Effect of Continuous Trio Breeding Compared with Continuous Pair Breeding in ‘Shoebox’ Caging on Measures of Reproductive Performance in Estrogen Receptor Knockout Mice

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Some performance standards for continuous trio breeding in ‘shoebox’ cages for inbred stocks and outbred strains of mice challenge the minimum floor space recommendations in the 8th edition of the *Guide for the Care and Use of Laboratory Animals*. In our study, we evaluated whether continuous trio breeding could be successfully applied to a breeding colony of genetically engineered mice housed in shoebox cages with a floor area of 67.6 in². Mice heterozygous for genetically engineered mutations to estrogen receptors and their wildtype counterparts were continuously bred as trios or pairs. Confounding environmental factors were controlled through standardized husbandry practices and husbandry, and all mice were bred simultaneously to control for temporal factors. Several measures of reproductive performance—including number of litters per female, production index, interlitter interval, litter size at birth, litter size at weaning, weaning rate, and body weight of pups at weaning—were evaluated over approximately 6 mo. Regardless of genotype, interlitter interval, litter size at birth, and litter size at weaning were significantly lower for trio-bred mice than for pair-bred mice. In addition, significant interactions emerged between genotype and breeding strategy for these reproductive measures. Furthermore, significant differences between genotypes occurred for interlitter interval and weaning rate, regardless of breeding strategy. Underlying mechanisms to account for effects of genotype on interlitter interval and the interaction of genotype with breeding strategy were unclear but may reflect effects of overcrowding and reproductive suppression.

Abbreviations: α BERKO, estrogen receptor α / β knockout; β ERKO, estrogen receptor β knockout; GEM, genetically engineered mice; PI, production index

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Continuous trio breeding is a common strategy for mice in which a single male mouse is housed with 2 females and thereby mates with them both on an ongoing basis. This breeding strategy has been proposed for rapid colony expansion, for propagating genes from a valuable male, or when valuable males are low in number.^{8,31,38} Continuous trio breeding may enhance reproductive performance by taking advantage of postpartum estrus, thereby decreasing the interlitter interval. In addition, trio breeding allows mice to be reared in communal nests, which has been associated with increased pup growth rate and adult body weight^{15,16,33} but also with reproductive suppression,¹³ a process in which successful reproduction is inhibited or prevented in sexually mature and healthy animals.

The 8th edition of the *Guide for the Care and Use of Laboratory Animals* created significant challenges to researchers wishing to use continuous trio breeding. This edition introduced a minimum floor space recommendation of 51 in.² for a female mouse with a litter.²¹ According to this recommendation, continuous trio breeding would thus require 117 in.² of floor

space (51 in.² for each female plus 15 in.² for a mature male weighing at least 25 g). However, technical specifications from many mouse caging manufacturers indicate that commercially available ‘shoebox’ mouse cages have a maximum of 83 in.² of floor space^{2–4,20,24,34}—too small for continuous trio breeding as mandated in this edition of the *Guide*. Mouse colony managers using continuous trio breeding when the 8th edition of the *Guide* was published thus were required to alter their breeding strategy if the institutions were unable to provide them with larger cages. One option was to remove females from the trio once they were identified as gravid. This option, however, does not allow research personnel to take advantage of postpartum estrus. It also requires single-housing of the separated females until parturition, precluding them from social housing, another recommendation of the *Guide*. Another option was to change the breeding strategy to continuous pair breeding, in which a single male is housed with a single female. Although this strategy takes advantage of postpartum estrus and meets the *Guide*’s recommendations for social housing, it requires twice as many male mice as does trio breeding, and it does not accommodate communal nesting.

