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Ultrasonic Vocalization Analysis as a Novel Metric to Assess Cage Enrichment in Rats

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Laboratory rodent housing conditions vary significantly across laboratories and facilities. Variation in housing can be associated with animal stress leading to study variability and the subsequent inability to replicate experimental findings. Optimization and standardization of animal housing are necessary to promote animal welfare and data consistency, thereby reducing the number of animals necessary to detect treatment effects. While interest in environmental enrichment is increasing, many studies do not examine the behavior of animals in the home cage, neglecting important aspects of enrichment. To determine how increased vertical home cage area affects animal welfare, double-decker cages (enriched), which allow rats to upright stand, were compared with standard single-level cages, which impede the ability to upright stand. Home cage welfare was assessed by analyzing ultrasonic vocalizations, fecal corticosterone, upright standing, and fighting. Ultrasonic vocalization was further explored by analyses of call type as defined by a 14 call-type schematic. Rats housed in enriched cages spent more time fighting, produced fewer 50 kHz calls, and had higher levels of fecal corticosterone. Rats in standard cages attempted to upright stand more often but remained upright for a shorter amount of time due to the height limitation imposed by standard cages. In addition, standard cages restrict some naturalistic behaviors such as upright standing and reduce fighting, which may be attributable to their single-tier organization and floor space. Enriched cages permit rats to engage in normal ethological behavior but also increase fighting. This study demonstrates that housing conditions have a meaningful impact on multiple measures of animal affect. When considering study design, researchers should be aware of how housing conditions affect animal subjects.

Abbreviations: IRR, Interrater reliability; kHz, kilohertz; USV, ultrasonic vocalization

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Introduction

Despite the consensus that housing conditions affect the wellbeing of research animals, ideal housing conditions and enrichment options have not been rigorously defined in controlled experiments.[51] Cage space requirements and optimal enrichment for common research animals are still debated, and further refinement is required.[1,2,19,23] Housing parameters are an experimental variable that can alter experimental outcome.[18,45,47] Differences in outcome across laboratories may require the use of more animals to reach experimental conclusions. The ability of the environment to affect experimental outcomes necessitates standardization that addresses animal welfare concerns while being practical for use.

Environmental enrichment can be by both physical and social means. Physical enrichment refers to added complexity to the environment in the forms of cage structure modification, tunnels, toys, running wheels, and so forth. Social enrichment refers to providing interactions with conspecifics. Rats are known to be social animals,[31,42,48,50] and some authors have speculated that housing rats in groups with maximal physical enrichment may be optimal for animal welfare;[3] however, the literature provides conflicting reports concerning the benefits of physical and social enrichment.[4,22] For example, male rats are reported to show suppressed growth, feeding, and locomotion in enriched (social and physical) environments.[52] In addition, rats, especially males, can experience stress due to crowding.[6,44]

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Commonly, environmental enrichment studies assess the behavior of animals in paradigms outside of the home cage,[43] without assessment of the animals in the home environment. Difficulty in drawing conclusions from existing enrichment literature arises from the vast variance in study design including but not limited to strain, sex, number of cage mates, duration of enrichment, age at onset of enrichment, and type of enrichment. The ability to draw conclusions from these studies is further compromised by non-standardized behavioral testing protocols and interpretation of results.

Ultrasonic vocalizations (USVs) are a sensitive method of assessing rodent communication and affective state[9,33] and is showing promise as a novel metric to assess rodent welfare in the home environment. USVs in rats can be categorized into 2 main groups: positive/"happy" 50 kHz USVs and negative/"distress" 22 kHz USVs.[7-9,24,33,38,39,41] In recent years, further delineation of 50 kHz calls has occurred, with some authors suggesting as many as 14 distinct USVs.[49] Exploration of distinct USVs has revealed a linkage between certain call types and behaviors.[14] Measuring USVs of rats subjected to different housing conditions can determine if they are in the positive 50 kHz range or the negative 22 kHz range and may provide insight into the affective state of rats. Furthermore, the complex classification of 50 kHz calls may reveal more detail about the affective state of the rat and provide additional information regarding the importance of specific call types in communication.[10] One of the greatest benefits of USVs is their ability to reflect affective state or stress status continuously and noninvasively, making USVs preferable to other measures of stress or anxiety, such as fecal corticosterone, in rats.[21,26,27,30]

Many ethologically relevant behaviors are not accommodated by standard rodent housing conditions, including behaviors restricted by available cage space, such as upright standing. Previous guidelines from the Canadian Council on Animal Care (CCAC) mandated a minimum cage height that did not allow upright standing for most rats; however, the current guidelines recommend a minimal height equivalent to the nose-to-tail-base distance plus 4 cm, which would allow upright standing.[16,28] Movement of guidelines toward increased vertical space could improve animal welfare. Consistent with the implied benefit of increased vertical space, rats that have the opportunity will stand upright; when unable to do so, they will stretch laterally more frequently, suggesting that the ability to extend the body is important. Rats may lateral stretch more frequently in circumstances where general mobility is restricted.[28] Given the changes recommended by the CCAC, further research into the effect of physical housing enrichment on ethological-relevant behaviors of laboratory animals such as communication and naturalistic behaviors (such as upright standing) should be conducted.

