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Effect of Individually Ventilated Bi-Level Caging on Anxiety-Like Behavior and Breeding Performance in Rats

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Research Article

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ABSTRACT

Introduction: Rats housed in complex, environmentally-enriched caging exhibit behavior changes indicative of improved welfare; however, limited studies have been done specifically on bi-level cage design.

Objective: To determine if bi-level caging would affect anxiety in rats, three cage types were examined: standard 140 sq in single-level cages, larger 232 sq in single-level cages, and larger 232 sq in bi-level cages.

Methods: Male Hsd: SD rat offspring born into and housed in each of the three conditions were tested at 45 and 95 days of age using social interaction, locomotion, and center locomotion behavioral analyses. Breeding success and serum corticosterone levels were used as physiological parameters.

Results: No differences in behavior or corticosterone levels were found among groups. Modest non-statistically significant breeding benefits were observed with bi-level caging in the form of shorter time to litters and higher average pup weights at two weeks of age.

Conclusion: Housing in bi-level caging in laboratory rats does not affect anxiety, which is an important finding when employing novel cages in behavioral research. Additionally, increased area for normal bi-pedal posture and species-specific behaviors adds to good welfare.

INTRODUCTION

The Guide for the Care and Use of Laboratory Animals states that animals must be provided enough space to express natural postures without touching enclosure ceilings. The Guide further states a specific aim of environmental enrichment should be to facilitate expression of species-specific behaviors [1]. Bi-pedal posture in rats is a well-known documented species-specific behavior that can be either limited or completely prevented in many modern individually ventilated cages [2-5]. Anxiety in research animals, whatever the cause, can have negative impacts on research studies, with outcomes affecting breeding, experimental results, variability among studies, and most importantly, negative effects on animal well-being [6-12]. Reproducibility of studies has become a topic of significant interest, and housing in current standard caging has been suggested as a contributing factor in variability and validity of studies [12]. A possible source of underlying anxiety and variability in recent research involving rats may be attributed to limiting of normal species-specific postures and behaviors. Studies examining environmental enrichment via bi-level housing for rats in the research setting are warranted.

Environmental enrichment benefits of individually ventilated bi-level caging has been minimally studied to this point [13-15]. Several bi-level caging options are now commercially available; they provide the potential for enrichment through many different facets including increased complexity, area for exercise, height to allow for natural bi-pedal posture, areas for separation, and overall space. Although most strains of rats used for research typically show outward signs of general good health such as appropriate growth, breeding, and stable health status, more specific behaviors may manifest differentially across cage types. The object of this study was to determine the effects of cage type on well-characterized behavioral assays in rats (social interaction test, locomotion, and center locomotion) [16-22] and to assess physiologically relevant markers of stress (corticosterone levels in serum

and breeding parameters) [8,23-26]. We hypothesized that the bi-level cage design would serve as a type of built-in environmental enrichment, allowing species-specific behaviors and postural adjustments; this would lead to lower anxiety levels in rats born and weaned into bi-level caging when compared to a standard caging configuration. Additional hypotheses were that physiological parameters would support behavioral data with lower baseline serum corticosterone levels and improved breeding performance, such as increased numbers of pups per litter, higher weekly pup and weaning weights, and a shorter number of days between litters in bi-level cages.

MATERIALS AND METHODS

Animals and Housing

24 Hsd: SD rats (12 females, 12 males) were purchased at 10 to 11 weeks of age (Envigo, USA), given ad libitum access to food (Teklad, USA breeder chow for breeders with pup access during the first 3 weeks of life, and Teklad, USA maintenance diet thereafter) and chlorinated, autoclaved water, and exposed to a 12 hrs light-dark cycle (on at 0700 and off at 1900). Animal housing rooms were temperature-and humidity-controlled at 70°F to 74°F and 30-70% humidity. Health checks were performed daily. Animals were considered negative for *Mycoplasma pulmonis*, Respiratory Syncytial Virus, parvoviruses (RMV, KRV, and H1), Pneumonia Virus, Sialodacryoadenitis Virus, Theilovirus, and Sendai virus through thrice-yearly sentinel testing. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (protocol 14-148).