Several studies have reevaluated the floor space recommendations provided by the 8th edition of the *Guide*, emphasizing performance standards over engineering standards. However, we have not found any studies in the literature that examined

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the effect of breeding strategy in shoebox cages on the reproductive performance of specific genotypes of genetically engineered mice (GEM). One study demonstrated an overall similarity in litter survival and growth rates between trio- and pair-bred mice housed in shoebox caging; the study included mice of numerous inbred strains and genotypes, including GEM, but did not examine the effects of breeding strategy on reproductive performance by genotype.²² Other authors concluded that reproductive performance measures were not significantly different between mice housed in shoebox cages as breeding trios compared with breeding pairs; that study included an inbred strain (C57BL/6NCr) and an outbred stock (CrI:CD1[ICr]), neither of which was genetically altered.¹⁴ A case study yielded similar findings from unspecified strains, stocks, or genotypes of mice.³⁶ Although the mice included in these studies reproduced equally well whether bred as trios or as pairs in shoebox cages, this result should not be assumed to apply directly to specific genotypes of GEM. GEM may express distinct adverse reproductive phenotypes as an unintended consequence of genetic manipulation.⁸ The effects of these alterations might only be determined through direct evaluation of the different breeding strategies of the genotype in question.

For example, mice lacking functional estrogen receptor α (ER α) and estrogen receptor β (ER β) have been created to help discern the role of estrogen receptors in various tissues. Mice heterozygous for both mutations are bred to generate double-knockout mice for experiments ($\alpha\beta$ ERKO), given that female mice homozygous for mutations in ER α and in ER β (β ERKO) are infertile; male mice homozygous for mutation in ER α are also infertile, but β ERKO male mice can reproduce.^{23,26,35} These breeders are reported to have similar reproductive indices as their wildtype counterparts and do not require special husbandry. However, mice bred as trios compared with pairs have not been compared. In this pilot study, we investigated whether breeding strategy affects the reproductive performance and overall welfare of mice bred to generate $\alpha\beta$ ERKO offspring. We hypothesized that for mice bred to generate $\alpha\beta$ ERKO offspring as well as their wildtype counterparts, measures of reproductive performance and animal welfare would not differ significantly between mice bred continuously as trios compared with pairs in shoebox cages.

Materials and Methods

The study was conducted in accordance with the *Guide*. All work was approved by the Oregon Health and Science University IACUC and performed in an AAALAC-accredited facility.

Animals. Age-matched virgin female and male mice (age, 8 to 12 wk) were obtained from a breeding colony managed by 3 of the authors (NL, SLM, SJM). To generate $\alpha\beta$ ERKO mice, female B6.129P2-Esr1^{tm1Ksk/+}-Esr2^{tm1Unc +/-} mice were mated with male B6.129P2-Esr1^{tm1Ksk/+}-Esr2^{tm1Unc -/-} mice (that is, HET breeders). To generate WT control mice, female B6.129P2-Esr1^{tm1Ksk/+}-Esr2^{tm1Unc+/+} mice were mated with male B6.129P2-Esr1^{tm1Ksk/+}-Esr2^{tm1Unc+/+} mice (that is, WT breeders). Pups were weaned at 21 d after birth. Mice were free from the following infectious agents, according to health surveillance program reports, as determined by a combination of serology, PCR analysis, and direct observation for ecto- and endoparasites: mouse parvovirus, minute virus of mice, mouse hepatitis virus, enzootic diarrhea of infant mice virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus, *Mycoplasma pulmonis*, *Aspiculuris tetraptera*, *Syphacia* spp., *Myobia* spp., *Mycopetes* spp., and *Radfordia* spp.

Housing and husbandry. Mice were housed on racks in commercially available IVC (Maxi Miser 1; 7.70 in. $\times$ 12.17 in. $\times$ 5.25 in.; total floor space, 67.6 in.²; Thoren Caging, Hazelton, PA). Mice were housed on processed cellulose bedding (ECOfresh, Absorption Corporation, Ferndale, WA). Cages were changed every 2 wk. Room conditions were temperature of 20.0 to 22.2 °C, humidity of 30% to 70%, and a 12:12-h light:dark photocycle. Mice had unrestricted access to food (5LOD PicoLab Laboratory Rodent Diet, Purina Mills, St Louis, MO) and reverse-osmosis-purified water. Nesting material (Cotton squares, Ancare, Bellmore, NY) was provided.