Fighting is common in male rats and is thought to be of 2 varieties: playful and aggressive. A previous study differentiated the 2 types of fight behavior based on the target and type of contact.[34] Excess agonistic engagement between cage mates may be harmful to wellbeing while playful fighting may be beneficial.[36,37] Play fighting between rats, as well as play between a rat and a human, are known to elicit intense 50 kHz calling,[20,25] while threatening scenarios, such as an intruder or exposure to a predator is more commonly associated with 22 kHz calls.[46] Both 50 and 22 kHz calls are critical in guiding the nature of interactions between rats, suggesting an important communicative role for USV in guiding social interactions.[12,13] USV analysis, including complex call analysis, may be a useful way to assist in determining the dynamics of fighting behavior in rats.

The current study aimed to determine the effects of physically enriched compared with standard housing conditions in adult rats by monitoring parameters indicative of animal welfare and affective state, including naturalistic behavior and fecal corticosterone. The indicated measures were also compared with USVs as a means of further validating the utility of USVs as an instantaneous measure of animal welfare under different housing scenarios.

Materials and Methods

Animals and acclimation. All animals in the study were male CD rats acquired from Charles River Laboratories (Saint-Constant, Quebec). The experimental design comprised 4 control (standard) and 4 treatment (enriched) cages, with 3 rats /cage for a total of 12 rats per treatment group. Rats were approximately 80 d old at the time of allocation into groups. Before usage in the current study, rats were housed in standard cages. All rats were allowed at least 1 wk of acclimation to the facility prior to any manipulations. After acclimation, rats were handled for 5 min every other day for 1 wk by the individual performing behavioral testing. Handling involved retrieving the rat from the cage with one hand lightly gripping the tail and the other hand, palm up, supporting the rat's ventral surface. The rat rested in the arms of the handler, with the handler's arms cradling the rat. The week before testing and allocation, rats were transported to the testing suite daily as a means of habituating them to the transportation process and the testing suite. The testing suite, which was adjacent to the colony room, contained 2 lamps with red bulbs at the opposite ends of the room. The lamps faced into the corner to minimize glare in the recordings. There was no overhead lighting. All procedures were conducted consistent with the guidelines of the Canadian Council on Animal Care and were approved in advance by the University of Prince Edward Island Animal Care Committee.

Environment. The average daily high temperature in the colony room was 21.3 ± 1.2 °C (70.3 ± 2.2 °F) and average daily low temperature was 19.6 ± 0.4 °C (67.28 ± 0.72 °F). The humidity daily high was 48 ± 12% and the humidity daily low was 31 ± 80%. Light was provided by overhead fluorescent lighting. Within the colony room, the light intensity during the light phase was 83.7 ± 66.9 lx and during the dark phase, with red lamps on, was 0.6 ± 0.4 lx. The light intensity in the hallway in which the rats were transported for behavioral testing was 0.6 ± 0.4 lx. The light intensity in the behavioral testing suite was 3.7 ± 0.2 lx. The colony room was on a 12-h reverse light cycle with the lights going off at 0600 and coming on at 1800. All behavioral testing was conducted during the dark phase. When the colony room was entered during the dark cycle, a red lamp was used. The ambient colony room sound was 70 to 80 dB. All rats were maintained on a diet of Laboratory Rodent Diet 5001 (LabDiet, Saint Louis, MO) and had ad libitum access to food and tap water for the duration of the study.

Cages. After acclimation and handling, rats were randomly allocated to standard (508 × 406 × 216 mm) (Ancare Corporation, Bellmore, NY) or enriched (GR1800 Double Decker; 381 × 305 × 394 mm) (Tecniplast, Montreal, Quebec) cages (Figure 1); both were maintained under static conditions. The standard cages were slightly larger in terms of total floor space (enriched: 1862 cm^2, standard: 2062 cm^2); however, the total volume of the enriched condition (approximately 45780 cm^3) was greater than the standard condition (approximately 44550 cm^3). The enriched cage consisted of 2 levels with the upper level characterized as a shelf, overhanging approximately half of the lower level. Both levels of the enriched cage allowed rats to obtain a full vertical stretch. The standard cage consisted of one level. Each cage was equipped with 2 water bottles and a black opaque PVC tube measuring 140 mm in length, an internal diameter of 76 mm, and a total diameter of 89 mm. In both cages, food was accessible without upright standing by means of a floor level ceramic food dish. Rats remained in their respective conditions for 33 d. Cages were bedded with Hardwood Beta Chips (North Eastern Products, Warrensburg, NY) and were changed twice per week. Cages in both conditions were randomly distributed on the rack at the time of cage changing. Cage changes and daily maintenance were the responsibility of the laboratory technician.

USV and behavioral recording. On testing days, the rats were transported to the testing suite in their home cages and left undisturbed for 5 min. After the 5 min acclimation, USV and video recordings were performed in the home cages. USV-recordings were made every 3 d for 18 d using Avisoft Ultrasound Gate (Glienicke/Nordbahn, Germany). Video-recordings were made every 3 d for 15 d using Google Pixel 2 XL Smartphone (Mountain View, CA). The frame rate was 120 FPS. Both the video and USV-recordings were 5 min long. On day 33, a single 30-min USV and video recording was performed to assess the relationship between fighting and 22 kHz calls. The entire 30-min period for each animal was analyzed for both USV and video recording. After all recordings, the cages were returned to the colony room. For USV recordings, the microphone was connected to a tripod with an attached extender. For the enriched condition, the microphone was placed approximately 125 mm away from the front of the wire lid. For the standard cages, the microphone was suspended 125 mm above the wire lid in the

