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Assessment of Routine Procedure Effect on Breathing Parameters in Mice by Using Whole-Body Plethysmography

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We used whole-body plethysmography to investigate the effect of restraint, ear marking, tail vein and retroorbital blood sampling, and tail clipping on respiration in Balb/c × TCR-HA$^{+/-}$ F1 hybrid mice (F1h). Baseline values of breathing parameters were determined. During the experiment, mice experienced a procedure and then plethysmographic recordings were obtained immediately and at 4, 24, and 48 h afterward. Baseline breathing parameters showed significant differences between sexes. Restraint affected minute volume differently than did handling in male mice and to a lesser extent in female mice. Ear marking significantly changed minute volume compared with handling but not restraint in male mice and in the opposite manner in female mice. Tail vein blood sampling changed minute volume in a significant manner compared with restraint but not compared with handling in both sexes. Retroorbital blood sampling significantly changed minute volume compared with values for both handling and restraint in male mice but only compared with handling in female mice. Tail clipping modified minute volume significantly compared with handling in male mice and compared with restraint in both sexes. Analysis of data showed that routine procedures affect minute volume in mice depending on invasiveness of maneuver and in a sex-biased manner for as long as 24 h after the procedure. Our experiment shows that procedures performed on laboratory mice can change respiratory parameters and can be investigated by plethysmography.

Abbreviation: F1h, Balb/c × TCR-HA$^{+/-}$ F1 hybrid mice.

Handling, restraint, identification methods, and blood or tissue sampling are regarded as routine procedures in animal experiments. This aspect is particularly important when working with transgenic animals, where the need for genotyping demands the frequent use of these procedures, which can readily yield DNA samples.[5] The effect of routine procedures on physiologic parameters varies depending on method, frequency, and duration, but these procedures generally are considered to be acute stressors.[2,5,26,27] A large body of research has focused on the effect of routine procedures on animals' physiology. These effects were assessed by studying changes in behavior,[1,23,36,37] body weight,[29,37] food and water intake,[29,37] body temperature[35] and heart rate by radiotelemetry,[5,10,19,26,27] corticosterone[11,12,24,25,29] and glucose levels,[33] and blood variables.[29,36] Several differences in the response of laboratory animals to stress have been correlated to breed,[2,33] strain,[11,14,37] age,[24] and sex.[12,23,24] Some authors[24] have noted that female mice show a lower overall stress level due to the modest effect of social competition. Another study[12] showed a different, sex-specific response to hypoxic ventilation in rats, which was higher in male than female rats. In addition, these authors[12] suggested that female ovarian hormones are prime candidates for stress regulation.

Whole-body plethysmography is a noninvasive, indirect method of studying respiratory function in conscious, unrestrained animals. This method has been used particularly in pharmacologic and toxicologic studies in diverse animal species including mice,[3,9,14,15,38] rats,[8,20] cats,[22] dogs,[16,34] and pigs.[13] The present study was designed to investigate the extent to which routine procedures affect breathing parameters in a transgenic mouse colony. Male and female mice were exposed to 4 routine procedures that are used for DNA sampling and identification of transgenic mice (ear marking, tail clipping, and tail vein and retroorbital blood sampling). Respiratory parameters were recorded by whole-body plethysmography immediately and at 4, 24, and 48 h after the procedure. Handling and restraint were used as control procedures.

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Materials and Methods

The study protocol was reviewed and approved by the Internal Ethics Committee of the Cantacuzino National Institute for Research and Development in Microbiology and Immunology (Cantacuzino NIRDMI). All animal care was in accordance with standards set forth in the Council Directive 86/609/EEC.[6]

Animals and housing conditions. Male and female Balb/c × TCR-HA$^{+/-}$ F1 hybrid mice (F1h; age, 8 to 10 wk) from the transgenic breeding colony of the Cantacuzino NIRDMI animal facility were conventionally housed, free of *Mycoplasma* spp. and *Pasteurella* spp., and used throughout the study. This transgenic animal model has been described previously.[4,21,30] The mice were housed in solid-bottom polycarbonate cages with wire-grid lids (Techniplast, Varese, Italy) with wood-chip bedding (Lignocel 3/4 S, JRS, Rosenberg, Germany). Untreated 8-mm pelleted diet (SB 780, NIRDMI Cantacuzino, Bucharest, Romania) and tap water were provided ad libitum. Cages, bedding, and water bottles were changed once weekly.

