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Effect of Cage Change Frequency on Perinatal Mortality in C57BL/6J Mice

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Perinatal mortality is a common problem in mouse breeding colonies. Few studies have examined the influence of environmental changes on mouse pup survival. In this study, monogamous breeding cages of C57BL/6J mice were set up and randomized into 3 cage change groups: 1) cage change at 8 d after parturition, 2) cage change at 3 d after parturition, or 3) cage change at 3 d after parturition with the addition of a polycarbonate hut in the cage. Pairs were bred to produce a minimum of 4 litters. Pup survival to weaning relative to experimental cage change date, and survival rates after cage change were evaluated. The results revealed no significant differences between experimental groups. The majority of pup loss occurred within the first 24 h after birth for those pups that were alive at birth. Overall, the postpartum day of cage change did not affect the perinatal survival of mouse pups.

Abbreviations and Acronyms: D3NH, postpartum day 3 without a cage hut; D3H, postpartum day 3 with a cage hut; D8, postpartum day 8 without a cage hut

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Introduction

Perinatal mortality, defined as the sum of stillbirths and early neonatal death (newborn mortality before 7 d of age), is a common concern in mouse breeding colonies but limited scientific literature leaves this phenomenon poorly understood. Documented causes of perinatal mortality in mice have been related to strain, first parity, genetic lethality, advanced dam age, and litter overlap.[3,4,6,7,8,10,12,18] Significant pup losses often result in the need for more breeders to supply experimental needs. The *Guide for the Care and Use of Laboratory Animals*[5] states that frequent bedding changes may be contraindicated in the prepartum or postpartum period yet provides no minimum frequency for changing bedding. Having a better understanding of pup mortality and its relationship to cage changing will provide guidance for mouse breeding colony management and husbandry.

Neonatal handling is known to affect maternal care and alter mother-pup relationships, potentially leading to preweaning mortality.[1,4,9,11] Despite some evidence of cannibalism, the literature indicates that infanticide is rare and that the dams eat mouse pups after they die but do not actively kill them.[17,19] In the authors' experience, many researchers believe that environmental disruption of early neonatal mouse litters due to cage changes is associated with greater perinatal mortality. To minimize disruptions of the home environment, many facilities limit the handling of dams and their litters during the early postnatal period. This is often accomplished by postponing cage changes until 7 d after parturition regardless of when the last cage change had occurred.

The aim of this study was to investigate the effects of cage changing on pup survival by comparing the effects of changes at 3 d and 8 d postpartum, thus testing the practice of delaying cage change until after the first week postpartum. Our expectation was that we would not find a statistical difference in pup deaths after cage changes on day 3 and day 8 postpartum, and that the addition of structural enrichment in the form of a polycarbonate hut would increase the percentage of pups successfully reaching weaning age by allowing dams to use the hut as a supplement to a nest. Secondary aims investigated the correlation between litter number (parity) and cage rack location to pup survival.

Materials and Methods

Animals. The mice used in this study were housed in an AAALAC-accredited animal facility at Yale University. Study subjects were nulliparous 8-wk-old female (n = 20) and male (n = 20) C57BL/6J mice obtained from the Jackson Laboratory. All mice were screened for by Jackson Laboratory and 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, reo-virus 3, rotavirus, and Sendai virus, *Bordetella* spp., *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Filobacterium 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*) Twenty monogamous mating pairs were established and housed on an individually ventilated caging system (IVC Sealsafe Plus GM500 Rack, Tecniplast, Italy) with ¼-in. corncob bedding (Teklad 7097 Corncob Bedding, Envigo, Madison, WI) and a single compressed cotton square (NesletsTM, Ancare, Bellmore, NY) for enrichment. The room was maintained at 70 ± 2 °F (21 ± -17 °C) with 30 to 70% relative humidity and a

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12:12 h light:dark cycle. Standard rodent chow (Teklad global 18% protein diet 2018, Envigo, Indianapolis, IN) and water (reverse osmosis, hyperchlorinated at 4 to 6 ppm supplied by an automated watering system Avidity Science, Waterford, WI) were available ad libitum. Daily health and husbandry checks were performed by trained study staff, including on weekends and holidays. All procedures and housing were compliant with the *Guide for the Care and Use of Laboratory Animals*[5] and animal use was approved by the Institutional Animal Care and Use Committee at Yale University.