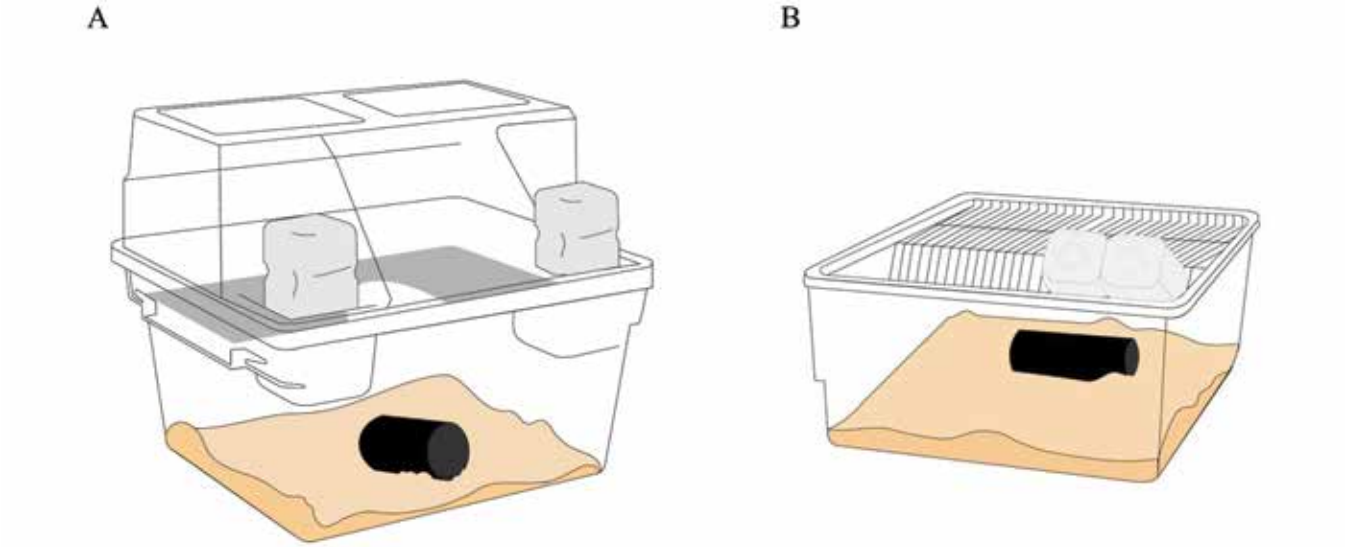

Figure 1. Housing conditions. Rats were placed in either (A) an enriched cage or (B) a standard cage.

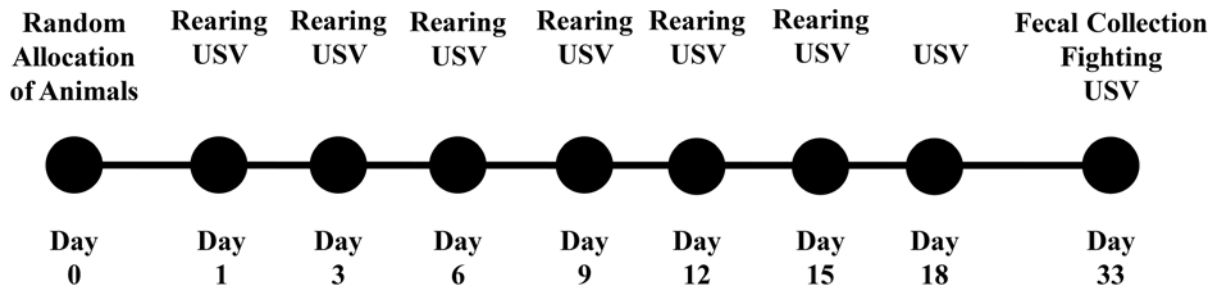

Figure 2. Timeline of events.

center of the cage. All behavioral testing was conducted between 0730 and 1400 h, with an effort to keep the time of day as consistent as possible. The order of testing each day was random.

USV analysis. USVs were analyzed using Avisoft SASLab pro [(Glienicke/Nordbahn, Germany) (FFT = 512, Frame Size = 100%, Window = Flattop, Overlap = 87.5%, Peak Frequency Interpol.=Auto)]. Element separation for 50 kHz calls used the following parameters: max change = 3, hold time = 20ms, and minimum duration = 5ms. Element separation for 22 kHz calls used the following parameters: max change = 3, hold time = 20ms, and minimum duration = 50ms. The automatic scoring was cross checked by researchers with extensive experience "hand scoring" USVs to determine the number of false positives. The program was 89% accurate for 50 kHz calls, and 100% accurate for 22 kHz calls.

Complex call analysis. Due to the labor-intensive nature of complex call classification, only recordings from days 1, 9, and 18 were analyzed. Complex call classification was performed according to a modified classification schematic.[49] Interrater reliability (IRR) was assessed by 2 analysts for consistent duration (within 25% of call average) and call type consistency. IRR was 67% based on the classification of 231 calls. Analysts were consistent in scoring the call duration and in recognizing that a call had occurred (IRR = 89%), but demonstrated some discrepancies in classifying the call (IRR = 69%). Errors most frequently arose during the classification of modulated pitch calls. Calls were not categorized as 22 kHz or 50 kHz calls during the complex call classification. Calls that could not be adequately discerned were referred to as unclassifiable; calls that did not fit any of the categories were referred to as miscellaneous.[49]

Behavioral scoring. Upright standing was manually scored using randomly selected, 1-min intervals from video recordings on days 1, 3, 6, 9, 12, and 15. Upright standing was defined as the rat's front paws being elevated off the ground while not touching the cage wall, with the rat supported only by its hind legs. The hind legs did not have to be extended. The back could be either convex, concave, or flat. Given the character of the standard cages, very brief vertical stretches were possible. Counts of each upright standing event and the duration were recorded for every rat in the cage and summed.