Mice were assigned to 12 groups (3 mice per group per sex for each procedure) and allowed 3 d to accommodate to the new social environment. Male mice were kept in littermate groups.

At the end of the experiment the mice were not euthanized and remained available for other experiments, thereby serving the imperative of the 3Rs.[31]

Plethysmography. A single-chamber whole-body plethysmograph for mouse (model PLY 310, EMMS, Hampshire, UK) was used, and flow-derived parameters were recorded. The system was calibrated prior to each recording session. The preamplifier was zeroed and balanced, and calibration was performed by injecting 1 mL of air into the system and reading the effective range, according to the manufacturer's instructions. The box pressure signals were analyzed by using eDaq version 1.7 software (EMMS) to obtain values for tidal volume, inspiration time, expiration time, respiratory rate (no. of breaths per minute), minute volume, end-inspiratory pause, end-expiratory pause, and enhanced pause.

Mice were brought into the plethymography room 48 h before the experiments were performed and housed there for duration of the experiment. During the habituation period, each mouse was placed into the plethysmography chamber for two 10-min sessions to familiarize it with the enclosed surroundings. All of the plethysmographic measurements were performed between 0900 to 1100 and 1300 to 1500 for the 4-h recordings. Temperature interval was maintained between 19 to 21 °C.

Plethysmographic protocol. Mice were put in the plethysmography chamber by lifting the cage lid, picking up the mouse by the tail, easing the animal into the overturned superior part of the chamber, turning the lid with the mouse inside, and fixing the superior part onto the inferior part of the chamber by rotating it. Special care was taken to not trap the tail or feet of the mouse between the 2 parts of the chamber. Each mouse-containing chamber was checked before measurements were started and throughout the protocol. Breathing patterns were evaluated by measuring flow-derived parameters in 15-min sessions (5-min acclimation period and 10-min log period) at 0, 4, 24, and 48 h after completion of the procedure and comparing them to previously recorded (24 h before the procedure) baseline values.

Baseline values. Mice were put into the plethysmography chambers, and baseline values were recorded for 10 min after the 5-min acclimation period.

Postprocedure flow-derived parameters. For the 0-h measurement, mice from each group underwent their designated procedure (that is, handling, restraint, ear marking, tail clipping, or tail vein or retroorbital blood sampling) and were placed into the plethysmography chamber for 15 min. After the 10-min recording session, the mice were returned to their home cages. At the 4-, 24-, and 48-h time points, mice were put into the plethysmography chambers, and values were recorded as for the 0-h time point.

Experimental procedures. ***Handling and restraint.*** Because all of the other procedures included handling and then restraint, mice in these groups were considered to be 2 separate controls for the other procedures. For these control groups, the mouse was grasped by the tail, taken out of its home cage, and placed onto the lid of another empty cage. The mouse then was restrained by the scruff of its neck, lifted from the lid, and held in one hand firmly so that the animal could not bite or struggle. Each of these procedures lasted about 1 min.

Ear marking. This procedure was a modification of the ear-notching technique.[19] The mouse was restrained, and a 2-mm band of tissue was cut off the edge of one ear by using a pair of sharp scissors. The scissors were rinsed with distilled water after every marking. The procedure lasted about 2 min. The mice in this group had not been marked previously.

Tail clipping. The modified tail-clip technique was performed as previously described.[1] In brief, the mouse was placed on an unfamiliar surface (an overturned cage lid) and allowed to explore while the handler held the base of the tail. The distal 1 mm of the tail was clipped with a pair of sharp scissors. Gentle pressure was applied to the tail tip to stop the bleeding. The scissors were rinsed thoroughly with distilled water after every clip. The procedure lasted about 3 min.

