito *et al*. (2016) found that USV playback prior to a judgement bias test had a stimulus-specific effect on performance, with 50 kHz USV playback leading to a more "optimistic" response compared with more "pessimistic" responses after playback of 22 kHz calls.

Previous work using the radial maze playback paradigm provides evidence for the contrasting effect of USVs on rat behaviour (Wöhr *et al*., 2016). This paradigm assesses the locomotory behaviour of an individual rat in an eight-armed maze in relation to a speaker that presents the acoustic stimuli. Experiments using this paradigm consistently report that in males rats, 50 kHz ‘positive communicative' USVs induce approach behaviour towards the speaker and increase exploration (Wöhr, 2017; Wöhr and Schwarting, 2012, 2009), whereas 22-kHz ‘negative' alarm USVs induce behaviours indicative of the fight/flight system, *e.g.,* reduced locomotor activity and freezing (Schwarting *et al*., 2007; Schwarting and Wöhr, 2012).

Whilst the radial maze playback paradigm has successfully been used as a behavioural assay for animal models of human neurodevelopmental disorders, it is limited for study in relation to positive animal welfare. In particular, behavioural expression may be confounded by a number of stressors introduced when using the radial maze as rats are exposed to handling and isolation, both of which are known to increase behavioural and physiological

markers of stress in rats (Begni *et al*., 2020; Gärtner *et al*., 1980; Korte and Boer, 2003; Nathiya and Vanisree, 2010).

As such, we aimed to develop a novel home cage playback paradigm to induce a positive affective state in rats. This aim was met through three independent experiments; Experiment 1 acted as a proof-of-concept test to establish whether rats could perceive the playback stimulus. Following support from the proof-of-concept test, in Experiment 2, rats were presented with three acoustic stimuli (50 kHz USVs, Background Noise and White Noise) in a pseudo-randomised Latin square experimental design across three days. In Experiment 3, rats were presented with one of the three acoustic stimuli for five consecutive days. Across experiments, we hypothesised that when rats are exposed to playback of 50 kHz USVs, they would themselves produce more 50 kHz USVs, and show increased approach behaviour towards the speaker and more play behaviour than when exposed to Background Noise or White Noise indicative of an enhanced affective state.

Methods

Ethics

This study was conducted in accordance with the European Union Directive of 22nd September 2010 (2010/63/EU) and the UK Animals (Scientific Procedures) Act 1986. The protocol was approved by the local animal experimentation ethics committees (Comité d'Ethique en Expérimentation Animale INRA IdF-Jouy-en-Josas/AgroParisTech (Comethea); permission #16–17), Roslin Institute Animal Welfare and Ethical Review Body (AWERB) andThe Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (VERC) at The University of Edinburgh and Scotland's Rural College (SRUC) Animal Ethics Committee.

Animals, housing, and husbandry

Experiment 1 was conducted at the National Research Institute for Agriculture, Food and Environment (INRAE) at the Île-de-France-Jouy-en-Josas Research Centre in France between 28th March and 5th April 2019. Experiments 2 and 3 were conducted at the Roslin Institute, Edinburgh, UK, between 4th and 12th of August 2020 and 25th August and 7th September 2021 respectively. The change in location of these experiments was due to the Covid-19 outbreak and the closure of the INRAE animal facility. This offered the advantage of testing the home cage playback paradigm in rats of a different population pool and in a different laboratory environment.

For Experiment 1, male Wistar rats were bred and raised in-house at INRAE from primiparous females. For Experiments 2 and 3, Wistar male rats were imported from females of unknown parity from Charles River Laboratories, Margate, UK. Wistar rats are a commonly used strain for experiments examining USV production (*e.g.,* Wöhr and Schwarting, 2009), with a bias towards males (Olszyński et al., 2020; Seffer et al., 2014). Juveniles were used as they show more pronounced changes in behaviour in response to acoustic playback compared to adults (Wöhr and Schwarting, 2007). For Experiment 1 as a proof-of-concept test, we used a cohort of 12 juvenile male Wistar rats who were 37 days old at testing. These animals were then used in an unrelated electrophysiology study at INRAE, to reduce the use of experimental animals in research, in the spirit of the 3Rs of experimental research. Experiments 2 and 3 involved pair-housed juvenile males (N = 28 in Experiment 2; N= 40 in Experiment 3). Within pairs, rats were randomly selected across litters and were unrelated to reduce any confounding effects of a shared life experience. Rats were also matched by weight within 2g as body condition has been shown to impact play behaviour in rats (Hammond *et al.*, 2019). Data collection began when rats were 37 days old as play shows the highest absolute levels between 32 and 40 days of age

depending on strain, the peak of play being on the latter end of this range for albino rats (Panksepp, 1981; Thor and Holloway, 1984).

Rats were housed in clear plastic cages with a wire lid (France: 42.5 cm × 26.6 cm × 18.5 cm and UK: 48 cm x 26.3 cm x 20.5 cm; both from Techniplast, Italy). Each cage was supplemented with wood chip bedding and *ad libitum* access to food and water. Within each experiment, cage cleaning was carried out by the same caretaker once a week, ensuring no disturbance occurred at least two hours prior to any experimental procedure. The home room was maintained on an inverted 12 h light/dark cycle (lights on at 21:00) and at a constant temperature (23 ± 0.2 °C) and relative humidity (37.5 ± 6.7 %). During the dark interval, red lighting was used to allow behavioural recording (4.5 lx measured using an Isotech digital light meter Lux-1337). For Experiment 1, cages were situated on two tiers across four standard cage racks, with four cages per rack. For Experiments 2 and 3, cages were situated on a single rack across four tiers. Both rats within a cage received marks on the tail using a non-toxic black marker pen for individual identification.

Experimental design

Experiment 1: Each pair of rats received the single 50 kHz playback stimuli in the home cage in a randomised order on the same day, with all pairs tested within three hours. This short period of testing was to minimise the effects of circadian rhythm on natural activity patterns. For Experiment 1, no record of body weight was taken.

Experiment 2: Each pair of rats received all acoustic stimuli treatments in the home cage in a pseudo-randomised Latin square experimental design across three days. To achieve this, cages were assigned to one of five groups (3 cages per group), with groups balanced according to total cage weight on the day before playback began so that average group weight was within 1.63 g of each other. Overall mean weight and standard deviation on the day before playback was 103.2 ± 6.3 g for Experiment 2. The order in which each group

received each treatment was pseudo-randomised in that all cages would receive each treatment once across three days. Within each day, the order of each cage was randomised, making sure the same treatments did not follow each other to prevent the potential build-up of response to one treatment.

Experiment 3: Across the 20 cages of paired rats, seven cages were assigned to receive 50 kHz acoustic stimuli, seven cages received White Noise acoustic stimuli and six received Background Noise. This division of cage numbers per treatment was randomly assigned. Treatment groups were balanced according to total cage weight three days before playback began, so the groups' weights were within 0.8 g of each other. The overall mean weight and standard deviation three days before playback was 193.8 ± 16.7 g. Within each day, the order of each cage was randomised, making sure the same treatments did not follow each other to prevent the potential build-up of response to one treatment.

Rats received playback of acoustic stimuli in the home cage for five days according to the assigned treatment (50 kHz, White Noise and Background Noise). Within these five days, days one and five were assigned as recording days, with home cage behaviour recorded for during playback of acoustic stimuli. These recordings allowed for investigation of the effect of playback on play and other spontaneous behaviour in the home cage. On days two to four, rats were exposed to playback according to the assigned treatment but were not recorded in the home cage before and after playback. Data were analysed from days one and five only. On the evening of day 5, all rats were given a sucrose preference test.

Experimental setup

Rats were accustomed to an inverse light cycle, so experimental testing could occur during the dark period when the rats are naturally active (lights off at 09:00 with testing between 10:00 and 15:00). All testing was conducted within the holding room at least 3 meters away from other rats to minimise the potential for emotional contagion (Hammond *et al*., 2019;

Hatfield *et al*., 1993). This room was lit with red lighting, which delivered a low-intensity illumination (4.5 lx) to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998).

All testing was performed in the home cage, with an ultrasonic microphone placed above the cage (see the recording of USVs section below) and an ultrasonic speaker replacing the food hopper (**Figure 1**). One camera was positioned at 90 degrees to the home cage to capture behaviour.

<Figure 1>

Experimental procedures

Habituation to equipment: All rats were habituated to the experimental setup before being tested over three days. The playback speaker was fitted to the home cage by removing the food hopper and water bottle and then placing the speaker behind a metal wire lid (**Figure 1**). The equipment was left for an increasing time each day, up to a total of seven minutes, to match the maximum time of each playback trial. Following a session, the wire cage lid was covered with cardboard for ten minutes to create a barrier for USVs and odours affecting other rats in the room. Rats were not individually handled during transport or during experimentation except when weighed and marked at the start of each experiment.

