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Effects of Refined Handling on Reproductive Indices of BALB/cJ and CD-1 IGS Mice

Margaret A Hull,[1,*] Elizabeth A Nunamaker,[2] and Penny S Reynolds[3]

Current mouse handling methods during cage change procedures can cause stress and potentially compromise animal welfare. Our previous study of breeding C57BL/6J mice found modest increases in pup production and a significant reduction in preweaning litter losses when mice were handled using a tunnel as compared with a tail-lift with padded forceps. The current study evaluated how these 2 handling methods affected reproduction by 2 additional mouse strains, BALB/cJ (a low- to intermediate-fecundity strain) and CD-1 IGS (a high-fecundity stock). We predicted that refined handling would have minimal effects on the high-fecundity line with a satisfactory production rate and greater effects on the low-fecundity line. Handling method (tunnel compared with tail-lift) was randomly assigned to monogamous breeding pairs of mice. Reproductive metrics (litter size at birth and weaning, numbers of litters, litter attrition, between-litter intervals, pup weaning weight, and sex ratio) were prospectively monitored for 80 BALB/cJ and 77 CD-1 pairs that were bred continuously for 6 mo. Both strains of mice were highly productive, exceeding previously published breeding data. However, neither strain demonstrated operational or statistically significant differences between handling methods for any reproduction metric. As we detected no negative effects in these 2 strains and the benefits are clear in other strains, refined handling should be considered for all breeding mice.

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Introduction

Mice are commonly handled by the tail, either by hand or with padded forceps.[4,11] However, routine tail handling by either method has documented adverse effects, including strong and persistent aversion and anxiety associated with handling.[22] As such, a growing body of literature is focused on alternative handling practices for mice. These handling practices have a variety of names—nonaversive, low-stress, or refined handling—but they usually refer to handling mice using either cupped hands or a tunnel. These methods reduce anxiety associated with handling in individual mice and are simple and inexpensive refinement measures for improving rodent welfare.[11,28]

Previously, we demonstrated that refined handling of breeding C57BL/6J mice during cage change was associated with a modest but operationally relevant increase in the number of pups produced per pair and statistically and operationally significant reductions in preweaning litter loss.[27] Preweaning mortality is a major consideration for the management of rodent breeding colonies.[41] Both environmental and induced stressors affect the maternal behavior of nursing females and can result in increased rates of pup loss.[45] Therefore, procedural changes that minimize preventable pup death can be an important animal welfare refinement. Reducing pup losses can also result in considerable cost savings for large colonies, including vendor colonies. This provides a major financial incentive for using refined handling methods due to lower lost opportunity costs and greater animal availability for sales.[27]

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[1]Animal Care Services, University of Florida, Gainesville, Florida; [2]Global Animal Welfare and Training, Charles River Laboratories, Wilmington, Massachusetts; and [3]Statistics in Anesthesiology Research Core, Department of Anesthesiology, College of Medicine, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Florida
**Corresponding author. Email: ma.hull@ufl.edu*

The objective of the current study was to determine whether our previous findings would also apply to 2 additional mouse lines, BALB/cJ and CD-1 IGS. Logistics and cost issues associated with the introduction of refined handling in breeding operations have been discussed in detail elsewhere.[27,47,54] Our choice of mouse lines was based on the best information we could find on strain breeding performance. BALB/cJ is an inbred strain that is commonly used for antibody production and cancer, immunologic, and cardiovascular investigations. Reproductive performance is characterized as 'low' to 'intermediate,' with litter sizes averaging 5 to 6 pups.[15,31,33,40,50] In contrast, CD-1 IGS is a robust outbred multipurpose stock, often used for aging, toxicology, carcinogenesis, and pharmacological research. Reproductive performance is characterized as 'good' to 'high,' with average litter sizes of approximately 12, and high pup survival.[15,19] We hypothesized that refined handling of breeding mice during routine husbandry would result in higher productivity as compared with mice handled by the standard method of tail-lift with forceps. This hypothesis generated 2 specific predictions: 1) productivity of CD-1 pairs (high-fecundity line) would be relatively insensitive to handling method, because pup production for this strain is expected to be close to high; and 2) in BALB/cJ mice (low-fecundity line), refined handling would result in improved productivity (especially with later litters) relative to the tail-lift method (Figure 1).