Experimental design. Mice were housed one breeding group per cage, either as 1 male and 2 females (trio) or 1 male and 1 female (pair). The experimental groups consisted of HET breeding trios, HET breeding pairs, WT breeding trios, and WT breeding pairs. Breeding trios and pairs were maintained continuously. Cages were evaluated weekly for up to 31 wk. Number of litters per female, production index (PI; number of pups weaned per female per week), and interlitter interval (number of days between births of consecutive litters) were evaluated for each breeding cage. The following measures of reproductive performance were evaluated for each litter: litter size at birth; litter size at weaning; weaning rate (number of pups weaned by number of pups born); and body weight of pups at weaning. In addition, nest building score¹⁸ was evaluated 1 wk after each cage change as an additional measure of general animal welfare. Briefly, nests were evaluated on height and degree of complexity and then given a score ranging from 0 to 5: 0, nesting material undisturbed; 1, nesting material disturbed but not gathered to a nest site; 2, nesting material gathered to a nest site and has flattened saucer shape with no or incomplete walls; 3, nest has identifiable walls that form a cup or bowl shape; 4, nest forms an incomplete dome; and 5, nest forms complete dome.

Sample size. An initial power analysis (desired power, 0.8; number of groups, 4; α , 0.05) determined that 10 breeder females per experimental group in each colony were needed to detect significant differences in these reproductive measures. However, the final number of breeding mice available for the study was lower. In particular, only 3 of 16 of all mice generated in the HET breeding colony were of the correct genotype for breeding. Therefore, at the time the pilot study was initiated, there were only enough HET breeders available to create 3 HET breeding trios (6 females, 3 males) and 3 HET breeding pairs (3 females, 3 males). Two WT breeding trios and 3 WT breeding pairs were created to provide equivalent control sample sizes.

Statistical evaluation. Data were analyzed by using commercially available statistical analysis computer software (version 24, SPSS, IBM, Armonk, NY). The number of litters per female and PI for each experimental group were compared by using 2-way ANOVA, with genotype and breeding strategy set as fixed factors. Interlitter interval, litter size at birth, litter size at weaning, weaning rate, body weight of pups at weaning, and nest building score for each experimental group were compared by using linear mixed effects modeling, with genotype and breeding strategy set as fixed effects and cage set as a random effect. In all analyses, a *P* value of 0.05 was considered significant.

Results

Number of litters per dam and PI. Mean number of litters per dam and mean PI did not differ significantly (*P* < 0.05) between HET and WT breeders or between breeding trios compared with pairs (Table 1). Regardless of genotype, breeding trios tended to have lower PI than pairs. The results did not reveal a significant interaction between genotype and breeding strategy.

Table 1. Number of litters per dam and production index for cages of HET and WT mice bred in pairs or trios

Genotype	Breeding strategy	No. of cages	Time (wk) maintained	No. litters per female	Production index
HET	All	6	26.3	4.75 ± 1.06	0.68 ± 0.18
	Pair	3	29.0	6.33 ± 0.33	0.96 ± 0.15
	Trio	3	23.7 ^a	3.17 ± 1.74	0.41 ± 0.25
WT	All	5	28.2	5.20 ± 0.37	0.94 ± 0.16
	Pair	3	28.7	5.00 ± 0.58	1.00 ± 0.28
	Trio	2	27.5	5.50 ± 0.50	0.85 ± 0.14
All pairs		6	28.8	5.67 ± 0.42	0.98 ± 0.14
All trios		5	25.2	4.10 ± 1.12	0.58 ± 0.18

Data are given as mean ± SEM.

^aOne cage of HET mice bred in trios produced no litters after 15 wk and subsequently was removed from the study.

Interlitter interval. Results for interlitter interval are summarized in Table 2. Overall, the interlitter interval was shorter ($df = 32.794$, $F = 9.406$, $P = 0.004$) in HET breeders than WT breeders. Breeding trios overall had significantly shorter interlitter intervals than breeding pairs ($df = 32.794$, $F = 60.414$, $P < 0.001$). In addition, genotype and breeding strategy showed significant ($df = 32.794$, $F = 19.308$, $P < 0.001$) interaction. The difference in interlitter interval was smaller between HET breeding trios and HET breeding pairs than between WT breeding trios and WT breeding pairs.

Litter size at birth. Results for litter size at birth, litter size at weaning, and weaning rate are summarized in Table 3. Litter size at birth was not overall significantly different between HET breeders and WT breeders. However, overall, breeding trios had smaller litters at birth than breeding pairs ($df = 45.192$, $F = 14.204$, $P < 0.001$). This difference was greater for HET breeders than for WT breeders, reflecting significant interaction between genotype and breeding strategy ($df = 45.192$, $F = 5.920$, $P = 0.019$).

