Sex- and Strain-related Differences in the Stress Response of Mice to CO₂ Euthanasia

Michelle A Creamer-Hente,^{1,*} Fironica K Lao,¹ Zala P Dragos,² and Linda L Waterman³

The 2013 edition of the AVMA *Guidelines for the Euthanasia of Animals* recommends a 10% to 30% volume displacement rate (VDR) per minute for CO₂ euthanasia of rodents. Here we sought to evaluate behavior and plasma catecholamine levels in multiple strains of male and female mice, euthanized individually or in a group, with CO₂ VDR of 10% to 100%. Behavioral observations included ataxia, labored breathing, time to recumbency, time to surgical plane of anesthesia, and the number of jumps or paws at the face during the euthanasia process. Behaviors did not differ significantly between male and female mice at any of the VDR, but interstrain differences occurred. Slower VDR resulted in longer periods of ataxia and labored breathing regardless of euthanasia as a group or as an individual. In addition, mice jumped and pawed at the face more often with slower VDR than higher. At all VDR, mice euthanized as a group had lower catecholamine levels than mice euthanized individually, but there were no significant differences between VDR. Time to recumbency and time to surgical plane anesthesia were longer with slower displacement rates; in addition, these parameters were prolonged for mice euthanized as a group compared with individually. Overall, faster VDR do not appear to be more distressful than slower rates. In fact, faster VDR shorten the time during which mice might experience distress prior to recumbency.

Abbreviation: VDR, volume displacement rate

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CO₂ inhalation is a commonly used method of euthanasia for mice. CO₂ is relatively inexpensive, has minimal safety risks for human personnel handling the gas, and is simple to administer to small or large quantities of mice. The AVMA *Guidelines for the Euthanasia of Animals*, 2013 Edition recommends a 10% to 30% volume/min displacement rate (VDR) for euthanasia of small rodents.¹ Mice, rats, hamsters, guinea pigs, gerbils, degus, and cotton rats are grouped together under this category of small rodents however the majority of research cited with the 2013 recommended VDR was performed using rats.¹ Mice are the most widely used animal in research and may behave differently than rats during exposure to CO₂ for euthanasia.¹⁸

CO₂ gas forms carbonic acid when exposed to moisture and can irritate the nasal mucosa and cause pain when it is inhaled.¹⁷ Behavioral indicators of pain or aversion and distress to inhaled CO₂ may include pawing at the nose and face and escape behaviors such as jumping.^{9,22} High and low concentrations of CO₂ cause labored breathing which is distressing in humans and may also be distressing in mice.⁹ Distress is defined as an aversive, negative state in which coping and adaptation processes fail to return an organism to physiologic or psychologic homeostasis.¹⁵ The body responds to acute stress through the sympathetic-adrenal-medullary system and the HPA axis. These pathways produce different hormone responses and include norepinephrine and epinephrine (sympathetic-adrenal-medullary system) as well as corticotropin-releasing hormone, adrenocorticotrophic hormone, and corticosterone (HPA axis).

Euthanasia should be performed in a manner that minimizes or eliminates pain or distress.¹ Although it may be impossible to eliminate all sources of pain or distress during euthanasia, all efforts to minimize pain and distress should be considered when selecting a euthanasia method. The potential for pain and distress associated with a euthanasia method can only occur during a conscious state. Determining the point of unconsciousness in animals is difficult.³ In the current study, we equated the loss of consciousness to the point of recumbency when mice ceased all forward movement and all 4 feet and the chin resting were on the cage floor. The overall aim of this study was to investigate multiple VDR of CO₂ euthanasia and their effects on mouse behavior and the stress response. We hypothesized that slower VDR would result in greater distress than faster VDR. Mice of multiple strains and both sexes were used to provide additional information regarding strain- or sex-associated differences in response to CO₂ euthanasia.

Materials and Methods

Mice. All animal care and use for this study were performed in accordance with a protocol reviewed and approved by the IACUC at The Jackson Laboratory (Sacramento, CA), an AAALAC-accredited animal program.