Methods

Ethical oversight. The study was conducted at a single academic AAALAC-accredited institution. The protocol was approved by the University of Florida IACUC (no. 202111320), and a statistical analysis plan was prepared before the study began; the study was not otherwise preregistered. Animal care was in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals*.[43]

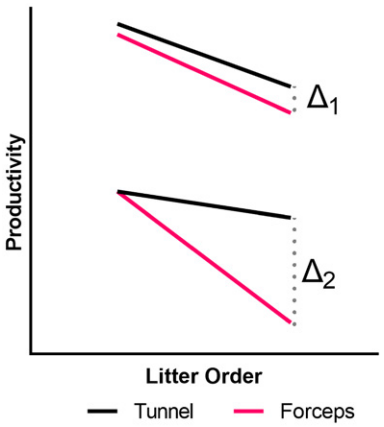

Figure 1. Predicted differences in productivity (Δ) associated with parity and handling method (tunnel handling compared with tail-lift handling using padded forceps). CD-1 is a high-fecundity strain. We predicted that we would find minimal differences in productivity between handling methods (Δ_1) and only minor reductions in productivity with parity. BALB/cJ is a historically low-fecundity strain. We predicted that we would find large differences in productivity between handling methods (Δ_2) and that productivity of tail-handled mice would decline more with parity as compared with tunnel-handled mice.

Animals. Breeding mice were 6-wk-old males and nulliparous females. BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, strain no. 000651), and CD-1 IGS mice from Charles River Laboratories (Raleigh, NC, stock no. 022). Based on vendor report and in-house surveillance, all mice were SPF of the following agents: lymphocytic choriomeningitis virus, ectromelia, murine rotavirus, minute virus of mice, murine adenovirus 1 and 2, mouse cytomegalovirus, mouse hepatitis virus, mouse parvovirus, pneumonia virus of mice, reovirus 3, Sendai virus, Theiler murine encephalitis virus, ectromelia, *Mycoplasmia pulmonis*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Filobacterium rodentium*, *Salmonella* sp., *Streptobacillus moniliformis*, pinworms, and fur mites. Health status was confirmed by quarterly environmental PCR testing that was performed approximately 2 to 3 wk before a complete cage change. All samples were analyzed by the Charles River Diagnostic Laboratory.

Housing and husbandry. Details of animal care, housing, husbandry, colony pathogen status, and health management procedures were reported in detail previously.[27] In brief, mice were acclimated for 7 d after arrival at the facility before pairing. Mice were housed in monogamous pairs in individually ventilated cages (484-cm^2 floor area) with microbarrier tops (75 JAG mouse caging, Allentown Caging, Allentown, NJ). Breeding pairs were maintained at 21 to 25 °C, 30 to 70% humidity, and 14:10-h light:dark cycle, with corncob bedding (7097, Envigo, Indianapolis, IN) and commercial nesting material (cotton square, Lab Supply, Fort Worth, TX). Food (Teklad Extruded Diet 2019, Envigo, Indianapolis, IN) and water (Edstrom automatic watering system; Avidity Science, Waterford, WI) were provided ad libitum. Cage bottoms, including bedding and cotton squares, were changed every 2 wk. Additional cage components such as the wire bar feeder and cage lid were changed every 12 wk. Water bottles, if used, were changed weekly. A small portion of soiled bedding and nest material was transferred to new caging at every cage change event. To minimize disturbing the dam, cages were not opened or changed for 7 d after parturition. If a scheduled cage change fell within this window, it was performed on the eighth day after parturition or on the next working day after the eighth day.

Adult mice were transferred to clean cages using either tail-lift (our facility's standard of care) or transfer tunnels. Tail-lift (control) mice were transferred using rubber-tipped forceps to gently grasp the base of the tail. Forceps were stored in liquid disinfectant (Peroxigard; Virox Technologies, Oakville, ON, Canada) between being used on consecutive cages. Tunnel-handled mice were gently guided by hand into a tunnel that was then transferred to the clean cage. Tunnels were 8.9 × 6.4 × 5.1-cm clear medical-grade polycarbonate square tubes (no. J-1002; Petro Extrusion Technologies, Middlesex, NJ). Tunnels remained in the cage and were replaced with new autoclaved tunnels when visibly soiled or every 12 wk. Pups were moved with cupped hands.