Daily procedure during testing: The same procedure was followed during testing in all three experiments. Once the equipment was set up, the video and USV recording were started, and the playback track began to play. The experimenter left the room during recording of home cage behaviour and during playback to prevent any human disturbance which could alter rat behaviour.

Acoustic stimuli

All recordings used for playback were produced using the same equipment; the USVs were from rats of the same developmental stage, sex, and strain as the rats in this experiment (juvenile male Wistar rats between 37 and 49 days old), Background Noise was recorded from the experimental room without the presence of rats and White Noise was generated from Audacity (https://www.audacityteam.org; a free and open-source digital audio editor and recording application software; Audacity, Version 2.1.3, Pennsylvania, United States of America). Recordings had a sampling rate of 384 kHz in 16-bit format. Examples of the three types of acoustic stimuli used are shown in **Figure 2**. Playback tracks were produced in Audacity. All the acoustic stimuli were presented through the Avisoft-RECORDER USGH software (Avisoft Bioacoustics, Berlin, Germany) using an ultrasonic dynamic speaker (Vifa, Avisoft Bioacoustics) with a frequency range of 1 – 120 kHz (at 12 dB) and an ultrasonic power amplifier with a frequency range of 1–180 kHz (UltraSoundGate Player 216H, Avisoft Bioacoustics).

Experiment 1: Experiment 1 was a proof-of-concept test to establish whether rats perceive the playback stimulus. We presented rats with 1) 150 seconds of habituation to the equipment set up with no playback of USVs, followed by playback tracks of 2) 30 seconds of Background Noise, 3) 30 seconds of 50 kHz USVs, and 4) 30 seconds of Background Noise. The total 30 second 50 kHz stimuli consisted of 152 USVs, consisting of four different sequences between five and ten seconds long. The 50 kHz USVs were recorded from individual rats during playful handling. Playful handling is a novel approach to positive interactions between humans and rats which aims to incorporate the diversity and unpredictability of juvenile rat social play (*Bombail et al, 2021*). The USVs had a mean call duration of 69 ms with a mean dominant frequency of 63.2 kHz.

Experiments 2 and 3: Playback stimuli were generated by collecting USVs from rats used in other studies. The 50 kHz USVs were recorded from individual rats during playful handling

and from pairs of rats during spontaneous social play in the home cage. The White Noise acoustic stimuli track was generated by matching the time and intensity of the natural USVs in the 50 kHz track and replacing them with artificial White Noise generated within Audacity (Wöhr and Schwarting, 2012). A high-pass filter was applied to all stimuli to remove all sounds below 30 kHz. Background Noise was recorded under the same experimental conditions with no rats in the room.

Each track consisted of a one-minute habituation interval and three sets of alternate presentations of the acoustic stimuli and pauses for one minute each. The total playback track was seven minutes long. 50 kHz USVs, White Noise and Background Noise were presented at approximately 60 dB (measured from 30cm). In Experiment 3, to prevent habituation over the five days of testing (Wöhr and Schwarting, 2012), the playback track within each treatment was different for each day. Each one-minute playback section was split into 15 second phrases and reordered to achieve this.

We carried out a survey of the USV contained in the playback tracks by observation of spectrograms on Audacity, as per the criteria of Wright *et al.* (2010). Most abundant USV types in the auditory stimulation tracks were flat (25.8%), upward ramps (18.7%), complex (12.3%), short (11.6%) and trills (10.3%).

<Figure 2>

Recording and analysis of behaviour

Behaviour in the home cage before, during, and after playback were recorded using a Sony HD camcorder (HDR- PJ810E) and subsequently analysed using Observer XT 14 software (Noldus Information Technology, Wageningen, the Netherlands). The videos were captured in Full HD resolution at 1920 x 1080 pixels, utilising an MPEG-4 format and supporting a frame rate of 50p. Frequency and duration were coded according to the following ethograms

for Experiment 1 (**Table 1a**) and Experiments 2 and 3 (**Table 1b**). During the collection of data, the human coder (TH) was blinded to treatment to prevent bias. Recording of subject-produced USVs during USV playback was done using a high-quality USB microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik; Sweden) connected via a USB interface to a PC laptop. This microphone was placed over the centre of the home cage (height 51 cm), pointing downwards towards the home cage floor (see Figure 1). Recorded sound was digitised at a sampling rate of 384 kHz and a bit depth of 24 bit using Audacity.

USVs were manually counted from spectrograms produced by Audacity and labelled as 50 kHz USVs (peak frequency between 30 and 80 kHz and duration between 10–150 ms) or 22 kHz USVs (bandwidth of > 4 kHz, peak frequency between 20-29 kHz and duration of 300 ms or more; Brudzynski, 2009; LaFollette *et al.*, 2018b; Wright *et al.,* 2010*)*. Overlapping USVs were counted individually, as two rats were present during the recording. Vocalisations were counted according to the habituation, playback and pause intervals of the acoustic playback track. USV rate was expressed as the number of calls emitted per minute (calls/min.).

After counting the number of USVs produced by the rats, the human coder compared the recorded subject produced USV track directly in time with the relevant acoustic stimuli track for all experiments. This was to prevent the double-counting of USVs, which may have been recorded from the playback track rather than produced by the rats themselves. If a vocalisation was deemed as a duplicate of the playback track, it was not counted. During the collection of data from spectrograms, the human coder was blinded to treatment to prevent bias.

<Table 1>

Statistical analysis

Tests of intra-observer reliability were conducted prior to statistical analysis. As only one observer scored all behaviours, inter-observer reliability tests were unnecessary.

All data were analysed in Genstat 19.1 using General Linear Models (GLM). Figures were generated using GraphPad Prism (ver 9.2). Assumptions of the GLMs were tested using Bartlett's test for homogeneity of variances and the Anderson-Darling test for normality. Tukey's honest significance difference (HSD) procedure was conducted for multiple comparisons on significant differences (a = 0.05). Means (M) and standard errors of the mean (SEM) were reported as backtransformed to the original scale for biological significance. For all tests, the level of statistical significance was set at $p < 0.05$.

Across all three experiments, USV and other behavioural data were collected during each session. USV counts were converted into a rate per minute to compare USV production between different experiments. The frequency and duration (sec.) were calculated for most behaviours with two exceptions: general locomotor activity was measured by the frequency of crossing over from one side to the other, and solitary play was only measured using the frequency due to the short duration of these events. Of the eleven behaviours measured in Experiments 2 and 3 (**Table 1b**), four (resting, freezing, other social and non-social behaviours) were not fitted to a General Linear Model as they occurred too infrequently to allow statistical analysis (only eight total occurrences displayed by 3 % of rats). As rats were tested together in the home cage and were likely to influence each other's behaviour, the frequency and duration of each rat's USV production and behaviour were summed to give a cage level of expression. To account for this, cage was included as a blocking factor in all models.

For Experiment 1, to investigate the effect of a single presentation of acoustic stimuli on USV production and behaviour, interval type (playback of acoustic stimuli or pause intervals) was

modelled as the main effect, with cage as the blocking factor. For Experiment 2, to investigate the effect of acoustic stimuli on rat USV production and behaviour, acoustic treatment (50 kHz, Background Noise or White Noise) and interval type (playback of acoustic stimuli or pause intervals) were modelled as the main effects with an additional interaction between the two main effects. The blocking factor was (Group/Cage_number)*Day to reflect that each cage was nested within a group, with this identification remaining unchanged across all test days. For all models, the appropriate frequency or duration of the behaviour of interest including USVs during the one minute of habituation for each day were fitted as covariates. This was to account for any baseline differences in cages that were not dependent on treatment. Effects of treatment order were accounted for within the Latin square design. For Experiment 3, USV and behavioural data were taken from the first and fifth consecutive days of playback. To investigate USV production and behaviour during playback of different acoustic stimuli, interval type (playback of acoustic stimuli or pause intervals) and day (one or five) were modelled as the main effects with additional interactions between the three main effects. Cage was the blocking factor. As rats were tested together in the home cage, the frequency and duration of each rat's behaviour were summed to give a cage level of expression. Of the eleven behaviours measured, four (resting, freezing, other social and non-social behaviours) were not fitted to a General Linear Model as they occurred too infrequently to allow statistical analysis.

To investigate individual variation in USV responses to playback of acoustic stimuli, we present the coefficient of variation as a statistical measure of the difference in variation between treatments. The higher the coefficient of variation, the greater the level of dispersion around the mean and thus, the higher the individual variation.