We evaluated 4 strains (NSG, NU/J, BALB/cJ, and C57BL/6J) of experimentally naïve male and female mice (age, 6 to 10 wk; The Jackson Laboratory). All animals were negative for the following pathogens and opportunists: ectromelia virus, Theiler mouse encephalomyelitis virus, Hantaan virus, K virus, lactic dehydrogenase elevating virus, lymphocytic choriomeningitis virus, murine adenovirus, murine cytomegalovirus, murine hepatitis virus, murine minute virus, murine parvovirus, murine thymic virus, murine norovirus, pneumonia virus of mice, polyoma virus, reovirus 3, rotavirus, Sendai virus, *Bordetella*

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¹Comparative Medicine and Quality and ²Animal Husbandry and Performance, The Jackson Laboratory, Sacramento, California; and ³Comparative Medicine and Quality, The Jackson Laboratory, Bar Harbor, Maine

*Corresponding author. Email: michelle.hente@gmail.com

spp., *CAR* bacillus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Corynebacterium bovis*, *Pasturella pneumotropica*, *Helicobacter* spp., β -hemolytic *Streptococcus* spp., *Klebsiella* spp., *Pneumocystis murina*, *Pseudomonas* spp., *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus moniliformis*, *Encephalitozoon cuniculi*, fleas, fur mites, lice, follicle mites, pinworms, roundworms and other helminths, tapeworms, and opportunistic protozoa (for example, *Giardia*, *Spiroplasma*). Mice were group-housed on individually ventilated racks (Thoren Caging Systems, Hazleton, PA) supplied with 60 air changes hourly. Microenvironmental conditions were kept at 69 °F (20.6 °C) and 45% to 55% relative humidity. Mice were housed in steam-sterilized polysulfone caging (Thoren Caging Systems) on bedding composed of steam-sterilized aspen wood chip (Nepco, East Providence, RI) and shavings (PJ Murphy, Montville, NJ), provided unrestricted access to steam-sterilized rodent chow (LabDiet JL 6% 5K0Q, Purina, St Louis, MO) and steam-sterilized, filtered, and acidified water. The combination of aspen chips and shavings provides mice an opportunity to gather the shavings and incorporate them into a nest. Additional environmental enrichment in the cages included tunnels (Bio-Tunnels, Bio-Serv, Flemington, NJ) or domes (Bio Domes, Shepherd Specialty Papers, Watertown, TN) or both. Mice were kept under a 14:10-h light:dark cycle, and euthanasia took place during the same 3 h window each day (1200 to 1500). Mice were transported to the euthanasia station, which was located in a procedure room, and acclimated to the room for approximately 3 h before euthanasia.

Euthanasia of individual mice. Euthanasia was performed by using an inhouse-designed euthanasia system at VDR of 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. A programmable logic controller (Barry-Wehmiller Design Group, Roseville, CA) was used to automatically set the desired flow rate for CO₂ in a standardized, empty euthanasia chamber (model 2, Weaning Cage, Thoren Caging Systems) that measured 112.9 in.² The flow rate was regulated by a mass flow controller (Sierra Instruments, Monterey, CA), and CO₂ was directed into the top of the euthanasia chamber. Weep holes were drilled into the euthanasia chamber lid to allow room air to escape as CO₂ filled the chamber. We used 100% compressed CO₂ gas to euthanize mice. After euthanasia, the chamber was cleaned with 70% ethanol and allowed to dry. For each VDR, strain, and sex, 10 mice were euthanized individually or as a group.

Each euthanasia trial was video recorded by using a high-definition video camera (model CX560V, Sony, Tokyo, Japan). Videos were scored for onset of ataxia, labored breathing, recumbency, surgical plane of anesthesia, the number of observed jumps, and pawing at the face (Figure 1). One person, who was blinded to the VDR, scored all of the videos.

Pilot studies (data not shown) were conducted to determine the average time to reach a surgical plane of anesthesia after recumbency to minimize the number of times the investigator would need to test for a response to a noxious stimulus, thus minimizing the risk of artificially manipulating the catecholamine response from the tail pinch. Once a mouse reached recumbency, the investigator waited 10 s (determined from pilot data) before clamping the base of the tail with 9-in. intestinal Doyen hemostatic forceps (Skylar Surgical Instruments, West Chester, PA) inserted through a weep hole in the euthanasia chamber lid to test for a response to a noxious stimulus (Figure 2).