Blood sampling from the tail vein. The mouse was eased into a restrainer (a perforated 50-mL centrifuge tube) while the handler held the base of the tail. The tail was swabbed with 70% alcohol, and the lateral tail vein was penetrated with a 25-gauge needle as for intravenous injection. A total of 3 or 4 drops of blood was allowed to drip from the needle hub. The needle was removed, and gentle pressure was applied at the puncture site. The mouse was removed from the restrainer and put in the plethysmography chamber. The procedure lasted about 4 min.

Blood sampling from the retroorbital plexus. The mouse was restrained and gently scruffed to make the eyes bulge slightly. A sterile Pasteur pipette was inserted at the internal angle of the eye and gently rotated to puncture the retroorbital sinus so that blood was allowed to flow by capillary action. The pipette was withdrawn when blood reached the end of the capillary part of the pipette to ensure a small volume of sampled blood, and gentle pressure was applied to the eye with saline-moistened sterile gauze. An antibiotic ointment was applied to the eye to prevent postprocedural infection. The mouse was put in the plethysmograph chamber and carefully supervised during the measurement. After plethysmography, the mouse was returned to its home cage and checked at 30 min and 2 h after blood sampling for recovery and signs of discomfort or complications. Because the experimental endpoint was represented by breathing parameters, the procedure was performed without the use of anesthesia to prevent interference with results. The procedure lasted approximately 5 min.

Data processing and statistical analysis. Flow-derived parameters were recorded during 10-min sessions, with a mean value for every 1-min interval. The means for flow-derived parameters at the time points analyzed were calculated for each group by using the 1-min mean values from each mouse at each time point. Baseline values were obtained by using values from all groups at baseline determination. Baseline flow-derived parameters values of male compared with female mice were analyzed by using 2-tailed paired *t* tests. Values from different procedures were compared with handling and with restraint for each sex by using 2-way ANOVA, and posthoc analysis to identify the time points at which differences occurred was performed by using Tukey Honestly Significant Differences. A 95% confidence interval was calculated, so that *P* values for ANOVA and *t* tests and adjusted *P* values (from posthoc analysis) of less than 0.05 were considered statistically significant. Statistical analysis was performed and graphs generated by using the R software package, version 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline breathing parameters. Baseline breathing parameters were recorded for each single-sex group of F1h mice before the experiment, and the respiratory values for each sex were pooled and mean values analyzed. Mean respiratory values (Table 1) show significant ($P < 0.05$) differences between sexes for most parameters except expiration time and end-expiratory pause. The greater values for tidal volume (0.39 ± 0.04 mL) and minute volume (200 ± 36 mL) recorded for male mice was in accordance

Table 1. Baseline values of respiratory parameters measured by whole-body plethysmography in F1h mice

	Female	Male
Body weight (g)	22.4 ± 1.4	27.4 ± 2.3
Tidal volume (mL)	0.37 ± 0.0[a]	0.39 ± 0.04
Inspiration time (s)	0.04 ± 0.01[a]	0.05 ± 0.01
Expiration time (s)	0.09 ± 0.03	0.09 ± 0.03
No. of breaths per minute	549 ± 7[a]	529 ± 93
Minute volume (mL)	185 ± 32[a]	200 ± 36
End inspiratory pause (s)	3.84 ± 0.17[a]	3.93 ± 0.32
End expiratory pause (s)	3.58 ± 2.55	3.32 ± 1.45
Enhanced pause	0.89 ± 0.23[a]	0.97 ± 0.19

Data are shown as mean ± 1 SD (n = 36; 18 male, 18 female). Minute volume is the volume (mL) inspired in one breath.
[a]Significant ($P < 0.05$) difference between sexes.

with body weight values (27.42 ± 2.34 g) of male compared with female mice, which were considerably smaller (22.4 ± 1.37 g). This difference is further emphasized by the decreased respiratory rate of male (529 ± 93 breaths per minute) compared with female (549 ± 79 breaths per minute) mice, evidence that corroborates data presented by other groups.[22]

