Defining characteristics of immersion carbon dioxide gas for successful euthanasia of neonatal and young broilers

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ABSTRACT This study investigated how the carbon dioxide (**CO_2**) concentration within a chamber affects the efficacy of CO_2 euthanasia and how the efficacy of CO_2 induction methods changes as birds age. In experiment 1, pairs of broiler chicks (n = 192; 0, 3, and 6 D of age) were immersed into a chamber prefilled with 70, 80, 90, or 100% CO_2. For experiment 2, 3- and 6-day-old broiler chicks (n = 88) were immersed in pairs into 100% CO_2 or exposed to CO_2 gradual fill in a chamber with a displacement rate of 28% chamber volume per minute. Latency to performance of headshaking (**HS**) and gasping (**GS**) as potential indicators of distress, loss of posture indicative of insensibility, and the cessation of rhythmic breathing (**CRB**) and cessation of movement (**COM**) as the indicators of death were monitored (live focal sampling/video recordings). The duration and frequency of HS and GS were assessed. For both experiments, behavior data were analyzed for CO_2 method and age (4 × 3 factorial). Age and CO_2 concentration interacted for latency to CRB and COM, with longer latencies for 0-day-old chicks immersed into 70% CO_2 than other concentrations and ages. CO_2 concentration did not affect latency to HS, GS, or loss of posture but affected CRB and COM, with latencies longest for 70% and shortest for 90 and 100% CO_2. Newly hatched chicks had a longer latency to CRB and COM and longer duration and frequency of distress behaviors than older chicks. At all ages, initiation of all behaviors occurred later with gradual fill compared to immersion. There was an increased duration and frequency of distress behaviors with gradual induction compared with immersion. Overall, immersion into 90 to 100% CO_2 resulted in the shortest time to insensibility and death, with a decreased duration and frequency of distress behaviors. Chicks immersed into 70% CO_2 had the longest duration of GS and time to death. Age affects the efficacy of CO_2 euthanasia, with increasing age decreasing time to death and the duration and frequency of distress behaviors.

Key words: euthanasia, insensibility, distress, welfare, poultry

2020 Poultry Science 99:4408–4416
https://doi.org/10.1016/j.psj.2020.05.039

INTRODUCTION

Newly hatched broiler chicks that are moribund, injured, or unviable require euthanasia. At present, both the Canadian Codes of Practice (NFACC, 2016) and American Veterinary Medical Association (**AVMA**) (AVMA, 2013) accept, with conditions, the use of maceration and gaseous euthanasia with carbon dioxide (**CO_2**) in the first 72 h of life as methods of euthanasia in the hatchery and on farm. Despite having National Farm Animal Care Council and AVMA approval, many hatcheries are looking for an alternative method of euthanasia to maceration owing to public concern. Recently, researchers have investigated a number of alternative methods for neonatal chicken euthanasia, including CO_2 gaseous euthanasia (Gurung et al., 2018; Baker et al., 2019). Gaseous euthanasia is used for culling adult and neonatal poultry, during which exposure to high or prolonged concentrations of an inhalant agent, in this case CO_2, results in insensibility and death (Galvin et al., 2005; Raj et al., 2006;

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Received January 20, 2020.
Accepted May 22, 2020.
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AVMA, 2013; Baker et al., 2019). Carbon dioxide inhalation induces insensibility and death, as the elevated concentration of inspired CO_2 and reduced oxygen availability cause hypercapnia and hypoxia, respectively (AVMA, 2013; Terlouw et al., 2016). When neonatal chick euthanasia via CO_2 was compared with euthanasia via N_2 inhalation or low atmospheric pressure stunning, Gurung et al. (2018) found euthanasia with CO_2 resulted in the shortest time to insensibility and death. The authors also reported bird stress, as measured by serum corticosterone and serotonin levels, was similar for all the 3 methods (Gurung et al., 2018).

One concern with the use of CO_2 for euthanasia of poultry neonates is that loss of sensibility is not instantaneous with CO_2 inhalation, and chicks may experience distress before insensibility. Carbon dioxide is an acidic gas that when inhaled, reacts with water molecules in the mucosal tissues forming carbonic acid, causing irritation of the nasal mucosa (Iwarsson and Rehbinder, 1993; Gerritzen et al., 2007) and inducing pain as the gas stimulates nasal and trigeminal nociceptors (Lambooij et al., 1999; McKeegan et al., 2005; Hawkins et al., 2006; Turner et al., 2012). The nociceptive threshold of nasal and trigeminal nociceptors is ∼40 to 50% CO_2 for adult laying hens (McKeegan et al., 2005); thus, inhalation of CO_2 concentrations more than 40% would be painful. The nociceptive threshold for CO_2 is unknown for neonate broiler chicks. Gaseous CO_2 is also pungent (Lambooij et al., 1999; Gerritzen et al., 2000). The inhalation of CO_2 can result in further distress, as when inhaled, it stimulates arterial and central chemoreceptors causing respiratory depression (Raj et al., 2006; McKeegan et al., 2007). This may also induce a feeling of dyspnea or breathlessness (Hawkins et al., 2006; Raj et al., 2006; Gerritzen et al., 2007; McKeegan et al., 2007), an unpleasant and distressful experience due to an increase in respiratory effort and a feeling of "air hunger" (McKeegan et al., 2007; Beausoleil and Mellor, 2015). Birds experience high concentrations of CO_2 as negative, as research has found birds avoid or leave CO_2 atmospheres if able (Raj et al., 2006; Bandara et al., 2018) and perform gasping and struggling when exposed to CO_2 at concentrations of 40% and higher (McKeegan et al., 2005).