The hemostatic forceps contained 3 notches that provide increasing pressure on clamped materials. With all tests, the first

notch was clamped to provide a standardized level of pressure during noxious stimulus testing. If a mouse responded to the noxious stimulus with purposeful movement, the investigator waited an additional 10 s prior to clamping again. The time to surgical plane of anesthesia was recorded when a mouse did not respond to 2 clamps in succession.

Blood collection and catecholamine analysis. Once mice reached a surgical plane of anesthesia, 500 μ L of whole blood was collected by using cardiocentesis, mixed with EGTA, and processed to plasma. After blood collection, cervical dislocation was performed to ensure death. Plasma was stored at -80 °C until it was analyzed to evaluate catecholamine levels (norepinephrine and epinephrine). Catecholamine levels were determined by using HPLC (Hormone Assay and Analytical Services Core, Vanderbilt University, Nashville, TN; supported by NIH grants DK059637 and DK020593).

Group euthanasia. Group euthanasia was performed similarly as described earlier for the individual method. For group euthanasia, 10 mice of the same strain or sex were placed together in the euthanasia chamber and euthanized at the appropriate VDR. Loss of response to noxious stimulus was tested on 2 of the 10 mice in rapid succession prior to collecting blood. The time to surgical plane of anesthesia was recorded relative to the time measured from the noxious stimulus test on the second mouse. Blood was collected from all mice simultaneously by using decapitation and processed as described earlier. Catecholamine levels were averaged across all mice in each cage. Pawing at the face and jumping behaviors were recorded on individual mice in each group and averaged across the group. Ataxia and labored breathing behaviors were averaged across the group.

CO₂ concentration. A CO₂ gas data logger (model no. CM-003, CO₂ Meter, Ormond Beach, FL) was placed in the euthanasia chamber without mice and a baseline level of CO₂ in atmospheric air was recorded. At each VDR (without mice), the percentage concentration of CO₂ was measured at 2-s intervals for 2 min. The time course of CO₂ concentration was correlated with the average time to recumbency and the average onset of labored breathing.

Statistics. All statistical tests were performed in Prism 6 (GraphPad Software, La Jolla, CA) for comparison of the means between VDR by using 1-way or 2-way ANOVA. Interactions between sex and strain were evaluated also. Where main effects were significant, posthoc comparisons were performed by using Tukey analysis. Data are presented as mean $\pm$ SEM, and differences were considered significant at a *P* value less than 0.05.

Results

Behavior. Behavior did not differ significantly ($F_{11,960} = 0.92$, $P = 0.51$) between male and female mice at any of the VDR. Although no single strain showed widespread differences in all behaviors, individual behaviors differed between specific strains. NU/J mice had significantly ($F_{3,960} = 87.43$, $P < 0.01$) shorter periods of ataxia for all VDR compared with the other 3 strains (Figure 3). BALB/cJ mice had significantly ($F_{3,960} = 148.1$, $P < 0.01$) longer periods of labored breathing at all VDR compared with the other 3 strains (Figure 4). C57BL/6J mice reached a surgical plane of anesthesia significantly ($F_{3,960} = 97.19$, $P < 0.01$) faster than all other strains at VDR 10% through 30% except 25%, for which all strains were similar (Figure 5), as they were for all VDR greater than 30%. In addition, C57BL/6J mice also became recumbent faster ($F_{3,960} = 100.6$, $P < 0.01$) than all other strains for VDR of 10% through 30% and 70% (Figure 6). There were no significant differences between the strains at the remaining VDR.

Behavior	Definition
Recumbency	Cessation of forward movement, with all four feet and the head resting on the floor of the chamber.
Surgical plane of anesthesia	Lack of purposeful movement in response to a pinch on the base of the tail.
Jump	Rapid upward movement towards the top of the euthanasia chamber with all four feet leaving the floor.
Ataxia	Presence of abnormal, uncoordinated, staggering movements.
Labored breathing	Increased or difficulty breathing.
Paws at face	Rapid wiping of the muzzle with both front paws.