We then assessed breathing parameters recorded at various intervals in the handling groups (Table 2) and compared these data with baseline values, to assess parameter reliability between experimental time points. In light of the data obtained, interindividual variation likely led to the variability observed in respiratory times and pauses at different time points; therefore, we will not address these parameters further. Among female mice, differences in breathing parameters peaked at the 4-h point, at which tidal volume reached 0.38 ± 0.06 mL ($P < 0.05$) and respiratory rate decreased to 470 ± 107 breaths per minute ($P < 0.05$) compared with baseline values. In male mice, differences started to appear at 24 h, as for tidal volume (0.41 ± 0.02 mL; $P < 0.05$) and at 48 h, as for minute volume (231.64 ± 34 mL; $P < 0.001$). Because minute volume, which represents a direct relationship between respiratory rate and tidal volume, shows the greatest homogeneity among the various parameters and different time points (except for the 48-h point in male mice), we chose this parameter to monitor the effect of the different routine experimental procedures.

Standard procedure induced changes in minute volume. Compared with handling and with restraint, routine procedures altered minute volume of F1h mice immediately and/or at 4, 24, and 48 h afterward (Figure 1). Throughout the evaluation period, sex-associated differences in minute volume were significant for handling ($F_{1,290}$ = 11.171, $P > 0.001$), restraint ($F_{1,290}$ = 5.035, $P > 0.05$), tail vein bleeding ($F_{1,290}$ = 8.152, $P > 0.01$), retroorbital bleeding ($F_{1,290}$ = 172.53, $P > 0.001$), and tail clipping ($F_{1,290}$ = 62.236, $P > 0.001$) but not for ear marking. Time point×sex interactions were significant only for handling ($F_{4,290}$ = 2.692, $P > 0.05$), restraint ($F_{4,290}$ = 11.700, $P > 0.001$), and tail vein bleeding ($F_{4,290}$ = 3.516, $P > 0.01$).

Compared with handling only, restraint significantly affected minute volume in male mice ($F_{1,290}$ = 21.554, $P < 0.001$) and to a lesser extent in female mice ($F_{1,290}$ = 9.482, $P < 0.01$). Time point×procedure interactions were significant in male ($F_{4,290}$ = 4.380, $P < 0.01$) and female ($F_{4,290}$ = 4.509, $P < 0.01$) mice, with significant ($P < 0.05$) differences only between baseline and 48 h.

Ear marking in male mice significantly changed minute volume compared with handling ($F_{1,290}$ = 8.561, $P > 0.01$) and, to a lesser extent, with restraint ($F_{1,290}$ = 4.148, $P > 0.05$; Figure 1 A). Time point×procedure interactions were significant in male mice when compared with handling ($F_{4,290}$ =7.501, $P > 0.001$) and restraint ($F_{4,290}$ = 6.789, $P < 0.001$). In female mice, routine procedures appeared to change minute volume compared with restraint ($F_{1,290}$ = 8.447, $P < 0.01$) but not handling (Figure 1 B). Time point×procedure interactions were significant ($F_{4,290}$ =4.810, $P > 0.001$) at the 48-h point ($P < 0.01$) in both male and female mice.

In both sexes of mice, tail-vein blood sampling significantly altered minute volume compared with restraint (female mice, $F_{1,290}$ = 25.888; male mice, $F_{1,290}$ = 27.121; $P < 0.001$) but not handling (Figure 1). Recording time point×procedure interactions were significant ($F_{4,290}$ =2.738, $P < 0.05$) only in male mice. Differences from the values obtained with restraint only were apparent at the 24-h recording ($P < 0.05$) in male mice and at both 0 and 48 h ($P < 0.01$) in female mice.

Retroorbital blood sampling changed minute volume in a significant manner compared with handling in both sexes (female mice: $F_{1,290}$ = 4.668, $P < 0.05$; male mice: $F_{1,290}$ = 43.830, $P < 0.001$). Compared with restraint, differences were significant only in male mice ($F_{1,290}$ = 167.850, $P < 0.001$; Figure 1). Recording time point×procedure interactions were significant only in male mice for both handling ($F_{4,290}$ =12.140, $P > 0.001$) and restraint ($F_{4,290}$ =11.520, $P > 0.001$). In male mice, differences in minute volume relative to that with handling only were apparent only at the 4 h ($P < 0.001$) and 24 h ($P < 0.01$) and compared with that due to restraint at 4, 24, and 48 h ($P < 0.001$).