Study design. Upon arrival from the vendor, mice were assigned to 3 study groups: cage change on postpartum day 3 without a cage hut (D3NH), postpartum day 3 with a hut (D3H) (K3327 Mouse Hut Red Certified, Bio-Serv, Flemington, NJ), or postpartum day 8 without a hut (D8) (Figure 1). Each pair was bred to produce a minimum of 4 litters.

Cages were strategically placed on the IVC rack in a Latin square design to assess for differences in survival relative to cage rack location (comparison of top, middle, and bottom rows). Cages were undisturbed except for experimental cage changes, food replenishment, or evaluation of health concerns. Breeding cages were assessed daily for litters; both live and dead pups were counted by study staff without opening cages by lifting the cage card holder. Cages were removed completely from racks for closer observation when multiple pups appeared pale, unresponsive, or if blood and/or cannibalized pups were seen in the cage. If more than a third of a litter was dead, those dead pups were removed from the cage. Otherwise, cages were not opened, and dead pups were left in the cage to minimize the unscheduled handling of mice. When pups were removed, a stillborn assay was performed by visualization of the presence of a milk spot and a lung float test. The lung float test was used to discern live birth from stillbirth and shows whether lungs were inflated by respiration. Lung lobes and liver tissue were removed at necropsy and placed in a water-filled tube to assess the buoyancy of the 2 tissues. A neonate was determined stillborn if the lungs sank, whereas inflated, floating lung tissue indicated a live birth. The liver lobe acted as a control to determine the absence of gas producing bacteria in the tissues. To transfer mice at cage changes, study personnel used gloved hands to scoop and transfer adult mice and neonates in their nests to clean cages. All cages were changed 2 wk after experimental cage changes.

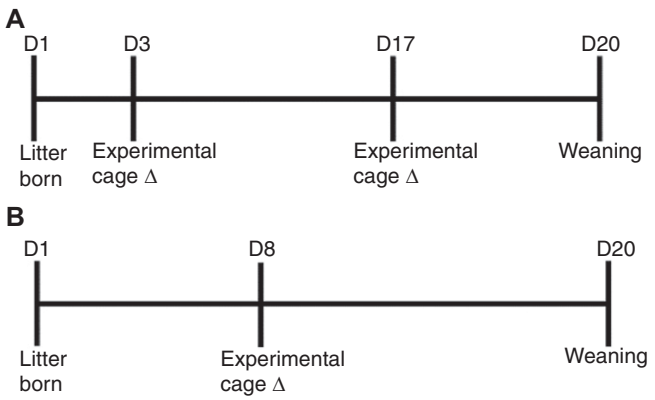


Figure 1. (A) Experimental timeline for cage change groups postpartum day 3 (D3NH and D3H). A total of 7 monogamous breeding pairs of C57BL/6J mice were used for these two study groups. (B) Experimental timeline for cage change group postpartum day 8 (D8). A total of 6 monogamous breeding pairs of C57BL/6J mice were used for this study group.

Pup survival was calculated as the number of pups that survived to weaning age compared with number of pups present at the experimental cage change and was compared across study groups.

Mouse pups were weaned from the parent cage at 19 to 20 d of age to avoid litter overlap. In keeping with the 3 Rs,[15] weanlings and breeders at the end of study were made available to interested labs for further study use.

Statistical analysis. At least 72 litters were determined necessary to detect significant differences across all dependent variables: (postpartum day 3 cage change without hut, postpartum day 3 cage change with a hut, postpartum day 8 cage change without a hut) at a 95% confidence level with 0.8 power using G*Power software version 3.1.9.4.[2] Outliers were maintained within the analysis and did not impact statistical significance. Two dams of different parity were excluded from the analysis because they produced no litters.

