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Lifetime Reproductive Efficiency of BALB/c Mouse Pairs after an Environmental Modification at 3 Mating Ages

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The aim of this study was to evaluate the effects of an environment change and the age at which mating pairs were formed on the lifetime reproductive performance of BALB/c mice. We assigned 60 monogamous pairs to a randomized design in a 2 × 3 factorial arrangement (with or without an environmental modification and with 3 mating ages: 28, 45, or 60 d). Autoclaved cardboard tubes (length, 10 cm; diameter, 4 cm) were used as the environmental modification. Data were collected from a total of 456 litters over a period of 10 mo. The mice tore the cardboard tube and used its parts both as shelters and as nesting material. The presence of a cardboard tube decreased the preweaning litter mortality rate in the first 6 reproductive cycles. Mating at 28 or 45 d of age also decreased the preweaning mortality rate in the first 6 reproductive cycles, compared with monogamous pairs formed at 60 d of age. Treatments did not affect age at first parturition, number of litters, time between litters, or litter size and weight at birth and weaning. In addition to contributing to animal wellbeing, providing a cardboard tube improved productivity by decreasing the preweaning mortality rate. BALB/c siblings should be paired for mating when no older than 28 d, to reduce preweaning mortality of the offspring.

Each year, our institute uses numerous BALB/c mice for research and manufacture of immunobiologic products. Therefore, concern has grown regarding reducing the numbers of animals used in research and testing and maximizing their welfare.[8]

Laboratory rodent breeding facilities are strictly controlled settings, in which space is limited. Although environmental enrichment and the provision of nesting material have been recommended,[15] laboratory mice are often kept in small, barren cages with no additional elements other than bedding, pelleted feed, and water.[36] In such an environment, mice lack the possibility to perform motivated behaviors such as exploring, foraging, and nesting, because all of their physical needs are supplied. In addition to inactivity, they are routinely exposed to brief, but stressful, husbandry procedures, including restraint and cage changing.[24] Taken together, these factors may cause endocrine changes and the suppression of normal behavior patterns, thereby affecting reproduction and survival and potentially compromising the quality of research data.[4]

Modification of the environment, including increasing the complexity of mouse cages by providing structures that can be used as shelters and the provision of nesting materials, has been used in attempts to minimize stress in laboratory mice.[16,24] When these changes exert favorable effects on the animals' behavior and ability to cope with captivity, they can be viewed as environmental enrichment.[24] Several materials have been tested, some of which were used by the animals for nesting, such as compressed cotton squares, paper tissues, paper towels, paper stripes, and 'wood wool,'[10,13,16,30,32] or as shelters, such as nest boxes, plastic tubes, and plastic igloos.[16,26,32] These studies showed that, in addition to the breeding animals, adult nonbreeding mice from both sexes manipulated and combined the materials to make nests used for resting.[30,31] Depending on the strain, the provision of shelter and nesting material may increase or decrease aggressiveness in groups of male mice.[19,20,34] In addition, increasing complexity in laboratory cages may stimulate territorial behavior in some strains.[21]

Providing nesting material increased the number of pups born and weaned in nude mice,[11] but various types of nesting material (paper towel, aspen wood wool) and environmental enrichment (paper tissue, cotton squares and plastic igloos) did not affect reproductive performance or pup survival in other inbred mouse strains.[6,26] Information regarding the application of enrichment materials in long-term studies of reproductive performance is scarce. In the Brazilian market, no certified nesting or enrichment materials for laboratory rodents are available; therefore, studying alternative materials is of interest.

Most inbred mouse strains reach puberty between 6 and 8 wk of age,[28,29] but there are marked differences in litter size, time between litters, and survival among strains and substrains.[1,7] Low reproductive performance was reported in BALB/cJ mice when compared with other important inbred strains: BALB/cJ mice were 8 wk old at first mating, had a mean litter size of 5.2 pups, and produced only 3.8 litters on average.[28] In the expansion and multiplication of inbred mouse colonies, there is a choice regarding the age of mice used to form sibling mating pairs. Whether this mating age affects lifetime reproductive performance is unclear.

The objective of the current study was to evaluate the influences of an environmental modification, that is, the presence of a cardboard tube in the cage, and of the age at which mating pairs were formed on the lifetime reproduction performance of BALB/c monogamous pairs.

