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Evaluation of Best Practices for the Euthanasia of Zebra Finches (*Taeniopygia guttata*)

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Although zebra finches (*Taeniopygia guttata*) have been used in biomedical research for many years, no published reports are available about euthanizing these small birds. In this study, we compared 5 methods for zebra finch euthanasia: sodium pentobarbital (NaP) given intracoelomically with physical restraint but no anesthesia; isoflurane anesthesia followed by intracoelomic injection of NaP; and CO_2 asphyxiation at 20%, 40%, and 80% chamber displacement rates (percentage of chamber volume per minute). Birds undergoing euthanasia were videorecorded and scored by 2 observers for behaviors potentially related to discomfort or distress. Time to recumbency and time until respiratory arrest (RA) were also assessed. RA was achieved faster by using NaP in a conscious bird compared to using isoflurane anesthesia followed by NaP; however, neither method caused behaviors that might affect animal welfare, such as open-mouth breathing, to any appreciable extent. Among the CO_2 treatment groups, there was an inverse correlation between the chamber displacement rate used and the duration of open-mouth breathing, onset of head retroflexion, and time to RA. The results demonstrate that the intracoelomic administration of NaP in an awake, restrained zebra finch is a rapid and effective method of euthanasia. If CO_2 is used to euthanize these birds, a high displacement rate (for example, 80%) will minimize the duration of the procedure and associated behaviors.

Abbreviations: MSA, Measurement System Analysis; NaP, sodium pentobarbital; OMB, open-mouth breathing; RA, respiratory arrest

Procedures for the euthanasia of laboratory animals are of utmost importance to animal welfare and are the subject of ongoing research, especially in rodents. For example, different CO_2 displacement rates,[2,8,10] mixing CO_2 with other gases,[13] and the value of inhalant anesthesia prior to CO_2[10,14] have recently been examined in mice. In contrast, the euthanasia of zebra finches (*Taeniopygia guttata*), a songbird used in neuroscience and other areas of biomedical research,[9] has not been investigated. Recommendations for avian species in the *AVMA Guidelines for the Euthanasia of Animals, 2013 edition*[1] are primarily based on experiences with the poultry industry and in wildlife. Although CO_2 asphyxiation of birds is acceptable according to the *AVMA Guidelines*, earlier publications focused on mixtures of CO_2 and inert gases, and an optimal CO_2-only displacement rate in small birds has not been established. In addition, birds are highly sensitive to a number of aerosolized agents, with one report indicating that chickens exhibit headshaking and open-mouth breathing (OMB) at CO_2 concentrations as low as 10%.[7] In the avian section of the *AVMA Guidelines*, intracoelomic sodium pentobarbital (NaP) is approved only when birds are unconscious or anesthetized. However, with the permission of our IACUC, we have administered NaP intracoelomically to well-restrained, awake zebra finches for several years; the method appears to be humane as long as the injection does not enter the air sacs.

To evaluate various methods of euthanasia in individual zebra finches, we compared intracoelomic NaP in conscious birds, isoflurane anesthesia prior to intracoelomic NaP, and CO_2 asphyxiation at 3 different chamber displacement rates (20%, 40%, and 80% chamber volume per minute). Videorecordings of the birds undergoing euthanasia were examined for behaviors that might indicate discomfort or distress and to determine the onsets of recumbency and respiratory arrest (RA).

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Materials and Methods

Animals. Adult zebra finches (n = 51; 20 male, 31 female) were included in the study; the birds had good body condition scores (2/5 or greater), were without clinical signs of respiratory disease or any coelomic mass, and were scheduled for culling as part of routine colony management. The finch colony is maintained in an AAALAC-accredited facility under a 12:12 light:dark cycle, temperatures between 73 to 77 °F (22.8 to 25.0 °C), and ambient humidity set at 30% to 70%. A millet–canary grass seed–oat mix fortified with amino acids and vitamins (Supreme Fortified Daily Diet: Finch, Kaytee Products, Chilton, WI) constitutes the main diet, with a high-protein supplement (High Potency Mash, Harrison's Bird Foods, HBD International, Brentwood, TN, moistened and mixed with minced hard-boiled eggs) fed several times each week; fresh water is provided without restriction. Finches are group-housed with same-sex conspecifics in flight cages (maximum, 20 birds) or in breeding cages (parents and offspring or 4 compatible adults). Health monitoring for ectoparasites and enteric pathogens is performed semiannually. All animal work was approved by the Massachusetts Institute of Technology's IACUC.

