Carbon dioxide, but not isoflurane, elicits ultrasonic vocalisations in female rats

Short title: Ultrasonic vocalisations in rats

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Gradual chamber fill with carbon dioxide is currently listed by the Canadian Council of Animal Care guidelines as a conditionally acceptable method of euthanasia for rats. Behavioural evidence suggests, however, that exposure to carbon dioxide gas is aversive. Isoflurane is less aversive than carbon dioxide and may be a viable alternative, though objective data during the period leading up to loss of consciousness is lacking. It has been shown that during negative affective states, such as pain and distress, rats produce ultrasonic vocalisations. The objective of this study was to detect ultrasonic vocalisations during exposure to carbon dioxide gas or isoflurane as an indicator of a negative affective state. Specialized recording equipment was used to register these calls during administration of each agent. Nine female Sprague-Dawley rats were exposed to either carbon dioxide or isoflurane on two different occasions. All rats vocalised in the ultrasonic range (30 to 70 kHz) during exposure to carbon dioxide. When exposed to isoflurane, no calls were detected from any of the animals. The frequent occurrence of ultrasonic vocalisations during carbon dioxide exposure furthers concerns that the common practice of carbon dioxide euthanasia is aversive to rats and that isoflurane may be a preferred alternative.

Keywords: ultrasonic vocalisation, euthanasia, carbon dioxide, isoflurane, refinement

With over 2.5 million animals used annually in Canada and the European Union, rats are one of the most common species in biomedical research.[1,2] The great majority of these animals will be euthanised using an overdose of carbon dioxide gas. Despite evidence

from behavioural studies showing that carbon dioxide gas is aversive to rats, the practice remains popular because it is cheap, effective, widely available and poses a minimal health risk to personnel.[3] Euthanasia with isoflurane is deemed an acceptable method when it is followed by a secondary method of euthanasia such as cervical dislocation (mice only) or carbon dioxide (rats and mice).[4] Current national guidelines are largely based on evidence from approach avoidance studies, with the unavoidable limitation that data cannot be collected between the onset of aversion and loss of consciousness.[5] Ultrasonic vocalisations (USV) in rats have been shown to reflect a negative affective state (such as pain or distress) and may provide a novel tool for identifying pain and distress during euthanasia.[6] Vocalising in the ultrasonic range is a strategy rats have developed to adapt to a high predatory pressure. Therefore USV allows communication with conspecifics but is inaudible to many predators.[7] In general, lower frequency USV (18-32 kilohertz [kHz]) have been associated with negative affective states, and higher frequency USV (32-92 kHz) with positive affective states. Lower frequency USV (so-called 22 kHz calls) act as alarm calls and have been associated with pain, distress, and fear.[6,8] For these reasons, the recording of USV has been suggested as a measure of pain and fear in laboratory animals.[9,10] Oliveira and Barros (2006) assessed USV as a behavioural measure of pain and recorded a significantly increased number of low frequency USV from rats during the formalin test.[6] High frequency USV (32-92 kHz) have been recorded during positive affective states such as tickling and mating.[11,12] We conducted a pilot study to evaluate the application of USV recording as a reflection of pain or distress (or both) experienced by rats during exposure to carbon dioxide or isoflurane.

Nine female Sprague-Dawley rats (Health Sciences Animal Resource Centre, University of Calgary) between the ages of 7 to 9 weeks old and weighing 195-312g were used in this experiment. Animals were housed in groups of two or three in a standard rat cage (47 x 25 x 21 cm) with commercially available wood shavings (Aspen chip, NEPCO, Warrensburg, NY, USA) and a plastic tube for enrichment. Rats received water and food (Prolab 2500 Rodent 5P14, LabDiet, PMI Nutrition International, St-Louis, MO, USA) ad libitum and were kept on a 12 hour light-dark cycle (lights off at 7 pm). All experiments were performed between 3 pm and 6 pm with a minimum of 24 hours between treatments to allow the rats to recover.

Six animals were exposed to each gas on different occasions. The order of these treatments was determined by a random draw. Three other rats received only carbon dioxide as a treatment. Each animal was tested individually and exposed to the gas in a purpose made closed Perspex test chamber (3000 mL volume) while remaining within sight of its cage mate(s) throughout the experiment. The test chamber had 3 openings fitted for instrument connection (microphone, gas analyzer and gas inflow tube). The following standardized protocol was used: five minute acclimatisation period in room air, then five minutes with oxygen in-flow at 1 liter per minute (L/min), which equals 30% chamber volume per minute (CV/min), and finally (once oxygen concentration had returned to 21%) exposure to the treatment agent. Carbon dioxide (100%), or isoflurane (2.5% carried in oxygen) was delivered at a flow rate of 1.0 L/min (30% CV/min). Sound recordings were performed with an ultrasound microphone (Condenser ultrasound microphone and UltraSoundGate CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) from the time the animal was placed in the test chamber. Carbon dioxide and oxygen

were delivered with agent- specific calibrated flowmeters, and the isoflurane and oxygen concentrations monitored with a calibrated gas analyser (Datex Ohmeda s/5 monitor, GE Health Care, Waukesha, WI, USA). The experiment was terminated and the animal allowed to recover when a loss of righting reflex occurred. Vaginal swabs were taken during recovery and smears were prepared for cytologic examination (slides were air dried and stained with Diff Quik) to determine if there was a correlation between the stage of oestrous cycle and the presence or absence of USV. All recordings were visually inspected twice for USV identification by a blinded observer. Vaginal smears were evaluated by a blinded observer. Descriptive statistics are reported and data shown as median and range.

