Journal of the American Association for Laboratory Animal Science
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Vol 62, No 2
March 2023
Pages 116–122

Effects of Housing Density on Reproductive Performance, Intracage Ammonia, and Welfare of Mice Continuously Housed as Breeders in Standard Mouse and Rat Caging

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Maintaining compliance with cage density recommendations in *The Guide for the Care and Use of Laboratory Animals* precludes continuous trio breeding in standard-sized mouse cages. This study evaluated and compared several parameters of reproductive performance, intracage ammonia concentration, and fecal corticosterone levels in 2 strains of mice, C57BL/6J (B6) and B6.129S(Cg)-*Stat1*^{tm1Dlv}/J (STAT1$^{-/-}$), housed as continuous breeding pairs or trios in standard-sized mouse cages, and continuous breeding trios in standard-sized rat cages. Reproductive performance data indicated that STAT1$^{-/-}$ trios raised in rat cages weaned significantly more pups per litter than did STAT1$^{-/-}$ trios raised in mouse cages, and B6 mice had higher pup survival rates at weaning than did STAT1$^{-/-}$ mice in mouse cages housing continuous breeding trios. In addition, the Production Index was significantly higher for B6 breeding trios in rat cages than for B6 trios in mouse cages. Intracage ammonia concentration increased with cage density, with significantly higher ammonia concentrations in mouse cages housing trios compared with rat cages housing trios. However, fecal corticosterone levels did not differ significantly regardless of genotype, breeding configuration, or cage size, and daily health checks revealed no clinical abnormalities under any of the conditions evaluated. These results suggest that, although continuous trio breeding in standard-sized mouse cages does not seem to compromise mouse welfare, it offers no advantage in reproductive performance compared with pair breeding, and in some cases, it might be disadvantageous in this regard. Further, high intracage ammonia in mouse cages containing breeding trios might necessitate more frequent cage changes.

Abbreviations and Acronyms: B6, C57BL/6J; STAT1$^{-/-}$, B6.129S(Cg)-*Stat1*^{tm1Dlv}/J, signal transducer and activator of transcription 1; ILI, Interlitter Interval; MP, mouse cage paired breeders; MT, mouse cage trio breeders; PI, Production Index; RT, rat cage trio breeders

DOI: 10.30802/AALAS-JAALAS-22-000069

Introduction

For several reasons, including lack of commercial availability of certain genetically engineered mice (GEM), production of littermate controls for experiments using GEM, and cost savings, many of the mice used in research are generated from inhouse breeding colonies. These colonies are often managed by investigators and their staff. Despite the advantages of inhouse breeding, the generation and maintenance of research mouse colonies from a set of founder mice can be time consuming, expensive, and require a large amount of housing space. Therefore, both investigators and animal resource managers strive to optimize colony production and reduce costs while using vivarium space efficiently. To this end, breeding schemes that use a single male and multiple females in a single cage are sometimes implemented. However, the number of animals that can be housed in a mouse cage is limited by regulatory standards for cage density, specifically, the recommendations set by the eighth edition of *The Guide for the Care and Use of Laboratory Animals* (The Guide). *The Guide* recommends a minimum floor space of 51 in^2 (330 cm^2) for each female and litter, and an additional 15 in^2 (97 cm^2) for each mouse weighing 25 g or more.[23] Standard mouse cages used at the authors' institution provide 77.66 in^2 (501 cm^2) of floor space, which is adequate for housing a breeding pair and a single litter according to these recommendations. Pair breeding typically involves continuously housing one male and one female together. The continued presence of the male allows for breeding during the postpartum estrus, which occurs 14 to 24 h after the female gives birth,[42] thereby maximizing production from the female and reducing the interlitter interval (ILI). However, this approach requires a larger number of male mice than does the use of a single male to service multiple females.