Litter size at weaning. Results for litter size at weaning followed a similar pattern as litter size at birth (Table 3). Litter size at weaning did not differ overall between HET breeders and WT breeders but was smaller overall ($df = 17.194$, $F = 5.158$, $P = 0.036$) for breeding trios than for breeding pairs. This difference was smaller for HET breeders than for WT breeders, again reflecting significant ($df = 17.194$, $F = 9.595$, $P = 0.006$) interaction between genotype and breeding strategy.

Weaning rate. Overall litters from HET breeders had a lower weaning rate than litters from WT breeders (Table 3; $df = 9.786$, $F = 15.757$, $P = 0.003$). However, overall weaning rate did not differ between trio breeders and pair breeders; neither did genotype and breeding strategy interact significantly.

Body weight of pups at weaning. Body weight of pups at weaning did not differ between experimental groups (Table 4).

Nest building score. Overall, breeding pairs overall had higher nest building scores than breeding trios (Table 5; $df = 118.894$, $F = 4.229$, $P = 0.042$). Nest building score did not differ between HET breeders and WT breeders overall, nor did genotype and breeding strategy interact.

Discussion

In our study, breeding HET compared with WT in trios compared with pairs was associated with significantly different outcomes for some reproductive measures. Breeding strategy did not affect the number of litters per dam, PI, body weight of pups at weaning, or nest building score. However, interlitter

Table 2. Interlitter interval for HET and WT mice bred in pairs or trios

Genotype	Breeding strategy	No. of intervals	Interlitter interval (d)
HET	All	33	23.76 ± 2.02 ^a
	Pair	16	29.44 ± 2.81 ^c
	Trio	17	18.41 ± 2.27 ^c
WT	All	31	25.10 ± 2.86 ^a
	Pair	12	40.67 ± 3.14 ^c
	Trio	19	15.26 ± 1.97 ^c
All pairs		28	34.25 ± 2.39 ^b
All trios		36	16.75 ± 1.49 ^b

Data are given as mean ± SEM.

^aSignificant difference between HET and WT breeders overall ($df = 32.794$, $F = 9.406$, $P = 0.004$).

^bSignificant difference between breeding trios and breeding pairs overall ($df = 32.794$, $F = 60.414$, $P < 0.001$).

^cSignificant interaction between genotype and breeding strategy ($df = 32.794$, $F = 19.308$, $P < 0.001$).

interval, litter size at birth, and litter size at weaning were lower for trio breeders than for pair breeders; in addition, genotype and breeding strategy interacted significantly for these reproductive measures. Interlitter interval and weaning rate were the only reproductive parameters that differed significantly between genotypes.

The small number of cages available for this study was a limitation imposed by the size of the breeding colony to generate $\alpha\beta$ ERKO mice. The breeding colony was maintained at a size appropriate to meet the minimal animal number requirements for other ongoing experiments. The number of cages available for this study could have been increased to meet the required number determined by the initial power calculation, but doing so would have introduced additional confounding variables. For example, our study tracked all breeding cages simultaneously over a period of about 6 mo. One way to increase the number of cages would have been to include additional generations of breeders after the original 6-mo period. However, doing so would have introduced temporally associated confounding variables, such as time of year, personnel changes, and intermittent noise and vibration associated with new construction at our institution. Another approach to ensure a larger sample size could have been to collaborate with other investigators

Table 3. Litter size at birth, litter size at weaning, and survival rate for HET and WT mice bred in pairs or trios

Genotype	Breeding strategy	No. of litters	Litter size (no. of pups)		Weaning rate (%)
			At birth	At weaning	
HET	All	38	5.79 ± 0.34	4.00 ± 0.45	0.63 ± 0.06 ^e
	Pair	19	6.47 ± 0.43 ^b	4.37 ± 0.62 ^d	0.65 ± 0.08
	Trio	19	5.11 ± 0.50 ^b	3.63 ± 0.67 ^d	0.62 ± 0.09
WT	All	37	5.97 ± 0.34	4.86 ± 0.45	0.76 ± 0.06 ^e
	Pair	15	6.47 ± 0.50 ^b	5.73 ± 0.69 ^d	0.85 ± 0.07
	Trio	22	5.64 ± 0.45 ^b	4.27 ± 0.57 ^d	0.75 ± 0.08
All pairs		34	6.47 ± 0.32 ^a	4.97 ± 0.47 ^c	0.74 ± 0.05
All trios		41	5.39 ± 0.34 ^a	3.98 ± 0.43 ^c	0.66 ± 0.06

Data are given as mean ± SEM.