Experimental design. The study was conducted as separate 2-arm prospective randomized controlled trials on each mouse line. The tail-lift with forceps or tunnel handling method was randomly allocated to pairs of each line. Intervention allocation could not be concealed from technical staff, but allocation was concealed from the data analyst. The unit of analysis was the breeding pair.

Pairing and randomized treatment allocation for BALB/cJ mice were performed on the same day. Because of space constraints and to minimize overproduction of pups relative to researcher demand, CD-1 mice were obtained from the vendor in 2 time blocks. The first block of 37 CD-1 mice was paired at the same time as all BALB/cJ pairs, and the second block of 38 CD-1 mice was paired 3 mo later. To minimize potential environmental effects of cage position on breeding productivity, cages were randomized to rack position (identified by horizontal and vertical location) using a replicated 2 × 2 Latin square design.[32] Randomization plans were generated separately for each line (PROC PLAN; SAS v.9.4, SAS, Cary, NC).

Productivity metrics assessed for each pair were number of pups born, number of pups weaned, between-litter interval (d), number of litters, litter attrition (all pups in a litter died or disappeared before weaning, yes/no), sex at weaning, and total litter weight at weaning (g). Pups were weaned at approximately 18 d after birth. The entire litter was weighed to the nearest 0.1 g at the time of weaning. Pups in each litter were then individually sexed, and sex ratio for each litter was calculated as the number of females/(total number of pups weaned). Pup weight was estimated as the total litter weight divided by litter size at weaning. Productivity index per litter was calculated as the between-litter interval divided by the number of pups weaned.

Pairs were retired after approximately 6 mo (approximately 185 d) of continuous breeding. However, mice that were gravid or tending a litter were allowed to continue until pups from that litter were weaned. Maximum production time was 202 d. Pairs were removed from the study and breeding was classified as 'unsuccessful' if the dam was euthanized for dystocia, found dead or moribund, had 2 consecutive litter attrition events ('litter failed to wean': all pups in a litter disappeared or found dead before weaning), or was nonproductive for greater than 60 d or if pups could not be weaned (no incisors, pup too small to reach a wire bar feeder on its own, pup unthrifty, or death of the dam). Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Weaned pups were donated to institutional researchers or the internal Animal Care Services unit for personnel training.

Statistical analysis. ***Sample size justification.*** Sample size calculations were based on the primary outcome of number of pups weaned per litter. Based on vendor information, mean litter

Table 1. Productivity of 80 BALB/cJ pairs handled with either refined (tunnel) handling or the facility standard (tail-lift with padded forceps)

Productivity metric	Tunnel	Tail-lift	Total
Number of pairs	40	40	80
Number of litters	224	220	444
Number pups born	1,417	1,434	2,851
Number pups weaned	1,319	1,277	2,596
Weaning success (pups weaned: born) (%)	93	89	91
Number of females at weaning	611	613	1,224
Number of males at weaning	708	664	1,372
Entire litter failed to wean [*n* (%)]	23 (10)	25 (11)	48 (11)
Number of pups disappeared or found dead	19	24	43
All-cause dam mortality (dystocia, found dead)	5	4	9
Nonproductive pairs > 60 d	2	5	7

Result are total counts.

sizes were assumed to average 6 pups for BALB/cJ mice and 12 pups per litter for CD-1 mice and were expected to produce 6 litters over an expected reproductive lifetime of 6 mo. Sample size calculations were performed for Poisson-distributed count data analyzed by generalized linear mixed models. An exemplary dataset was generated from litter sizes estimated from reported data and a projected cumulative increase of 4 to 5 pups over 6 litters, or 0.7 to 0.8 pups per litter, for the tunnel-handling relative to the tail-lift method. The noncentrality parameter and corresponding degrees of freedom were generated from analysis of the exemplary data in SAS PROC GLIMMIX, and the inverse function was used to estimate power.[35,51] Sample size required to detect a 4- to 5-pup increase in lifetime pup numbers per pair with $\alpha = 0.05$, and power greater than 0.8 was approximately 80 pairs for BALB/c and 75 pairs for CD-1. An operationally significant change in productivity associated with handling method was defined as one extra pup over the reproductive lifespan of each breeding pair.[27]