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Materials and Methods

Mice. All of the animal procedures used in this study were in accordance with the recommendations of the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research*[14] and were approved by the IACUC of the Faculdade de Medicina Veterinária e Zootecnia (UNESP, Botucatu, São Paulo, Brazil) under protocol number 136/2009.

The study population comprised 60 full-sib monogamous pairs that originated from 39 BALB/cJ mouse litters that were used in this study from weaning (at 28 d) until they were 339 d old, that is, for a period of approximately 10 mo. The experiment was performed at the Inbred Mouse Production Unit (Instituto Butantan, São Paulo, Brazil). The mouse colony follows a health monitoring program that involves testing quarterly for parasitic and bacterial pathogens according to guidelines of the Federation of European Laboratory Animal Science Associations;[22] the mice used in this study were free from these pathogens. The mice were housed in standard polypropylene cages (300 × 200 × 130 mm) with approximately 35 g autoclaved wood shavings bedding (JR Maravalha, Conchal, Brazil). During the entire period, they had free access to a balanced commercial feed (220 g Crude Protein/kg and 3000 kcal Metabolizable Energy/ kg; Nuvilab CR1, Nuvital, Curitiba, Brazil) and filtered drinking water. Animal rooms were protected by barriers such as an air-filtration system (Absolut, HEPA H 13 filter, Camfil, São Paulo, Brazil) and an air-pressure differential. Ambient temperature was maintained at 22 ± 2 °C. In addition, an exhaust system installed at cage level provided 15 to 20 filtered air changes hourly. The mice were exposed to a 12:12-h light:dark cycle, with lights on from 0600 to 1800. There was a generator for protection of the whole system against power failures. Fresh drinking water was provided 3 times each week.

Experimental design. Mouse pairs were assigned to a randomized design in a 2 × 3 factorial arrangement (with or without cage modification; 3 mating ages: 28, 45, and 60 d) comprising 6 treatments and 10 replicates. Cardboard tubes (length, 10 cm; diameter, 4 cm; Damapel, São Paulo, Brazil) were chosen as the environmental modification because their shape, availability, and cost are satisfactory, and they can easily be autoclaved. They were autoclaved before use and were removed and replaced twice weekly, at the same time as the cage and bedding were changed.

Mice in the initial group were all born on the same day and were weaned on day 28, when littermates were separated by sex into 2 cages; 20 full-sib monogamous pairs were formed randomly on this same day. These monogamous pairs were housed in standard polypropylene cages (300 × 200 × 130 mm), half of which received a cardboard tube. Another 20 monogamous pairs were formed according to this same procedure on day 45, and a final 20 on day 60. The age at which the monogamous pairs were formed was considered as the mating age, for the purpose of this study. From weaning until the breeding pairs were formed, the mice were kept in standard cages, in company with their littermates of the same sex. During this period, no mouse was housed alone; 2 to 5 mice were maintained in each cage.

The monogamous pairs were formed either at 28, 45, or 60 d of age, as explained earlier, and were maintained until they were 339 d old, unless one of the mice died. When one of the mice from a pair died, the corresponding mate was removed from the study also, but the previous production from that pair was still considered. The cages were inspected daily during this entire period, and a number of traits related to reproductive performance and litter survival were recorded. Weaning age was set to 20 to 21 d; accordingly, 95% of the litters were weaned within this time span. Four litters were weaned before 20 d due the birth of the next litter, and 10 litters were weaned after 21 d of age because there was no weaning on Sundays.

Two sets of traits were considered. The first set was used to evaluate lifetime parental productivity from mating until 339 d of age. The following traits were recorded during this 10-mo-period (from 22 May 2009 [when the first 20 pairs were formed at 28 d of age] until 29 March 2010 [end of the experiment]), always on a by-cage basis: age at first parturition (in days), number of litters, total duration (expressed as the number of days from mating until weaning of the last litter), age at weaning of the last litter (in days), and the total numbers of live neonates and weanlings. The total number of pups dead from birth to weaning was computed as the difference between the total numbers of live neonates and weanlings. Data were averaged per cage. Litters that had not been weaned by the time the experiment was finished were excluded from the dataset.