The entire 30-min video recording from day 33 was scored for fighting behavior. Fighting behavior was defined as any time 2 rats were wrestling, one contacting the other's nape of the neck or face, or one pinning another on the ground. To be classified as a bout of fighting, the duration of the episode had to be 5 seconds or more. The total duration of fighting was calculated for all rats in the cage over the 30-min period.

Fecal corticosterone. Fecal collection occurred on day 33 after behavioral testing (Figure 2). Rats were placed in a clean holding cage with a strip of paper towel in the bottom. The rats remained in the cage until defecation occurred. If a rat did not defecate during the allotted 6-h time period, it was returned to the colony room. No further attempts at fecal collection were made due to concerns regarding temporal sampling consistency. Fecal matter was retrieved using sterile forceps and placed in sterile collection tubes. The tubes were then frozen at -80 °C (-112 °F). In preparation for the ELISA, samples were thawed, flash frozen in liquid nitrogen, and homogenized using mortar and pestle. The fecal sample was combined with 80% methanol at 0.1g: 1ml. The sample was

vortexed for 30 min and then centrifuged for 15 min at 2500xg. The supernatant was collected and stored at -80 °C. Sample supernatants were heat inactivated at 95 °C for 15 min immediately before use. Sample supernatants were diluted 1/20 in assay buffer and fecal corticosterone concentration determined with commercial ELISA kits (Arbor Assay kit K014-H1, Ann Arbor, MI).

Data scoring and analysis. Ultrasonic vocalization analysis and fecal corticosterone analysis data were assessed by investigators who were blind to experimental conditions. Experimenters could not be blind in the analysis of in-cage behaviors. A single analyst scored the in-cage behaviors; therefore, IRR was not performed. All videos were scored using a VLC media player (VideoLAN, Paris, France), a counter, and a timer.

Statistical analysis. Statistical analysis was performed on GraphPad Prism Version 6.01 for Windows (Graphpad Software, La Jolla, California). When comparing fighting and fecal corticosterone, an unpaired 2-tailed Student *t* test was used. Fecal corticosterone analysis was performed on 7 samples from enriched cages and 8 samples from standard cages. When comparing the number of 50 kHz and 22 kHz calls, upright standing, and complex calls, a 2-way ANOVA was performed with day and condition as levels. For 50 kHz calls, an unpaired 2-tailed Student *t* test comparing the 2 conditions on day 18 was performed after the detection of a significant day x treatment interaction. We assumed that differences between the groups would most likely occur at the latest observation point in the study. When correlating fighting with the number of 22 kHz calls, a linear regression was performed. All descriptive data was expressed as mean ± SEM and the threshold for significance was $\alpha = 0.05$.

Results

USV analysis. For 50 kHz calls, a 2-way ANOVA for treatment (cage type) and day revealed a near-significant treatment effect ($F(1, 6) = 5.75$, $P = 0.0535$), a significant day effect ($F(6, 36) = 3.31$, $P = 0.0107$), and a significant treatment x day effect ($F(6, 36) = 3.27$, $P = 0.0115$). On day 18, rats in the standard cages produced more 50 kHz calls than did rats in enriched cages ($t(6) = 2.66$, $P = 0.0378$) (Figure 3A).

For 22 kHz calls, a 2-way ANOVA revealed a significant treatment effect ($F(1, 6) = 9.84$, $P = 0.0202$), a nonsignificant day effect, $F(6, 36) = 0.767$, $P = 0.601$, and a nonsignificant day x treatment interaction, $F(6, 36) = 0.690$, $P = 0.659$). Rats made

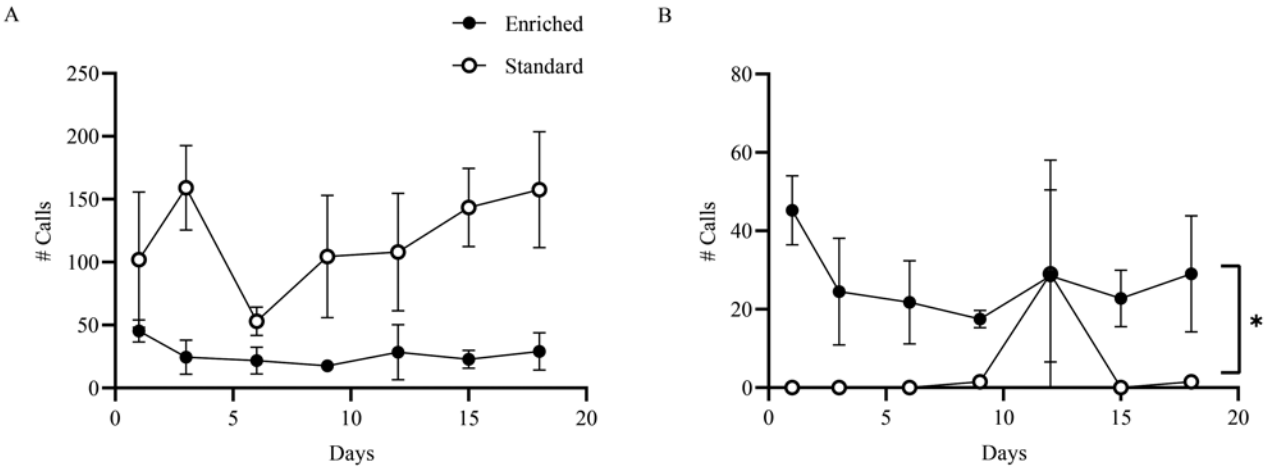

Figure 3. Ultrasonic communication. Number of (A) 50- and (B) 22-kHz calls produced by rats housed in standard and enriched cages. The treatment effect for 50 kHz calls approached significance ($P = 0.0535$) and a significant treatment effect was detected for 22 kHz calls ($P = 0.0202$). Data shown as Mean ± SEM, $n = 4$.