Harem breeding involves housing one male with 2 or more females, thus limiting the number of males required in the colony, reducing the cost to investigators by increasing cage-level production, and using housing space more efficiently. However, in most cases the harem breeding strategy is more labor intensive because compliance with *Guide* cage density recommendations for standard sized mouse cages requires the removal of pregnant females prior to parturition and the reestablishment of the harems after litters are weaned.[23] In addition, production might be limited if males are not present for copulation with the isolated females at the postpartum estrus.[18]

Submitted: 15 Jul 2022. Revision requested: 02 Sep 2022. Accepted: 30 Oct 2022.
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In recent years the Institutional Animal Care and Use Committee (IACUC) at the authors' institution has seen more requests for departures from cage density requirements to allow trio breeding in which one male and 2 females are housed continuously in a standard-sized mouse cage (77.66 in^2 (501 cm^2)), which, according to *The Guide* recommendations, would require a minimum of 117 $in.^2$ (523.4 cm^2).[23] These requests have been justified based on anecdotal reports of better reproductive performance under these conditions due to communal rearing of pups and a desire to standardize the gut microbiome of cohorts of mice that have been reared in the same cage through weaning. This trio breeding strategy combines the advantages of harem breeding (fewer males needed and higher cage-level production) with the advantage of having a male present for the females at the postpartum estrus, reducing the ILI. This strategy also provides greater cage-level production while making more efficient use of housing space, which is often limited in contemporary animal facilities, and reduces per diem costs to the investigator. Furthermore, the occurrence of alloparenting is common in communal breeding cages of research mice,[19] and may result in pups that are more robust than those raised by paired breeders or a single dam.[4,8,22]

Such requests for departures from our cage density policy have undergone intense scrutiny by the IACUC due to potential animal welfare concerns, in addition to a general reluctance to depart from regulatory guidelines without compelling justification. Exposure to higher ammonia levels and stress associated with higher stocking densities can compromise animal welfare, and litter overlap in traditional caging during continuous trio breeding has been associated with greater pup mortality.[28]

Previous studies comparing trio or pair breeding schema have been inconsistent in their findings and recommendations. These studies have largely compared 2 or more breeding configurations by measuring reproductive parameters to assess productivity and clinical and physiologic changes in mice maintained at a greater housing density. Commonly used parameters for reproductive performance include litter survival rates, pup weight at weaning, Production Index (PI; number of pups weaned per dam per week), and ILI.[4,8,19] Multiple studies found that continuous trio breeding in traditional mouse caging had few or no deleterious effects on pup growth and pup survival; and that *The Guide*'s recommendations for housing density deserve reconsideration regarding welfare concerns in continuous trio breeding in traditional caging.[23,25,30,32,41,43] Conversely, one study compared reproductive performance for continuous trio and pair breeding, and found that trios, as compared with pairs, had a prolonged ILI, a smaller litter size at birth, and a lower ratio of pups surviving to weaning.[8] Studies that compared weights of weanlings from pair- and trio-bred systems reported that pups raised in trio cages, which allowed alloparenting, had higher weights at weaning as compared with those raised by paired breeders.[4,8,22,32,42]

Intracage ammonia concentration and corticosterone metabolite levels are commonly assessed when studying the effects of increased housing density that results from continuous trio breeding. Due to the increased housing density, ammonia levels accumulate at a faster rate in cages that house continuous trio breeders, possibly to levels high enough to require weekly cage changes.[7] However, frequent cage disturbances can be stressful to mice,[7] perhaps countering any production benefits derived from continuous trio breeding. High intracage ammonia concentration is a mucosal irritant and can lead to nasal pathology.[7,11,27,40] Corticosterone levels and adrenal gland size[1,3,9,17,20,24] have also been assessed as indicators of compromised animal welfare in the context of increased housing density, with both parameters increasing with increasing cage density.[10,26,29]

The current study compares continuous pair and trio breeding with respect to reproductive performance, microenvironmental quality, and animal welfare in C57BL/6J (B6) and a GEM strain purported to demonstrate higher production under continuous trio conditions at our institution. We hypothesized that continuously housing breeding trios in standard mouse cages would not result in a significant improvement in reproductive performance, despite anecdotal reports to the contrary. We further hypothesized that increased housing densities would result in higher intracage ammonia levels, possibly resulting in a need for more frequent cage changes.