A second set of traits was used to evaluate average parental productivity in the first 6 reproductive cycles and consisted of: average interval between litters (days), average litter size at birth and at weaning, average litter weight at birth and weaning (grams), and average litter mortality rate from birth to weaning (%), which was computed by subtracting the number of weanlings from the number of pups born alive and expressed as percentage relative to the number of pups born alive. The reasons for considering the first 6 reproductive cycles were that 90% of the mating pairs produced at least 6 litters, and there was a sharp decrease in this proportion afterward (Figure 1). In addition, an overall litter index was computed that considered the actual number of litters born to a maximal parity of 6, divided by the possible number of litters to parity 6, and expressed as a percentage.[7]

Statistical analyses. Lifetime productivity traits were analyzed according to a model that included the fixed effects of cage modification, mating age, and the cage modification × mating age interaction plus the random error, by using the GLM procedure of SAS.[25] For traits measured at weaning, average weaning age was included in the models as a covariate. Age at mating means were compared by using Tukey test whenever needed, with $\alpha = 0.05$.

Litter performance traits in the first 6 reproductive cycles were analyzed according to repeated-measures models,[17] implemented by using the MIXED procedure of SAS.[25] These models included the fixed effects of cage modification, breeding age, the cage modification × breeding age interaction, parity order, and all interactions. Two random errors were defined: 1) the variance between mice (subjects) within treatments and 2) the variance between measurements (litters from each pair) within mice. The first random error was used to test the effects of cage modification, breeding age, and the cage modification × mating age interaction. The second random error was used to test the parity effect and the effects of the interactions involving parity order. An autoregressive covariance structure [AR(1)] was used. Only litters that had at least one pup weaned were considered in these analyses. For traits measured at weaning, age at weaning was included in the models as a covariate. Appropriate contrasts were computed to compare age at mating means.

χ^2 tests were applied to compare number of litters produced to parity 6, according to environmental modification and mating ages. Exact P values for F tests are provided to facilitate interpretation of statistical data.

Results

Overall performance. The lifetime parental productivity of BALB/cJ mice was evaluated over a period of 10 mo. All 60

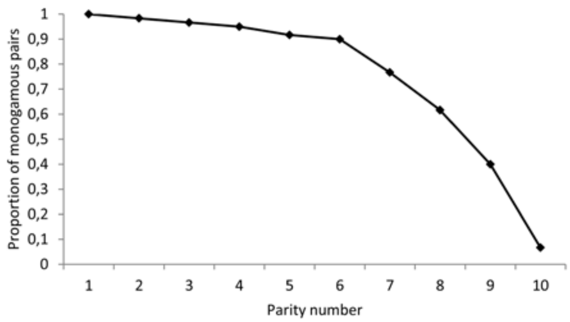

Figure 1. Proportions of the 60 initial monogamous pairs that produced a litter, according to parity number

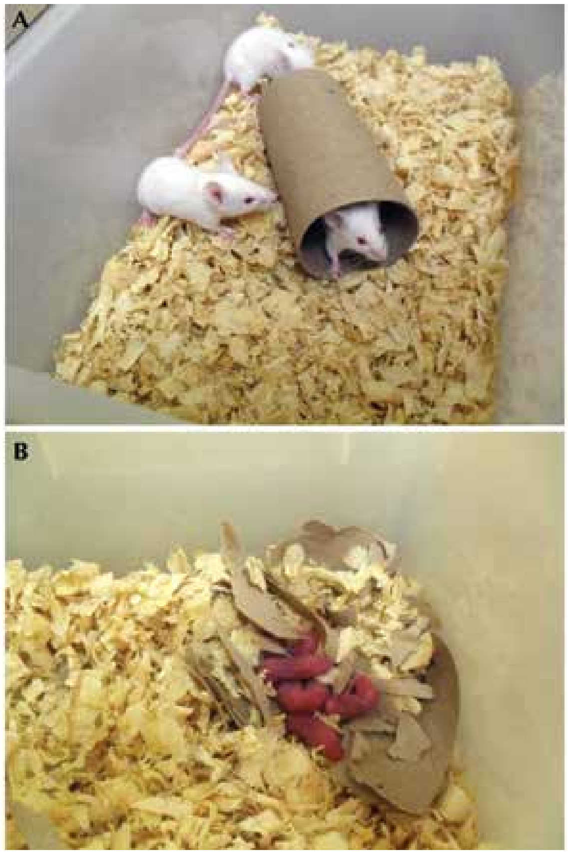

Figure 2. The cardboard tube was used as (A) shelter and (B) nesting material. These photos were taken (A) a few minutes and (B) approximately 5 h after the tube was introduced into the cages.