^aSignificant difference in litter size at birth between breeding pairs and breeding trios overall (df = 45.192, F = 14.204, $P < 0.001$).

^bSignificant interaction between genotype and breeding strategy (df = 45.192, F = 5.920, $P = 0.019$).

^cSignificant difference in litter size at weaning between breeding pairs and breeding trios overall (df = 17.194, F = 5.158, $P = 0.036$).

^dSignificant interaction between genotype and breeding strategy (df = 17.194, F = 9.595, $P = 0.006$).

^eSignificant difference in weaning rate between HET and WT breeders overall (df = 9.786, F = 15.757, $P = 0.003$).

using $\alpha\beta$ ERKO mice. However, this approach would have introduced confounding variables associated with differences in personnel, location within the vivarium, and differences between institutions. Finally, additional mice could have been bred specifically to expand the colony to meet the aims of this study, but this strategy would have generated more mice than required for research needs. In considering the 3 Rs,³² expanding a breeding colony of double-knockout mice for the sole purpose of this study was difficult to justify.

Although we believe the decision to accept a smaller sample size was well-justified, one of the HET breeding trio cages never produced a litter and was removed from the study after 15 wk. The lack of litters in this cage was most likely the result of an incorrectly genotyped male being selected for breeding, although perhaps both females had an incorrect genotype, or perhaps the male's ability to reproduce was otherwise compromised. Data from this cage were included in the results for number of litters per female, PI, and nest building score. The results for this cage introduced considerable variability into the results for number of litters per female and PI, which could account for why significant differences were not observed between genotypes or between breeding strategies for these cage-level reproductive measures. In contrast, significant differences emerged for a number of other reproductive measures, including nest building score.

Using the data from this pilot study, we performed a posttest power analysis. With a confidence interval of 95% and a power of 0.8, 4 cages were required to detect differences in number of litters per female between HET and WT breeders, and 3 cages were required to detect differences in number of litters per female between breeding pairs and breeding trios. Using the same confidence interval and power level, 5 cages were required to detect differences in PI between HET and WT breeders, and 2 cages were required to detect differences in PI between breeding pairs and breeding trios. According to the results of these power analyses and the fact that significant differences emerged for several other reproductive measures, the number of breeding cages available for this study may, in fact, have been adequate. We will use these power analyses and results to inform the design of follow-up studies.

The results of our study confirm findings by other investigators that show that differences in breeding strategy within

Table 4. Pup weight at weaning (mean ± SEM) for HET and WT mice bred in pairs or trios.

Genotype	Breeding strategy	No. of pups weaned	Pup weaning weight (g)
HET	All	150	8.34 ± 0.13
	Pair	83	8.89 ± 0.16
	Trio	67	8.78 ± 0.21
WT	All	177	9.12 ± 0.12
	Pair	83	9.00 ± 0.18
	Trio	94	9.22 ± 0.16
All pairs		166	8.94 ± 0.12
All trios		161	9.04 ± 0.09

shoebox caging does not appear to affect the number of litters per female^{1,36} or PI.¹⁴ Number of litters per dam was not expected to differ between the various experimental groups, given that this reproductive measure varies primarily between different strains of mice,³¹ and all mice in this experiment were of the same background strain. In addition, PI is a reproductive measure that directly reflects weaning success and indirectly reflects interlitter interval, both of which differed significantly between breeding trios and breeding pairs. Therefore, one explanation for why PI did not significantly differ between breeding strategies is that any benefits from trio breeding gained in shorter interlitter interval were effectively negated by lower weaning rates.