Analysis methods. Descriptive summary statistics for group data are reported as means (SD), medians (IQR), and counts (percentages), as indicated. Results are reported as means adjusted for pair, parity, and litter size and 95% CI.

Productivity outcomes per litter per pair and per pair cumulative totals for pups, pups weaned, and litters that failed to wean were analyzed by intention to treat. Differences between handling methods were assessed by 2-level hierarchical generalized linear mixed models[1,12,26] in SAS PROC GLIMMIX, with handling method (tunnel compared with tail-lift) as the fixed effect, pair as the random effect, litters nested within pair, and parity as a repeated effect (with autoregression AR-1 covariance structure). Models providing the best fit to the data were chosen based on model convergence, goodness of fit statistics, and residual plots. Preliminary analyses showed no detectable spatial effects due to cage position for per-pair total pups born and weaned, total number of litters, or litter weight.

Results

BALBc/J productivity. Summary data are presented in Table 1. Eighty BALB/cJ pairs produced a total of 2,851 pups in 444 litters with 2,596 pups weaned for an overall weaning success rate of 91%. Median litter size at birth was 7 (IQR: 4, 9; range: 0 to 15), and median litter size at weaning was 6 (IQR: 4, 8; range: 0 to 15), with an average of 6 litters per pair. The average productivity index per litter was 1.15 (95% CI: 1.10, 1.20). Interlitter interval averaged 38.4 d (95% CI: 37.6, 39.2). Forty pairs (50%) exceeded 185 d of productivity.

Approximately half of all pairs (41/80; 52%) successfully weaned all litters produced, 30 pairs (15 in each group) lost one entire litter before weaning, and 9 pairs (4 in the tunnel group and 5 in the tail-lift group) lost 2 entire litters. Of all pups born, approximately 11% did not survive to weaning. Nine dams (5 tunnel and 4 tail-lift) were found dead or were euthanized for dystocia. Seven pairs were removed from the study due to 2 consecutive losses of complete entire litters (2 tunnel and 5 tail-lift). Seven pairs (2 tunnel and 5 tail-lift) were nonproductive for greater than 60 d.

After adjusting for parental and parity effects, we found no statistically or operationally significant differences between handling methods for any production metric (Table 2). Pooled litter size averaged 6.6 pups per litter at birth (95% CI: 6.4, 6.8) and 6.5 pups per litter at weaning (95% CI: 6.3, 6.8). Litter size at birth showed a weak negative association with parity ($r = -0.13$; 95% CI: −0.23, −0.03; $P = 0.015$), but the number of pups weaned was not significantly associated with parity ($r = -0.03$; 95% CI: −0.14, 0.09; $P = 0.63$). Total litter weight at weaning

Table 2. Mean (95% CI) for BALB/cJ mouse productivity metrics adjusted for parental effects and parity

	Tunnel			Tail-lift			
	Adjusted mean	95% CI		Adjusted mean	95% CI		*P* Value
Pups born per litter	6.4	6.0	6.8	6.5	6.1	6.9	0.56
Pups weaned per litter	5.9	5.5	6.3	5.8	5.4	6.2	0.72
Total litter weight at weaning (g)	52.0	48.7	55.4	51.5	48.1	54.8	0.81
Pup weight at weaning (g)	8.3	7.8	8.8	8.2	7.7	8.7	0.80
Interlitter interval (d)	38.3	37.2	39.4	38.4	37.3	39.6	0.89
Sex ratio F/(M + F)	0.46	0.44	0.49	0.48	0.45	0.51	0.39

Pup weights were also adjusted for litter size. *P* values test the effect of intervention (tunnel compared with tail-lift) on each metric.