Results

Experiment 1: Proof-of-concept experiment to establish whether rats hear the playback stimulus

Rats increased USV production in response to playback of 50 kHz acoustic stimuli

There was a significant main effect of interval type on subject-produced USVs, with rats producing approximately four-fold the number of 50 kHz USVs during the 30 sec. of 50 kHz stimuli USVs compared with the Background Noise before and after playback (16 vs 36 vs 3 ± 0.8 USVs/min. $F_{2,17} = 74.81$, $p < 0.001$). Tukey's HSD revealed that the number of USVs produced in the intervals of Background Noise before and after playback were not significantly different from each other ($T = 1.97$, $p = 0.32$). The coefficient of variation for USVs did not differ between the three acoustic stimuli. On day one, the coefficient of variation for rats exposed to 50-kHz stimuli was 90.4%, White Noise was 92.3%, and Background Noise was 88.4%. On day five, the coefficient of variation for rats exposed to 50-kHz stimuli was 76.6%, White Noise was 81.2%, and Background Noise was 93.4%.

Other behaviours were not influenced by a 30 second playback of 50 kHz acoustic stimuli

When exposed to the playback track, there was no significant main effect of interval type on the frequency or duration of approach behaviour towards the speaker (frequency: $F_{2,35} = 0.11$, $p = 0.92$ and duration: $F_{2,35} = 0.19$, $p = 0.98$), undirected rearing (frequency: $F_{2,35} = 0.29$, $p = 0.77$ and duration: $F_{2,35} = 0.52$, $p = 0.56$) or inactivity (frequency: $F_{2,35} = 0.14$, $p = 0.10$ and duration: $F_{2,35} = 0.12$, $p = 0.93$) during the 30sec. period of acoustic stimuli compared with the before and after Background Noise intervals.

Experiment 2: Playback of 50 kHz, White Noise, and Background Noise in a Latin Square experimental design.

Subject-produced 50 kHz USVs increased in response to playback of 50 kHz acoustic stimuli and White Noise

There was a significant main effect of acoustic stimuli treatment on subject produced USVs, with rats producing more USVs in response to 50 kHz and White Noise compared to Background Noise ($F_{2,239} = 6.05$, $p < 0.05$; **Figure 3**). Tukey's HSD test indicated that the mean number of USVs produced during the 50 kHz stimuli (T = 11.45, $p = 0.02$) and White Noise (T = 13.47, $p = 0.02$) differed significantly from Background Noise but not from each other (T = 3.79, $p = 0.23$). There was no main effect of interval type on USV production ($F_{1,239} = 3.63$, $p = 0.06$). The coefficient of variation for USVs was highest in response to White Noise at 89.9%, Background Noise at 63.2%, and then 50-kHz stimuli at 52.9%, respectively.

<Figure 3>

Rats exposed to White Noise conducted more social play than rats exposed to 50 kHz or Background Noise

There was a main effect of treatment on the frequency and duration of social play, with rats conducting more social play in response to White Noise, followed by Background Noise and then 50 kHz (frequency: 2.9 vs 2.6 vs 1.9 ± 0.3 events/min; $F_{2,239} = 2.98$, $p < 0.05$ and duration: 21.9 vs 17.8 vs 13.3 ± 2.2 sec/min; $F_{2,239} = 3.55$, $p = 0.03$). Tukey's HSD test indicated that the frequency of social play during each treatment was significantly different from each other (50-kHz vs White Noise: T = 11.10, $p = 0.03$, 50 kHz vs Background Noise: T = 12.07, $p = 0.03$, White Noise vs Background Noise: T = 9.88, $p < 0.05$). Similarly, Tukey's HSD test indicated that the durations of social play during each treatment were

significantly different from each other (50-kHz vs White Noise: T = 12.63, $p = 0.02$, 50-kHz vs Background Noise: T = 11.71, $p = 0.03$, White Noise vs Background Noise: T = 8.06, $p < 0.05$). There was no significant effect of interval type on social play (frequency; $F_{5,239} = 0.40$, $p = 0.06$ and duration; $F_{5,239} = 0.53$, $p = 0.8$).

<Figure 4>

There was a tendency towards a main effect of treatment on the duration, but not the frequency ($F_{2,239} = 0.20$, $p = 0.82$), of approach behaviour towards speaker, with rats exposed to 50-kHz and White Noise conducting the longest durations of approach behaviour, followed by rats exposed to Background Noise (26.4 vs 25.7 vs 21.4 ± 1.7 seconds per minute; $F_{2,239} = 2.51$, $p = 0.008$). Tukey's HSD test indicated that the duration of approach behaviour produced during the 50-kHz stimuli (T = 10.91, p = 0.04) and White Noise (T = 9.89, p = 0.05) differed significantly from Background Noise but not each other (T = 2.65, p = 0.98). There was no significant effect of interval type on the frequency or duration of approach behaviour towards the speaker play (frequency; $F_{5,239} = 0.18$, $p = 0.67$ and duration; $F_{5,239} = 0.38$, $p = 0.54$).

There was a significant main effect of interval type, but not treatment ($F_{2,239} = 0.76$, $p = 0.47$), on solitary play, with rats conducting more play during the pause than during playback intervals (1.4 vs 0.9 ± 0.1 events per minute; $F_{5,239} = 7.75$, $p = 0.006$).

There was no significant main effect of treatment on locomotor activity (frequency: $F_{2,239} = 1.87$, p = 0.16), undirected rearing (frequency: $F_{2,239} = 0.17$, p = 0.84 and duration: $F_{2,239} = 6.68$, p = 0.12), self-grooming (frequency: $F_{2,239} = 0.17$, p = 0.83 and duration: $F_{2,239} = 6.67$, p = 0.3) and digging (frequency: $F_{2,239} = 1.39$, p = 0.25 and duration: $F_{2,239} = 2.51$, p = 0.08).

Experiment 3: Repeated presentation of acoustic stimuli for five days

USV production was dependent on an interaction between acoustic stimuli and day

There was significant main effect of acoustic stimuli treatment ($F_{2,479} = 0.75$, $p < 0.05$) and day ($F_{1,479} = 4.25$, $p = 0.04$) on subject produced USVs (**Figure 4**). Rats produced the most USVs in response to 50-kHz USVs, followed by White Noise and Background Noise and on day 1 compared to day 5. On day 1, rats produced more USVs in response to 50-kHz and White Noise compared to Background Noise. Tukey's HSD test indicated that on day 1, the mean number of USVs produced during the 50-kHz stimuli (T = 10.67, p = 0.02) and White Noise (T = 12.58, p = 0.03) differed significantly from Background Noise but not each other (T = 2.03, p = 0.97). On day 5, USV production increased in rats exposed to 50-kHz acoustic stimuli, with no change in response to Background Noise. Tukey's HSD test indicated that on day 5, the mean number of USVs produced during the 50 kHz stimuli (T = 10.67, p = 0.02) differed significantly from White Noise (T = 12.98, p = 0.003) and Background Noise (T = 14.37, p = 0.002) which were not different from each other (T = 2.03, p = 0.84).There was no significant main effect of interval type on subject-produced USVs ($F_{1,479} = 1.69$, $p = 0.19$).

<Figure 4>

Playback of 50 kHz acoustic stimuli increased approach behaviour towards the speaker and social play

Treatment had a significant effect on approach behaviour towards the speaker, with rats exposed to 50 kHz acoustic stimuli conducting the highest frequency and duration of approach behaviour, followed by those exposed to White Noise and then Background Noise (frequency: 15.3 vs 13.4 vs 12.5 ± 1.2 events per minute; $F_{2, 479} = 4.07$, $p = 0.04$) and duration: 90.8 vs 73.2 vs 56.5 ± 7.2 seconds per minute; $F_{2, 479} = 10.55$, $p < 0.01$). Tukey's HSD test indicated that the mean duration of approach behaviour significantly differed between all three treatments across both days (50 kHz vs White Noise: T = 10.12, p = 0.04, 50-kHz vs Background Noise: T = 13.56, p = 0.01, White Noise vs Background Noise: T = 9.80, p = 0.04). There was a significant effect of day on the frequency and duration of approach behaviour, with rats conducting more approach behaviour on day 1 compared to

day 5 (frequency: 2.8 vs 1.9 ± 0.1 events/min; $F_{1, 479} = 4.08$, $p = 0.02$ and duration: 11.2 vs 8.9 ± 0.6 sec./min; $F_{1, 479} = 7.57$, $p = 0.008$). There was also a main effect of interval type on the frequency and duration of approach behaviour, with rats conducting more approach behaviour during the playback intervals compared with the pauses (frequency: 2.1 vs 1.9 ± 0.1 events/min; $F_{5, 479} = 3.16$, $p < 0.05$ and duration: 11.1 vs 8.9 ± 0.6 sec./min; $F_{5, 479} = 7.03$, $p < 0.05$).