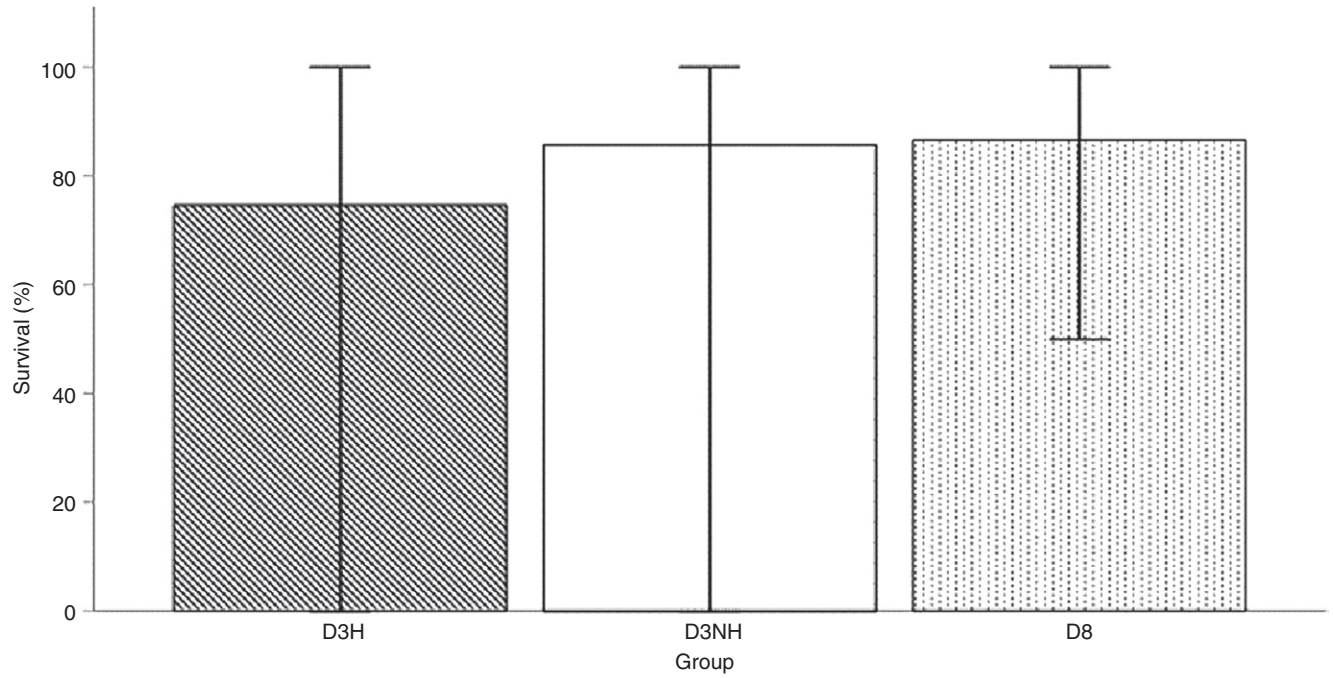
Data were analyzed by using commercially available SPSS software (SPSS 28, IBM, Armonk, NY). Nonparametric Kruskal-Wallis H tests were used for all analyses except for survival of pups from birth to the experimental cage change, and from the cage change to weaning. When the distributions of the dependent variable were dissimilar across the 6 groups, mean-ranks were used to conduct the analysis with reported medians. Post hoc pairwise comparisons with Bonferroni correction for multiple tests were employed when results were significant. Wilcoxon Signed-Rank tests were used to determine a difference in percent survival of the pups from before the experimental cage change until weaning. A P value of 0.05 is considered statistically significant unless otherwise stated. Descriptive data are presented as means with SD and as medians.