Table 3. Total count productivity of 77 CD-1 IGS pairs handled with either tunnel or tail-lift with padded forceps

	Tunnel	Tail-lift	Total
Number of pairs	39	38	77
Number of litters	268	267	535
Number pups born	4,099	4,053	8,152
Number pups weaned	3,874	3,840	7,714
Weaning success (pups weaned: pups born) (%)	95	95	95
Number of females at weaning	1,894	1,911	3,805
Number of males at weaning	1,980	1,929	3,909
Entire litter failed to wean [*n*(%)]	8 (3)	9 (3)	17 (3)
Number of pups that died or disappeared	228	216	444
All-cause dam mortality (dystocia, found dead)	3	2	5
Nonproductive pairs > 60 d	0	2	2

averaged 58 g (95% CI: 56, 60), and average weaning pup weight was 9.3 g (95% CI: 9.0, 9.5). Parity was not associated with either total litter weight ($r = 0.03$) or average pup weight ($r = -0.03$).

CD-1 productivity. Summary data for CD-1 mice are presented in Table 3. Seventy-seven CD-1 pairs produced a total of 8,152 pups in 535 litters and weaned 7,714 pups, for an overall weaning success rate of 95%. The median litter size at birth was 16 (IQR: 13, 18; range, 0 to 24), and the median litter size at weaning was 15 (IQR: 13, 17; range: 0 to 23), with an average of 7 litters per pair. The average productivity index was 2.69 (95% CI: 2.61, 2.77). The interlitter interval for all mice averaged 38.4 d (95% CI: 37.6, 39.2). Forty-seven pairs (59%) exceeded 185 d of productivity.

Two-thirds of all pairs (52/77, 68%) successfully weaned all litters produced. Thirteen pairs (7 pairs in tail-lift and 6 in tunnel groups) lost one entire litter before weaning, and 2 pairs (1 in each group) lost 2 entire litters. Approximately 3% of all litters failed to wean. Five dams (3 tunnel and 2 tail-lift) were found dead or euthanized for dystocia. One pair in the tail-lift group was removed from the study after losing 2 consecutive litters. Two pairs, both in the tail-lift group, were removed for nonproductivity for over 60 d.

No statistically or operationally significant differences were detected between handling methods for any production metric after adjustment for parental effects and parity (Table 4). Pooled litter size at birth averaged 15.2 pups per litter (95% CI: 14.8, 15.7) and 14.9 pups at weaning (95% CI: 14.4, 15.4). Total litter weight at weaning averaged 134.6 g (95% CI: 131.0, 138.2), with an average pup weight of 10.0 g (95% CI: 9.7, 10.3). Weak positive correlations with parity were observed for per-pair litter size at birth ($r = 0.19$; 95% CI: 0.10, 0.27; $P < 0.0001$), number of pups at weaning ($r = 0.21$; 95% CI: 0.12, 0.30; $P < 0.0001$), total litter weight at weaning ($r = 0.18$; 95% CI: 0.09, 0.26; $P < 0.0001$), and average weaning pup weight ($r = 0.14$; 95% CI: 0.05, 0.23; $P < 0.0001$).

Discussion

This study compared 2 mouse handling methods—refined (tunnel) handling or standard (tail-lift with padded forceps)—of breeding BALB/cJ and CD-1 IGS mice. Our results supported our prediction that the high-fecundity CD-1 stock would be relatively insensitive to handling method. Overall breeding performance of CD-1 mice in this study was better than expected. Compared with previous reports for this stock,[10,15] mice in this study produced 2 to 3 pups more on average, with no decline in fecundity by 6 mo and preweaning litter losses of 3%. However, contrary to our expectations, we found no operational or statistical differences between handling methods for BALB/cJ productivity. Average BALB/cJ litter sizes at birth and weaning were similar to or exceeded those reported elsewhere[16,40,42,58] with no apparent decline in litter size with parity. Preweaning litter loss for BALB/cJ was approximately 11%, compared with other reports of 4%[40] to 20% loss of entire litters.[55,56]