Materials and Methods

Humane care and use of animals. This study was performed in an AAALAC-accredited facility and approved by the Yale University IACUC. With the exception of higher housing density in groups housing breeding trios continuously in mouse cages, all aspects of this study were compliant with *The Guide*.[23]

Animals. Age-matched, 6- to 8-wk-old, nulliparous C57BL/6J (JAX stock #000664) and B6.129(Cg)-*Stat1*^{tmDlv}/J mice (STAT1$^{-/-}$; JAX stock #012606) were obtained from Jackson Laboratories (Farmington, CT). All mice were free of Ectromelia virus, Theiler virus, Hantaan virus, K virus, LDH elevated virus, lymphocytic choriomeningitis virus, mouse adenovirus, mouse cytomegalovirus, mouse hepatitis virus, mouse chapparovirus, mouse minute virus, mouse norovirus, mouse parvovirus, mouse thymic virus, pneumonia virus of mice, polyoma virus, reovirus 3, rotavirus, and Sendai virus, *Bordetella* spp., *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Filbacterium rodentium*, *Mycoplasma pulmonis*, *Mycoplasma* spp., *Salmonella* spp., *Streptobacillus moniliformis*, *Encephalitozoon cuniculi*, ectoparasites, tapeworms, pinworms, and other helminths, follicle mites, and protozoa (*Giardia* spp., *Spironucleus* spp., and *Toxoplasma gondii*).

Housing and husbandry. Mice were housed in individually ventilated cages (75 air changes per hour). GM500 mouse caging (77.66 in^2) on DGM80 racks or GR900 rat caging (140.12 in^2) on 2GR35 racks, (Tecniplast, West Chester, PA) were used. Cages contained 1/8 in. (3.175 mm) of corncob bedding (Envigo, Somerset, NJ), ad libitum rodent chow (Global Rodent Diet 2018S, Envigo Teklad, Somerset, NJ), and nesting material (Cotton squares, Ancare, Bellmore, NY). Cages and components (wire tops, feed, bedding, and filter tops) were preassembled and autoclaved prior to use. Mice had ad libitum access to hyperchlorinated (4 to 6 ppm) water delivered via an automated watering system (Avidity, Waterford, WI). Room temperature and relative humidity were maintained at 72 ± 2 °F (22 °C) and 50% ± 10% respectively, with 10 to 15 air changes hourly and a 12:12-h light:dark cycle. At each cage change, a portion of the nesting material from the soiled cage was transferred into the new cage, as is standard practice at our institution to mitigate stress associated with the clean cage.

Experimental design. All breeding configurations were maintained continuously. Mice were evaluated daily for the duration of the study. Cages were changed every 2 wk, with no exception for cages containing pups (< 7 d). Mice and cages were not handled or otherwise disturbed between cage changes, other than for replenishing chow and weaning 21-d old litters. Mice were evaluated daily using a cage-side approach to assess general health and appearance. More thorough examinations were performed during cage changes and weaning, including identifying and counting fetal remnants. New litters that were

born between cage changes were noted, and pups were counted without opening the cage to avoid causing additional stress.

Mice were grouped by strain and breeding configuration, and cages were evenly distributed across the rows of IVC racks to ensure equal distribution of differential light levels and mechanical noise from rack blowers between groups. Upon arrival, mice were randomly assigned within each strain to one of 3 experimental groups defined by breeding configuration and cage size: pairs (1F, 1M) in mouse cages, trios (2F, 1M) in mouse cages, or trios (2F, 1M) in rat cages, with breeding females (n = 12) assigned to each group. Therefore, female (n = 36) and male (n = 24) mice of each strain were assigned to 12 breeding pairs (n = 12 females, n = 12 males) in mouse cages (MP), 6 breeding trios (n = 12 females, n = 6 males) in mouse cages (MT), and 6 breeding trios (n = 12 females, n = 6 males) in rat cages (RT), for a total of 72 females and 48 males. We initiated the study with 3 B6 MP cages, 2 B6 MT cages, and 1 B6 RT cage, created from an initial shipment of mice. Upon arrival, the STAT1$^{-/-}$ mice were allocated into the following housing conditions:3 STAT1$^{-/-}$ MP, 2 STAT1$^{-/-}$ MT, and 2 STAT1$^{-/-}$ RT. Offspring from these cages were randomly assigned across groups to reach our target of 12 MP, 6 MT, and 6 RT cages of STAT1$^{-/-}$. To achieve the same target number of cages, additional B6 mice were purchased and allocated into cages. Data were collected over a period of one to 5 mo for each cage. Over the course of the experiment, 15 male STAT1$^{-/-}$ mice were replaced due to penile injuries sustained from female breeding partners, and 2 female B6 breeders were replaced after euthanasia due to dystocia. When losses occurred, new mice of equivalent age and parity were assigned to the affected cage to maintain statistical integrity.