Results

Percent of pups surviving to weaning (Figure 2). A total of 85 litters from nulliparous dams were used for the analysis. Each study group produced at least 24 litters [day 8 cage change (n = 24), day 3 cage change with no hut (n = 29), and day 3 cage change with a hut (n = 32)] (Figure 1). A Kruskal-Wallis H test was used to compare groups. The median percent survival to weaning was 75 ± 44% for day 3 cage change with a hut, 86 ± 45% for day 3 cage change with no hut, and 87 ± 87% for day 8 cage change; these differences were not statistically significant, $\chi^2(2) = 1.485$, $P = 0.476$.

Percent survival of pups from birth to experimental cage change (Figure 3). Differences in percent survival of pups from birth to experimental cage change between the 3 cage change groups were analyzed by using the Kruskal-Wallis H test, as follows: day 8 cage change (n = 24, median = 87%, mean = 70 ± 38%), day 3 cage change with no hut (n = 29, median = 86%, mean = 62 ± 43%), and day 3 cage change with a hut (n = 32, median = 86%, mean = 64 ± 42%). Percent survival did not change significantly among groups between birth and the cage change [$\chi^2(2) = 0.556$, $P = 0.757$].

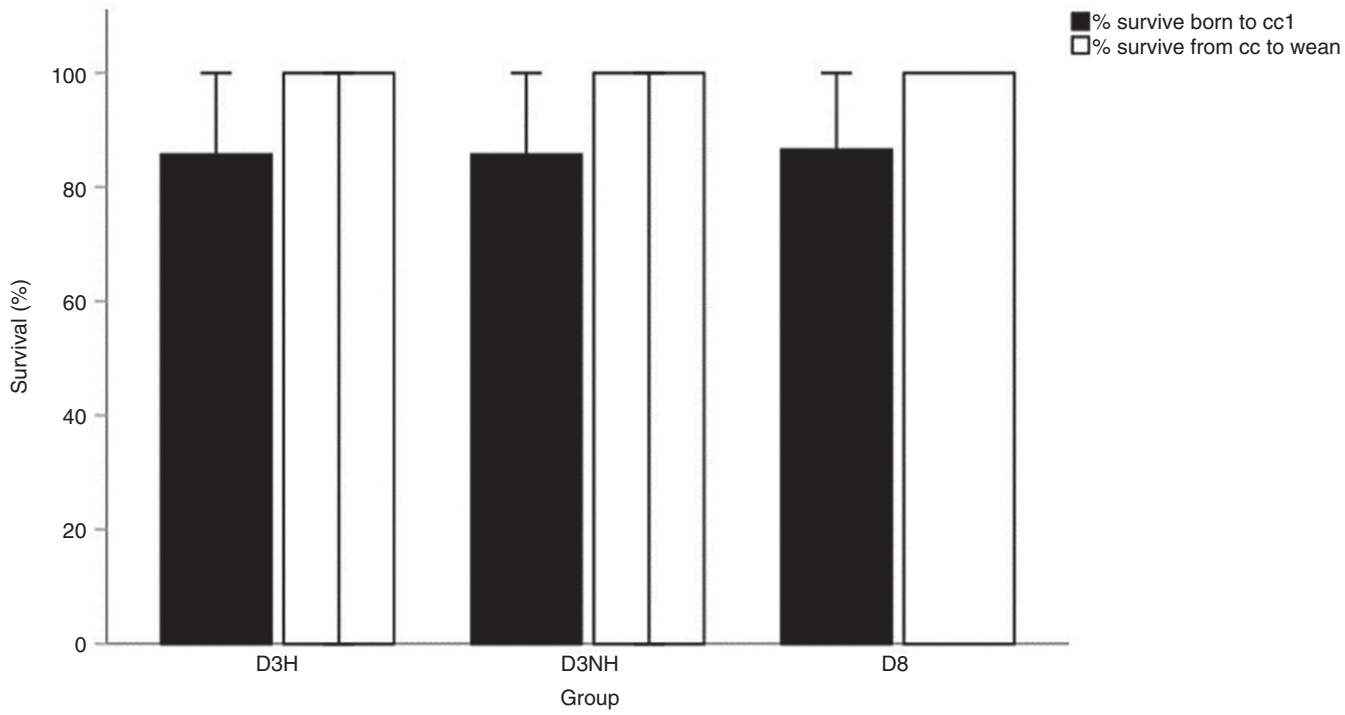
Percent survival of pups from experimental cage change until weaning (Figure 3). A Kruskal-Wallis H test was used to compare survival of pups between the experimental cage change and weaning for the 3 cage change groups, as follows: day 8 cage change (n = 24, median = 100%, mean = 83 ± 38%), day 3 cage change with no hut (n = 29, median = 100%, mean = 66 ± 48%), and day 3 cage change with a hut (n = 32, median = 100%, mean = 64 ± 48%). Percent survival of pups was not significantly different among groups between the experimental cage change and weaning [$\chi^2(2) = 3.391$, $P = 0.183$].