Rats were randomly paired for breeding and placed in one of three specified experimental caging groups, with four pairs of rats per cage type. The three caging types utilized were: 'blue-line' cages ('BL,' 232.5 sq in total area, dimensions 10.5 × 19 × 15 inch; **Figure 1A**), 'bi-level' cages ('DD,' 232.5 sq in of total area with part of the area provided via an upper level, dimensions 16 × 15 × 18 inch; Figure 1B), and 'green-line' cages ('GL, 140 sq in, the standard caging currently used for housing rats at UNC, dimensions 8.5 × 15 × 13.5 inch; **Figure 1B and 1C)** (Tecniplast, USA). Each pair was maintained in its respective cage type through weaning of its third litter making for three generations. All caging types were individually ventilated with 70 air changes per hour, changed every other week, and supplied with brown crinkle paper nesting material (FiberCore LLC, Cleveland, OH) and corncob bedding (Bed-o-Cobs, Maumee, Ohio) throughout the study.

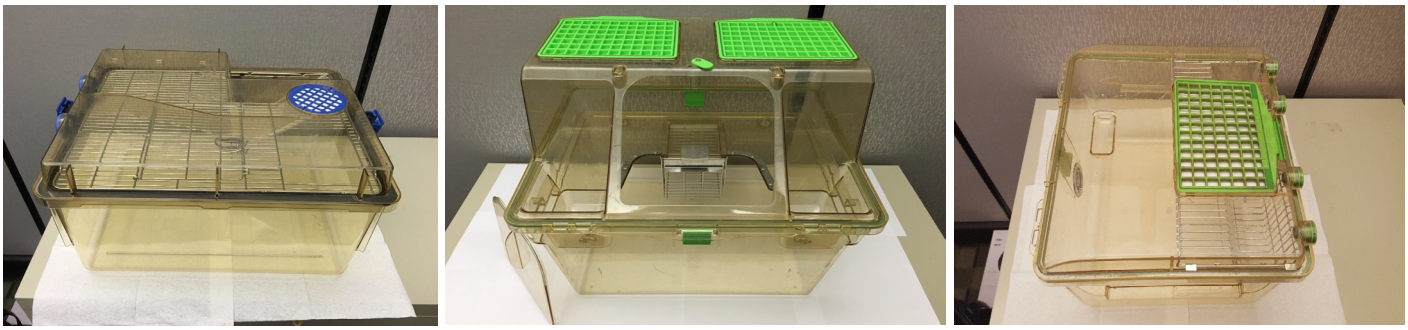

Figure 1. Pictures of 232.5 sq in "blue-line" (BL) (10.5 × 19 × 15 inch) (a), 232.5 sq in "bi-level" (DD) (16 × 15 × 18 inch) (b), and 140 sq in "green-line" (GL) (8.5 × 15 × 13.5 inch) (c) cages used in this study.

After recording original litter size, litters were culled to ten pups across experimental groups on postnatal day 1 to 3 to ensure equal access to nutrition and parental resources (warmth, grooming, etc.) [27,28]. Pups were weighed once weekly starting at two weeks of age through the end of the study in a nearby procedure room that remained consistent throughout the study. Recorded parameters included weekly pup weights starting at two weeks of age, number of pups per litter, and days to the first litter and then between subsequent litters. Male pups were weaned at 21 to 23 days of age into the same caging type they were born into and housed with three to four rats per cage. This provided rats in BL and DD cages between 77.5 and 116.25 sq in of space per rat and rats in GL cages between 35 and 46.67 sq in of space per rat. Any female pups within the weaned pups comprising a group of ten were separated at the time of weaning and either transferred to another approved protocol or euthanized. One of the pairs of rats from the BL caging remained paired throughout the study but did not produce any litters; necropsy did not explain the lack of successful procreation, and these rats were excluded from the results for analysis. Only one rat of the 157 male offspring died during the study, and a large number of bladder stones were found on necropsy. No breeding adults or offspring required humane euthanasia during the course of the study.