There was a significant interaction effect on the frequency and duration of social play between treatment and day (frequency: $F_{2,479} = 4.22$, 0.02 and duration: $F_{2,479} = 4.57$, p = 0.01). On day 1, rats exposed to White Noise and Background Noise conducted the most social play, followed by 50 kHz (frequency: 0.5 vs 0.5 vs 0.4 ± 0.2 events per minute and duration: 3.8 vs 3.2 vs 2.1 ± 2.4 seconds per minute). Tukey's HSD test indicated that on day 1, the mean duration of social play during playback of White Noise (T = 12.76, p < 0.05) and Background Noise (T = 13.54, p < 0.05) differed significantly from 50 kHz but not each other (T = 2.30, p = 0.76). On day 5, this effect was reversed, with rats exposed to 50 kHz conducting the most social play, followed by White Noise and Background Noise (frequency: 0.8 vs 0.5 vs 0.4 ± 0.2 events per minute and duration: 4.0 vs 2.3 vs 2.1 ± 2.4 sec./min). Tukey's HSD test indicated that on day 5, the mean duration of social play during playback of White Noise (T = 13.50, p = 0.03) and Background Noise (T = 11.93, p = 0.02) differed significantly from 50-kHz but not each other (T = 3.01, p = 0.81).

<Figure 6>

There was no significant main effect of treatment on locomotor activity (frequency: $F_{2,479} = 0.89$, 0.43), undirected rearing (frequency: $F_{2,479} = 0.14$, $p = 0.87$ and duration: $F_{2,479} = 0.30$, $p = 0.75$), self-grooming (frequency: $F_{2,479} = 1.32$, $p = 0.31$ and duration: $F_{2,479} = 3.27$, $p = 0.07$), digging (frequency: $F_{2,479} = 0.13$, $p = 0.87$ and duration: $F_{2,479} = 0.33$, $p = 0.76$), inactivity (frequency: $F_{2,479} = 0.81$, $p = 0.46$ and duration: $F_{2,479} = 1.64$, $p = 0.18$) and solitary play (frequency: $F_{2,479} = 0.21$, $p = 0.61$). There was a significant main effect of interval type

on locomotor activity and self-grooming, with rats becoming more active during the playback intervals than the pause intervals (2.0 vs 1.8 ± 0.04 events/min; $F_{5,479} = 10.55$, $p = 0.001$) and conducting more self-grooming during the pause intervals than the playback intervals (frequency: 0.4 vs 0.2 ± 0.04 events/min; $F_{5,479} = 10.73$, $p = 0.001$ and duration: 3.0 vs 1.4 ± 0.5 sec./min; $F_{5,479} = 9.63$, $p = 0.002$). There was a significant main effect of day on locomotor activity, with rats becoming more active on day 5 compared to day 1 (1.8 vs 2.9 ± 0.04 events/min; $F_{1,479} = 8.4$, $p = 0.004$).

Discussion

The potential of using playback of positively valenced vocalisations to induce a positive affective state and promote positive animal welfare has yet to be fully explored. Playback of vocalisations to manipulate affective state is particularly promising for rats, with some previous work demonstrating consistent differences in approach behaviour towards positively valenced vocalisations (Schwarting *et al*., 2018; Wöhr *et al*., 2016; Wöhr *et al*., 2008). The home cage playback paradigm was designed to be conducive to measuring more of the full behavioural repertoire of rats for the purpose of welfare assessment. Explicitly, this paradigm was designed to meet four criteria (in no specific order of importance): the provision of sufficient space, a conspecific with whom to enact social behaviours, the removal of potential stressors (*i.e.,* bright lighting, handling, and novelty) and the ability to measure USV production as an indicator of affective state. Here, we found support for the use of the home cage playback paradigm to manipulate affective state; rats produced more USVs in response to 50 kHz and White Noise acoustic stimuli than Background Noise. After five days of repeated playback of these stimuli, USV production in response to White Noise dropped to that of Background Noise and increased in response to 50 kHz stimuli. Rats also conducted the most approach behaviour towards the playback speaker and social play in response to 50 kHz acoustic stimuli. This suggests that while rats became habituated to the

White Noise stimuli, the positive response to 50 kHz stimuli increased after the five days of presentation. Overall, these results illustrate the potential of the home cage playback paradigm to induce a positive affective state in rats.

Do rats perceive the acoustic stimuli? Confirmation through USV production

Experiment 1 was a proof-of-concept test, using a single 30 second presentation of 50 kHz USVs to cages of paired rats in between two intervals of Background Noise. A rapid increase in subject produced USVs during this 30 second playback interval confirmed that rats could perceive the acoustic stimulus presented by the speaker. The production of 50 kHz USVs and the absence of 22 kHz USVs also provided evidence that the rats did not find the equipment set up or protocol aversive, even with minimal habituation. However, the effect of playback was restricted to USV production, as rats showed no change in other behavioural expressions. This could be because this experiment used a short exposure to the 50 kHz stimuli, which did not allow sufficient time for rats to process or locate the source. For example, in the radial maze paradigm, rats are exposed to a minimum of 60 sec. in total, with rats consistently exhibiting increased approach behaviour and social exploration compared to background noise in response to 50 kHz USVs (Wöhr *et al*., 2016; Schwarting *et al.,* 2018).

The playback of 50-kHz stimuli induced a positive affective state in rats, as indicated by USV production, approach behaviour towards the speaker and social play

Playback paradigms are based on the premise that as vocalisations are associated with a particular affective state, the playback of these vocalisations will have valence-matched effects on receiving conspecifics (Briefer, 2018; Manteuffel *et al*., 2004). The process through which the affective state of the producer and receiver converges is termed emotional contagion (Hatfield *et al*., 1993; Waal, 2007). In rats, 50 kHz USVs are the primary vocalisation type associated with positive affect (Barker, 2018; Browning *et al*., 2011;

Burgdorf *et al*., 2007) and, thus, are most likely to facilitate emotional contagion and induce a positive affective state in receivers. Consistent with this hypothesis, there was evidence that the presentation of 50 kHz USVs in the home cage playback paradigm successfully facilitated the transfer of positive emotional contagion; in all three experiments, there was a significant increase in subject-produced 50 kHz calls in response to 50 kHz USVs compared to Background Noise.

Whilst the positive effect of 50 kHz acoustic stimuli on USV production during a single exposure is consistent with previous work (Olszyński *et al*., 2020; Engelhardt et al., 2017), this is one of the first studies to also monitor the change in USV production or other behaviours after repeated presentations of acoustic stimuli in rodents. In Experiment 3, rats were presented with the same acoustic stimuli treatments for five consecutive days. After five days of exposure, rats exposed to the 50 kHz stimuli USVs produced significantly more 50 kHz USVs than when exposed to Background Noise. This consistent effect of 50 kHz acoustic stimuli on USV production suggests that 50 kHz USVs retain their appetitive properties after five days of repeated exposure (Wöhr, 2017). Overall, the increase in subject produced USVs in comparison to Background Noise suggests that exposure to 50 kHz acoustic stimuli in the home cage playback paradigm successfully induces a positive affective state in juvenile male Wistar rats during both single and repeated presentations.

The use of approach behaviour to measure an animal's perception of certain stimuli is based on the theory that animals will approach or avoid a stimulus according to the perceived valence of the given stimulus (Kurt, 1936). Acoustic stimuli had a consistent effect on the frequency and duration of approach behaviour towards the speaker across days one and five; rats conducted the most approach behaviour in response to 50 kHz acoustic stimuli, followed by exposure to White Noise and then Background Noise. In Experiment 3, whilst there was a decrease in time spent conducting approach behaviour towards the speaker on day 5 compared to day 1, this was constant across all acoustic stimuli, suggesting a

universal habituation effect. This result is analogous to that found in the radial maze paradigm, where playback of 50 kHz USVs consistently increases approach behaviour towards the sound source (Berz et al, 2022; Schwarting *et al*., 2018; Wöhr and Schwarting, 2007). However, this habituation effect does not uniformly apply to all rat strains, as variations have been observed between Wistar and Sprague-Dawley rats (Berz *et al,* 2021). Overall, these results provide evidence that rats are most motivated to approach the 50 kHz acoustic stimuli.