Table 1. Complex call ANOVA table. USV recordings were performed every 3 d for 18 d in enriched and standard cages.

Call-Type	Condition	Day	Condition x Day
Complex	$F(1, 6) = 5.00$, $P = 0.0667$	$F(2, 12) = 1.50$, $P = 0.262$	$F(2, 12) = 3.50$, $P = 0.0635$
Composite	$F(1, 6) = 4.99$, $P = 0.0669$	$F(2, 12) = 2.00$, $P = 0.178$	$F(2, 12) = 3.42$, $P = 0.0668$
Downward ramp	$F(1, 6) = 3.10$ $P = 0.129$	$F(2, 12) = 2.42$, $P = 0.131$	$F(2, 12) = 4.74$, $P = 0.0304$
Flat	$F(1, 6) = 0.599$, $P = 0.468$	$F(2, 12) = 0.429$, $P = 0.661$	$F(2, 12) = 2.22$, $P = 0.151$
Flat-Trill	$F(1, 6) = 17.4$, $P = 0.00590$	$F(2, 12) = 13.34$, $P = 9.00e\text{-}4$	$F(2, 12) = 10.8$, $P = 0.0020$
Inverted-U	$F(1, 6) = 6.50$, $P = 0.0435$	$F(2, 12) = 3.30$, $P = 0.0723$	$F(2, 12) = 3.49$, $P = 0.0638$
Miscellaneous	$F(1, 6) = 1.43$, $P = 0.276$	$F(2, 12) = 2.24$, $P = 0.149$	$F(2, 12) = 1.43$, $P = 0.277$
Multi-Step	$F(1, 6) = 6.12$, $P = 0.0482$	$F(2, 12) = 2.04$, $P = 0.173$	$F(2, 12) = 2.50$, $P = 0.124$
Short	$F(1, 6) = 5.03$, $P = 0.0660$	$F(2, 12) = 0.767$, $P = 0.486$	$F(2, 12) = 0.775$, $P = 0.483$
Split	$F(1, 6) = 5.79$, $P = 0.0528$	$F(2, 12) = 0.234$, $P = 0.795$	$F(2, 12) = 0.234$, $P = 0.795$
Step-Down	$F(1, 6) = 1.76$, $P = 0.233$	$F(2, 12) = 0.988$, $P = 0.401$	$F(2, 12) = 1.02$, $P = 0.391$
Step-Up	$F(1, 6) = 3.60$, $P = 0.107$	$F(2, 12) = 0.583$, $P = 0.573$	$F(2, 12) = 0.250$, $P = 0.783$
Trill	$F(1, 6) = 7.89$, $P = 0.0308$	$F(2, 12) = 3.31$, $P = 0.0719$	$F(2, 12) = 3.49$, $P = 0.0638$
Trill-Jump	$F(1, 6) = 0.600$, $P = 0.468$	$F(2, 12) = 0.857$, $P = 0.449$	$F(2, 12) = 0.857$, $P = 0.449$
Unclassifiable	$F(1, 6) = 5.13$, $P = 0.0642$	$F(2, 12) = 0.386$, $P = 0.688$	$F(2, 12) = 0.144$, $P = 0.867$
Upward Ramp	$F(1, 6) = 1.28$, $P = 0.301$	$F(2, 12) = 3.19$, $P = 0.0777$	$F(2, 12) = 5.41$, $P = 0.0212$

Complex call analysis was performed on days 1, 9, and 18.