monogamous pairs gave birth to at least one litter, whereas 90% (54 of 60) of the mating pairs produced at least 6 litters, 40% (24 pairs) produced 9 litters, and only 6.7% (4 pairs) produced 10 litters during this time period (Figure 1). One male parent that was mated at 28 d and did not receive a cardboard tube died after producing the first litter, and one female parent that was mated at 60 d and received a cardboard tube died after weaning 6 litters. No other parental deaths were recorded during the 10-mo period. The total number of litters born was 456. Loss of entire litters amounted to 17 (3.7%) during the 10 mo period, 6 of which occurred at birth (all pups were stillborn) and 11 from birth to weaning. In general, losses were of very small litters of 1 or 2 pups and were most likely due to abandonment, starvation and hypothermia. Cannibalism was recorded only in one litter. From birth to weaning, 3 litters in modified cages and 8 in nonmodified cages were lost, as were 2 litters in pairs formed at 28 d, 4 litters in pairs formed at 45 d, and 5 litters in pairs formed at 60 d of age. Considering parity order, we recorded only one litter lost from each of parities 4 through 6; 2 litters lost at parity 8, and 3 litters lost at each of parities 2 and 7.

No effect of the cage modification × mating age interaction was detected on any of the lifetime parental productivity traits or of the average parental productivity traits in the first 6 reproductive cycles. Therefore the effects of these 2 main factors were considered separately.

Environmental modification. The mice tore the cardboard tubes and used their parts both as shelters and as nesting material (Figure 2). The presence of a cardboard tube in the cage decreased ($P = 0.012$) the total number of pups dead from birth to weaning (Table 1) but did not affect the age at first parturition, number of litters produced, duration from mating until weaning of the last litter, age at weaning of the last litter, or total number of pups born alive or weaned per mating pair.

The overall litter index (maximal parity, 6) was 96.4%; the specific number of litters produced did not differ between modified or nonmodified cages (97.8% compared with 95.0%, respectively, $P = 0.788$) or mating age (94.4% for 28 d, 100% for 45 d, and 94.2% for 60 d, $P = 0.883$). The presence of the cardboard tube in the cage decreased ($P = 0.020$) the preweaning mortality rate during the first 6 reproductive cycles (Table 2) but did not affect other traits, such as average litter size at birth and weaning, average litter birth and weaning weights, and average interval between litters.

Age at mating. Age at mating had no effect on age at first parturition or on the number of litters produced by the mating pair (Table 1). The proportion of mating pairs that was older than 3 mo before a litter was born was 20% for the pairs mated at 28 and 45 d and 35% for pairs mated at 60 d of age. The number of days from mating until weaning of the last litter from a pair decreased ($P = 0.032$), as expected, as mating age increased (Table 1). Therefore, it was highest for pairs mated at 28 d, intermediate for pairs mated at 45 d, and lowest for pairs mated at 60 d of age, but age at weaning of last litter did not differ among mating-age groups. No effect of age at mating was detected regarding the total number of pups born alive and weaned, but increasing the age at mating increased ($P = 0.012$) the total number of pups that died from birth until weaning (Table 1). Preweaning mortality rate was lowest for pairs mated at 28 d, intermediate for pairs mated at 45 d, and highest for pairs mated at 60 d of age.

Age at mating had a pronounced effect on preweaning mortality during the first 6 reproductive cycles ($P = 0.007$), which was higher when the mating pairs were formed at 60 d of age (Table 2), as compared with both 28 and 45 d. Average litter size at birth and at weaning, average litter weight at birth and weaning, and average litter interval were not influenced by age at mating.