Experimental procedures. Birds were assigned to 1 of 5 experimental groups using block randomization:[12] conscious NaP (9 males, 1 female), unconscious NaP (1 male, 9 females), and CO_2 asphyxiation at a displacement rate (percentage of chamber volume per minute) of 20% (4 males, 7 females), 40% (2 males, 8 females), and 80% (4 males, 6 females). An IMPAC 6 machine (VetEquip, Livermore, CA), which was configured for both CO_2 and isoflurane, was used with a 2-L induction chamber (VetEquip) for the NaP treatment groups, whereas a polysulfone

Figure 1. Intracoelomic injection of sodium pentobarbital in a zebra finch.

Behavior or event	Definition (parameter evaluated)
Headshake	Quick side-to-side movement of head (duration)
Open-mouth breathing	Beak wide open during inspiration and expiration (duration; Figure 3)
Wing flapping	Wing movement with full extension of both wings (duration)
Total wing movement	Any wing movement (duration)
Tail bob	Rhythmic tail movement with every breath (duration)
Head retroflexion	Tilting of the head backward (time to onset; Figure 3)
Recumbency	Positioning of the body on the enclosure floor (time to onset)
Respiratory arrest	Cessation of respiration involving the keel (time to onset)

Figure 2. Definitions of behaviors and events evaluated during the euthanasia of zebra finches.

standard mouse-cage bottom (Ancare, Bellmore, NY) was used for the birds receiving CO_2. Both containers were positioned to ensure a consistent angle and distance from the video camera (Hero3+ Silver, GoPro, Riverside, CA). Videorecording started at the beginning of each procedure and was continued for a minimum of 10 s after cessation of respiratory movements.

Intracoelomic injection of 0.05 mL (19.5 mg) NaP (Fatal-Plus Solution, Vortech Pharmaceuticals, Dearborn, MI) was performed by using a ½-mL tuberculin syringe with a 28-gauge, 0.5-in. needle (Figure 1). The needle was inserted 6 mm intracoelomically at a 45° angle, on the midline and 5 mm cranial to the vent; before injection, the plunger was aspirated slightly to check for coelomic contents. Immediately thereafter, the bird was placed into the induction chamber. Birds in the unconscious NaP group underwent induction with 4% isoflurane in oxygen at a rate of 1 L/min until recumbency, followed by intracoelomic NaP and placement back into the induction chamber. The isoflurane induction chamber was flushed with oxygen for 10 s and cleaned between birds. For all CO_2 groups, clean mouse-cage bottoms were used to ensure that the chamber was not prefilled from the previous procedure.

Behavioral scoring. Two experienced observers evaluated the videorecordings of all euthanasia procedures for the presence and duration of various behaviors that might represent distress (headshaking, OMB, wing flapping, total wing movement, tail bob, head retroflexion) and for the times to recumbency and RA (Figures 2 and 3). For the purposes of this study, time to recumbency was taken to indicate loss of consciousness, and time of RA was taken to represent the time of death. Although it was not feasible to blind observers regarding which birds received the NaP treatments, the observers were blinded to the different CO_2 chamber displacement rates.

Statistical analysis. To assess consistency between the 2 observers, we tested whether their scores of the videorecordings were correlated with each other. For each behavior and event noted, agreement between observers was ranked on a scale from low (0.0) to high (1.0) by using Measurement Systems Analysis

Figure 3. A male zebra finch displaying open-mouth breathing and head retroflexion.

(MSA) implemented in JMP Pro 12.2.0. (SAS Institute, Cary, NC). Only behaviors and events that were highly correlated between observers (scores of 0.8 to 1.0) were included in further analyses.

A standard least-squares regression was used to evaluate significant differences in the onset of recumbency because of its parametric distribution, as determined by the Shapiro–Wilk normality test. In the standard least-squares regression, the euthanasia treatment was treated as an independent variable, the timing of recumbency was treated as a dependent variable, and observer and animal were treated as random effects. In cases where a significant difference between treatments was detected, Tukey posthoc testing of pairwise comparisons was performed. The remaining behaviors and events (headshake, OMB, wing flapping, total wing movement, tail bob, head retroflexion, and RA) were distributed nonparametrically, and Kruskal–Wallis testing was applied to those with high MSA scores that were

Table 1. Summary of experimental groups and Measurement Systems Analysis scores of 0.8 to 1.0 (indicating high correlation between observers)