Table 1. Tabulation of days to first litter and days between subsequent litters, number of pups per litter, and average pup weight at two weeks of age by cage type.

	Blue Line	Double Decker	Green Line
Days from Pairing to Litter 1	29.33	24.75	35.25
Days Between Litters 1 and 2	40.33	36	43.75
Days Between Litters 2 and 3	32	33.25	36.5
Pups Produced Litter 1	Total No. Male 13	Total No. Male 23	Total No. Male 26
	Total No. Female 18	Total No. Female 20	Total No. Female 19
Pups Produced Litter 2	Total No. Male 11	Total No. Male 16	Total No. Male 9
	Total No. Female 12	Total No. Female 13	Total No. Female 15
Pups Produced Litter 3	Total No. Male 21	Total No. Male 22	Total No. Male 16
	Total No. Female 5	Total No. Female 13	Total No. Female 8
Average Pup Weight at 2 Weeks of Age (grams)	29.77	31.51	28.02

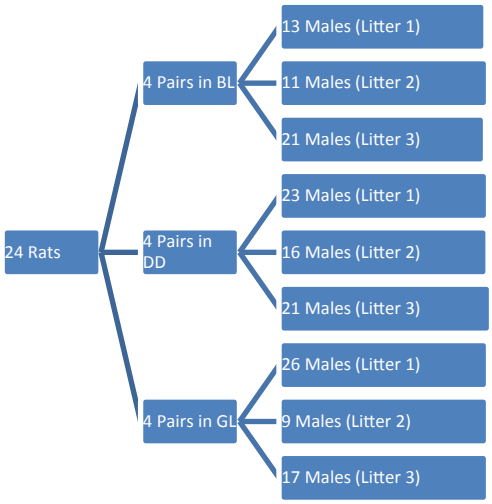

Figure 2. Schematic of study breeding pairs of rats and resulting experimentally-usable male offspring.

Behavioral Testing

Male offspring were exposed to 1-2 min of handling during weekly weightings until approximately 45 days of age, at which time behavioral testing was performed. A 2 ft by 2 ft open field arena constructed of black Plexiglass lined with brown butcher paper was used to examine social interaction and locomotion. Pairs of rats were matched by body weight, randomly assigned using a random number generator, and placed simultaneously in the apparatus [29,30]. Optimal pairs were considered rats from different breeding pairs and the same cage type, whereas non-optimal pairs were considered rats from the same breeding pair but different weaning cages or rats from different cage types. There was no statistical difference or difference in conclusions between optimal or non-optimal pairing. The 5 min testing period was video-recorded and projected onto a monitor approximately five feet from the testing arena for counting/scoring in order to decrease observer interference with behavior. The observer was blinded to the experimental conditions, and the apparatus was cleaned following each experimental run. During the testing period, lighting conditions were low in order to generate an intermediate level of social interaction [30,31], and the time each rat engaged in social interaction (conspecific grooming, sniffing, following, crawling over/under, or boxing) with its partner [32-34] during a 5 min testing period was recorded. Previously completed experiments revealed that recording scores for each member of a pair of rats provided the same statistical outcome and identical conclusions as treating the pair as a unit [29,34-36]. Locomotion activity was simultaneously recorded as the number of squares entered with the front two paws during the 5 min session and provided a measure independent of social interaction [29,32-37]. Center locomotion was recorded as the number of centerline crosses that occurred during the 5 min session. The testing was conducted at the same time of day and on the same two days of the week so as to reduce variability and avoid cage change days.

Corticosterone Quantification

To allow for re-acclimation to caging in order to assess baseline corticosterone levels, we waited one week after the second social interaction test was completed, and approximately 700 µl to 900 µl of whole blood was collected via tail clip from each rat. Rats were brought to the same location for this sampling as that of all weights and behavioral tests so as to keep a consistent routine and limit stress and variability. Collected blood was centrifuged at 3500 rpm, and resultant serum was run in duplicate with a corticosterone ELISA kit per manufacturer's instructions (Enzo Life Sciences, ADI-901-097).