Headshaking and gasping are 2 behavioral responses interpreted to be indicative of distress (Raj and Gregory, 1990; Lambooij et al., 1999; Gerritzen et al., 2007), as these may indicate breathlessness and irritation resulting from CO_2 inhalation (Gerritzen et al., 2000; Abeyesinghe et al., 2007). Headshaking has also suggested to be an alerting response to novel stimuli or an attempt to regain alertness as CO_2 anesthetic effect results in disorientation or dizziness (Hughes, 1983; Gerritzen et al., 2007; McKeegan et al., 2007). It is possible that headshaking is both indicative of irritation and an attempt to counteract the disorientation or dizziness (which itself may be an unpleasant or distressful experience). Headshaking is [one of] the first behavioral responses to CO_2, its onset occurs rapidly after CO_2 introduction, and the behavior starts before the onset of gasping in both neonate (Baker et al., 2019) and adult broilers (Gerritzen et al., 2000). Broiler chicks euthanized by CO_2 on the day-of-hatch demonstrate headshaking and gasping at concentrations of CO_2 lower than 1.5% and continue to do so until insensibility occurs at 12 to 18% CO_2 (Baker et al., 2019). In this period, before loss of sensibility with CO_2, birds may be in distress (Raj et al., 2006; Gerritzen et al., 2013; Baker et al., 2019). Baker et al. (2019) investigated methodology for CO_2 euthanasia of neonatal chicks and determined chicks perform headshaking and gasping with CO_2 inhalation regardless of induction method (gradual vs. immersion) or fill rate used. To minimize the potential for distress and suffering associated with CO_2 euthanasia, the time between initial exposure and loss of sensibility should be as short as possible. Baker et al. (2019) studied the efficacy and welfare impact of different CO_2 induction methods and reported that of the different induction methods tested, immersion into 100% CO_2 caused the shortest latency to insensibility. Immersion into 100% CO_2 also caused the shortest duration and lowest frequency of performance of headshaking and gasping.

However, ensuring CO_2 concentration with the euthanasia chamber is exactly 100% CO_2 may not be feasible in the hatchery or on farm, as it may be difficult to source pure CO_2 or the equipment may not be airtight. The effect of a lower CO_2 concentration in the chamber on the efficacy and welfare impact of neonatal euthanasia via immersion is unknown. Research has determined that with gradual fill, in which neonatal chicks are placed into the chamber and then CO_2 is gradually introduced, death occurs between 70 and 79% CO_2 (Baker et al., 2019), and a final concentration of 75% CO_2 is sufficient to result in death (Gurung et al., 2018). These concentrations are specific to gradual fill and are not directly applicable to immersion, as physiological response to the immediate and significant increase in inhaled CO_2 with immersion may differ from the response to a slow buildup of CO_2 and thus affect the minimum CO_2 concentration requirement.

In addition, neonatal chicks have an increased tolerance to CO_2 and hypercapnia and thus respond to CO_2 differently than adult poultry (Jaksch, 1981; Raj et al., 1992). Young birds require both longer exposure and higher CO_2 concentrations for successful euthanasia (Raj et al., 1992; Raj and Whittington, 1995). When comparing final concentrations with gradual fill, 75% CO_2 is required for chicks on the day of hatch (Gurung et al., 2018), whereas for adult birds, a final concentration of 40% CO_2 is required (Gerritzen et al., 2007). Neonates also perform behaviors indicative of distress at lower concentrations than older birds (Baker et al., 2019). Little is understood of the relationship between CO_2 tolerance and age and how this relates to the distress experienced by the birds. Gaining an understanding on how age impacts the effects of CO_2 exposure on indicators of distress and times to insensibility and death would increase our knowledge of gaseous euthanasia of young birds and help develop recommendations on the best methodologies for using CO_2 euthanasia.

This study aimed to determine the efficacy and welfare impact of euthanasia of young broiler chicks by immersion into various CO_2 concentrations. The specific objectives were to 1) evaluate the effect of immersion into different CO_2 concentrations on the efficacy of euthanasia and 2) determine the effect of age on the efficacy of CO_2 euthanasia via immersion or gradual fill.

MATERIALS AND METHODS

Animals

This research was approved by the Animal Care Committeeof the University of Saskatchewan and followed the recommendations of the Canadian Council of Animal Care (1993, 2009). Mixed-sex Ross 308 broiler chicks (n = 280) were sourced from a local commercial hatchery, with 192 chicks in experiment 1 and 88 chicks in experiment 2. Healthy chicks were housed under commercial conditions recommended by the breeding company management guide (Ross, 2014), fed a commercial broiler starter diet, and given ad libitum access to water until the test.