Play has been proposed as a promising indicator of positive emotions and welfare in domesticated animals (*e.g.,* Boissy *et al*., 2007; Held and Špinka, 2011) and play tends to occur when animals are under favourable conditions, as it is reduced or disappears when the animal's potential ability to survive is challenged (Held and Špinka, 2011; Boissy *et al*., 2007; Lawrence, 1987). In Experiments 2 and 3, there was an effect of acoustic stimuli on social play behaviour during presentation of 50 kHz USVs and White Noise. In Experiment 2 and on the first day of Experiment 3, 50 kHz and White Noise stimuli was not universally matched with increased play; social play was reduced in response to 50 kHz stimuli, whilst social play during White Noise was similar to that observed during Background Noise. However, after five days of presentation, rats exposed to 50 kHz USVs conducted more social play than when exposed to Background Noise and White Noise, consistent with the changes in USV production. The repeated need for presentations to induce social play in rats, may be attributed to a shift in motivational priorities from exploration on day 1 to a decreased emphasis on exploration by day 5, allowing for low-risk engagement in social play as an expression of positive affective state.

White Noise had an unexpected effect on affective state

In Experiments 2 and 3, there was an unexpected effect of White Noise on subject-produced 50 kHz USVs during a single exposure; subject-produced USVs increased in response to White Noise to a similar level to those produced on exposure to 50 kHz acoustic stimuli. The

White Noise stimulus used in these studies was generated with the initial aim of providing an additional control stimulus that provides the same level of an auditory signal without the modulated properties of USVs. As previous work has suggested that white noise in the range of 0 to 27 kHz produces behavioural indicators of aversion (Wöhr and Schwarting, 2012), the White Noise presented here was filtered to be above 30 kHz. As a result of this process, the White Noise acoustic stimuli might serve as an ambiguous stimulus, subject to varying interpretations among individuals, akin to cognitive bias, i.e. it could be perceived either positively or negatively depending on the internal state of the animal (Brydges *et al*, 2011). In this sense White Noise would be different to the general noise in the facility and the Background Noise acoustic stimuli which are perceived as neutral stimuli.

Considering that rats show rapid habituation to complex acoustic stimuli, *i.e.,* 50 kHz USVs (Wöhr and Schwarting, 2012), it is likely that exposure to a less complex stimulus such as White Noise would result in decreased interest and arousal over repeated presentations. Although the White Noise acoustic stimulus underwent the same process as the 50 kHz USVs, the lack of complexity or relevance for social communication is also likely to contribute to rapid habituation to White Noise. Consistent with this, the effect of White Noise on USV production by subjects did not remain consistent after five days of repeated exposure; on day 5, USV production in response to White Noise dropped to a similar level to that produced in response to Background Noise. Overall, the increase in subject produced USVs on day 1 suggests that exposure to White Noise acoustic stimuli successfully induces a positive affective state in juvenile male Wistar rats during a single but not repeated presentation. The lack of communicative information in the White Noise acoustic stimulus suggests that the increase in positive affective state is likely to result from positively-valenced arousal rather than emotional contagion.

Conclusion

Overall, this work provides evidence that the home cage playback paradigm holds promise as a method for inducing a positive affective state in rats. The consistent positive emotional contagion effects, as demonstrated by increased subject produced USVs, approach behaviour, and social play in response to 50 kHz stimuli, persist even after five days of repeated exposure. However, the unexpected impact of White Noise on subject produced USVs suggests its role as an ambiguous stimulus potentially through its arousing properties. These findings emphasise the nuanced influence of different acoustic stimuli on affective states, providing valuable insights for utilising playback paradigms in the assessment of animal welfare.

Funding

Alistair Lawrence, Simone Meddle and Sarah Brown acknowledge Roslin Institute strategic grant funding from the U.K. Biotechnology and Biological Sciences Research Council (BB/P013759/1). Alistair Lawrence also receives funding support from the Scottish Government's Rural and Environment Science and Analytical Services Division (RESAS).

The work was also supported by funding from the Animal Physiology and Livestock Systems (PHASE) department at the French National Research Institute for Agriculture, Food and Environment (INRAE) to Birte Nielsen and Vincent Bombail and from NC3Rs to Alistair Lawrence, Simone Meddle, Sarah Brown and Vincent Bombail.

Acknowledgements

We gratefully acknowledge Iain Nevison for his statistical advice. No study would have been possible without the excellent care and knowledge of the technical staff of the Biological Resource Facility at INRAE and the Biological Research Facility at the Roslin Institute, the University of Edinburgh. We thank Lamia Benabed for her help with the qualitative analysis of USV recordings.

References

Alvarez, E.O., Alvarez, P.A., 2008. Motivated exploratory behaviour in the rat: The role of hippocampus and the histaminergic neurotransmission. Behav Brain Res 186, 118–125. https://doi.org/10.1016/j.bbr.2007.07.038

Barker, D.J., 2018. Chapter 24 - Ultrasonic Vocalizations as an Index of Positive Emotional State, in: Brudzynski, S.M. (Ed.), Handbook of Behavioral Neuroscience. Elsevier, pp. 253–260. https://doi.org/10.1016/b978-0-12-809600-0.00024-x

Bayne, K., Würbel, H., 2014. The impact of environmental enrichment on the outcome variability and scientific validity of laboratory animal studies. Revue Sci Et Technique Int Office Epizootics 33, 273–80. https://doi.org/10.20506/rst.33.1.2282

Begni, V., Sanson, A., Pfeiffer, N., Brandwein, C., Inta, D., Talbot, S.R., Riva, M.A., Gass, P., Mallien, A.S., 2020. Social isolation in rats: Effects on animal welfare and molecular markers for neuroplasticity. PLOS One 15, e0240439. https://doi.org/10.1371/journal.pone.0240439

Berz, A., de Souza, C. P., Wöhr, M., & Schwarting, R. K., 2021). Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats. IScience, 24(5).

Berz AC, Wöhr M, Schwarting RKW. Response Calls Evoked by Playback of Natural 50-kHz Ultrasonic Vocalizations in Rats. Front Behav Neurosci. 2022 Jan 14;15:812142. doi: 10.3389/fnbeh.2021.812142. PMID: 35095442; PMCID: PMC8797927.

Boissy, Alain, Manteuffel, G., Jensen, M.B., Moe, R.O., Spruijt, B., Keeling, L.J., Winckler, C., Forkman, B., Dimitrov, I., Langbein, J., Bakken, M., Veissier, I., Aubert, A., 2007. Assessment of positive emotions in animals to improve their welfare. Physiol Behav 92, 375–397.

Bombail, V., Brown, S.M., Hammond, T.J., Meddle, S.L., Nielsen, B.L., Tivey, E.K.L., Lawrence, A.B., 2021. Crying With Laughter: Adapting the Tickling Protocol to Address Individual Differences Among Rats in Their Response to Playful Handling. Frontiers Vet Sci 8, 1–5. https://doi.org/10.3389/fvets.2021.677872

Briefer, E.F., 2018. Vocal contagion of emotions in non-human animals. Proc Royal Soc B Biological Sci 285, 20172783. https://doi.org/10.1098/rspb.2017.2783

Briefer, E.F., 2012. Vocal expression of emotions in mammals: mechanisms of production and evidence. J Zool 288, 1–20. https://doi.org/10.1111/j.1469-7998.2012.00920.x

Browning, J.R., Browning, D.A., Maxwell, A.O., Dong, Y., Jansen, H.T., Panksepp, J., Sorg, B.A., 2011. Positive affective vocalizations during cocaine and sucrose self-administration: A model for spontaneous drug desire in rats. Neuropharmacology 61, 268–275. https://doi.org/10.1016/j.neuropharm.2011.04.012

Brudzynski, S., 2013. Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. Curr Opin Neurobiol 23, 310–317. https://doi.org/10.1016/j.conb.2013.01.014

Brudzynski, S.M., 2007. Ultrasonic calls of rats as indicator variables of negative or positive states: Acetylcholine–dopamine interaction and acoustic coding. Behav Brain Res 182, 261–273. https://doi.org/10.1016/j.bbr.2007.03.004

Brydges, N. M., Leach, M., Nicol, K., Wright, R., & Bateson, M. 2011. Environmental enrichment induces optimistic cognitive bias in rats. Animal Behaviour, 81(1), 169–175. https://doi.org/10.1016/j.anbehav.2010.09.030

Burgdorf, J., Kroes, R.A., Moskal, J.R., Pfaus, J.G., Brudzynski, S.M., Panksepp, J., 2008. Ultrasonic Vocalizations of Rats (Rattus norvegicus) During Mating, Play, and Aggression: Behavioral Concomitants, Relationship to Reward, and Self-Administration of Playback. J Comp Psychol 122, 357–367. https://doi.org/10.1037/a0012889

Burgdorf, J., Wood, P.L., Kroes, R.A., Moskal, J.R., Panksepp, J., 2007. Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies. Behav Brain Res 182, 274–283. https://doi.org/10.1016/j.bbr.2007.03.010

Cait, J., Cait, A., Scott, R.W., Winder, C.B., Mason, G.J., 2022. Conventional laboratory housing increases morbidity and mortality in research rodents: results of a meta-analysis. Bmc Biol 20, 15. https://doi.org/10.1186/s12915-021-01184-0

Carbone, L., 2021. Estimating mouse and rat use in American laboratories by extrapolation from Animal Welfare Act-regulated species. Sci Rep-uk 11, 493. https://doi.org/10.1038/s41598-020-79961-0

Engelhardt K.A, Fuchs E, Schwarting R.K.W, Wöhr M. 2017 Effects of amphetamine on pro-social ultrasonic communication in juvenile rats: Implications for mania models. Eur Neuropsychopharmacol. 27(3):261-273. doi: 10.1016/j.euroneuro.2017.01.003.