Statistical Methods

A split-plot design was used, with the caging type assigned to the mating pairs, and the response of interest measured on the

pups using general linear mixed models. The cage type, litter effects, pairing types (optimal or non-optimal), and their interactions were considered fixed, while nested random pair effects were used as the whole plot error. Post-hoc tests were run for statistically significant effects and Tukey's adjustment was used to correct for multiple comparisons. P-values less than 0.05 were considered to be statistically significant. All analyses were run in SAS, version 9.4 (Cary, NC).

RESULTS

Experimentally usable male subjects for behavioral testing are summarized in **Figure 2**. We measured social interaction, locomotion, and center locomotion, as they are all well-developed and accepted measures of anxiety-like behavior in rats that have been used for over a quarter of a century [18]. No statistically significant differences in social interaction times between rats from different cage types at 45 or 95 day time points were found **(Figures 3A and 3B)**, least-squares means with Tukey's multiple comparisons post hoc). We did see a decrease in social interaction from the 45 to 95 day time points across all cage types, which is not unexpected, as rats in the adolescent stage are inherently more interactive at this life stage than adult rats. Likewise, there were no statistically significant differences in locomotion or center locomotion scores amongst rats from different cage types at either time points **(Figures 4 and 5)**, least-squares means with Tukey's multiple comparisons post hoc).

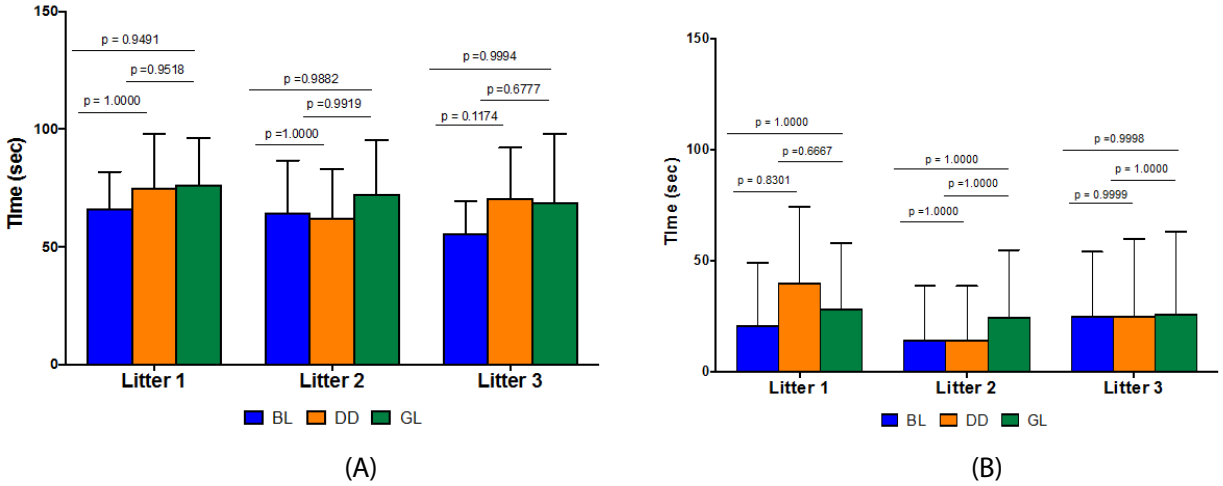

Figure 3. Social interaction testing. Measure of social interaction at 45 days of age (A) and 95 days of age (B) in BL, DD, and GL cages with the sum total of interaction during a 5 min testing interval reported in seconds. Least-squares means were calculated for each treatment combination. Post hoc comparisons were made using Tukey's adjustment to control the Type I error rate. Error bars equal the standard deviation of the mean.