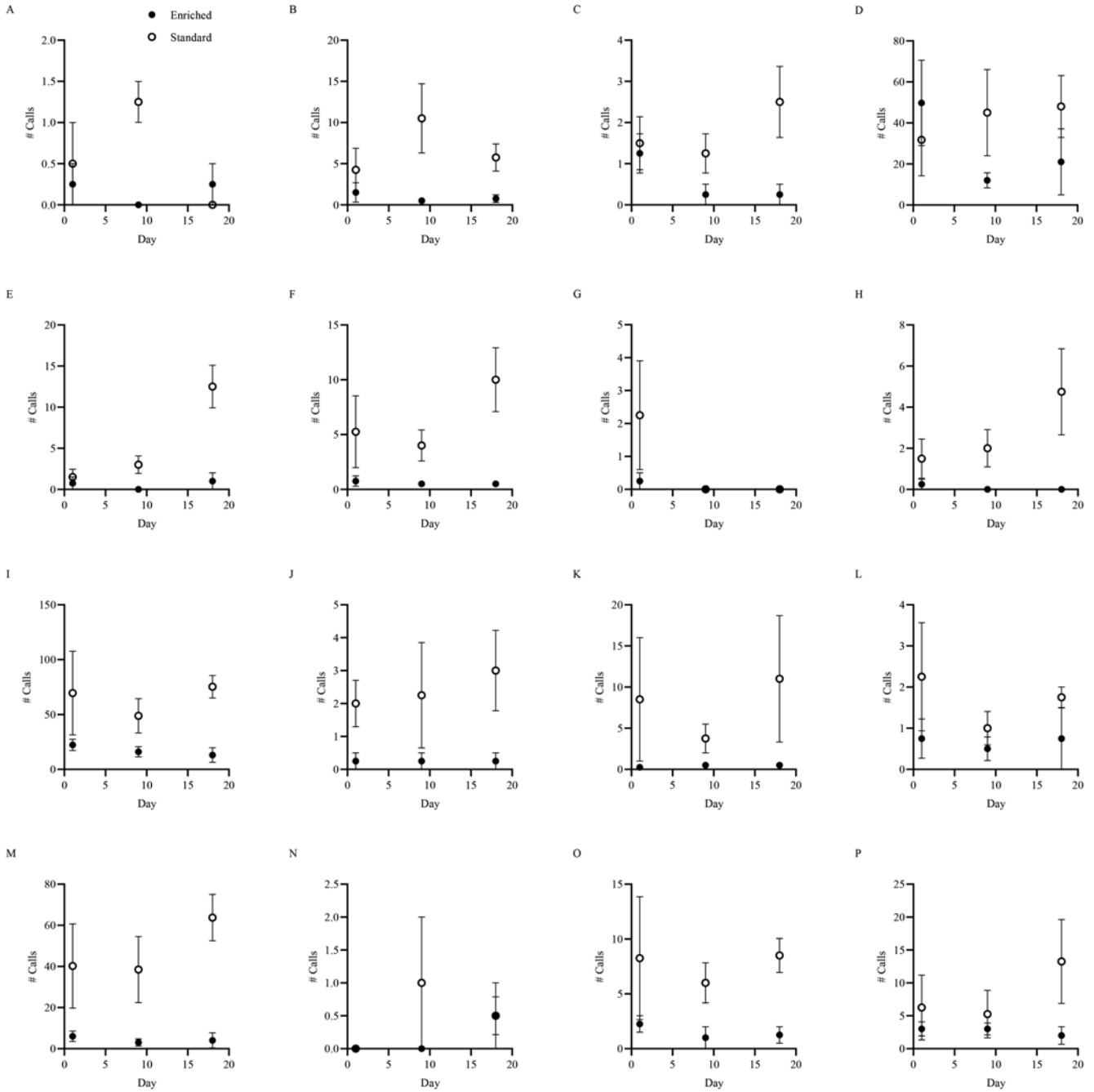

Figure 4. Complex call classification. Comparison of call types in standard compared with enriched cages for (A) Complex, (B) Composite, (C) Downward Ramp, (D) Flat, (E) Flat-Trill, (F) Inverted-U, (G) Miscellaneous, (H) Multi-Step, (I) Short, (J) Split, (K) Step-Down, (L) Step-Up, (M) Trill, (N) Trill-Jump, (O) Unclassifiable, and (P) Upward Ramp. Call classification was based on a modification of a previous schematic.[48] Data shown as Mean ± SEM, $n = 4$.

more 22 kHz calls in the enriched cages as compared with standard cages (Figure 3B).

Several effects were observed for complex call analysis with condition effects observed for several call types including flat-trill (P = 0.00590), inverted-U (0.0435), multi-step (0.048) and trill ($P = 0.0308$) (Table 1 and Figure 4).

Fighting No difference was detected in the total duration of fighting between rats in enriched and standard cages (t (6) = 2.13, $P = 0.0775$) (Figure 5A). However, a significant simple linear regression relationship was found between the duration of fighting and the number of 22 kHz calls (F (1,6) = 23.3, $P = 0.00290$, R^2 of 0.795) (Figure 5B).[17]

Corticosterone. Rats in enriched cages had higher levels of fecal corticosterone than did rats in standard cages (t (11) = 3.70, $P = 0.00350$) (Figure 6).

Upright standing. A 2-way ANOVA revealed a significant treatment (cage type) effect for upright standing (F (1, 6) = 28.8, $P = 0.00170$), a nonsignificant day effect (F (5, 30) = 0.592, $P = 0.706$), and a nonsignificant treatment x day interaction (F (5, 30) = 0.496, $P = 0.777$). Rats in standard cages stood upright more frequently than did rats in enriched cages (Figure 7A). The total duration of standing upright showed a significant treatment effect (F (1, 6) = 34.7, $P = 0.00110$), a nonsignificant day effect (F (5, 30) = 1.15, $P = 0.358$), and a