Experimental Design

The effect of CO_2 euthanasia induction methods and age were evaluated in 2 experiments: the first investigated the effect of immersion into CO_2 concentrations of 70, 80, 90, and 100% CO_2 at 0, 3, and 6 D of age, and the second investigated the effect of immersion or gradual fill on birds at 3 and 6 D of age. In both experiments, chicks were tested in pairs.

Experiment 1—Immersion Concentration Chicks (0, 3 and 6 D of age) were immersed (see the following) into 70, 80, 90, or 100% CO_2 to determine the effects of the CO_2 concentration and age. Originally, the experiment was designed with a fifth treatment of immersion into 60% CO_2, but during a pilot experiment to validate the treatments, the 60% immersion treatment did not result in death within 20 min and was therefore removed from the trial.

Each treatment was tested 8 times (determined as appropriate using a randomized complete block design power test with estimated means and SD from a previous experiment (Baker et al., 2019)), with each run of 4 treatments used as a block. The order of treatments within a block was randomized using a computer-generated random number table, and birds were randomly assigned to each treatment.

Experiment 2—Gradual Fill vs. Immersion In the second experiment, chicks at 3 and 6 D of age were euthanized by either immersion into 100% CO_2 or exposed to gradual fill with CO_2 using a displacement rate of 28% chamber volume per minute. Each treatment was tested 11 times (sample size calculated using a similar power test as described previously), with each group of treatments used as a block. Treatment randomization and assignment was conducted as described for experiment 1.

Experimental Apparatus and Procedure

A 42.5-L EZ-197 induction chamber (dimensions: 46 × 30.5 × 30.5 cm; Euthanex Corp., Palmer, PA) with a EP-1305 LPM CO_2 regulator (Euthanex Corp., Palmer, PA) was used with 99.998% research grade CO_2 (Praxair Canada Inc., Calgary, AB, CA) for the CO_2 euthanasia. The CO_2 concentration was measured at the bird level within the chamber using a CM-0121 COZIR Wide-Range 100% CO_2 sensor (CO2meter.com, Inc., Ormond Beach, FL) and corresponding GasLab software (CO2Meter.com, Inc., Ormond Beach, FL). The CO_2 sensor was located on the chamber wall parallel to the gas inlet at chick height and raised with age. An iButton Hygrochron DS1923-F5# data logger (Maxim Integrated; San Jose, CA) was located on the wall adjacent to the gas inlet, to record the environmental conditions at chick level without hindering live or video observation. Chamber temperatures remained within 22°C to 28°C for the duration of both experiments.

Before treatment, in both experiments, 1 bird was marked for identification with a small ink mark on the head, chest, and back. For the immersion treatments, the chamber was then filled to the desired concentration of 70, 80, 90, or 100% CO_2 after which both birds were placed into the prefilled chamber. Chamber concentration was maintained by addition of CO_2 when necessary. Whilst, for the gradual fill treatments (used in experiment 2), birds were placed into the chamber and allowed to habituate for 30 s; then, CO_2 was introduced into the chamber at a displacement rate of 28% of the chamber volume per minute. In both experiments, the birds' behavioral responses were recorded via both live observation and video cameras (Canon Vixia HFR700 camcorders; Canon Canada, Mississauga, ON, Canada). Birds were removed from the chamber 1 min after final observed movement from either of the chicks. Insensibility was confirmed by a lack of response to corneal blink reflex and pedal reflex withdrawal test (Erasmus et al., 2010) and cessation of heartbeat was confirmed via auscultation (Stethoscope; Littmann Classic, 3M; London, ON, Canada). The chicks were monitored for 5 min to ensure sensibility did not return, after which death was reconfirmed via the absence of heartbeat, corneal blink, and pedal reflex. Cervical dislocation was performed as a secondary euthanasia method.

Behavioral Observations

Continuous focal sampling was used to observe chicks' behavioral responses, with the performance of specific behaviors measured from point of gas introduction until total cessation of movement (Table 1). The behaviors measured in the experiments were latency to headshaking and gasping, loss of posture, cessation of rhythmic breathing, and cessation of all movement. All the

Table 1. Ethogram for behavioral indicators of distress, insensibility, and death for broiler chicks.

Measure	Description
Gasping	Deep breaths with open mouth and out of sync with normal breathing rhythm
Headshaking	Vigorous side-to-side movement of the head and stretched neck
Loss of posture	Inability to remain in initial upright posture combined with a visual loss of neck tension
Cessation of rhythmic breathing	Loss of the rhythmic movement up and out of the rib cage and keel associated with expansion for inhalation, followed by movement of keel and rib cage back down with exhalation. Movement may slow with insensibility but should remain rhythmic with a consistent time between breaths with a maximum of 3–4 s between 2 breaths
Cessation of movement	Complete absence of all movement for a minimum of 1 min

Adapted from Lambooij et al., 1999, Gerritzen et al., 2004, and Erasmus et al., 2010.

behaviors have been previously used in research studying CO_2 euthanasia and neonatal poultry (Baker et al., 2019). The intensity and duration of a negative experience are important when evaluating the welfare impact of aversive conditions (Cook et al., 2000; Conlee et al., 2009), so to assess the negative implication of CO_2 euthanasia the duration and frequency of distress behaviors, headshaking and gasping (Lambooij et al., 1999; Gerritzen et al., 2004), were also measured. Duration was measured from first performance to final performance of behavior or until loss of posture, depending which occurred earlier. Loss of posture was interpreted as an indicator of insensibility, and the cessation of rhythmic breathing and cessation of movement were used as indicators of death. The latency to occurrence of behaviors was recorded from the moment the bird's feet touched the chamber floor for immersion and as time from introduction of CO_2 into the chamber for gradual fill treatments, until the performance of the behavior.