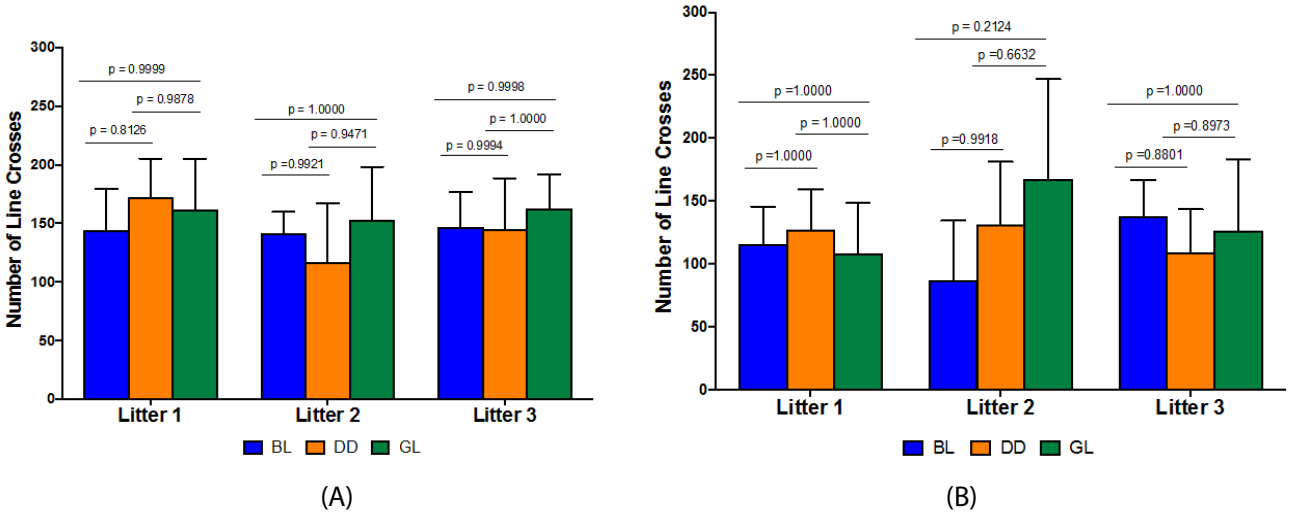

Figure 4. Locomotion testing. Total number of line crosses at 45 days of age (A) and 95 days of age (B) in BL, DD, and GL cages was measured during a 5 min testing interval from a grid composed of nine equal squares within the testing arena. Least-squares means were calculated for each treatment combination. Post hoc comparisons were made using Tukey's adjustment to control the Type I error rate. Error bars equal the standard deviation of the mean.

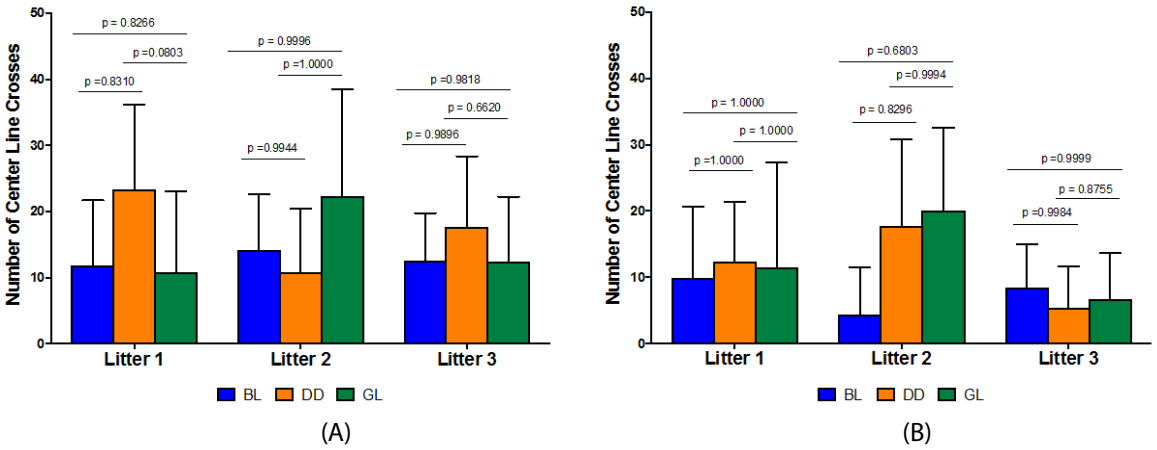

Figure 5. Locomotion center testing. Total number of center line crosses at 45 days (A) and 95 days (B) of age in BL, DD, and GL cages were measured during a 5 min testing interval from a grid composed of nine equal squares within the testing arena and the center four lines counting towards center line crosses. Least-squares means were calculated for each treatment combination. Post hoc comparisons were made using Tukey's adjustment to control the Type I error rate. Error bars equal the standard deviation of the mean.