Compared with restraint, tail clipping altered minute volume in both sexes (female mice: $F_{1,290}$ = 16.129, $P < 0.001$; male mice, $F_{1,290}$ = 64.266, $P > 0.001$), but compared with handling, tail clipping changed minute volume only in male mice ($F_{1,290}$ = 6.812, $P > 0.01$; Figure 1). Recording time point×procedure interactions were significant for tail clipping compared with handling in female mice ($F_{4,290}$ =10.128, $P < 0.001$), with differences ($P < 0.001$) in the 48-h recordings, and in male mice ($F_{4,290}$ =4.337, $P < 0.01$), for which posthoc analysis showed differences ($P < 0.01$) at 4 h. Compared with restraint, procedure×time point interactions were significant only in male mice ($F_{4,290}$ =4.150, $P < 0.01$) at the baseline ($P < 0.01$), 24-h ($P < 0.001$), and 48-h ($P < 0.01$) time points.

Discussion

Although procedures like handling, restraint, ear marking, caudal venipuncture, tail clipping, and retroorbital blood sampling are considered routine[2] and are used frequently, especially in transgenic mice husbandry,[5] increasing evidence has associated these procedures with stress.[2,5,26,27] Furthermore, despite the accumulating data on the stressful effects of routine procedures and reported differences in the physiology and stress response between sexes,[12,23,24] we did not find any published study that had investigated these sex-associated differences in procedure-associated stress. The effect of routine procedures has been investigated by monitoring behavioral,[1,23,36,37] somatic and physiologic,[24,29,32,37] and various biochemical parameters.[25,33,36] However, to our knowledge, no reports address these effects from the point of view of respiratory parameters. We therefore assessed the effect of routine experimental procedures used in the husbandry of a transgenic mouse colony on plethysmographic parameters.

Because handling is an unavoidable procedure, we assessed it first and subsequently compared all other procedures (including restraint) with handling. Restraint significantly altered minute volume in both sexes, but because restraint is necessary for all of the other procedures we evaluated, we considered it to be a

Table 2. Respiratory parameters in female and male F1h mice by whole-body plethysmography

		Baseline	0 h	4 h	24 h	48 h
Tidal volume (mL)						
	Female	0.36 ± 0.02	0.36 ± 0.05	0.38 ± 0.06[a]	0.37 ± 0.03	0.33 ± 0.03
	Male	0.39 ± 0.03	0.38 ± 0.06	0.37 ± 0.06	0.41 ± 0.02[a]	0.39 ± 0.05
Inspiration time (s)						
	Female	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.01[a]	0.04 ± 0.01	0.04 ± 0.00
	Male	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00[a]
Expiration time (s)						
	Female	0.09 ± 0.02	0.10 ± 0.03	0.10 ± 0.03	0.09 ± 0.02	0.08 ± 0.02[a]
	Male	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.04	0.09 ± 0.02	0.07 ± 0.01[a]
No. of breaths per minute						
	Female	534 ± 73	512 ± 90	470 ± 107[a]	538 ± 75	583 ± 61
	Male	543 ± 67	529 ± 65	475 ± 107	503 ± 60	609 ± 49[a]
Minute volume (mL)						
	Female	187 ± 32	179 ± 46	178 ± 59	193 ± 28	190 ± 28
	Male	203 ± 31	196.41 ± 40	172 ± 59	202 ± 27	232 ± 34[a]
End-inspiratory pause (s)						
	Female	3.93 ± 0.18	3.98 ± 0.20[a]	4.10 ± 0.24[a]	3.94 ± 0.19[a]	3.87 ± 0.19
	Male	4.18 ± 0.54	3.95 ± 0.42	3.99 ± 0.47	3.92 ± 0.26	3.82 ± 0.27
End-expiratory pause (s)						
	Female	3.34 ± 1.63	3.30 ± 0.82	2.48 ± 0.50[a]	2.88 ± 0.87[a]	2.66 ± 0.85[a]
	Male	4.29 ± 1.86	3.03 ± 0.97	3.27 ± 0.86	2.60 ± 0.93[a]	3.04 ± 0.90
Enhanced pause						
	Female	0.92 ± 0.27	0.90 ± 0.18	0.82 ± 0.20	0.86 ± 0.16	0.88 ± 0.19
	Male	1.17 ± 0.16	0.99 ± 0.20	0.97 ± 0.21	0.80 ± 0.11[a]	1.02 ± 0.20