Error Bars: 95% CI

Figure 2. Overall percent pups surviving from birth to weaning. There were no significant differences between cage change groups for D8 (postpartum day 8), D3NH (postpartum day 3 without hut), and D3H (postpartum day 3 with hut).

Percent pup survival before and after experimental cage change (Figure 4). A total of 85 litters were included in the analysis to compare percent survival before and after the experimental cage change. Data are expressed as medians unless otherwise stated. Of the 85 litters, 28 had a % survival that was greater between the experimental cage change and weaning as compared with survival from birth to the experimental cage change. Six litters had a % survival that was lower from the experimental cage change to weaning than it was from birth to the experimental cage change. Fifty-one litters had no change in % survival from either time point. The survival of pups from before the first cage change until weaning (median = 86, ± 41%)



Error Bars: 95% CI

Figure 3. (Black) Pup survival from birth to experimental cage change one by group. (White) Pup survival from experimental cage change to weaning by group. Error bars demonstrate 95% CIs.

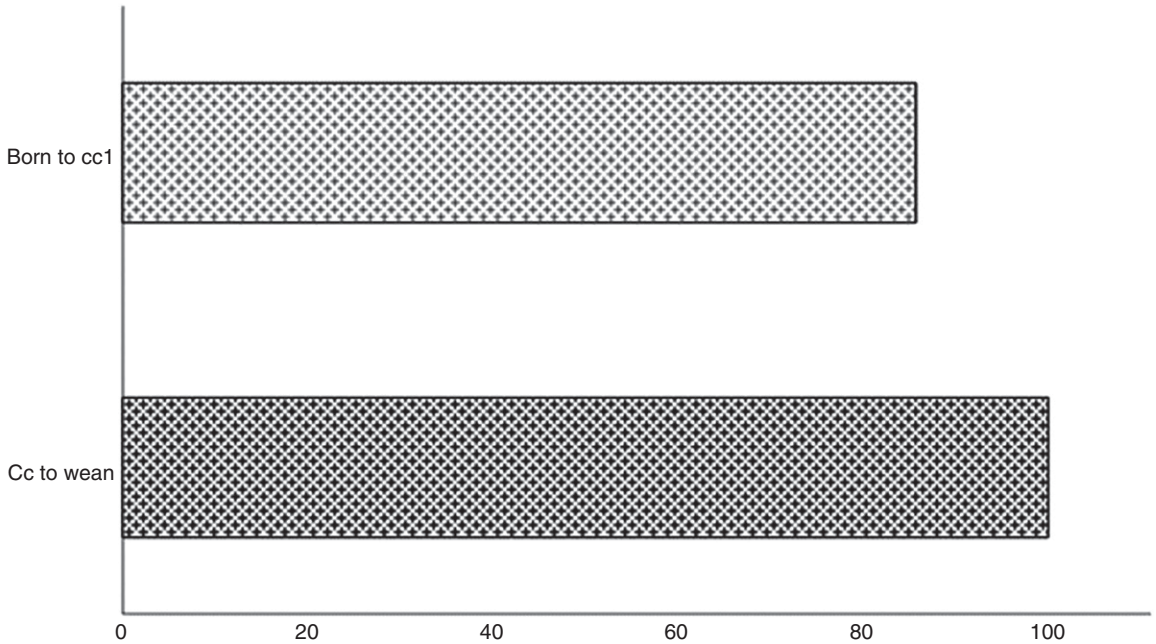


Figure 4. Percent pup survival from birth to cage change one and cage change one to wean.

was significantly lower than survival after the cage change until weaning (median = 100 ± 46%) $z = 3.601$, $P < 0.001$.