This experimental protocol was reviewed and approved by the Animal Care Committee at the University of Calgary, Canada, which operates under the auspices of the Canadian Council on Animal Care.

Control recordings made during the acclimatisation period and oxygen inflow (performed before each gas exposure) resulted in one rat that vocalized once during both oxygen exposures, and data from this animal was not included in the analysis. Exposure to isoflurane did not elicit USV from any rat (0 out of 6 animals). In contrast, during exposure to carbon dioxide, we recorded USV from all animals (8 out of 8 animals). Of these, a median of two calls per rat (range 1 to 8) were recorded. The frequency ranged from 30 to 70 kHz (median 51 kHz) with a median duration of 0.05s (0.014 to 0.26s). Results are summarised in Table 1. Vaginal cytology revealed that vocalising rats were at various stages in the oestrus cycle and that there was no association with the occurrence of USV.

To our knowledge, these preliminary data show for the first time that female rats vocalise during exposure to carbon dioxide but not when exposed to isoflurane. Though 22 kHz calls are usually associated with negative affective states such as distress or pain, our findings in the context of evidence from approach avoidance studies indicate that USV in a higher frequency range may also be reflective of these states. In a series of experiments Wöhr et al. (2008) showed that 50 kHz calls are not strictly attributed to positive experiences but are also emitted when rats were separated from a cage mate, during an open field test, elevated plus maze test and introduction to a novel cage.[13] While approach avoidance studies have shown that isoflurane is aversive, our results indicate that it may be an acceptable alternative to carbon dioxide.[3] Further work is necessary to assess if different administration techniques or alternative agents provide more humane alternatives. As millions of rodents are euthanised by carbon dioxide each year the implications are widespread.

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Fig 1. Example of a typical ultrasonic vocalisation emitted by a female rat exposed to 100% carbon dioxide at a fill rate of 30% chamber volume per minute. All rats exposed to carbon dioxide gas (8 out of 8 animals) produced ultrasonic vocalisations. No rats (0 out of 6 animals) exposed to isoflurane (2.5% carried in oxygen at a fill rate of 30% chamber volume per minute) produced ultrasonic vocalisations.

Table 1. Occurrence and properties of ultrasonic vocalisations during exposure to room air, oxygen alone, isoflurane (2.5% carried in oxygen) and carbon dioxide (100% carbon dioxide at a fill rate of 30% chamber volume per minute) in female rats. Frequency and duration are reported as median (range). NA = not applicable.

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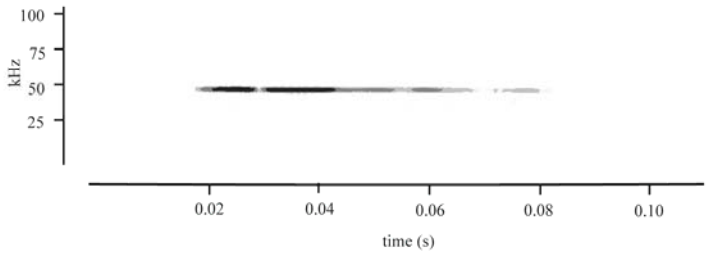
Treatment	Number of animals vocalizing	Number of calls	Frequency, kHz	Duration, seconds
Room air	0 out of 8	0	NA	NA
Oxygen	0 out of 8	0	NA	NA
Isoflurane	0 out of 6	0	NA	NA
Carbon dioxide	8 out of 8	23	51 (30-70)	0.05 (0.014-0.26)



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160 Figure 1.

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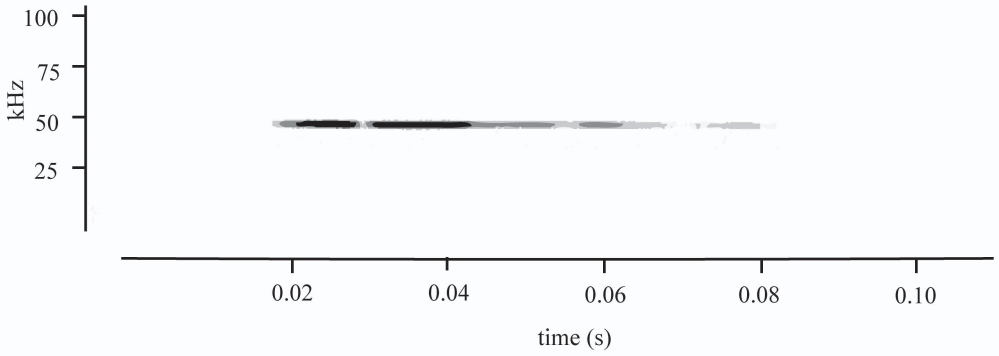
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