Figure 1. Description of behaviors evaluated within this study.

Figure 2. Sketch of euthanasia chamber schematic and testing of surgical plane of anesthesia by using a noxious stimulus (hemostatic forceps).

Figure 3. Duration (s; mean $\pm$ SEM) of ataxia in mice grouped by strain and VDR. *, $P < 0.05$.

Figure 4. Duration (s; mean $\pm$ SEM) of labored breathing in mice grouped by strain and VDR. *, $P < 0.05$.

When comparing all mice euthanized individually, 10% and 70% VDR caused significantly ($F_{11,960} = 4.87$, $P < 0.01$) longer periods of ataxia than all other VDR (Figure 7). Mice euthanized as a group experienced significantly ($F_{11,120} = 6.761$, $P < 0.01$) longer periods of ataxia at 10% and 15% VDR (Figure 8). Mice

Figure 5. Time (s; mean $\pm$ SEM) until surgical plane of anesthesia in mice grouped by strain and VDR.

Figure 6. Time (s; mean $\pm$ SEM) until recumbency in mice grouped by strain and VDR.

euthanized individually (Figure 9) or as a group (Figure 10) experienced significantly ($F_{11,960} = 32.64$, $P < 0.01$ and $F_{11,120} = 11.88$, respectively; $P < 0.01$ for both) longer periods of labored breathing at VDR of 15% or less and 20% or less, respectively. Group-euthanized mice took longer to reach recumbency and a surgical plane of anesthesia than individually euthanized mice (Table 1). In addition, compared with individually euthanized mice, group-euthanized mice experienced significantly ($F_{11,960} = 14.97$, $P < 0.01$ and $F_{11,960} = 9.708$, respectively; $P < 0.01$ for both) longer periods of labored breathing at a 15% VDR and ataxia at 10%, 15%, 25%, and 30% VDR (Table 2).

Mice euthanized individually at a 10% VDR pawed at the face and jumped significantly ($F_{11,960} = 3.853$, $P < 0.01$) more than at all other VDR (Figures 11 and 12). Mice euthanized as a group did not show increased pawing or jumping at any VDR ($F_{11,120} = 0.7883$, $P = 0.65$; Figures 13 and 14).

Plasma catecholamine concentrations. Mice euthanized as a group had lower plasma norepinephrine ($F_{11,960} = 2.424$, $P < 0.01$) and epinephrine ($F_{11,960} = 1.889$, $P < 0.01$) levels at all VDR than mice euthanized as individuals (Figures 15 and 16). However, catecholamine levels did not differ ($F_{11,960} = 1.394$, $P = 0.1688$) between VDR. In addition, all VDR elicited

Figure 7. Duration (s; mean $\pm$ SEM) of ataxia in individually euthanized mice. *, $P < 0.05$.

Figure 9. Duration (s; mean $\pm$ SEM) of labored breathing in individually euthanized mice. *, $P < 0.05$.

Figure 8. Duration (s; mean $\pm$ SEM) of ataxia in group-euthanized mice. *, $P < 0.05$.

Figure 10. Duration (s; mean $\pm$ SEM) of labored breathing in group-euthanized mice. *, $P < 0.05$.

marked catecholamine responses, which did not differ ($F_{1,960} = 0.9786$, $P = 0.32$) between male and female mice at any VDR. However, in general, NSG and BALB/cJ mice had higher catecholamine levels at most VDR than did the C57BL/6J and NU/J strains.

CO₂ concentration. Individually euthanized mice began exhibiting signs of labored breathing when CO₂ concentrations averaged $7.7\% \pm 0.4\%$ (range, 6% to 10%) across all VDR. Group-euthanized mice exhibited signs of labored breathing when CO₂ concentrations averaged $5.7 \pm 0.5\%$ (range, 3.4% to 10.0%) across all VDR. Mice became recumbent at CO₂ concentrations ranging from 25% to 34% for individually euthanized mice and 30% to 44% for group-euthanized mice (Table 3).