The shorter interlitter interval for breeding trios compared with breeding pairs was expected. Gestation length should have remained relatively constant regardless of breeding strategy, but the presence of 2 females in cages of breeding trios produced staggered litters, resulting in shorter interlitter intervals in cages of breeding trios than in cages of breeding pairs. However, interlitter interval was not expected to differ between HET breeders and WT breeders. Aside from strain differences, factors that have been associated with differences in interlitter interval in laboratory mice include environmental temperature³¹ and litter parity.³⁹ However, we controlled for these variables in our study, in that all mice were housed in the same room, and all breeding

Table 5. Nest building score (mean $\pm$ SEM) for HET and WT mice bred in pairs or trios

Genotype	Breeding strategy	No. of observations	Nest building score
HET	All	81	2.53 $\pm$ 0.10
	Pair	44	2.64 $\pm$ 0.13
	Trio	37	2.41 $\pm$ 0.14
	All	75	2.64 $\pm$ 0.10
WT	Pair	46	2.80 $\pm$ 0.15
	Trio	29	2.38 $\pm$ 0.09
	All Pair	90	2.72 $\pm$ 0.10 ^a
	All Trio	66	2.39 $\pm$ 0.09 ^a

^aSignificant difference between breeding pairs and breeding trios overall (df = 118.894, F = 4.229, P = 0.042).

cages were established with nulliparous females. Lactation is known to delay gestation because of delayed implantation.³⁸ However, no differences in lactation or implantation between HET and WT breeder females have been reported.^{12,35} Heterozygous α ERKO mice express both functional and nonfunctional ER α proteins²⁶ and ER α levels that are partway between those of WT and knockout mice in certain tissues.⁹ Heterozygous β ERKO mice have a higher oocyte count than their WT counterparts.²³ But unlike that for knockout mice, the literature characterizing the reproductive behavior and performance of heterozygous ERKO mice is much less extensive.^{12,23,26,35} Perhaps the HET breeder females we used in this study differed from their WT counterparts in regard to reproductive behavior and performance, although what mechanism would have resulted in the exhibited shorter interlitter intervals is unknown. In addition, how this mechanism could be affected by breeding strategy to produce results in which the difference in interlitter interval between trio-bred HET mice and pair-bred HET mice was significantly smaller than the difference in interlitter interval between trio-bred WT mice and pair-bred WT mice is unclear. Interlitter interval has not been specifically characterized for ERKO mice, but β ERKO males nevertheless should be fertile and exhibit normal sexual behavior.^{12,19,23,35} Further studies are needed to determine the roles ER heterozygosity and ER β may play in affecting receptivity, courtship, gestation length, and other determinants of interlitter interval in ERKO mice. Additional work is needed to further examine how these factors interact with breeding strategy.

Litter size at birth can be affected by strain, seasonality, litter parity, age of the female, and degree of inbreeding depression.^{8,31,38} This reproductive measure was not expected to be significantly different between HET breeders and WT breeders overall, given that the study design controlled for these variables. Furthermore, female HET breeders should have been fertile.³⁵ However, the overall difference in litter size at birth between breeding trios and breeding pairs in our study differed from other reports in the literature.^{1,13,14,22} One difference between the current study and others was that all of our mice were bred continuously, whereas in other studies males were removed soon after parturition.^{1,14,22} This situation resulted in increased housing density, which has been associated with increased ammonia concentrations in the cage¹¹ and with plasma corticosterone levels in the cage occupants.^{25,27} These factors could have led to increased fetal resorption rates because of maternal stress⁵⁻⁷ in cages of continuously bred trios. Because

litter size at birth did not differ significantly different between genotypes, the reason underlying the significant interaction between genotype and breeding strategy is unclear. Perhaps HET breeders and WT breeders were, in fact, distinctly different (in degree of inbreeding, for example), but this difference was not biologically significant and remained unidentified until now. Future studies characterizing the potential effect of ER genotype on litter size will likely help to optimize colony management for these GEM.

α β ERKO mice are associated with distinct reproductive and nonreproductive phenotypes, such as shortened femur length, but do not appear to be associated with higher preweaning mortality.³⁵ Thus no difference in litter size at weaning between HET breeders and WT breeders was expected. The significant difference in litter size at weaning between breeding trios and breeding pairs overall is noteworthy and can be explained by a number of possible causes of preweaning mortality. Increased housing density in trio-bred cages may have led to deaths due to increased ammonia levels as well as trampling of newborn pups. Furthermore, reproductive suppression¹³ could explain the difference in litter size at birth between breeding trios and breeding pairs, if, for example, newborn pups from one dam were consumed by the other. The difference in male breeder genotype might explain the significant interaction between genotype and breeding strategy. Male HET breeders, effectively being β ERKO mice, were less likely than their WT counterparts to exhibit aggressive behavior.^{29,30} This difference may explain why the difference in number of pups weaned between breeding trios and breeding pairs was significantly less for HET breeders than for WT breeders.