Pup survival by parity (Table 1 and Figure 5). A Kruskal-Wallis H test was used to identify significant differences in percentage of pups surviving to weaning by parity (4-5 per dam). The survival percentages were significantly different with regard to parity [$\chi^2(4) = 20.989$, $P < 0.001$]. Pairwise comparisons were performed using a Bonferroni correction for multiple comparisons. Median, means with standard deviations are reported. This post hoc analysis revealed a statistically significant difference in percent survival between parity 4 (median = 0%, mean 37 ± 44%) and parity 1 (median = 100%, mean = 85 ± 25%) ($P = 0.007$), parity 4 and parity 3 (median = 100%, mean = 80 ± 36%) ($P = 0.01$), parity 1 and parity 2 (median = 33%, mean = 40 ± 41%) ($P = 0.016$), and parity 2 and parity 3 ($P = 0.023$), but not for any other group combination [$\chi^2(4) = 20.989$, $P < 0.001$].

Percent of pups surviving by cage rack location (Figure 5). Differences in percent survival to weaning of pups in the 3 cage change groups were determined with the Kruskal-Wallis H test as follows: bottom rack ($n = 27$, median = 86 ± 42), middle rack ($n = 32$, median = 82 ± 45), top rack ($n = 36$, median = 86 ± 43). The median percent survivals wean were not statistically significantly different between groups based on rack location [$\chi^2(2) = 0.380$, $P = 0.827$].

Table 1. Mean and median percent of pups surviving to weaning by litter number

Litter Number	Mean %	*N*	SD	Median %
1	85	20	25	100
2	41	19	41	33
3	80	19	36	100
4	37	18	44	0
5	64	9	48	86
Total	62	85	43	86

A total of 85 litters were analyzed ($n = 85$). Missing values or dams that produced no litters were excluded from the analysis.

Discussion

This study evaluated the effects of cage changes on mouse perinatal survival by comparing cages changed at either day 3 or day 8 postpartum. The results suggest that cage changing at either postpartum date did not significantly affect perinatal survival of C57BL/6J mice. In addition, provision of a hut and cage location on the rack did not significantly alter pup survival for groups changed at day 3 postpartum. These results refute the belief that limiting cage changing and pup handling within the first 7 d postpartum improves pup survival.

In this study, the majority of pup loss occurred before the experimental cage change, primarily during the initial 24 h after birth. For cases in which an entire litter was presumed dead ($n = 5$) on postpartum day 1 (0 to 24 h after birth), a stillborn assay was performed using the lung float test and milk spot check. All deceased pups in these 5 litters had no milk spot and their lungs failed the float test, indicating that they were stillborn. For litters in which less than a third of pups were found dead on either postpartum day 1 or before the experimental cage change ($n = 18$), cages were not disturbed in order to limit variability in the study, and a stillborn assay was not performed on those dead pups. Thus, only 5 cages were completely removed from the racks and opened for pup retrieval and clinical assessment. Further pathologic evaluation was not performed in this study but may provide further insight in potential future studies. Relatively few pups (11 of 529 pups) died after the experimental cage change. The chance of pup loss after cage change on day 3 was higher if the majority of their litter mates had died prior to cage change. Among all litters, only 6 instances were found of pups being cannibalized or missing at experimental cage change.