[a]Significant ($P < 0.05$) compared with baseline value of same-sex mice.

control procedure. Figure 1 shows a clear decrease in minute volume with restraint compared with handling, and a tendency of minute volume values to increase relative to handling-associated values after procedures such as ear marking, tail vein bleeding, retroorbital bleeding, and tail clipping, respectively. The decreased minute volume seen with restraint correlates with a decrease in heart rate after restraint, as has occurred in other studies in mice[11,27] and rats.[20] In addition, a depressive-like state has been reported to occur in rats after repeated injection stress.[18] This effect was much more pronounced in male rats.[18] Ear marking changed minute volume in both sexes of mice as compared with restraint only, with no significant difference between male and female mice.

Compared with restraint, tail vein bleeding elicits a marked effect on breathing—even greater than that due to ear marking—in both sexes but with differences between male and female mice. Male mice were affected by tail vein bleeding until the 24-h point, with a greater effect than that in females. We consider ear marking and tail vein bleeding to be less invasive procedures than retroorbital bleeding and tail clipping, as other studies have shown. [19, 25] The effect of tail vein bleeding on minute volume likely is not solely dependent on the invasiveness of the procedure but also on the prolonged time of restraint necessary to perform the method. Similarly, retroorbital blood sampling, a documented invasive procedure,[28] led to significant changes in minute volume in our male mice for as long as 24 h after the procedure, confirming through plethysmography the stressful effect reported previously.[36] Retroorbital bleeding only affected male mice. In response to tail clipping, another known invasive procedure,[5,32] male mice showed a rise in minute volume during the 4- to 24-h interval. As seen for retroorbital bleeding, the response to tail clipping was less intense in female mice.

Our data indicate that routine procedures, depending on their invasiveness, cause a decrease in respiration in response to restraint for the duration of the procedure and then for as long as 24 h afterward. This feature differs between sexes, in that the changes in minute volume of female mice are different from those of male mice. Our results show that the effects of the procedures are manifested in a more acute and less prolonged manner in female mice than in male mice. Studies[23, 24] have shown that stress levels are greater in male than in female mice and rats, even when the male rodents are kept in littermate groups. This difference has been attributed to hierarchical competition, social defeat, and submission[23] in male rodents, whereas in female rodents, agonistic interactions are relatively infrequent.[24] Considering this information, the differences in respiratory response to routine procedures between sexes as they manifest in our results are explainable as yet another form of physiologic dimorphism.

In conclusion, our results further underscore the necessity of considering the impact of routine procedures in the experimental design and point out that the recording of respiratory values in mice should be postponed for at least 24 h after ear marking, tail vein bleeding, retroorbital bleeding, and tail clipping to prevent their interference with experimental results. Minute volume may be useful as an indirect assessment of the stress response in F1h mice and perhaps other strains of mice, with the reminder that the stress response differs between strains.[11,14,18,37] Whole-body plethysmography is a valuable tool for assessing respiratory values, but it should be used with care and consideration of the method's limitations.[7,17] Our data show that routine procedures change respiratory parameters in laboratory mice and suggest that stress responses can perhaps be investigated by plethysmography.