Although both litter size at birth and at weaning were lower for trio breeders than for pair breeders, the proportion of number of pups weaned to number of pups born, as reflected in weaning rate, was not significantly different between breeding strategies. Instead, weaning rate differed by genotype, but this outcome was not unexpected. WT breeders produced only WT offspring, whereas HET breeders produced a mix of WT, heterozygous, and knockout offspring. Although not specifically described for ERKO mice, it is not uncommon for knockout mice to be more fragile than their WT or heterozygous littermates, and thus more prone to mortality before weaning.⁸ Our results for weaning rate were inconsistent with reports in the literature, in which weaning rate was increased for mice bred in trios compared with mice bred in pairs in one study²² but did not differ in others.^{1,14}

In studies that examined the effects of communal nesting, mice raised in communal nests were larger at weaning than mice reared in single litters.^{15,16,33} However, weaning weight of pups did not differ between those reared in breeding trios compared breeding pairs for either genotype. Why pups from trio breeders were not larger than pups from pair breeders at weaning might reflect unintended and as-yet uncharacterized phenotypes associated with mutation of the ER α and ER β genes. Maximum growth rate, for example, may be lower in ERKO mice, therefore any opportunities to increase growth rate, such as with communal nesting, were of no benefit. Alternatively, any benefits from communal nesting may have been counteracted by negative effects of overcrowding in trio cages. Although the reproductive consequences of ER gene alteration have been well described, additional studies are needed to discern the growth rate and other nonreproductive phenotypic characteristics of ERKO mice.

Nest building score has been used as a measure of overall animal welfare.^{13,14} But we found this measure to have limited

value for assessing differences in overall animal welfare between breeding trios compared with breeding pairs. Evaluation of nest building score was useful in confirming the presence of communal nesting in trio-bred cages. If breeding trios had built a single nest per litter rather than nested communally, there would have been more nest building scores per cage for breeding trios than for breeding pairs. The significant difference in nest building score between breeding pairs and breeding trios in our study was consistent with reported observations.¹⁴ The lower nest building score in breeding trios may be explained by the higher density of mice in these cages, resulting in reduced thermal stress as well as higher likelihood of nest trampling. Because of these inherent differences, nest building score does not appear to be a valuable measure of general animal welfare when comparing different breeding strategies. In addition, nest building score did not differ between genotypes, suggesting that HET breeders were as capable as their WT counterparts in the ability to build nests. ERKO mice display alterations in reproductive behaviors,^{10,28,30} but the nest building ability of these mice has not been characterized, unlike murine models of altered cognition, such as Alzheimer disease³⁷ and Down syndrome.¹⁷

In conclusion, breeding strategy affects the reproductive performance of ERKO mice housed in shoebox caging. Breeding ERKO mice in trios did not cause any increases in reproductive performance overall; in fact, this breeding strategy was associated with decreases in several measures of reproductive performance. Additional studies are needed to elucidate mechanisms for why breeding ERKO mice as trios resulted in smaller litter sizes at birth and at weaning, and why body weight of pups at weaning were not as high as we expected. Furthermore, future studies are needed to better characterize HET compared with WT α ERKO mice to determine how genotype may influence the interlitter interval and how this effect interacts with breeding strategy. Studies should be conducted to evaluate whether the decreases in reproductive performance of ERKO mice bred as trios are mitigated when they are housed in larger cages that meet the floor space recommendations of the *Guide*. Perhaps the results of our study reflect differences in cage density rather than breeding strategy itself. Given the performance standards that have been established in the literature for breeding different strains and stocks of mice as trios in shoebox caging, it may be tempting to apply this breeding strategy universally to GEM. However, researchers must be cautious before using trio breeding, because genetic alteration may have unknown consequences. Without sufficient insight into how breeding strategy influences the reproductive performance of specific genotypes, increasing colony productivity through trio breeding may be deleterious.

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