Franks, B., Champagne, F.A., Higgins, E.T., 2013. How Enrichment Affects Exploration Trade-Offs in Rats: Implications for Welfare and Well-Being. PLOS One 8, e83578. https://doi.org/10.1371/journal.pone.0083578

Fraser, D., Duncan, I.J.H., 1998. 'Pleasures', 'Pains' and Animal Welfare: Toward a Natural History of Affect. Animal Welfare 4, 383–396. no doi available.

Gärtner, K., Büttner, D., Döhler, K., Friedel, R., Lindena, J., Trautschold, I., 1980. Stress response of rats to handling and experimental procedures. Lab Anim 14, 267–274. https://doi.org/10.1258/002367780780937454

Hammond, T., Bombail, V., Nielsen, B.L., Meddle, S.L., Lawrence, A.B., Brown, S.M., 2019. Relationships between play and responses to tickling in male juvenile rats. Appl Anim Behav Sci 104879. https://doi.org/10.1016/j.applanim.2019.104879

Hatfield, E., Cacioppo, J.T., Rapson, R.L., 1993. Emotional Contagion. Cambridge University Press, Cambridge. https://doi.org/10.1017/cbo9781139174138.007

Held, S.D.E., Špinka, M., 2011. Animal play and animal welfare. Anim Behav 81, 891–899. https://doi.org/10.1016/j.anbehav.2011.01.007

Hubrecht, R.C., 2014. The Welfare of Animals Used in Research: Practice and Ethics, UFAW/Wiley-Blackwell Animal Welfare Book Series. John Wiley & Sons, Incorporated.

Kerkhof, L., Damsteegt, R., Trezza, V., Voorn, P., Vanderschuren, L., 2013. Social play behavior in adolescent rats is mediated by functional activity in medial prefrontal cortex and striatum. Neuropsychopharmacology 38, 1899–1909. https://doi.org/10.1038/npp.2013.83

Knutson, B., Burgdorf, J., Panksepp, J., 1998. Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. J Comp Psychol 112, 65–73. https://doi.org/10.1037/0735-7036.112.1.65

Korte, S.M., Boer, S.F.D., 2003. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol 463, 163–175. https://doi.org/10.1016/s0014-2999(03)01279-2

Kurt, L., 1936. A dynamic theory of personality-selected papers. Read Books Ltd. https://doi.org/10.1097/00005053-193611000-00051

LaFollette, Megan R., O'Haire, M.E., Cloutier, S., Gaskill, B.N., 2018a. A Happier Rat Pack: The Impacts of Tickling Pet Store Rats on Human-Animal Interactions and Rat Welfare. Appl Anim Behav Sci 203, 92–102. https://doi.org/10.1016/j.applanim.2018.02.006

LaFollette, Megan R , O'Haire, M.E., Cloutier, S., Gaskill, B.N., 2018b. Practical rat tickling: determining an efficient and effective dosage of heterospecific play. Appl Anim Behav Sci. https://doi.org/10.1016/j.applanim.2018.08.005

Lawrence, A.B., 1987. Consumer demand theory and the assessment of animal welfare. Anim Behav 35, 293–295. https://doi.org/10.1016/s0003-3472(87)80236-1

Lever, C., Burton, S., O'Keefe, J., 2006. Rearing on Hind Legs, Environmental Novelty, and the Hippocampal Formation. Rev Neuroscience 17, 111–134. https://doi.org/10.1515/revneuro.2006.17.1-2.111

Litvin, Y., Blanchard, D.C. and Blanchard, R.J., 2007. Rat 22 kHz ultrasonic vocalizations as alarm cries. Behavioural brain research, 182(2), 166-172. doi:10.1016/j.bbr.2006.11.038

Makowska, I.J., Weary, D.M., 2020. A Good Life for Laboratory Rodents? Ilar J 60, 373–388. https://doi.org/10.1093/ilar/ilaa001

Manteuffel, G., Puppe, B., Schön, P.C., 2004. Vocalization of farm animals as a measure of welfare. Appl Anim Behav Sci 88, 163–182. https://doi.org/10.1016/j.applanim.2004.02.012

Nathiya, V.C., Vanisree, A.J., 2010. Investigations on light induced stress model and on the role of Phyllanthus amarus in attenuation of stress related depression with focus on 5HT2Am- RNA expression. Ann Neurosci 17, 167–175. https://doi.org/10.5214/ans.0972.7531.1017405

Neville, V., Lecorps, B., Mendl, M., 2022. Good science requires better animal welfare. Science 376, 809–809. https://doi.org/10.1126/science.abq7198

Olszyński, K.H., Polowy, R., Małż, M., Boguszewski, P.M., Filipkowski, R.K., 2020. Playback of Alarm and Appetitive Calls Differentially Impacts Vocal, Heart-Rate, and Motor Response in Rats. Iscience 23, 101577. https://doi.org/10.1016/j.isci.2020.101577

Saito, Y., Yuki, S., Seki, Y., Kagawa, H., Okanoya, K., 2016. Cognitive bias in rats evoked by ultrasonic vocalizations suggests emotional contagion. Behav Process 132, 5–11. https://doi.org/10.1016/j.beproc.2016.08.005

Schwarting, R.K.W., Jegan, N., Wöhr, M., 2007. Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. Behav Brain Res 182, 208–222. https://doi.org/10.1016/j.bbr.2007.01.029

Schwarting, R.K.W., Kisko, T.M., Wöhr, M., 2018. Chapter 34 Playback of Ultrasonic Vocalizations to Juvenile and Adult Rats: Behavioral and Neuronal Effects, in: Brudzynski, S.M. (Ed.), Handbook of Behavioral Neuroscience, Handbook of Behavioral Neuroscience. pp. 357–369. https://doi.org/10.1016/b978-0-12-809600-0.00034-2

Schwarting, R.K.W., Wöhr, M., 2012. On the relationships between ultrasonic calling and anxiety-related behavior in rats. Braz J Med Biol Res 45, 337–348. https://doi.org/10.1590/s0100-879x2012007500038

Seffer, D., Schwarting, R.K.W., Wöhr, M., 2014. Pro-social ultrasonic communication in rats: Insights from playback studies. J Neurosci Meth 234, 73–81. https://doi.org/10.1016/j.jneumeth.2014.01.023

UK Home Office, 2016. Statistics of Scientific Procedures on living animals, Great Britain. At https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/627284/annual-statistics-scientific-procedures-living-animals-2016.pdf (2017).