Parity order. Parity order influenced litter size ($P = 0.032$) and weight at weaning ($P < 0.001$) but not litter size at birth, weight at birth, or time between litters (Figure 3). Litter size at weaning increased until parity 3 but seemed to show an oscillating pattern thereafter. Similarly, litter weaning weight increased until parity 3 but seemed to plateau thereafter. The cage modification × parity order interaction affected ($P = 0.039$) preweaning mortality (Figure 3). In the first parity, preweaning mortality was much lower ($P < 0.001$) in modified than in nonmodified cages, whereas from the second parity on, preweaning mortality rates did not differ between modified and nonmodified cages. In fact, preweaning mortality rate from the 2nd until 6th parity did not differ from 0. No other effects of interactions involving cage

Table 1. Lifetime accumulated mean parental productivity of BALB/c monogamous pairs according to cage modification and age at mating ($n = 60$)

	Cage modification				Age at mating				
	Yes	No	SE	*P*	28 d	45 d	60 d	SE	*P*
Age at first parturition (d)	93.0	88.9	3.6	0.418	90.2	87.4	95.2	4.4	0.440
No. of litters	7.70	7.50	0.35	0.689	7.45	7.80	7.55	0.43	0.840
Duration (d)[a]	270.9	256.6	6.8	0.144	279.2[c]	264.9[cd]	247.2[d]	8.3	0.032
Age at last weaning (d)	315.2	301.0	6.8	0.144	307.2	309.9	307.2	8.3	0.965
No. of live neonates	42.3	40.6	2.1	0.576	40.9	42.9	40.4	2.6	0.776
No. of weanlings	41.6	39.0	2.2	0.398	40.4	41.9	38.6	2.7	0.706
No. of dead pups[b]	0.62	1.68	0.31	0.018	0.49[d]	0.86[cd]	2.10[c]	0.38	0.012

[a]No. of days from mating until weaning of the last litter from a mating pair
[b]From birth to weaning
[c,d]Values with different letters in the same row differ significantly ($P < 0.05$).

Table 2. Parental productivity (mean ± SE) of BALB/c monogamous pairs in the first 6 reproductive cycles according to cage modification and age at mating ($n = 333$)

	Cage modification			Age at mating			
	Yes	No	*P*	28 d	45 d	60 d	*P*
Litter size at birth	5.49 ± 0.16	5.55 ± 0.16	0.774	5.66 ± 0.19	5.57 ± 0.19	5.32 ± 0.20	0.440
Litter size at weaning	5.44 ± 0.15	5.41 ± 0.16	0.877	5.60 ± 0.19	5.57 ± 0.19	5.11 ± 0.19	0.134
Litter birth weight (g)	9.12 ± 0.25	9.09 ± 0.25	0.931	9.48 ± 0.31	9.11 ± 0.31	8.71 ± 0.31	0.228
Litter weaning weight (g)	66.4 ± 1.4	65.2 ± 1.5	0.555	68.3 ± 1.8	66.7 ± 1.8	62.4 ± 1.8	0.067
Litter interval (d)	30.6 ± 1.1	29.6 ± 1.1	0.555	31.4 ± 1.4	29.4 ± 1.4	29.4 ± 1.4	0.518
Preweaning mortality (%)	0.71 ± 0.48	2.33 ± 0.48	0.020	0.74 ± 0.58[b]	0.72 ± 0.58 *b*	3.11 ± 0. 59[a]	0.007

[a,b]Values with different letters in the same row differ significantly ($P < 0.05$).

modification or age at mating and parity order were detected on traits evaluated in the first 6 reproductive cycles.

Discussion

This study evaluated the effects of an environmental modification, that is, the presence of a cardboard tube in the cage and of the age at first male–female pairing on lifetime reproductive performance of BALB/c pairs. Both factors independently affected preweaning mortality, although they did not influence other reproductive performance traits, either on a lifetime basis or in the first 6 parities. Average reproductive performance was satisfactory and pup survival was high, in general, in the current study compared with other reports involving BALB/c mice,[6,28,37] attesting to the quality of the genetic stock and of the environmental control. During 10 mo, only 17 (3.7%) litters were lost entirely in the present study, including 6 stillborn litters, whereas a 20% entire-litter loss in BALB/c mice was reported recently.[37]

Providing a cardboard tube in the cage decreased both overall pup mortality on a per-cage basis and average preweaning mortality in the first 6 reproductive cycles, but the cage modification × parity order effect on preweaning mortality showed that the modification was especially effective in reducing mortality in litters of primiparous female mice. A study involving 5 inbred strains of mice showed that primiparous BALB/c mothers engaged in maternal behavior less actively and showed less nursing and licking than did the other 4 strains,[27] but mortality rate was not investigated.