	Conscious NaP	Unconscious NaP	20% CO_2	40% CO_2	80% CO_2
Headshake			0.8–1.0	0.8–1.0	0.8–1.0
Open-mouth breathing	0.8–1.0		0.8–1.0	0.8–1.0	0.8-1.0
Wing flapping	0.8–1.0	0.8–1.0		0.8–1.0	
Total wing movement		0.8–1.0			0.8–1.0
Tail bob					
Head retroflexion	0.8–1.0	0.8–1.0	0.8–1.0	0.8–1.0	0.8–1.0
Recumbency			0.8–1.0	0.8–1.0	
Respiratory arrest	0.8–1.0	0.8–1.0	0.8–1.0	0.8–1.0	0.8–1.0

analyzed further. Dunn posthoc testing was used for pairwise comparisons. We excluded 6 of the 51 videos from MSA and additional analysis for OMB, head retroflexion, and RA because one or both observers commented that the bird's position in the chamber made it impossible to determine when respiratory movements ended. A *P* value of less than 0.05 was considered statistically significant.

Results

Interobserver correlation was high for the majority of behaviors and events (Table 1); in particular, the observers agreed on 4 of the 5 or all 5 of the treatment groups with regard to OMB, head retroflexion, and RA. The duration of headshaking, wing flapping, and total wing movement and the time when recumbency occurred were investigated for a subset of experimental groups. The MSA scores for tail bob-behavior were below the threshold for additional consideration and analysis.

The duration of OMB (Figure 4) and the onset time of head retroflexion (Figure 5) differed between euthanasia methods. Administration of NaP to conscious birds was associated with significantly ($P < 0.001$) less OMB than were 20% and 40% CO_2. Overall the 20% CO_2 group (median, 137 s; range, 118 to 171 s) had the longest duration of OMB, followed by 40% CO_2 (median, 89 s; range, 36 to 102 s) and then 80% CO_2 (median, 55 s; range, 50 to 66 s). In addition, the duration of OMB differed significantly between the 20% CO_2 group and the groups receiving 40% CO_2 ($P < 0.01$) or 80% CO_2 ($P < 0.001$). Head retroflexion (Figure 5) occurred only in the CO_2 treatment groups and its presence differed significantly ($P < 0.001$) between the 20% and 80% CO_2 displacement rates. The 80% CO_2 group had the earliest onset of head retroflexion (median, 28 s; range, 0 to 34 s), followed by the 40% CO_2 (median, 45 s; range, 0 to 57 s) and 20% CO_2 (median, 55 s; range, 42 to 70 s) groups.

All the euthanasia methods were successful in achieving RA (Figure 6), which was reached most rapidly (median, 60 s; range, 55 to 70 s) by using an 80% CO_2 displacement rate. The time to onset of RA for 80% CO_2 was faster ($P < 0.001$) than for conscious NaP (median, 122 s; range, 86 to 191 s), unconscious NaP (median, 334 s; range, 214 to 432 s), and 20% CO_2 (median, 147 s; range, 126 to 187 s) but not for 40% CO_2 (median, 98 s; range, 67 to 122 s). Except for 20% CO_2, unconscious NaP took significantly ($P < 0.001$) longer to achieve RA than any other euthanasia method.

Headshaking was evaluated in and did not differ significantly between all of the CO_2 euthanasia groups ($P = 0.50$). Wing flap duration was not significantly different for conscious NaP, unconscious NaP, or 40% CO_2, the only experimental groups that had high MSA scores for this behavior. The duration of total wing movement was longer in the unconscious NaP group (median, 16 s; range, 1 to 91 s) than the 80% CO_2 group (median, 5 s; range, 0 to 20 s; $P = 0.02$), but this difference disappeared

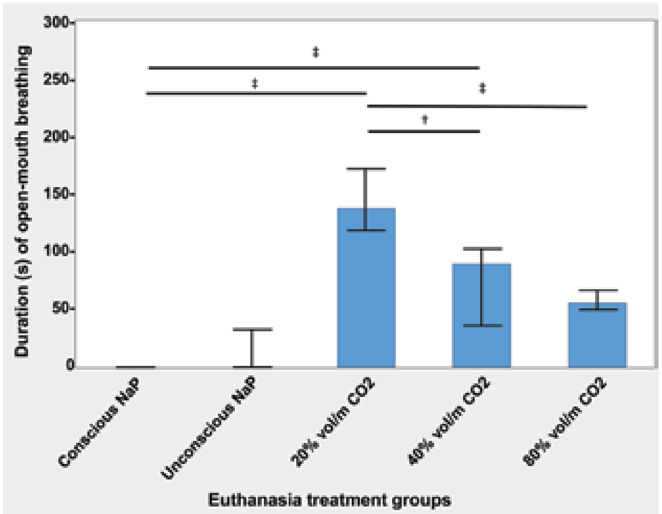

Figure 4. Median duration (s; bar, range) of open-mouth breathing in all 5 treatment groups. Kruskal–Wallis analysis with Dunn posthoc test; †, $P < 0.01$; ‡, $P < 0.001$.