As a physiologic measure of underlying anxiety levels and state of well-being, we assessed basic breeding parameters. When comparing number of pups born in each litter, days between litters, and pup weight starting at 2 weeks of age, modest, but non-statistically significant, differences were found across rats from different cage types **(Table 1)**. The average number of pups born in each litter was similar for Litters 1 and 2 when comparing DD and standard GL caging; however, DD cages produced 11 more pups for Litter 3 than GL cages, with an overall 14 more pups across all three litters when compared to breeding pairs from standard GL cages. Days to first litter was approximately ten days sooner in DD cages compared to standard caging, approximately eight days shorter in DD cages between Litter 1 and 2, and approximately three days shorter in DD cages between Litter 2 and 3. Finally, at 2 weeks of age, pups from DD cages weighed approximately nine percent more than pups from standard GL caging.

In addition to analyzing breeding parameters, we measured circulating corticosterone levels, another physiological parameter which is known to increase when animals are stressed [8,24]. Previous studies have shown that stressors will increase corticosterone levels, with the magnitude of the response dependent on many factors, including previous housing conditions [10,24]. We tested resting serum corticosterone levels in our rats from all three cage types in order to determine any baseline differences in circulating stress hormone levels. No significant differences were found in baseline corticosterone levels in rats from the different cage types **(Figure 6)**.

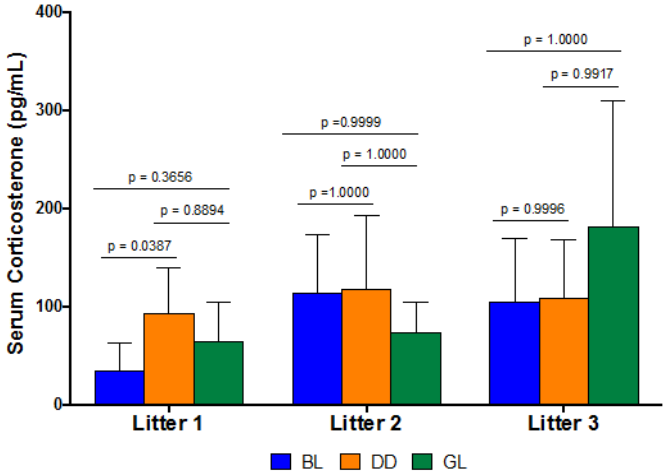

Figure 6. Serum corticosterone. Serum corticosterone was measured using a standard ELISA with concentration reported in pg/ml.

Least-squares means were calculated for each treatment combination. Post hoc comparisons were made using Tukey's adjustment to control the Type I error rate. Error bars equal the standard deviation of the mean.

DISCUSSION

In our attempt to determine potential differences in anxiety-like behavior of rats housed in bi-level cages when compared to standard cages, we assessed behavior by using social interaction, locomotion, and center locomotion tests at approximately 45 days of age (corresponding to a rat's late adolescent stage) and 95 days of age (corresponding to a rat's adult stage) [22,38].

Previous studies have shown evidence that decreased cage size leads to decreased locomotion in juvenile but not adult/post-pubertal rats [3], which is why it was important to evaluate rats at both time points. Although there was a discrepancy in space available per rat in the DD and GL cages, we addressed this variable by additionally using the BL cages, which provided the same available space per rat as the DD cages, just on a single level as opposed to on two levels.