Behavior observations were conducted using real-time observation and video recording and for each bird within the chamber. Throughout the experiments, 2 trained observers watched both birds during all live observations. Three video recorders continuously recorded behavior throughout all experiments. The cameras captured the entire chamber, with 2 perpendicular side views, and a top view to ensure different angles and views were available if a bird was not visible in 1 video. The video recordings were used to verify all live behavioral observations. For real-time observations, blinding of observers was not possible, but those viewing confirmatory videos were blinded to treatment.

Statistical Analyses

Behavior observations for each bird were averaged for the 2 observers, and the means were pooled for both birds in the chamber. Data were tested for normality and then (log + 1) transformed before analyses when necessary. Behavior data from experiment 1 were analyzed for main effects of CO_2 concentration and age (2-way factorial) as a randomized complete block design using PROC MIXED in SAS 9.4 (SAS Inst. Inc., Cary, NC), with each run of all 4 treatments as the random variable and chamber run as an experimental unit. To determine the effect of CO_2 treatment by age, regression analyses were performed on the behavior data in experiment 1, with PROC REG and RSREG to test for linear and quadratic effects, respectively. Data from experiment 2 were analyzed for the main effect of the CO_2 induction method and age via PROC MIXED, with each group of induction treatments as the random variable and chamber as experimental unit. For both experiments, means separation was performed using a Tukey–Kramer post hoc test when means were significantly different, with differences considered significant when $P < 0.05$.

RESULTS

Experiment 1

Latency to Behavioral Responses Latency to loss of posture decreased linearly as immersion concentration increased in newly hatched chicks (Table 2). Latencies to cessation of both rhythmic breathing and movement demonstrated a positive quadratic relationship with CO_2 concentration at 0 and 3 D and decreased linearly with increasing CO_2 concentration at 6 D.

Both headshaking and gasping were observed at all CO_2 concentrations and for every age; no effect of either CO_2 concentration or age was found in the latencies to observation of these behaviors (Table 3). The time to loss of posture was longest for 6-day-old chicks, whereas the time to death was the longest for 0-day-old chicks. Carbon dioxide concentration and age had an interactive effect on time to death, measured by the latency to cessation of rhythmic breathing and movement.

Table 2. The effect of increasing concentrations with immersion into a prefilled CO_2 chamber on the latency to behavioral indicators at 0, 3, and 6 D during CO_2 euthanasia of broiler chickens.

Indicators	Linear *P* value	Quadratic *P* value	Equation
Headshaking			
0 D	0.19	0.51	
3 D	0.63	0.17	
6 D	0.20	0.39	
Gasping			
0 D	0.45	0.73	
3 D	0.95	0.73	
6 D	0.67	0.14	
Loss of posture			
0 D	<0.01	0.80	$y = -0.10x + 21.18$
3 D	0.77	0.78	
6 D	0.93	0.99	
Cessation of rhythmic breathing			
0 D	<0.01	<0.01	$y = 0.91x^2 - 171.89x + 8069.71$
3 D	<0.01	<0.01	$y = 0.18x^2 - 32.74x + 1533.19$
6 D	0.02	0.41	$y = -2.59x + 297.94$
Cessation of movement			
0 D	<0.01	<0.01	$y = 0.93x^2 - 173.58x + 8134.55$
3 D	<0.01	<0.01	$y = 0.17x^2 - 31.37x + 1482.01$
6 D	<0.01	0.59	$y = -2.92x + 341.39$

Day-old chicks immersed into 70% CO_2 had the longest time to death, and time to death decreased for the other ages and CO_2 concentrations, with the shortest noted for 3-day-old chicks immersed into 80, 90, or 100% CO_2. Notably, the lower the CO_2 concentration used for immersion, the longer the latency to death, although no difference was found when chicks were immersed into either 90 or 100% CO_2.

Duration and Frequency of Behavioral Responses

Immersion into varying CO_2 concentrations at different ages had no interactive effect on the duration or frequency of headshaking or gasping (Table 4). The duration of gasping differed with CO_2 concentrations, with chicks immersed into 70% having a longer duration of gasping than chicks immersed into 80 or 100% CO_2. With respect to age, 0-day-old chicks had a higher

Table 3. Effect of immersion into 4 concentrations of CO_2 at 3 different ages on the latency to performance of behavioral indicators of distress, insensibility and death for young broilers (n = 97).