In the current study, the mice ripped the cardboard tube and used it as shelter and nesting material. An early study of behavior in inbred strains of mice described the ability of ancestor strains that gave rise to the BALB/c strain to fray the nesting material and building good spherical nests at the surface of the bedding.[35] The environmental change in the present study may have improved pup survival either by stimulating maternal behavior and nest building or by providing insulation and preventing heat loss or by a combination of these 2 factors. The importance of nest building for fitness was demonstrated in mouse lines divergently selected for thermoregulatory nest building: pup survival was higher in the high-selected line when the ambient temperature was set at 22 or 4 °C.[3] The provision of breeding mice with extra nesting material, such as cotton squares or paper tissue, is recommended to stimulate nest building, a typical rodent behavior.[5] Its beneficial effects were described as a refinement measure, but nest building could also lead to a reduction in the number of animals required for research, when fewer animals are lost due to preweaning mortality.[33] Our data support this assertion. In a previous study, providing 2 nesting materials (compressed cotton squares and 2-ply tissues) and a plastic igloo as shelter did not appear to affect mouse offspring growth and survival traits during the first 2 reproductive cycles; however a control group that lacked nesting material was not included in that study.[26] In addition, the cited study[26] looked at C57BL/6 mice, and important interstrain differences in physiology and maternal behavior have been reported.[21,27,35] When paper tissues were given as nesting material to weaned BALB/c and C57BL mice, body weight was higher and feed consumption was lower compared with those in the control group,[31] suggesting that the nesting material enabled the mice to regulate their body temperature behaviorally.

Experimental evidence suggests that the typical room conditions for mice are cooler than is the ambient temperature for ideal thermal comfort for outbred and inbred C57BL/6J mice.[9,12] Although thermal comfort depends on whether mice are housed singly or in groups, both single- and group-housed mice offered a temperature gradient selected 29 °C during the light phase, when motor activity was minimal, and 25 °C during the dark phase, when motor activity increased.[12] In the present study, the

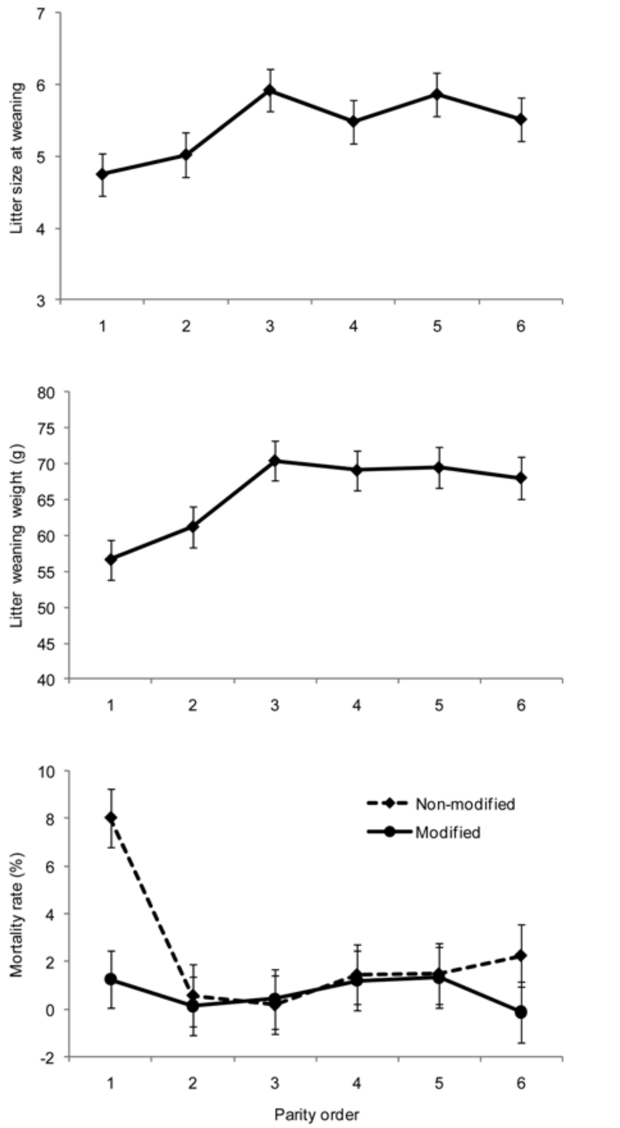

Figure 3. Effect of parity order on litter size and weight at weaning and of the cage modification (modified compared with nonmodified) × parity order on preweaning mortality.

ambient temperature was set at 22 ± 2 °C, which may be well below the ambient temperature preferred by the mice, especially during the light phase. Therefore, providing a cardboard tube may have led to the reduction in preweaning mortality through behavioral thermoregulation. In fact, nude mice provided with nesting material demonstrated the conservation of energy 'designated' for heat generation and its reallocation to improving breeding performance.[11] In contrast with the present study, despite the fact that the numbers born and weaned per litter consequently were increased in the previous study,[11] a reduction in mortality rate was not detected.