To evaluate anxiety-like behavior in rats housed in different caging types, three behavioral parameters were assessed. The social interaction test was first introduced in 1978 by File and Hyde and has been repeatedly validated as an index of anxiety-like behavior; test parameters decrease following anxiety-provoking stimuli, such as bright lights or exposure to predator odors, after administration of anxiogenic drugs, and following withdrawal from drugs of abuse [30,35]. In addition, social interaction is increased via prior exposure to a test arena or the administration of anxiolytic drugs [33,35,37], with File proving that the sedative effect of anxiolytic drugs can be distinguished from the anxiolytic effects [17]. Social interaction was scored by measuring the amount of time that rats spent involved in conspecific grooming, sniffing, following, crawling over/under, or boxing in a 5 min testing period. Analysis of locomotor activity was simultaneously examined as an independent behavioral parameter to social interaction testing. The correlation of locomotion with anxiety levels has proven especially true for the crossing of lines within the center area of an open field setting. A rat with lower anxiety and stress levels will tend to explore the center of an open field in addition to the periphery of the arena rather than spending the majority of the time in the periphery [21]. More activity in the center indicates an anxiolytic effect, and less activity in the center indicates an axiogenic effect.

Only male offspring were used in testing experiments due to the significant confounders that are introduced with the addition of female rats in behavioral research. Social interaction tests produce different results that should be interpreted differently when testing female rats [17]. Behavioral anxiety testing results vary with stage of estrus cycle [39-42], and control for or synchronization of this parameter while controlling numerous other variables goes beyond the scope of this study. Due to this variability, female rats are rarely used in behavioral research, providing little opportunity for comparison to previous studies. Future studies may attempt to include female rat subjects in addition to males.

Because anxiety-producing events significantly affect breeding performance in laboratory rodents, and animals that have better breeding production and success are thought to have lower anxiety levels, breeding parameters were assessed to help examine the effect of different cage types on animal well-being [6-9,11]. Breeding performance was also assessed to determine if the DD cages led to a higher pup weight via discriminatory nursing behavior, as other studies have proven that mothers 'escaping' their pups prior to weaning will drive the pups to eat solid food earlier and can lead to increased weaning weights [20]. Although none of our data showed statistically significant differences across cage types, some of the comparisons showed trends that may prove advantageous in high-output breeding operations or situations in which strains have difficulty with producing and rearing litters. For example, when comparing days to first litter and then in between subsequent litters/generation time, it may be beneficial to have DD caging in which time to first litter is about ten days sooner than standard caging. Likewise, having pups that weigh an average of nine percent more at two weeks of age is another helpful factor when working with poor breeders in certain strains. Larger caging also presents opportunities for co-housing larger numbers of rats per cage, in addition to the potential of trio mating with one male and two females per cage. Future studies evaluating the potential breeding benefits of these bi-level cages could be improved by including larger numbers of initial breeding pairs as our study was limited to three productive pairs in BL cages and 4 productive pairs in each of the DD and GL cages.

Previous studies have shown that environmental conditions during pregnancy can have a significant effect on offspring. For example, prenatal stress increases pup mortality and decreases pup weight; it also decreases ambulation during open field testing and increases latency to enter the open arms of an elevated plus maze–signs of higher anxiety. Likewise, postnatal stress, such as maternal separation, has been shown to induce effects on offsprings' stress response, ranging from behavioral to hormonal, that extends late into adolescence and adulthood. On the contrary, offspring that experience positive postnatal experiences, such as increased amounts of maternal contact and grooming, show lower fear and anxiety responses than offspring with reduced maternal care [43-45]. However, providing an area of 'escape' for mothers in bi-level caging is not deemed as reduced maternal care, as previous studies have found allowing discriminatory nursing behavior leads to increased pup weight as a result of earlier intake of solid food [20]. Our study did not show that bi-level cages provide either increased prenatal or decreased postnatal stress, as there were no significant differences in our behavioral testing or physiologic parameters measured. Although only serum corticosterone levels were used to evaluate stress levels in this study, future studies present the opportunity to examine other stress responsive hormones such as catecholamines or growth hormone, or to measure corticosterone levels in feces instead of serum to reflect a longer time-frame of stress evaluation.