Reproductive Parameters. Pups were counted through the cage at birth. At postnatal day 21, pups were counted, and each litter was weighed collectively. Litter weight was divided by the number of weaned pups in each litter to calculate the average pup weight at weaning. The total number of pups weaned was divided by the total number of pups born to calculate the ratio of pups weaned:born (that is, pup survival) for each strain and breeding configuration. The Production Index (number of pups weaned/female/week) was calculated for each breeding cage, then averaged across strain and breeding configuration. The ILI (time in days between the birth of each litter) was calculated for every litter, then averaged across strain and breeding configuration.

Cage ammonia quantification. Ammonia samples were collected before opening cages at the time of cage change (2-wk interval) as follows: the full length of the exposed portion of the ammonia detector tubes (105SE 1 to 200 ppm gas detector tube, Sensidyne, St. Petersburg, FL), connected to a sampling pump (Sensidyne Gas Sampling Pump Kit Model AP-20S, St. Petersburg, FL), were inserted through the cage automatic watering system grommets and samples of cage air were taken at a height of approximately 1 in. (2.54 cm) from the cage bottom, which approximates the height of the mouse breathing zone. These readings were recorded and used to calculate the average ammonia levels by strain and breeding configuration.

Fecal corticosterone metabolites. During cage changes, fresh fecal pellets were obtained directly from manually-restrained adult mice from each cage. At the time of pellet collection, the number of individuals per cage varied from 2 adults with no litter to up to 3 adults and 21 pups from 2 litters. The feces were stored at −20 °C and pooled by caging configuration pending submission for fecal corticosterone analysis (Arbor Assays, Ann Arbor, MI).

Statistical Analysis. *A-priori* sample size estimates calculated using G*Power[15] were used for each condition to detect significant differences across all dependent variables: ILI, PI, cage ammonia concentration, fecal corticosterone, number of pups weaned:pups born, and pup weights at weaning) at a 95% confidence level with 0.8 power. Outliers were maintained within the analysis except for the PI value for one female from a B6 trio mouse cage. Data were analyzed by using commercially available SPSS software (SPSS 26, IBM, Armonk, NY). Nonparametric Kruskal–Wallis H tests were conducted for all analyses with the exception of PI. If the distributions of the dependent variable were dissimilar across the 6 groups, mean-ranks were used to conduct the analysis. Posthoc pairwise comparisons with Bonferroni correction for multiple tests were employed when results were significant. Welch's ANOVA with post hoc Games-Howell tests were conducted to determine where the differences in production index lies across the 6 groups. A P value of 0.05 is considered statistically significant unless otherwise stated.

Results

Ratio of pups weaned:pups born. All data regarding the number of pups born and weaned are presented in Table 1. The ratio of the number of pups weaned:pups born (that is, pup survival) was statistically significantly different between the 6 groups, $\chi^2(5) = 14.280$, $P = 0.014$. Subsequently, pairwise comparisons were performed using Dunn's procedure.[12] A Bonferroni correction for multiple comparisons was made with the corrected statistical significance accepted at the $p < 0.003$ level. Adjusted P values and medians are presented. This post hoc analysis revealed a statistically significant difference in pups weaned:pups born between the STAT1$^{-/-}$/MT (0.339) and B6/MT (1.000; $P = 0.001$) groups. The total number of pups weaned was compared with the total number of pups born into each breeding cage configuration. B6 cages had higher pup survival rates compared with those of STAT1$^{-/-}$ cohorts. Among the STAT1$^{-/-}$ configurations MT cages weaned 43% of pups born, compared with 66% for MP and 61% for RT cages; however, these differences were not statistically significant.