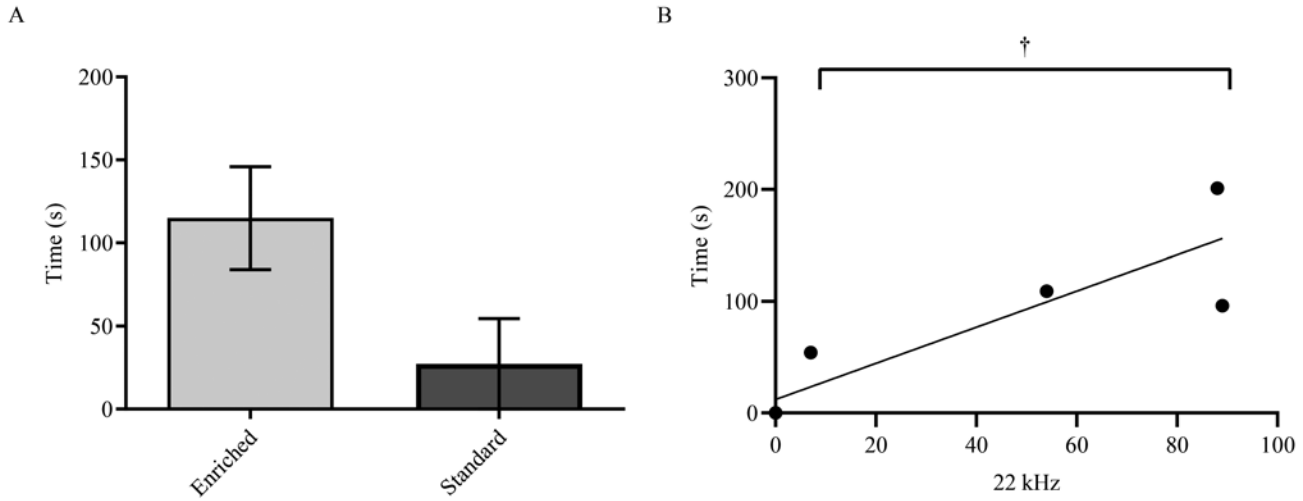

Figure 5. Fighting behavior in rats. (A) Rats in enriched cages spent more time fighting than did rats in standard cages (ns; $P = 0.0775$). Data shown as Mean ± SEM, $n = 4$. (B) Time spent fighting was related to the number of 22-kHz calls as determined by a significant linear regression equation ($P = 0.00290$). Data shown as Mean ± SEM, $n = 4$.

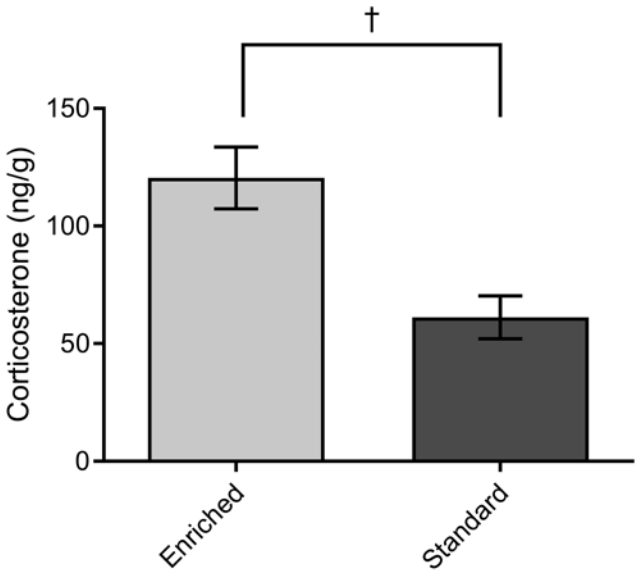

Figure 6. Fecal corticosterone. Rats under enriched housing conditions had significantly higher fecal corticosterone than did rats in standard housing ($P = 0.00350$). Data shown as Mean ± SEM, $n = 7$-8.

nonsignificant day x treatment interaction ($F(5, 30) = 0.911$, $P = 0.487$). Rats in enriched cages stood upright for a longer amount of time than rats in standard cages (Figure 7B).

Discussion

Rats in enriched cages stood upright for longer durations as compared with rats in standard housing, suggesting improved ethologically relevant welfare. However, rats in enriched housing had more 22 kHz USVs, fewer 50 kHz USVs, elevated fecal corticosterone, and more fighting behavior, which possibly indicate negative effects.

Rats in enriched cages produced fewer 50 kHz calls and more 22 kHz calls than did rats housed in standard caging. Several treatment differences were detected in the types of 50 kHz calls produced. While the field of 50 kHz call classification is relatively novel, evidence suggests that frequency-modulated calls, particularly those with a trill component, are indicative of positive affect.[7,11] A frequency-modulated call is defined broadly as a call containing directional changes of greater than or equal to 3 kHz; a frequency-modulated call with trills has the added component of 2 or more oscillations of greater than or equal to 3 kHz. Components of a trill call are typically less than 10 ms duration and occur less than 10 ms apart.[49] Two frequency-modulated calls with trills were recorded in higher proportion in the standard as compared with enriched cages, including trill and flat-trill (Figure 4, Table 1); this difference suggests a more positive affective state in rats housed in standard caging. Trill-jumps, the other frequency-modulated call type with trills, was not significantly affected by cage type. The greater number of 22 kHz calls in the enriched condition could also be associated with greater complexity of interactions, necessitating the use of a more elaborate vocabulary to communicate with cage mates appropriately.[12,13]

Fighting behavior was higher in enriched cages than in standard cages (Figure 5); the basis for this is uncertain. As rats age, play fighting becomes less common.[35] The rats in this study were more than 100 d old when social behavior was assessed. A previous study observed that 22 kHz vocalizations occurred after the first bite during resident-intruder scenarios that resulted in submission, suggesting an association between 22 kHz calls and non-play fighting.[32] Despite difficulty in determining which rat is producing the call, observation of thoracic movement suggests that it is primarily the submissive rat. In both cage types in this study, the number of 22 kHz calls was related to the amount of time spent fighting. Rats in the enriched condition produced more 22 kHz calls, which could be expected in a scenario with more play fighting. The 22 kHz calls are important in guiding play behavior, particularly to inform a partner that an interaction is becoming too intense.[13] Even though the majority of the interactions are not aggressive in nature, the number of 22 kHz calls would increase simply due to increased social interaction. Conversely, play-fighting or "rough and tumble play" is more closely associated with the emittance of 50 kHz calls.[5] While that type of interaction might not be aggressive, fewer 50 kHz calls occurred in the enriched condition, particularly frequency-modulated calls. Further detailed analysis of the fighting would be necessary to make decisive inferences about the nature of the calls.