In the present study, age at mating and lifetime total number of dead offspring were directly related. This effect was quite clear yet unanticipated and challenging to interpret. Surprisingly, no effect of age at mating was detected on age at first parturition, because parental age was similar at birth of their first litter, irrespective of the age at which the first male–female pairing occurred. It is generally accepted that early mating may impair reproductive performance, leading to the production of small litters. The recommendation has been to mate mice when they are 6 to 8 wk old.[28,29] However our findings with BALB/c mice do not agree entirely with this scheme. Not only did mating age not affect most reproductive performance traits, but when mating increased from 28 to 60 d, the lifetime total number of offspring that died prior to weaning increased proportionally. When we looked at the average mortality rate during the first 6 reproductive cycles, mating at 45 or 28 d resulted in lower pup mortality rate than did mating at 60 d of age. Therefore, mating BALB/c mice at as late as 60 d of age adversely affected parental ability.

The number of days that the parents spent in same-sex groups between weaning and subsequent mating was the main difference among the mating age groups. This duration was 0 d for pairs formed at 28 d, but lasted 17 and 32 d for pairs formed at 45 and 60 d, respectively. During this time period, these mice were housed in groups of 2 to 5 littermates of the same sex, in standard cages (300 × 200 × 130 mm), allowing at least 120 cm^2 floor area per mouse. No overcrowding was observed, considering that the recommended housing density for 15- to 25-g mice is 77.4 cm^2.[15] Potential hypotheses regarding the conditions that might affect neonatal mortality in future litters include physiologic factors such as a tendency to become less active or to accumulate body fat in female mice, effects on social behavior such as the establishment of hierarchy in the same-sex group, and decreased time for the adaptation of the mouse pairs once they were mated. Future studies should address these hypotheses.

Parental previous experience may contribute to parity-associated differences in offspring survival, litter weaning weight, and litter size at weaning. Newborns from altricial species, such as mice, are completely dependent on maternal care for survival, and pups are prone to die from starvation and hypothermia when the mother fails to provide appropriate care.[23,36] Therefore, the increased survival of second and third litters compared with the first litter from a mating pair and the increasing litter size and weight until the third litter that we observed in the current study may reflect improved maternal behavior due to previous experience.[36] More pups deaths in the first than in the second litter has been reported previously in both DBA/2J and C57BL/6J mouse strains.[2] In addition, in primiparous female rats, excessive energy expenditures were associated with the physiologic and behavioral performances of first-time reproduction, together with a small component of additional expenditure due to further growth.[18] In our case, the worse performance of primiparous female mice could be related to an unfavorable energy balance during the first parturition, especially in those in the nonmodified cages, where energy expenditure for thermoregulation was probably increased in mothers and litters. The higher pup weight gain from birth to weaning in BALB/c and C3H/He compared with DBA/2 mice previously was attributed to the higher food and water intakes of the mothers in the first 2 strains[27] and seems to support the hypothesis regarding negative energy balance.

Perinatal mortality in laboratory mice has been acknowledged as a serious welfare issue, even though it has been investigated minimally.[37] Working with BALB/c mice under well-controlled environmental conditions, we found that a change in the environment and mating at an early age both promoted consistent decreases in preweaning mortality, notably during the first litter. We believe that these 2 measures contribute to the wellbeing of BALB/c mice through a protocol refinement that leads to reduction in animal numbers. For example, as the number of pups lost during the preweaning period decreases, the number of parental pairs likely can be decreased as well.

We recommend providing cardboard tubes in cages of BALB/c mice under intensive reproduction, to improve productivity by decreasing preweaning mortality rates and to increase the wellbeing of the animals. In addition, siblings should be paired for mating no later than 28 d of age to increase preweaning survival of the offspring.

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