($P = 1.00$) when the data were normalized to the length of the procedure, that is, time of RA. Time to recumbency was analyzed for 2 of the 5 treatment groups; the results indicated that recumbency was delayed with the 20% CO_2 displacement rate (mean, 80 s; range, 55 to 133 s) compared with 40% CO_2 (mean, 54 s; range, 41 to 65 s; $P < 0.001$).

Discussion

When conscious zebra finches were held and injected intracoelomically with NaP, RA was achieved much faster than when NaP was used after sedation with isoflurane. In addition to isoflurane–NaP taking longer (334 s) to achieve euthanasia than NaP alone (122 s), birds placed in the isoflurane chamber for sedation hit the walls as they tried to escape; wing flapping and total wing movement were recorded during this time. Furthermore, some of the birds given isoflurane and NaP exhibited OMB. Conversely, with intracoelomic NaP in a restrained bird, the only unusual activity that occurred was some limited wing flapping when a few birds were placed in the induction chamber immediately after injection. At our institution, the operator holds the zebra finch until its respirations cease, so that even wing flapping is absent. Indeed the most stressful step with euthanasia using intracoelomic NaP in an awake, healthy bird is arguably its capture from a holding cage. As is always the case, training personnel to perform euthanasia correctly is paramount, particularly with intracoelomic NaP in small birds owing to the caudal extent of their air sacs. However, given this requirement for appropriate and specific training for intracoelomic injection of NaP, the procedure is comparable to using

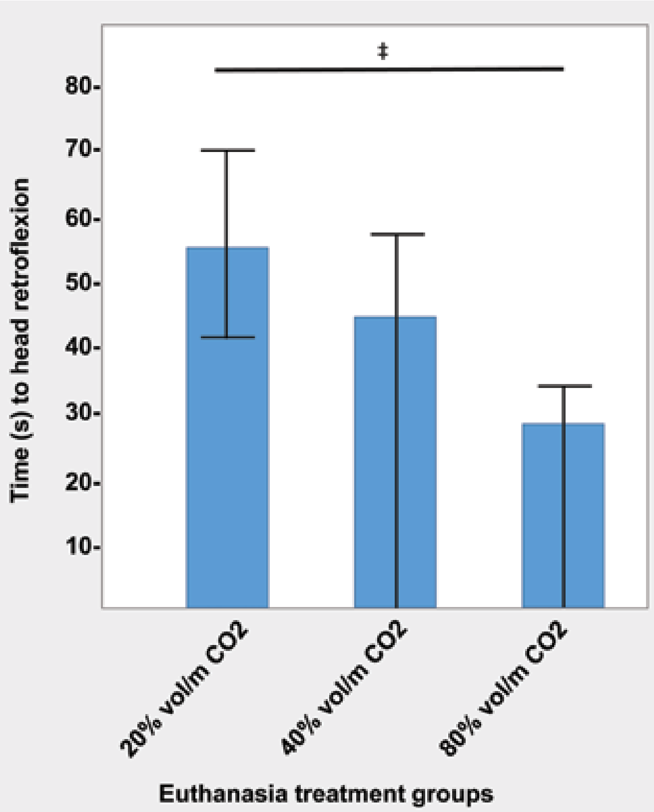

Figure 5. Median time (s; bar, range) to initiation of head retroflexion for the 3 CO_2 treatment groups. Kruskal–Wallis analysis with Dunn posthoc test; ‡, $P < 0.001$.