Average number of pups weaned per litter. All data regarding the average number of pups weaned among each of the different housing configurations are presented in Table 2. To calculate the average number of pups weaned per litter for each breeding configuration, the total number of pups weaned was divided by the total number of litters born throughout this study, including partial and lost litters On average, mice

Table 1. Number of pups born, pups weaned, and pups weaned:born ratio by genotype and breeding configuration

Genotype	Breeding strategy	Total # pups born	Total # pups weaned	Ratio of weaned to born pups
B6	MP	231	169	0.73
	MT	494	347	0.70*
	RT	412	287	0.70
STAT1$^{-/-}$	MP	161	106	0.66
	MT	191	82	0.43*
	RT	167	102	0.61

An analysis of the pup weaned to born ratios found statistically significant differences in all categories. A posthoc analysis revealed a statistically significant difference in pups weaned:pups born between the STAT1$^{-/-}$/MT (0.339) and B6/MT (1.000) ($P = 0.001$) groups (denoted with asterisks).

Table 2. Average number of pups weaned per litter by genotype and breeding strategy

Genotype	Breeding strategy	# pups weaned per litter (mean ± SD)
B6	MP	2.6 ± 0.4
	MT	3.3 ± 0.4
	RT	4.1 ± 0.5
STAT1$^{-/-}$	MP	4.0 ± 0.8
	MT	2.0 ± 0.5
	RT	4.0 ± 1.1
All Groups		3.4 ± 2.0

Table 3. Weaning weights (grams) by genotype and breeding strategy (n = # of cages)

Genotype	Breeding strategy	N	Pup weaning weight (g)
B6	MP	12	8.2 ± 1.6
	MT	12	8.1 ± 0.9
	RT	12	9.2 ± 0.9
STAT1$^{-/-}$	MP	10	7.94 ± 1.3
	MT	5	8.1 ± 1.2
	RT	5	8.4 ± 1.5
All Groups		56	8.2 ± 1.3

A Kruskal–Wallis H test was run to determine differences in pup weights between 6 groups of breeding, cage, and strain categories. Distributions of pup weights were not similar for all groups as assessed by visual inspection of a boxplot. The distribution of pup weights was not statistically significantly different between groups; $\chi^2(5) = 5.39$, $P = 0.37$.

Table 4. Production index (pups weaned/female/week) for each genotype and . Mean Interlitter Interval (N_{ILI} = days between litters per dam) breeding configuration with standard deviation

Genotype	Breeding strategy	PI	N_{ILI}
B6	MP	0.78 ± 0.34	26.4
	MT	0.53 ± 0.48*	26.7
	RT	1.00 ± 0.45*	41.1
STAT1$^{-/-}$	MP	0.78 ± 0.53	39.1
	MT	0.73 ± 0.29	28.4
	RT	0.82 ± 0.21	40.3

Production index was statistically significantly different between B6RT and B6MT groups Welch's $F(5, 39.156) = 2.785$, $P < 0.05$. Asterisk (*) denotes statistical significance. No statistically significant differences in Interlitter Interval between the groups.

Table 5. Median intracage ammonia concentration and median fecal corticosterone (pg/mg) by breeding configuration. n = number of cages sampled

Breeding strategy	N	Median [NH_3] (ppm)	Median fecal corticosterone (pg/mg)
MP	52	51	31.3
MT	55	72*	41.0
RT	41	30*	29.8

Post hoc analysis with Bonferroni correction revealed statistically significant differences in in median cage ammonia between the RT (30) and MT (72.5) ($P = 0.007$) groups, but not between the MP (51) or any other group combination.
Asterisk denotes statistical significance.

belonging to the RT group weaned more pups than did any other group, but the difference was not statistically significant $F(4,20.782) = 2.231$; $P = 0.101$).

Body weight of pups at weaning. All weanling body weight data are presented in Table 3. However, differences between groups were not significantly different.

Production Index. The PI for each genotype and breeding configuration is presented in Table 4. There was a statistically significant difference between B6/RT and B6/MT group PIs [Welch's $F(5, 39.156) = 2.785$, $P < 0.05$]. The average PI ranged from the B6/MT (0.53 ± 0.48) to STAT1$^{-/-}$/MT (0.73 ± 0.29), STAT1$^{-/-}$/MP (0.78 ± 0.53), B6/MP (0.78 ± 0.34), STAT1$^{-/-}$/RT (0.82 ± 0.21), and B6/RT (1.00 ± 0.45), groups, in that order from lowest to highest. Games-Howell post hoc analysis revealed that the difference in PI between 0.52 ± 0.47 in the B6/MT group and 1.00 ± 0.45 in the B6/RT group, an increase of 0.476 (95% CI, 0.1 to 0.85), was statistically significant ($p = 0.005$).