A statistically significant difference was detected in the percent survival based on parity. Across all study groups, parities 2 and 4 had survivals of 31% and 29%, respectively, as compared with survivals of 55% and 45% for parities 1, 3, and 5. This alternating pattern may be related to continuous breeding of dams without removal of the male from the cage after the confirmation of pregnancy. Leaving the males with the females permits subsequent breeding without allowing recovery time for the dam between litters. We did not assess the effect of continuous

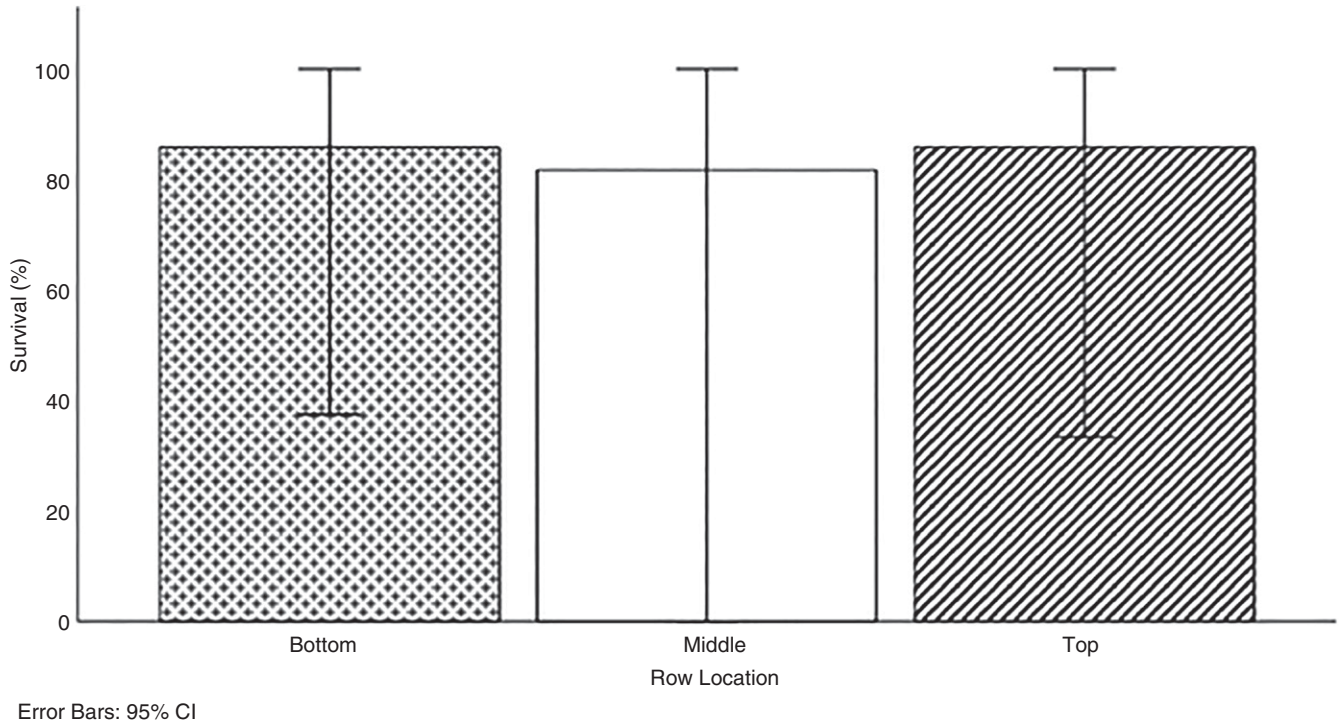


Figure 5. Percent of pups surviving by cage rack location (top row, middle row, bottom row).

compared with intermittent breeding in this study. Contrary to common beliefs, the first litters across all study groups had the highest rate of survival as compared with subsequent litters. Further study is needed to better understand difference in the survival rate between litter numbers.

Consistent with our results, previous studies have reported that pup survival in C57BL/6 mice is not altered by environmental stresses due to early postpartum cage change.[4,7,12,13,14,16,17,19]

In conclusion, our data support our hypothesis that litter survival is not significantly different in cages changed during early perinatal development as compared with cages that are changed later in development. Our results suggest that postponing cage change until after the early perinatal period does not improve pup survival in C57BL/6 mice. Further study is needed to determine causes and useful mechanisms for minimizing mouse mortality in early perinatal life.

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