	Behavioral indicator (s)				
Treatment effects	Headshaking	Gasping	Loss of posture	Rhythmic breathing	Cessation of movement
CO_2 induction treatment (%)					
70	1.2	2.1	15.3	251.1^a	260.8^a
80	1.0	2.0	13.8	98.4^b	112.2^b
90	1.0	2.3	15.7	38.8^c	49.5^c
100	1.0	1.9	13.8	37.5^c	49.2^c
P value	0.09	0.26	0.23	<0.01	<0.01
Age (D)					
0	1.1	2.1	12.4^b	190.0^a	197.0^a
3	1.1	1.9	13.9^b	52.8^b	65.1^c
6	1.0	2.2	17.8^a	78.5^b	93.7^b
P value	0.60	0.05	<0.01	<0.01	<0.01
Interaction (Age × CO_2 induction treatment)					
0 D–70%	1.2	2.2	14.2	532.5^a	536.5^a
0 D–80%	1.0	2.0	12.2	159.3^b	163.3^b
0 D–90%	1.1	2.2	12.2	$40.6^{c,d}$	$48.6^{d,e}$
0 D–100%	1.0	1.9	10.7	31.2^d	43.9^e
3 D–70%	1.2	1.9	13.4	113.5^b	123.2^b
3 D–80%	1.0	1.7	12.0	36.9^d	48.0^e
3 D–90%	1.0	2.1	17.4	33.2^d	48.1^e
3 D–100%	1.1	1.7	12.9	27.5^d	40.9^e
6 D–70%	1.1	2.2	18.0	123.3^b	138.0^b
6 D–80%	1.0	2.3	18.0	$90.3^{b,c,d}$	$120.0^{b,c,d}$
6 D–90%	1.0	2.5	17.5	$42.5^{c,d}$	$51.9^{d,e}$
6 D–100%	1.0	2.0	17.9	$53.7^{c,d}$	$62.9^{c,d,e}$
P value	0.90	0.99	0.24	<0.01	<0.01
SEM	0.03	0.07	0.50	15.05	14.77

[a–e]Means within a column and main effect with different superscripts differ significantly at $P \leq 0.05$.

Table 4. Effect of immersion into 4 concentrations of CO_2 at 3 different ages on the duration and frequency of the performance of behavioral indicators of distress during CO_2 euthanasia of young broiler chicks (n = 97).

	Behavioral indicator			
	Headshaking		Gasping	
Treatment effects	Duration (s)	Frequency (n)	Duration (s)	Frequency (n)
CO_2 induction treatment (%)				
70	5.8	3.3	7.9^{a}	4.8
80	5.5	3.2	5.4^{b}	4.2
90	4.8	3.3	6.3^{a,b}	4.8
100	5.6	3.7	5.3^{b}	4.2
P value	0.33	0.43	<0.01	0.17
Age (D)				
0	7.1^{a}	4.1^{a}	7.4^{a}	4.8
3	4.4^{b}	2.9^{b}	6.3^{b}	4.3
6	4.8^{b}	3.1^{b}	5.1^{b}	4.5
P value	<0.01	<0.01	<0.01	0.33
Interaction (age × CO_2 induction treatment)				
P value	0.61	0.22	0.28	0.46
SEM	0.23	0.13	0.34	0.16

[a,b]Means within a column and main effect with different superscripts differ significantly at $P \leq 0.05$.

frequency of headshaking and a longer duration of headshaking and gasping than older aged birds.

Experiment 2

Latency to Behavioral Responses An interaction between bird age and CO_2 induction method was noted for the latency to headshaking (Table 5). The latency to headshaking was shorter for chicks euthanized via immersion at both 3 and 6 D than that by gradual; for gradual fill, the latency to headshaking was shorter for 6-day-old than for 3-day-old chicks ($P = 0.03$; 1.1, 1.2, 9.2, and 12.5 s for immersion [6 D], immersion [3 D], gradual fill [6 D], and gradual fill [3 D], respectively). Latency to all behavioral responses was shorter for chicks euthanized via immersion, with the time to headshaking, gasping, loss of posture, cessation of rhythmic breathing, and cessation of movement being between 76 and 90% faster when chicks were euthanized by immersion as compared with gradual fill. The onset of gasping and cessation of rhythmic breathing occurred earlier at 6 D than at 3 D.

Duration and Frequency of Behavioral Responses Table 6 presents the duration and frequency of performance of headshaking and gasping. Gasping duration was shorter for birds immersed into 100% CO_2 at both 3 and 6 D than that of birds exposed via gradual fill at both ages ($P = 0.02$; 4.1, 4.4, 30.7, and 37.3 s for immersion [6 D], immersion [3 D], gradual fill [3 D], and gradual fill [6 D], respectively). With immersion, the durations of headshaking and frequency of headshaking and gasping were lower than when birds were euthanized with gradual fill. Headshaking duration and frequency were lowest at 6 D, whereas the frequency of gasping was lowest at 3 D.

DISCUSSION

The AVMA (2013) recommends using gradual fill induction during CO_2 euthanasia, despite it having a longer time to insensibility, as it is thought that with certain flow rates, the possible anesthetic effects of CO_2 may result in consciousness being lost or reduced before CO_2 inhalation could cause pain and distress

Table 5. Effect of gradual fill or immersion treatments and age on the latency to behavioral indicators of distress, insensibility, and death during CO_2 euthanasia of broiler chicks (n = 44).