Discussion

In this study, we used a combination of behavior assessments and biochemical markers of stress to evaluate VDR of CO₂ euthanasia. All VDR produced marked catecholamine responses, but catecholamine levels did not differ between slow and fast fill rates for euthanasia. This result suggests that fast VDR are no more distressing than slow VDR when catecholamine

response is assessed as the sole marker of stress. The similar catecholamine response to all VDR may be due to the body's natural reaction to rising CO₂ levels in the blood. Central and peripheral chemoreceptors for CO₂ can drive an increase in respiration when elevated pCO₂, hypercapnia, and metabolic acidosis are detected in the blood.¹⁶ Hypercapnia causes a marked increase in the firing rate of central norepinephrine neurons in the locus coeruleus and activates the systemic nervous system.¹⁶ However, this reaction is only part of the equation, because significant differences between individually and group euthanized mice occurred. This finding suggests that the stress response to CO₂ is tapered with social buffering, a phenomenon found in multiple species in which conspecifics show a better recovery from adverse events experienced as a group than individually.^{5,6,8,12,13,21} During highly stressful events, social species can display an isolation syndrome, in which an isolated animal shows an exaggerated stress response to a variety of stimuli.¹¹

Hormones can be measured to evaluate the stress response, but it is important to consider the time needed for a hormone to reach peak levels in the blood.^{8,10} The timing of the stressor, sample collection, and peak levels should all be considered

Table 1. Mean times (s) until recumbency and a surgical plane of anesthesia in mice ($n = 10$ per VDR) euthanized individually and as a group

VDR (%)	Individual euthanasia		Group euthanasia	
	Time to recumbency (s)	Time to anesthesia (s)	Time to recumbency (s)	Time to anestjsoa (s)
10	57.4	72.8	72.5	91.1 ^a
15	49.3	64.8	66.1 ^a	88.6 ^a
20	43.9	59.3	48.3	65.8
25	39.4	55.8	46.5 ^a	64.5 ^a
30	37.7	53.7	45.8 ^a	63.5 ^a
40	35.5	51.5	39.0	56.63
50	34.6	50.3	38.4	57.38
60	36.4	52.5	35.6	55.25
70	37.1	53.2	38.3	57.00
80	35.2	50.9	34.5	52.38
90	33.9	49.4	35.1	53.00
100	32.8	48.4	35.5	53.25

^aSignificant ($P < 0.05$) difference between values for individual and group euthanasia, by parameter.

Table 2. Mean durations (s) of ataxia and labored breathing in mice ($n = 10$ per VDR) euthanized individually or as a group

VDR (%)	Individual		Group	
	Ataxia (s)	Labored breathing (s)	Ataxia (s)	Labored breathing (s)
10	17.0	25.0	28.1 ^a	39.1
15	14.7	21.0	30.9 ^a	40.5 ^a
20	15.2	19.9	18.9	26.1
25	14.9	17.8	21.0 ^a	24.8
30	14.0	16.5	20.3 ^a	25.8
40	14.0	16.3	16.8	20.9
50	14.4	15.8	16.9	20.5
60	15.4	18.0	15.4	18.9
70	16.4	17.3	16.5	20.9
80	14.7	16.1	14.6	18.9
90	14.0	16.2	15.4	20.6
100	14.0	15.7	15.4	19.6

^aSignificant ($P < 0.05$) difference between values for individual and group euthanasia, by parameter.

Figure 11. Number of times (mean $\pm$ SEM) individually euthanized mice pawed at the face during the euthanasia process. *, $P < 0.05$. **Figure 12.** Number of times (mean $\pm$ SEM) individually euthanized mice jumped during the euthanasia process. *, $P < 0.05$.

when evaluating the stress response. Corticosterone can take several minutes to reach maximal levels, whereas ACTH and catecholamine levels peak within seconds after a stressor.^{4,7,19,20}

Given the wide range of VDR in the current study, we chose to assess catecholamines as the biochemical marker of stress to ensure that peak levels would be reached in all groups by the time blood was collected.

Figure 13. Number of times (mean ± SEM) group-euthanized mice pawed at the face during the euthanasia process.