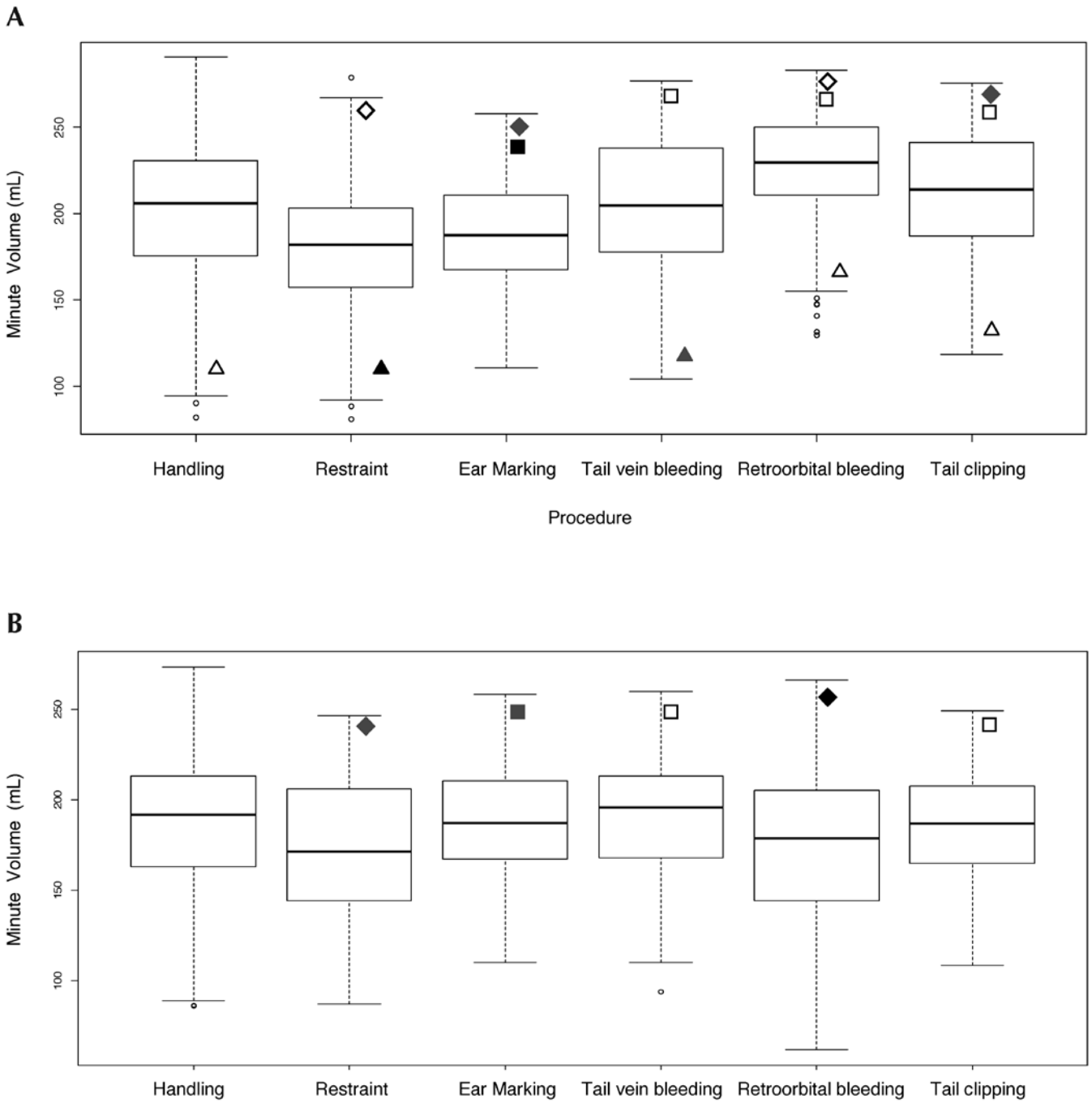

Figure 1. Boxplots of minute volumes (mL) obtained during handling, restraint, ear marking, tail vein bleeding, retroorbital bleeding, and tail clipping of (A) male and (B) female mice. Data are shown as median (horizontal line), first quartile (bottom of the box), third quartile (top of the box), 2 SD from mean (whiskers, dashed lines), and outliers (individual circles outside the whiskers). Significant (black diamond, $P < 0.05$; gray diamond, $P < 0.01$; white diamond, $P < 0.01$) difference between values for procedure and handling; significant (black square, $P < 0.05$; gray square, $P < 0.01$; white square, $P < 0.001$) difference between values for procedure and restraint; significant (black triangle, $P < 0.05$; gray triangle, $P < 0.01$; white triangle, $P < 0.001$) difference between values for procedure and sex.

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