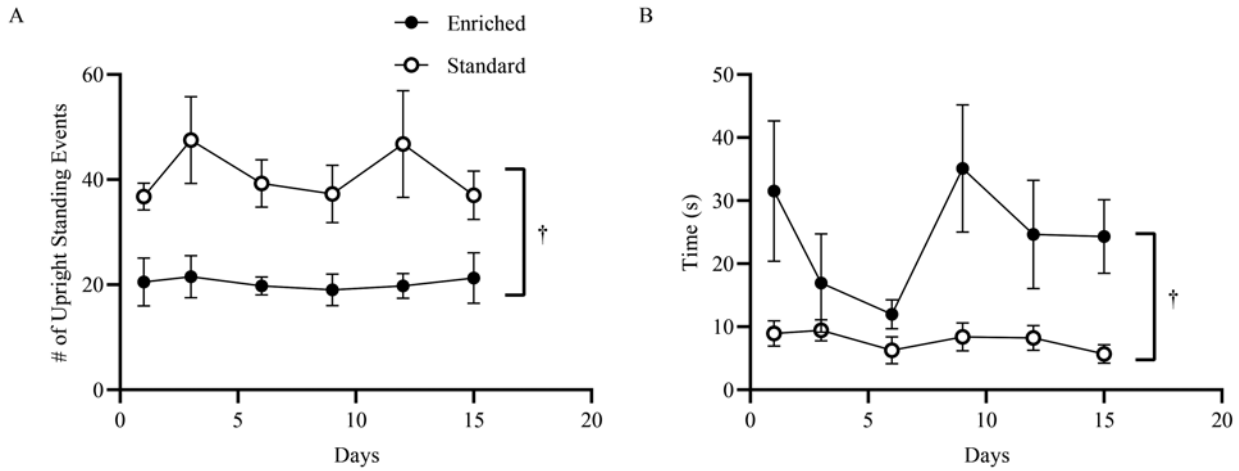

Figure 7. Upright standing behavior. (A) Number of upright standing events and (B) time spent standing upright in standard and enriched cages within random 1-min intervals each day. There was a significant treatment effect ($P = 0.00170$) for number of standing upright events and a significant treatment effect for duration of standing upright ($P = 0.00110$). Data shown as Mean ± SEM, $n = 4$.

Rats housed in the enriched environment also had higher levels of fecal corticosterone as compared with rats in standard housing. The observed elevation in fecal corticosterone was present at least 33 d after placement in the enriched environment. However, caution must be taken in assigning a physiologic state based on fecal corticosterone alone. Although fecal corticosterone may reflect an increased state of arousal, the arousal may not be negative.[29] Sexual contact, exercise, and playing reflect states of high arousal which are not necessarily negative. Prolonged elevation in corticosterone levels is implicated in damage to the central nervous system, particularly the hippocampus.[40] Neurologic damage can be potentiated by an elevation in glucocorticoids,[40] potentially complicating comparisons across labs. Future studies will investigate the change in fecal corticosterone throughout the housing period, although the fecal corticosterone was potentially persistently elevated throughout the study, as USV content, which was assessed every 3 d throughout the study, suggested increased negative affect and decreased positive affect in the enriched condition. Thus, the USV assessment appeared to predict the observed differences in fecal corticosterone. Whether the observed increase in fecal corticosterone was sufficient to affect performance in a test of affect, such as the open field or elevated plus maze, or a test of memory such as a radial arm maze, can be tested in the future.

Finally, rats in enriched cages stood upright for a longer duration than did rats in standard cages. Rats in the standard cages attempted to stand upright more frequently but perhaps did not remain upright for an extended period due to the limiting height of the standard cages, such that enriched rats could remain upright longer than rats in standard cages. The observed behavior is consistent with another study that also observed an increase in upright standing in a permissive environment.[28] However, the ability to stand upright did not result in an increase in 50 kHz calls or a decrease in 22 kHz calls. A comparison of USV data between groups with apparent differences in upright standing is complicated in that rats generally do not emit USVs when rearing.[14]

The enrichment feature in the enriched cages was the presence of a shelf (Figure 1A). The second floor could be accessed only by climbing from the lower level. We hypothesize that the second level, in combination with less floor space in enriched cages, contributes to the observed increased fighting, and is thus responsible for increased 22 kHz USVs and higher corticosterone levels. A rat occupying the second level of an enriched cage could defend territory more easily than rats in single-level standard cages. The decreased floor space results in an increase in proximity between rats, leading to increased fighting. Future studies will determine the effect of added vertical space in the absence of a shelf. The removal of the shelf will also allow more accurate assessment of upright standing without the confound of other sources of enrichment such as the shelf. Evidence suggests that females respond to housing conditions differently than males.[6] Given the sex differences in territoriality and fighting, studying the effects of the enriched cages in female rats will be informative. Another study of interest would include dams, which might benefit from the ability to isolate themselves from pups in order to rest. Another study could investigate the impact of different housing conditions on experimental outcomes. Different housing conditions may be at least partially responsible for the inability to replicate findings across laboratories.[15]

To summarize, this study aimed to validate the use of USV as a method of continuously assessing within-cage animal welfare. Although male rats may be able to stand upright in the enriched cages we used, the increase in corticosterone, more numerous 22 kHz calls, and reduction in 50 kHz calls in the enriched cages suggest that they may compromise welfare. Consequently, the elevated fighting in the enriched condition may not indicate play, but rather that the presence of the shelf created increased aggressive territorial behavior. Further investigation of USV in the contexts of play fighting and aggression could increase the value of this metric.

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