Interlitter interval. ILI were calculated for individual dams and averaged across genotype/breeding configuration as presented in Table 4. Pairwise comparisons with a Bonferroni correction for multiple comparisons revealed no statistically significant differences in ILI between the groups.

Intracage ammonia. Intracage ammonia data for each breeding configuration, combining both genotypes, are shown in Table 5. Median cage ammonia levels were statistically different between the MP, MT, and RT groups [$\chi^2(2) = 9.446$, $P = 0.009$]. Therefore, pairwise comparisons were performed using the Dunn procedure.[12] A Bonferroni correction for multiple comparisons was made with statistical significance accepted at the $P < 0.016$ level. Adjusted P values are presented. This posthoc analysis revealed statistically significant differences in cage ammonia between the RT (30) and MT (72.5) ($P = 0.007$) groups, but not between the MP (51) and RT or MT. No other group combinations were statistically different.

Fecal Corticosterone. Fecal corticosterone data for each breeding configuration, combining both genotypes, are shown in Table 5. Median fecal corticosterone did not differ statistically among the different types of housing: MP (n = 10, median = 31), MT (n = 10, median = 41), and RT (n = 10, median = 30) [$\chi^2(2) = 3.783$, $P = 0.151$].

Discussion

Our study compared reproductive, microenvironmental, and welfare parameters between 2 mouse strains maintained as continuous trio breeders and breeding pairs in 2 sizes of IVC caging. Based on data from 160 weaned litters from 65 breeding cages, B6 dams weaned a higher percentage of pups born (that is higher pup survival at weaning) compared with STAT1$^{-/-}$ dams. STAT1$^{-/-}$ mice housed in standard mouse cages as continuous breeding trios had the lowest pup survival rate at weaning of all groups (43%), while B6 in all 3 breeding configurations had the highest (70% to 73%). More specifically, the pup survival rate at weaning was significantly higher for B6 mice housed in mouse cages as continuous trios than for STAT1$^{-/-}$ mice under the same conditions. Under no condition (genotype or breeding configuration) did we find significantly greater pup survival for continuous breeding trios compared with breeding pairs, even with the provision of additional housing space. Other studies comparing trio and pair breeding had similar findings, with paired breeders having lower rates of mortality.[5,8] We speculate that perhaps fewer STAT1$^{-/-}$ MT pups survived to weaning age as compared with wild type B6 mice under the same conditions because although homozygous STAT1$^{-/-}$ mice are viable and fertile according to the Jackson

Laboratories website, the higher stocking density and ammonia concentrations that resulted from continuous trio housing could have resulted in higher pup mortality for this strain.[37]

Pup survival in MP configuration was not significantly different from that of the 2 trio-breeding configurations. Litter overlap has been associated with pup mortality.[28] Litter overlap occurred in our trio cages, but not in our MP cages, which always had plentiful resources for the pair and their litter. Conversely, during this study, we had 11 instances of MT cages requiring food replenishment at times other than cage change days, as we had anticipated due to the presence of multiple litters in MT breeding cages. Although our goal was to minimally handle all cages, our trio cages were disturbed more frequently than pair cages for litter weaning and food replenishment. This was done with the understanding that changes in behavior and levels of corticosterone metabolites could be affected by frequency of cage change/disturbance.[31,33] Higher frequency of cage changes and/or handling of breeding cages has been associated with increased pup mortality, and may represent a disadvantage of continuous trio breeding, at least in mouse standard cages.[5,32]

Pup and/or litter losses are common events in mouse breeding colonies. Both young[13] and primiparous dams[6] have lower rates of pup survival than do dams that were bred at a later age. Litter loss did occur during our study. Some litters were totally lost while others had partial loss of pups. All litters born were included when calculating our data

Weanling body weights did not differ significantly among groups regardless of genotype or housing configuration. In addition, STAT1$^{-/-}$ MT cages were not significantly different from STAT1$^{-/-}$ MP cages, and, despite the benefit of communal nesting and alloparenting. This result contrasts with anecdotal reports mentioned above paragraph regarding better reproductive performance of continuous STAT1$^{-/-}$ trio breeders in standard mouse ventilated caging. One could speculate that higher cage densities and ammonia concentrations observed under MT conditions may negate benefits of alloparenting and communal nesting. As such, RT raised pups likely prospered due to the benefits of a lower housing density, alloparenting, and a healthier cage microenvironment.