	Behavioral indicator (s)				
Treatment effects	Headshaking	Gasping	Loss of posture	Rhythmic breathing	Cessation of movement
CO_2 induction treatment					
Gradual	10.9^{a}	12.5^{a}	56.9^{a}	175.4^{a}	178.1^{a}
Immersion	1.1^{b}	2.1^{b}	13.9^{b}	31.1^{b}	43.4^{b}
P value	<0.01	<0.01	<0.01	<0.01	<0.01
Age (D)					
3	6.9^{a}	8.1^{a}	35.1	109.7^{a}	115.8
6	5.1^{b}	6.4^{b}	35.7	96.8^{b}	105.8
P value	<0.01	<0.01	0.91	0.03	0.26
SEM	0.82	0.85	3.34	11.57	10.95

[a–c]Means within a column and main effect with different superscripts differ significantly at $P \leq 0.05$.

Table 6. Effect of gradual fill or immersion treatments and age on the duration and frequency of the performance of behavioral indicators of distress during CO_2 euthanasia of young broiler chicks (n = 44).

	Behavioral indicator			
	Headshaking		Gasping	
Treatment effects	Duration (s)	Frequency (n)	Duration (s)	Frequency (n)
CO_2 induction treatment				
Gradual	16.1^{a}	5.9^{a}	34.0^{a}	12.8^{a}
Immersion	3.5^{b}	3.0^{b}	4.2^{b}	3.8^{b}
P value	<0.01	<0.01	<0.01	<0.01
Age (D)				
3	11.5^{a}	4.9^{a}	17.5	7.6^{b}
6	8.1^{b}	4.0^{b}	20.7	9.0^{a}
P value	<0.01	0.02	0.22	<0.01
SEM	1.186	0.317	2.375	0.732

[a–c]Means within a column and main effect with different superscripts differ significantly at $P \leq 0.05$.

(Gerritzen et al., 2004). As the pain threshold for adult bird nociceptors has been found to be 40% CO_2 or higher (McKeegan et al., 2005), then the bird would be insensible before CO_2 reaching painful levels. This assumption does not take into account other sources of distress resulting from CO_2 inhalation (i.e., breathlessness, irritation, disorientation) that can occur at lower concentrations. When gradual fill induction was compared with immersion into a chamber prefilled with 100% CO_2, immersion induction rapidly induced insensibility and death, with the shortest period before loss of sensibility (Baker et al., 2019). The latencies to all behavioral responses to CO_2 exposure in this study were shorter when chicks were euthanized via immersion than chicks euthanized via gradual fill, similar to previous findings (Gurung et al., 2018; Baker et al., 2019). Our results indicate immersion was the most effective method for chicks up to 6 D, as they exhibited shorter latencies to indicators of distress, insensibility, and death when euthanized via immersion compared with gradual fill.

The latencies to onset of behavioral responses and loss of posture were not affected by the concentration of CO_2 into which chicks were immersed. This observation is attributable to the CO_2 concentrations of the 4 immersion treatments being well above the concentration at which signs of distress and insensibility occur. In a previous study, when day-old chicks were killed using gradual fill induction, headshaking and gasping occurred at approximately 0.4 to 1.2% CO_2, loss of posture occurred at approximately 11 to 18% CO_2 and death occurred when concentrations were between 61 and 78% CO_2 (Baker et al., 2019). In the present study, latency to death differed with CO_2 concentration. With immersion into 70% CO_2, it took more than 4 min for all ages combined (more than 8 min at 0 D and around 2 min at 3 and 6 D), whereas immersion into 80% resulted in death in less than 2 min and immersion in 90 or 100% CO_2 resulted in death in less than 1 min. Immersion into 70% CO_2 falls within the range of CO_2 concentrations at which death occurs, whereas 80, 90, and 100% are higher than the minimum concentration needed. Immersion into higher CO_2 concentrations means the CO_2 levels within the body saturate the tissues, and subsequent neuronal dysfunction and cell death occur faster (Lambooij et al., 1999). When chicks were immersed into 60% CO_2, death did not occur within the 20-min time frame.

Euthanasia of day-of-hatch chicks by immersion into 70% CO_2 resulted in a longer latency to death than has been previously reported for chicks euthanized via gradual fill (Gurung et al., 2018; Baker et al., 2019). When 100% CO_2 was gradually introduced to neonatal chicks at a fill rate of 28% chamber volume added per minute, rhythmic breathing and total movement ceased within 4 min (Baker et al., 2019), whereas in the present study, neonates immersed into 70% CO_2 required longer than 8 min to cease rhythmic breathing and all movement. Notably, although immersion into 70% CO_2 caused a longer time to death than gradual fill, the latencies to headshaking, gasping, and loss of posture were shorter for neonatal chicks euthanized by immersion into 70% CO_2 than those euthanized by gradual fill. Gasping, one of the behaviors considered to be indicative of distress, was performed at 2.1 s after immersion into 70% CO_2 and 16 s after CO_2 introduction with gradual fill (Baker et al., 2019), thus the latency to gasping was longer (88%) with gradual fill. The duration of gasping was also longer (66%) for gradual fill, (8 s when immersed vs. 18 s with gradual fill) (Baker et al., 2019). Similarly, insensibility, as measured by loss of posture, occurred at 15 s with immersion into 70% CO_2 and at 51 s with gradual induction, a 70% increase in time. That only latency to death, and not distress and insensibility, was longer with immersion into 70% CO_2 than gradual fill, and that death occurred at 76% CO_2 with gradual fill (Baker et al., 2019), suggests that the increased time to death is related to the concentration of CO_2 being lower with the immersion treatment.