Figure 14. Number of times (mean ± SEM) group-euthanized mice jumped during the euthanasia process.

Figure 15. Plasma norepinephrine concentration (mean ± SEM) in individually or group-euthanized mice. *, $P < 0.05$.

Behaviorally, mice exhibited more signs of pain or distress, such as pawing at the face, jumping, and an increased period of labored breathing, with slow VDR ranging from 10% to 20%. Because behavior and biochemical responses were similar be-

Figure 16. Plasma epinephrine concentration (mean ± SEM) in individually or group-euthanized mice. *, $P < 0.05$.

tween sexes, male and female mice can be euthanized by CO_2 in the same manner. Individual strain-associated differences emerged in the behaviors we evaluated, and future studies using multiple strains should ensure that an equal number of animals of each strain is incorporated into study groups to avoid strain-associated bias. Comparisons between mice euthanized individually and those euthanized as a group showed that, at slower VDR, group-euthanized mice spent more time exhibiting labored breathing and ataxia than did individually euthanized animals. However, VDR above 30% did not produce any additional adverse behavioral responses. Perhaps the increased labored breathing and ataxia were due to more mice competing for the remaining oxygen within the euthanasia chamber. Although the CO_2 concentrations at the time of recumbency for fast VDR were generally higher than slower VDR, the increased time to reach recumbency with slow VDR meant that mice were exposed to aversive levels of CO_2 for a longer period of time overall.

Our findings are consistent with recent literature (published after the AVMA updated euthanasia guidelines in 2013) regarding slow compared with fast VDR for CO_2 euthanasia of rats or mice. One group concluded that low VDR had the potential to increase the distress experienced by rats, whereas moderate to high VDR minimized the potential distress experienced by rats by shortening the time they were exposed to concentrations of CO_2 that are known to induce panic and anxiety.⁹ The CO_2 concentrations at which mice began exhibiting labored breathing in our current study are similar to levels reported in the literature to be aversive to rodents.⁹ Although the concentration of CO_2 with slower VDR was lower than reported painful concentrations (40%),¹⁴ significantly more pawing at the face and jumping occurred at a 10% VDR. This finding suggests that the 10% VDR may not cause pain but is aversive and thus distressful. Another study in mice found that a VDR of 50% reduced the time mice experienced labored breathing compared with a VDR of 20%.¹⁴ Another group determined that a 50 or 100% VDR is no more painful or distressful than a 15% or 30% VDR and recommended the faster VDR as an acceptable method of euthanasia for mice.² In our current study, mice euthanized as a group at a 100% VDR were exposed to potentially painful CO_2 concentrations exceeding 40% prior to reaching recumbency, but we noted no behavioral indications that the 100% VDR was more distressing than slower VDR.

Our study suggests that a 30% VDR is the most ideal according to the current AVMA Guidelines for Euthanasia, given that it produces fewer behavioral signs of pain or distress than 10% to 20% VDR. However, higher VDR for CO_2 euthanasia with mice should be considered acceptable also, because no animal welfare

Table 3. Mean CO₂ concentration (%) at various time points during euthanasia

VDR (%)	Individual euthanasia (n=10)		Group euthanasia (n=10)	
	%CO ₂ at onset of labored breathing	%CO ₂ at recumbency	%CO ₂ at onset of labored breathing	%CO ₂ at recumbency
10	8.9	31.3	10.0	38.0
15	6.2	26.5	4.0	37.5
20	10.2	31.1	7.9	35.1
25	6.3	27.2	5.6	33.9
30	7.9	26.4	5.5	33.7
40	6.8	25.8	4.3	29.8
50	7.3	25.3	5.6	30.7
60	8.3	31.1	5.0	30.0
70	7.7	32.4	4.4	37.2
80	8.3	34.5	5.2	32.3
90	5.7	31.6	3.4	33.8
100	9.3	37.6	7.0	44.0

concerns occurred with these VDR. Furthermore, the very slow VDR of 10% to 20% should be considered for removal from the euthanasia recommendations, in view of animal welfare concerns associated with increased levels of distress.

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