PI is a calculation of the number of pups weaned per dam (or cage) per week.[35] We calculated PI for each dam and averaged those values for each breeding configuration and genotype in order to quantify overall reproductive efficiency despite differences in experimental durations of time for each active cage in the study. Overall, the PI of B6 mice (0.53 to 1.0) were similar to those reported by others (0.5 to 0.8 ± 0.2).[2,18,19,34] If the dams had been continuously bred for their entire reproductive life spans (from 6 wk of age to approximately 8 mo[16]), the values might be closer, given that reproductive efficiency declines with increasing age depending on strain, health, and other factors. In addition, PI values calculated under different breeding conditions and facility environments are likely to vary. Prolonged ILIs, which indicate fewer conceptions per dam, can be interpreted as a sign of reproductive suppression. A previous study[8] comparing MP and MT breeding in shoebox caging reported higher PI for MP, and shorter ILI for MT cages.

We also measured intracage ammonia concentration as a gauge of microenvironmental quality of the 3 breeding configurations. Within our 3 breeding configurations we found a statistically significant difference when comparing ammonia levels from MT and RT caging only. Behavioral changes have been observed in mice exposed to high ammonia concentrations.[14,21,36] Changes in nasal pathology were also reported, ranging from mild inflammation of respiratory epithelia to thickened and deformed nasal turbinates[7,11,27,32] proportional to the duration of time exposed to excessive intracage ammonia. A 2011 study reported that exposure to an ammonia concentration of 52 ppm for 13 d resulted in epithelial degeneration in IVC housed mice.[40] We did not observe clinical signs indicative of nasal irritation in any of our groups, and ammonia levels in MP and RT cages remained below the above mentioned thresholds. However, because we only measured intracage ammonia on the day of cage change, we do not know how long these mice were exposed to high concentrations. Other studies investigating ammonia concentration at higher population densities of mice reported that mouse cages containing trio breeders developed higher ammonia concentrations between cage changes.[7,11,32] Several MT cages in our study exceeded 100 ppm, which approaches or surpasses the intracage ammonia levels associated with changes in nasal pathology in other studies,[27,40] suggesting a potential welfare issue under these conditions and the potential need to introduce measures to limit exposure to high concentrations (for example, more frequent cage changes, separating litters, etc.).

Finally, we also measured fecal corticosterone levels as a general indicator of welfare or chronic stress in mice living at a higher housing density. However, significant differences were not noted among groups.

As a strategy for breeding colony expansion and maintenance, the use of continuous trio breeding saves mouse housing space in the vivarium and is more cost efficient than pair breeding if performed in standard mouse cages. However, this strategy may require more frequent cage changing to maintain intracage ammonia concentrations within acceptable limits.[32] More frequent cage changing could also result in higher per diem rates, thus offsetting cost savings associated with the use of fewer cages for trios. In addition, the overlap of litters in trio breeding cages might be associated with higher rates of pup mortality due to physical trauma from older pups, younger pups losing access to resources due to competition from older pups, and diminished cage microenvironmental quality.[28] On the other hand, balancing the frequency of cage changes with minimal cage disturbances (for example, timely weaning of pups and consideration of animal welfare and cage microenvironment), continuous trio breeding in standard mouse IVC can be a practical and efficient means of colony expansion. Furthermore, the use of this breeding strategy requires the purchase of fewer male mice, reduces cage per-diems by using fewer cages, and requires less space for housing. However, trio breeding seems to offer no significant advantage with respect to the productivity of individual dams or pup survival. In fact, housing trios in standard sized mouse cages might be a disadvantage in the context of both parameters. That said, our data does indicate that continuous trio breeding at a lower cage density (for example, in standard sized rat cages) might be useful because in our study, it resulted in statistically significant higher PI and in some cases greater pup survival, while improving the quality of the cage microenvironment as compared with trio breeding in standard sized mouse cages. The obvious downside of continuous RT breeding is the need for more vivarium housing space, as it requires approximately the same space per rack to house less than half the number of cages compared with mouse IVC racks. However, under certain circumstances, such as the foundation of a new breeding colony with strains known to be poor breeders, trio breeding in rat caging could be used to maximize breeding production.

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