Waal, F.B.M. de, 2007. The “Russian doll” model of empathy and imitation, in: Bråten, S. (Ed.), On Being Moved: From Mirror Neurons to Empathy. John Benjamins Publishing Company, pp. 49–69. https://doi.org/10.1075/aicr.68.06waa

Webber, E.S., Harmon, K.M., Beckwith, T.J., Peña, S., Burgdorf, J., Panksepp, J., Cromwell, H.C., 2012. Selective breeding for 50kHz ultrasonic vocalization emission produces alterations in the ontogeny and regulation of rough-and-tumble play. Behav Brain Res 229, 138–144. https://doi.org/10.1016/j.bbr.2012.01.012

Wöhr, M., 2017. Ultrasonic communication in rats: appetitive 50-kHz ultrasonic vocalizations as social contact calls. Behav Ecol Sociobiol 72, 14. https://doi.org/10.1007/s00265-017-2427-9

Wöhr, M., Houx, B., Schwarting, R.K., Spruijt, B., 2008. Effects of experience and context on 50-kHz vocalizations in rats. Physiol Behav 93, 766–776. https://doi.org/10.1016/j.physbeh.2007.11.031

Wöhr, M., Schwarting, R., 2013. Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 354, 81–97. https://doi.org/10.1007/s00441-013-1607-9

Wöhr, M., Schwarting, R., 2009. Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations. Pharmacol Biochem Behav 94, 285–295. https://doi.org/10.1016/j.pbb.2009.09.008

Wöhr, M., Schwarting, R.K., 2007. Ultrasonic Communication in Rats: Can Playback of 50-kHz Calls Induce Approach Behavior? PLOS One 2, e1365. https://doi.org/10.1371/journal.pone.0001365

Wöhr, M., Schwarting, R.K.W., 2012. Testing social acoustic memory in rats: Effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations. Neurobiol Learn Mem 98, 154–164. https://doi.org/10.1016/j.nlm.2012.05.004

Wöhr, M., Seffer, D., Schwarting, R.K.W., 2016. Studying Socio-Affective Communicationin Rats through Playback of Ultrasonic Vocalizations. Current Protocols in Neuroscience 75, 8–35. https://doi.org/10.1002/cpns.7

Wright, Jennifer M , Gourdon, J.C., Clarke, P.B., 2010. Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. Psychopharmacology 211, 1–13. https://doi.org/10.1007/s00213-010-1859-y

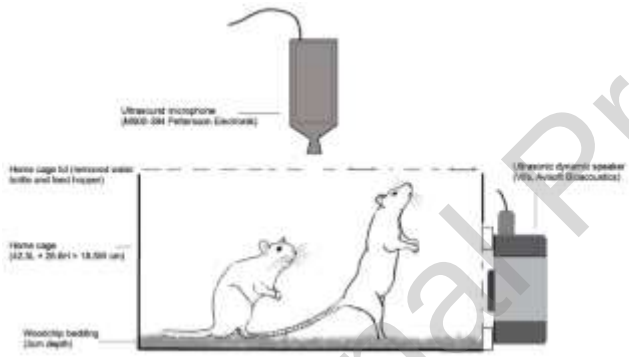


Figure 1. Equipment setup used for presenting different acoustic stimuli in the home cage to paired juvenile male Wistar rats. This figure is not to scale.

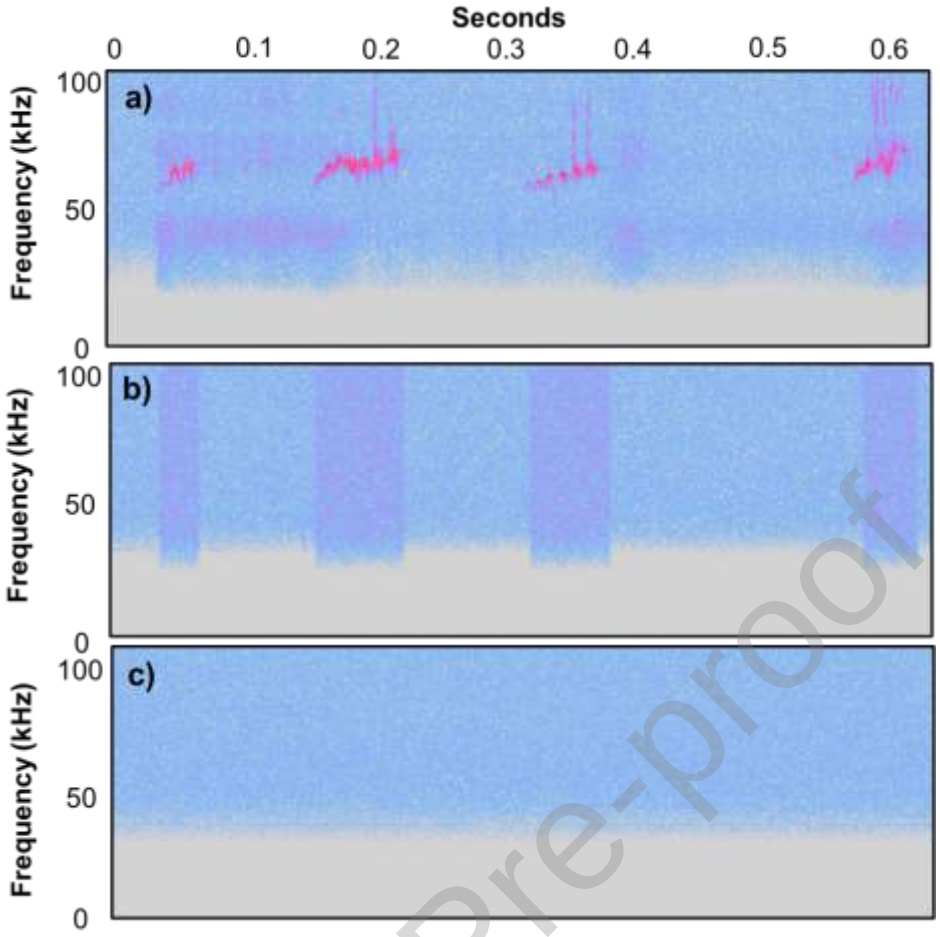


Figure 2. Example spectrograms of the three types of acoustic stimuli presented to juvenile male Wistar rats; a) 50-kHz USVs, b) White Noise, and c) Background Noise. All USVs were recorded from rats of the same developmental stage, sex, and strain used in other studies.

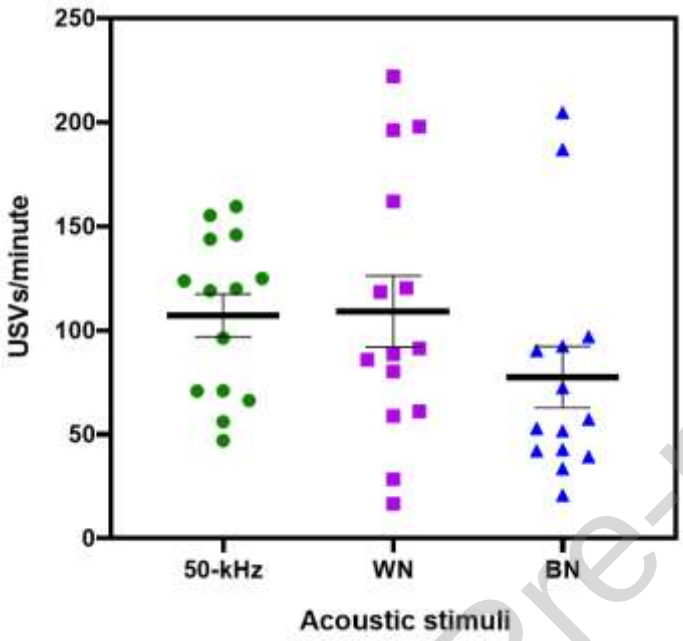


Figure 3. Mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs (N = 14) of juvenile male rats housed in pairs during Experiment 2. All rats were presented with all three treatments (50-kHz acoustic stimuli (green circles), White Noise (WN; purple squares), or Background Noise (BN; blue triangles)) in a Latin square design as pairs in the home cage. Markers are means for each pair with the black lines indicating the mean ± SEM for each treatment by day.

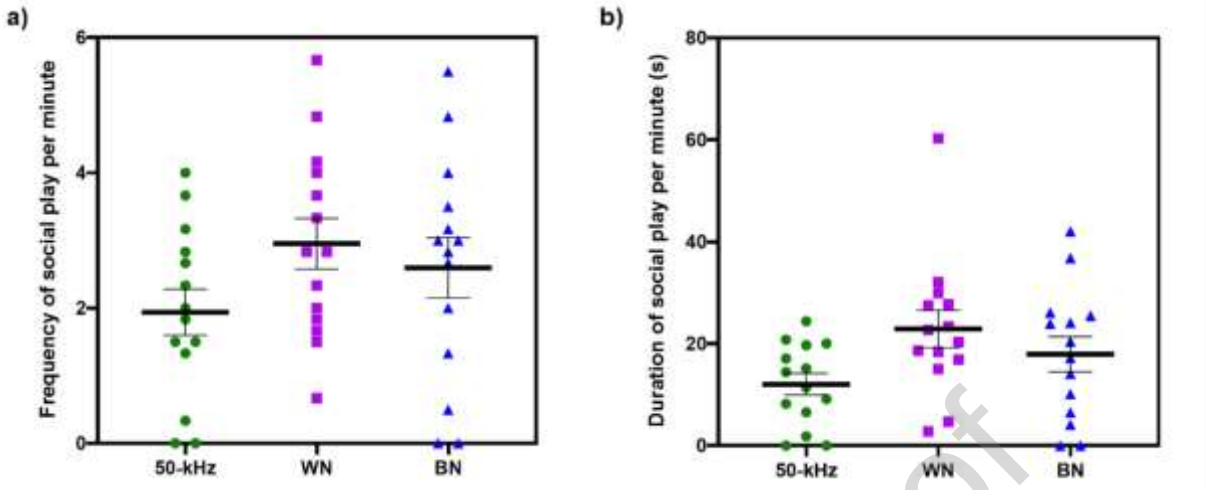


Figure 4. Panels representing the a) frequency and b) duration of social play events (s) of juvenile male rats (N = 28 rats housed in pairs; 14 cages) during Experiment 2. All rats were presented with all three treatments (50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares), or Background Noise (BN) (blue triangles)) in a Latin square design as pairs in the home cage. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day