Neonates are more tolerant to the effects of CO_2 than adult birds and require higher CO_2 concentrations and longer exposure times for successful euthanasia (Jaksch, 1981; Raj et al., 1992; Raj and Whittington, 1995). Our current findings demonstrate that a change in CO_2 sensitivity occurs as early as 3 D of age, with the time to death decreasing with bird age. The increased CO_2 tolerance of neonates also means, in regards to time of death, they are more sensitive to the concentration of CO_2 into which they are immersed. Time to death with immersion into 100% CO_2 was similar for the different ages, but with exposure to lower CO_2 concentrations, the time to death for day-old chicks was longer (longer than 8 min) than that of chicks at 3 or 6 D (just longer than 2 min). Baker et al. (2019) suggested that the CO_2 tolerance shown by neonates affects the onset of death and may affect the onset of insensibility, but it does not affect distress. Our research suggests that neonates have an increased tolerance to death by hypercapnia and hypoxia, but that tolerance does not apply to headshaking or gasping, as time to death was longer for neonates than older chicks, but no difference between ages was found for the latency to either behavior. Our findings also indicate that the frequency and duration of headshaking and gasping were increased for newly hatched chicks compared with the other ages tested. This suggests that neonatal tolerance to CO_2 does not extend to the distress behaviors and that the distress response to CO_2 may even be increased in neonates.

The present study used healthy chicks to study the efficacy of CO_2 euthanasia. When comparing these results with an earlier study by these authors using cull chicks (Baker et al., 2019), indicator values were similar to those seen here. When immersed into 100% CO_2, cull 0-day-old birds lost posture after 9 s, ceased rhythmic breathing after 18 s, and ceased moved after 56 s (Baker et al., 2019), compared with healthy 0-day-old birds that lost posture after 10.7 s and ceased rhythmic breathing and movement after 37.2 s and 44 s, respectively, in this study, which suggests this study is relevant for use with cull chicks.

There are still many questions surrounding the best method of using CO_2 for euthanasia, and further research would allow us to understand at what age the tolerance to CO_2 changes and at what point should immersion into high levels of CO_2 should change to slow gradual induction as previously suggested (AVMA, 2013). In addition, the research presented in the manuscript focused specifically on using CO_2 with broiler chicks; more research is needed to elucidate the best practice for using CO_2 with other poultry species.

CONCLUSION

At all the ages tested in these experiments, immersion into 100% CO_2 was the most efficacious method of euthanizing broiler chicks, as it resulted in rapid insensibility and death, with shortest duration and lowest frequency of distress behavior. However, immersion into 100% CO_2 may not always be feasible in practice. Immersion into 90% CO_2 was equivalent in efficacy to immersion with 100% CO_2, and immersion into 80% CO_2 had similar time to onset of distress behaviors and insensibility but with a longer time to death. Thus, it can be concluded that immersion into 80% CO_2 or higher concentration should be a requirement for euthanizing neonate chicks in a practical setting.

ACKNOWLEDGMENTS

The authors would like to thank Steve Laycock for his help with the project, as well as Chicken Farmers of Saskatchewan, Poultry Industry Council, Canadian Poultry Research Council, Egg Farmers of Canada, Hybrid Turkeys, the Ontario Ministry of Agriculture, Food and Rural Affairs, and Natural Sciences and Engineering Research Council for funding this study.

Conflict of Interest Statement: The authors declare that there is no conflict of interest.

REFERENCES

Abeyesinghe, S. M., D. E. F. McKeegan, M. A. McLeman, J. C. Lowe, T. G. M. Demmers, R. P. White, R. W. Kranen, H. van Bemmel, J. A. C. Lankhaar, and C. M. Wathes. 2007. Controlled atmosphere stunning of broiler chickens. I. Effects on behaviour, physiology and meat quality in a pilot scale system at a processing plant. Br. Poult. Sci. 48:406–423.

AVMA. 2013. Guidelines for the Euthanasia of animals: 2013 edition. Accessed Sept. 2015. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.

Baker, B. I., S. Torrey, T. M. Widowski, P. V. Turner, T. D. Knezacek, J. Nicholds, T. G. Crowe, and K. Schwean-Lardner. 2019. Evaluation of carbon dioxide induction methods for the euthanasia of day-old cull broiler chicks. Poult. Sci. 98:2043–2053.

Bandara, R., S. Torrey, R. Parsons, T. M. Widowski, and S. Millman. 2018. Assessment of aversion and time to insensibility to CO2 gas exposure in laying hens. Poult. Sci. 97(Suppl. 1) (Abstr.).