While not statistically significant, pups housed in bi-level housing trended towards increased weight at two weeks of age, suggesting a possible benefit in discriminatory nursing behavior. Rats weaned into an enriched environment have been shown to exhibit behavior changes indicative of good welfare (i.e. increased bouts of sleeping, reduced stereotypies, reduced aggression and decreased fighting) [46]. In one study, anticipation behavior of rats about to be moved to a familiar enriched cage was noted to be equivalent to the anticipation behavior of rats who were to be moved to a cage in which sexual activity would occur [47]. In a study with similar caging, rats were initially provided access to only the bottom half of the bi-level cages and then later exposed to both levels which resulted in a "positive affective state;" however, the same changes were not found in rats that were exposed to either bi-level or single-level cages without a change. In addition, the rats in this study were singly housed, so it would be inter-

esting to investigate anxiety levels in rats moving from a single-level cage to a bi-level cage in the context of cohousing in further studies [14]. While our study did not support markedly increased welfare benefits in anxiety-like behavior, there are numerous other welfare assessments, such as amount of sleeping, anticipatory behavior/cage choice, and quantification of rearing behavior, that could be conducted and may show a benefit. During this study, rats were observed to use the increased height for bi-pedal posture and the use of both caging levels for sleep, exploration, and play; this provides an area for possible future studies that may be able to quantify or qualify these types of behaviors.

With the increasing amount of rats being used in laboratory animal research, it is beneficial to assess co-housed situations, as space in laboratory animal facilities is always at a premium. Many previous studies have assessed environmental enrichment only with singly housed animals, which can present a confounder in rat behavior, as rats always prefer social housing over any other type of enrichment [46]. In addition, studies that have evaluated cage space typically increase the amount of space while concurrently increasing the number of animals housed in the space, creating a confounding evaluation of whether cage space itself indeed makes a difference [46]. In one study that looked at the tendency of rats to choose a larger cage size, the choice for this increased space did not vary with cages that had two or more rats per cage [48]. In consideration of these findings, we chose to co-house our rats in groups of three to four during our studies to control for increased space in combination with co-housed animals. In addition, our study provided co-housed rats with either increased uni-level space or more complex bi-level space to control for the possibility that better results from bi-level caging were simply a result of larger available space.

When deciding on the type of caging to use for housing laboratory rodents, practical factors should also be considered, including economic feasibility. The cost of a double-sided rack that holds 32 individually ventilated bi-level cages is approximately the same cost as a double-sided rack that holds 70 individually ventilated green-line cages. Taking into account the space requirement of 40 sq in per rat, this would allow a bi-level cage rack to hold 185 adult rats (~400 g each), compared to 245 adult rats in a green-line rack. Additional factors to consider include ease of handling and cleaning of cages, which tends to be more difficult in the bi-level cages due to their increased size and weight. Specialized equipment is available for purchase to help manipulation of the cages on the rack at an additional cost.

CONCLUSION

In conclusion, as we continue to strive to meet the recommendations of the Guide, it is important to continue to evaluate potential areas of good welfare for our laboratory animals. Although there were no statistically significant behavioral or physiological differences to support the use of bi-level cages over standard caging in the tests conducted in our study, bi-level caging may provide a more complex, environmentally enriched environment that allows natural postural adjustments and species specific behaviors. It is important that bi-level caging had no significant effect on anxiety-like behavior in rats, as researchers can take advantage of the welfare benefits of bi-level caging without concern for effects on behavioral research. We elected to use Sprague Dawley rats because of their common use in laboratory animal experiments; however, Gaskill et al evaluated both Sprague Dawley and Brown Norway rats in larger single-level caging and did see a difference between stocks/strains in some areas of the assessment, so the potential for varied rat stock or strain responses is an important area for future study [15]. In addition, the mild benefits in breeding performance may be useful in times of difficult breeding or high breeding output situations, and additional space allows for more rats per cage which may be beneficial with the increased use of rat models in research.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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