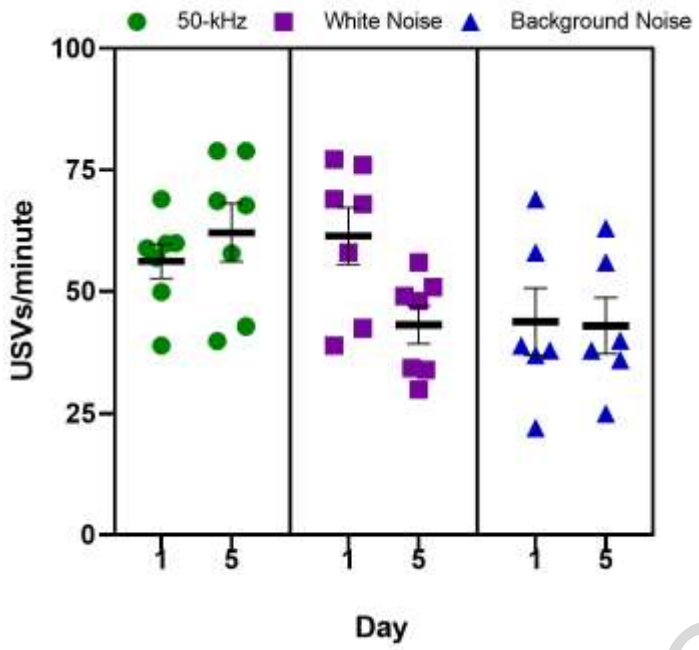


Figure 5. Mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs (N = 20) of juvenile male rats housed in pairs on days one and five of acoustic playback in Experiment 3. Rats were presented with either 50-kHz acoustic stimuli (green circles; n = 7), White Noise (purple squares; n = 7) or Background Noise (blue triangles; n = 6). Markers are means for each cage with the black lines indicating the mean ± SEM for each treatment by day.

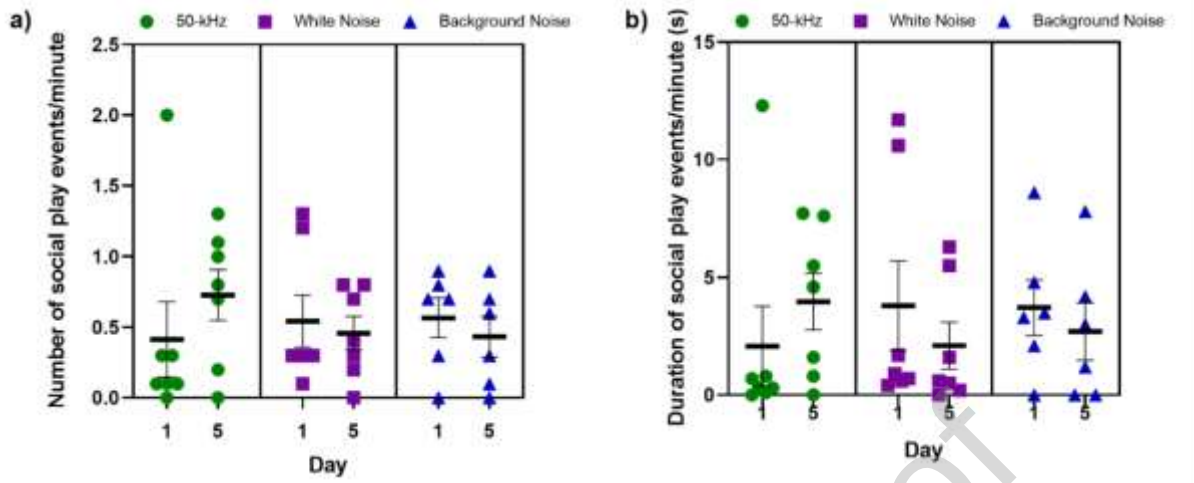


Figure 6. Panels showing a) the mean frequency and b) duration (s) of social play in pairs of juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) during presentation of one of three acoustic stimuli on the first and fifth day of playback in Experiment 3. Rats were presented with either 50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares) or Background Noise (BN) (blue triangles) for five days. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Table 1a. Ethogram used in Experiment 1 developed for recording the behaviour of pair-housed male Wistar rats (N = 7 cages) in the home cage during playback of 50-kHz USVs.

Behaviour	Description
Approach towards the speaker	Rat orients its body and head so that the nose is directed towards and within 1cm of the speaker (Lever *et al*., 2006).
Rearing (undirected)	Rat raises both front paws off the ground (can be in contact with the wall or not), standing up on hind legs. Includes all rears, except

	those with the rat's nose directed towards the speaker (Lever *et al.*, 2006).
Locomotor activity	Rat crosses the centre line of the cage with his whole body. The centre line was defined by a marker on the side length of home cage.
Inactive	Any behaviour where the rat's body is still and unmoving, such as freezing, resting, sitting, or lying still. The rat can make facial movements with eyes open or closed (LaFollette *et al.*, 2018).

Table 1b. Ethogram used in Experiments 2 (N = 14 cages) and 3 (N = 20 cages) developed for recording the behaviour of pair-housed male Wistar rats in the home cage during playback of three different acoustic stimuli (50-kHz, White Noise and Background Noise).

Behaviour	**Description**
Solitary play	Rat conducts fast locomotor movement involving at least one hop by an individual, where hops involve all four paws leaving the ground at the same time, not in the direction of a play partner or during a play bout. The behaviour starts with fast running or a hop from stationary or during locomotor movement and ceases when this movement stops (Hammond et al., 2019).
Social play	One rat jumps or lunges towards the partner's nape, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other), or rotating so that one rat is on his back with the other standing over him in a pin. The frequency of pinning and dorsal contacts within social play was also coded. Multiple pins and dorsal contacts can occur during a single social play bout. The behaviour starts with fast running, a

	jump or lunge towards a play partner and ceases when there is no chasing, rearing, pins or dorsal contacts between the play partners (Kerkhof *et al*., 2013; Webber *et al*., 2012).
Approach towards the speaker	Rat orients his body and head so that the nose is directed towards and within 1cm of the speaker (Lever *et al*., 2006).
Rearing (undirected)	Rat raises both front paws off the ground (can be in contact with the wall or not) standing up on hind legs. Includes all rears, except those with the rat's nose directed towards the speaker. (Lever *et al*., 2006)
Digging	Clear movement of bedding with the front or hind paws or face. The rat's front paws and/or face are not visible because they are beneath the bedding.
Self-grooming	All self-directed grooming behaviour including licking the fur, grooming with forepaws and scratching with any limb.
Locomotor activity	Rat crosses the centre line of the cage with his whole body. The centre line was defined by a marker on the side length of home cage.
Inactive	Any behaviour where the rat's body is still and unmoving, such as freezing, resting, sitting or lying still. The rat can make facial movements with eyes open or closed.
Freezing	The rat's body is motionless in a crouching position. Rat makes movements of the whiskers, gentle bobbing head movements, and has bulging eyes. (Brudzynski, 2007)

Resting	The rat's body is still and unmoving, either sitting or lying. The rat can make facial movements with eyes open or closed. (LaFollette *et al*., 2018)
Other non-social/ social	Includes urinating, defecating, chewing of any object and any other unclassified behaviour not directed towards the cage mate/includes any behaviour towards the cage mate such as social grooming.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- The novel home cage playback induces positive affect in rats through 50-kHz ultrasonic vocalisations (USVs).
- There was evidence of emotional contagion, under certain playback conditions, demonstrated through increased subject-produced USVs, approach behaviour, and social play in response to 50-kHz USVs.'
- We demonstrated the nuanced influence of different acoustic stimuli on affective states, providing valuable insights for the utilisation of playback paradigms in assessing and promoting animal welfare.