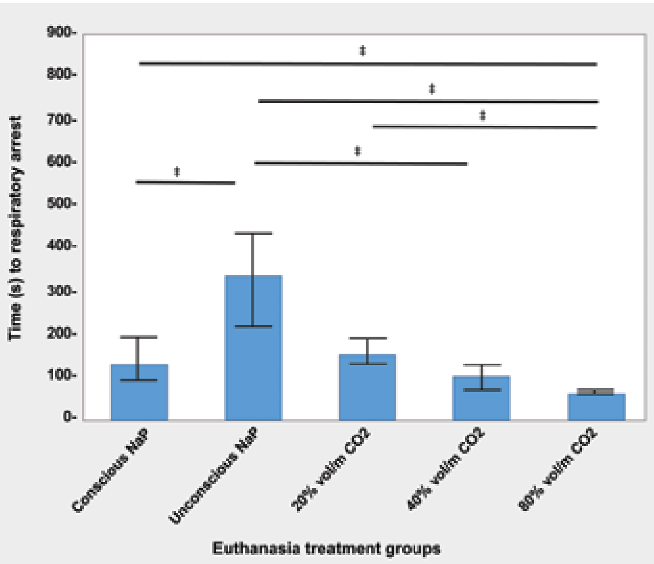

Figure 6. Median time (s; bar, range) to respiratory arrest for all 5 treatment groups. Kruskal–Wallis analysis with Dunn posthoc test; ‡, $P < 0.001$.

intraperitoneal NaP in rodents, which is a method with wide acceptance. Importantly, euthanasia guidelines from the Canadian Council for Animal Care[3] and the 1997 Working Party Report[4] for the European Commission both endorse intracoelomic NaP in unsedated birds. Although we have used intracoelomic NaP in birds with yolk peritonitis, a large ovarian tumor, and various other conditions, judgment is necessary regarding whether this method might be contraindicated in a given case. A drawback with using NaP, regardless of species and route, is that it is a controlled substance.

In the present study, the block randomization scheme did not take sex into account, thus resulting in a sex imbalance between experimental groups, principally for conscious NaP (9 males, 1 female) and unconscious NaP (1 male, 9 females), because birds became available for use regardless of sex. Although a balanced male:female ratio is desireable in theory, we did not note any sex-associated effect in response to a euthanasia technique subjectively, nor has one been described in the scientific literature.

Exposing zebra finches to CO_2 at any chamber displacement rate caused markedly longer periods of headshaking and OMB relative to birds in either NaP group. Headshaking in birds has been suggested to be an alerting response, a means of reducing stress, a reaction to mucosal acidification, or an aversive or distressful reaction.[5,6,7,15] Likewise, OMB has been described in other avian species exposed to CO_2 and has been postulated as a mechanism to reduce nasal nociception, as indicative of breathlessness, or as only a reflex, given that chickens with OMB will continue to eat in the presence of CO_2.[5,6,7,15,16] Due to the unclear physiologic basis for these behaviors, it cannot be proven unequivocally that they reflect real pain or distress; nevertheless, other avian researchers have associated them with the potential for decreased wellbeing.[6,15] In addition, headshaking and OMB in a zebra finch undergoing euthanasia is aesthetically unpleasant to witness (Figure 3). Head retroflexion was seen only in the CO_2 groups, usually with OMB, and always soon before the onset of recumbency.

Among the CO_2 groups, the duration of OMB, onset of head retroflexion, and time to RA were inversely correlated with the chamber displacement rate used. We recommend an 80% displacement rate for institutions that choose to euthanize zebra finches with CO_2 because this rate achieves RA rapidly and reduces the duration of OMB, similar to the situation in mice where higher flow rates result in a quicker death and a shorter period of dyspnea.[2,8] For zebra finch colonies, CO_2 could be the preferred method in an emergency situation where many birds need to be euthanized in a short time or when NaP is in limited supply.

The MSA scores demonstrated some lack of agreement between the observers for headshake, total wing movement, tail bob, and recumbency. Headshaking is difficult to measure because it occurs rapidly and in short bursts, with multiple shakes occurring in 1 s. Total wing movement was expected to be an indicator of aversion to CO_2, because birds try to escape irritating stimuli; however, among the treatments that were analyzed, CO_2 treatments did not lead to more wing movement. In the clinical setting, tail bob has been associated with respiratory distress in birds,[11] but it was not reliably discerned in our study. One possible explanation is that a tail bob is easier to detect when a bird is perched compared with standing on a flat-bottomed surface. Time to recumbency was evidently difficult for the observers to agree on, perhaps because determining when a 'body' was on the floor could be subject to interpretation; this was an unforeseen shortcoming in the study's design.

Our findings show intracoelomic administration of NaP in a conscious, well-restrained zebra finch is a rapid, effective method of euthanasia that causes few, if any, associated behaviors. The same technique might be useful in other bird species. If CO_2 asphyxiation is selected as the euthanasia method for zebra finches, RA is achieved more quickly at higher flow rates (for example, 80%), lessening the duration of potentially aversive behaviors.

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