Results for this study contrast with those from our previous study of C57BL/6J breeding mice. In the prior study, tunnel handling produced modest increases in litter size and greatly reduced rates of preweaning litter loss relative to tail-lift handling.[27] The finding of no differences between handling methods on BALB/cJ productivity was unexpected, as in general these mice exhibit more anxiety-like behaviors in cognitive tests and are considered to be more sensitive to chronic stress than C57BL/6 mice.[8,25,29,37,52] Strain differences in handling-induced anxiety[39,44] are due in part to environmental interactions, including husbandry practices.[9] Chronic

Table 4. Summary statistics (means, 95% CI) for CD-1 IGS mouse productivity metrics adjusted for parental effects and parity

	Tunnel			Tail-lift			
	Adjusted mean	95% CI		Adjusted mean	95% CI		*P* Value
Pups born per litter	15.3	14.9	15.8	15.2	14.7	15.7	0.74
Pups weaned per litter	14.5	14.0	15.0	14.4	13.8	15.0	0.85
Total litter weight at weaning (g)	138.3	135.3	141.5	138.8	135.7	142.0	0.83
Pup weight at weaning (g)	9.5	9.1	10.0	9.9	9.4	10.3	0.30
Interlitter interval (d)	38.4	38.0	38.8	38.2	37.8	38.6	0.54
Sex ratio F/(M + F)	0.49	0.47	0.51	0.50	0.48	0.52	0.48

Pup weights were also adjusted for litter size. *P* values test the effect of intervention (tunnel compared with tail-lift) on each metric.

and acute stressors are known to alter breeding productivity by reducing oocyte development,[10,17,36,59] delaying implantation and growth of embryos,[5,10,30,31] and increasing pup mortality.[2,24,45,49,56-58] Due to the combination of the effects of stress on BALB/cJ compared with C57BL/6 mice and the known effects of stress on breeding productivity, we expected that BALB/cJ mice would have the most significant change in breeding productivity due to refined handling. In both this study and the prior study on C57BL/6J mice, disturbance and handling of breeding pairs occurred only at biweekly cage changes and time of weaning. These more widely spaced disturbances to breeding pairs did not seem to cause enough stress to discriminate between handling methods for BALB/cJ mice in this study.

Limitations of this study include restrictions on visual assessment of nesting mice during daily health assessment and choice of mouse lines. For pup counts at or after parturition, we did not remove pups from the nest or disturb the dam. If we had done so, this might have provided more accurate estimates of event times and pup counts. However, more intrusive monitoring would increase the risk of maternal distress and possible harm to pups.[46]

Data from this study were used to determine if the breeding production increase previously observed for C57BL/6J mice would occur in other mouse strains used at our institution. We chose mouse lines in part due to researcher demands and ethical considerations. While other lines may be less fecund and potentially more susceptible to disturbance, we selected test strains that represented those most commonly used at our institution. This allowed us to use pups that were produced as part of the study and reduced production of unneeded mice. Historical data indicate that BALB/cJ mice are less fecund than other strains, including strains with a similar genetic background[13,15,16,31,40,42,58] CD-1 mice were chosen as the high-fecundity stock based on historical breeding data[10,14] and their common choice as an outbred stock. Additional strains such as immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) were considered to be low-fecundity strains but could not be included in this study due to space and cost constraints.

Interpreting the effects of handling method on breeding success is complicated by well-documented strain and subline differences in behavior[3] and different levels of response to interacting environmental factors. Cage size,[19] supplementary nesting material,[20,40] and ambient temperature[18,20] have been demonstrated to affect mouse productivity and maternal behavior. Because husbandry details are often not reported, evaluation of reported differences between studies is difficult. High fetal resorption rates,[30,34] reduced litter sizes,[30] and reduced maternal care behaviors[24,38] are associated with stress during gestation. Vibrations, such as those generated by moving and opening cages for cleaning, have been documented to adversely affect breeding performance,[6,7,48,53] and mitigation of vibrations is recommended.[53] Refined handling has been shown to provide positive benefits to adult mice[11,21,23,28] and to improve breeding productivity in at least one strain.[27] As few to no negative effects have been reported or observed for breeding mice, the use of refined handling for lines that are poor breeders, such as immunocompromised, transgenic, and certain knockout lines, should be considered.

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