Beausoleil, N. J., and D. J. Mellor. 2015. Introducing breathlessness as a significant animal welfare issue. N. Z. Vet. J. 63:44–51.

Conlee, K., M. Stephens, and A. N. Rowan. 2009. The minimization of research animal distress and pain: conclusions and recommendations. Lab. Exp. Collect. 23.

Cook, C. J., D. J. Mellor, P. J. Harris, J. R. Ingram, and L. R. Matthews. 2000. Hands-on and hands-off measurements of stress. Pages 123–147 in The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. G. P. Moberg and J. A. Mench eds. CABI Publishing, Oxon, UK.

Erasmus, M. A., P. V. Turner, and T. M. Widowski. 2010. Measures of insensibility used to determine effective stunning and killing of poultry. J. Appl. Poult. Res. 19:288–298.

Galvin, J. W., H. Blokhuis, M. C. Chimbombi, D. Jong, and S. Wotton. 2005. Killing of animals for disease control purposes. Rev. Sci. Tech. 24:711–722.

Gerritzen, M. A., E. Lambooij, S. J. W. Hillebrand, J. A. C. Lankhaar, and C. Pieterse. 2000. Behavioral responses of broilers to different gaseous atmospheres. Poult. Sci. 79:928–933.

Gerritzen, M., B. Lambooij, H. Reimert, A. Stegeman, and B. Spruijt. 2004. On-farm euthanasia of broiler chickens: effects of different gas mixtures on behavior and brain activity. Poult. Sci. 83:1294–1301.

Gerritzen, M., B. Lambooij, H. Reimert, A. Stegeman, and B. Spruijt. 2007. A note on behaviour of poultry exposed to increasing carbon dioxide concentrations. Appl. Anim. Behav. Sci. 108:179–185.

Gerritzen, M. A., H. G. M. Reimert, A. Lourens, M. B. M Bracke, and M. T. Verhoeven. 2013. Killing wild geese with carbon dioxide or a mixture of carbon dioxide and argon. Anim. Welf. 22:5–12.

Gurung, S., D. White, G. Archer, D. Zhao, Y. Farnell, J. A. Byrd, E. D. Peebles, and M. Farnell. 2018. Evaluation of alternative euthanasia methods of neonatal chickens. Animals 8:37.

Hawkins, P., L. Playle, H. Golledge, M. Leach, R. Banzett, A. Coenen, J. Cooper, P. Danneman, P. Flecknell, R. Kirkden, L. Niel, and M. Raj. 2006. Newcastle Consensus Meeting on Carbon Dioxide

Euthanasia of Laboratory Animals. University of Newcastle, Newcastle upon Tyne, UK.

Hughes, B. O. 1983. Headshaking in fowls: the effect of environmental stimuli. Appl. Anim. Ethol. 11:45–53.

Iwarsson, K., and C. Rehbinder. 1993. A study of different euthanasia techniques in Guinea pigs, rats and mice: animal response and post-mortem findings. Scand. J. Lab. Anim. Sci. 4:191–205.

Jaksch, W. 1981. Euthanasia of day-old male chicks in the poultry industry. Int. J. Stud. Anim. Prob. 2:203–213.

Lambooij, E., M. A. Gerritzen, B. Engel, S. J. W. Hillebrand, J. Lankhaar, and C. Pieterse. 1999. Behavioural response during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci. 62:255–265.

McKeegan, D., F. S. Smith, T. Demmers, C. Wathes, and R. Jones. 2005. Behavioral correlates of olfactory and trigeminal gaseous stimulation in chickens, Gallus domesticus. Physiol. Behav. 84:761–768.

McKeegan, D., J. McIntyre, T. Demmers, J. C. Lowe, C. Wathes, P. L. C. van den Broek, A. M. L. Coenen, and M. J. Gentle. 2007. Physiological and behavioural responses of broilers to controlled atmosphere stunning: implications for welfare. Anim. Welf. 16:409–426.

National Farm Animal Care Council. 2016. Code of practice for the care and handling of hatching eggs, breeders, chickens and turkeys. Accessed Dec. 2016. http://www.nfacc.ca/poultry-code-of-practice.

Raj, M., and N. G. Gregory. 1990. Effect of rate of induction of carbon dioxide anaesthesia on the time of onset of unconsciousness and convulsions. Res. Vet. Sci. 49:360–363.

Raj, M., and P. Whittington. 1995. Euthanasia of day-old chicks with carbon dioxide and argon. Vet. Rec. 136:292–294.

Raj, M., S. Wotton, and P. Whittington. 1992. Changes in the spontaneous and evoked electrical activity the brain of hens during stunning with 30 per cent carbon dioxide in argon with 5 per cent residual oxygen. Res. Vet. Sci. 53:126–129.

Raj, M., V. Sandilands, and N. Sparks. 2006. Review of gaseous methods of killing poultry on-farm for disease control purposes. Vet. Rec. 159:229–235.

Ross. 2014. Broiler management handbook. Ross. Accessed Dec. 2015. http://eu.aviagen.com/tech-center/download/18/Ross-BroilerHandbook2014-EN.pdf.

Terlouw, C., C. Bourguet, and D. Veronique. 2016. Consciousness, unconsciousness and death in the context of slaughter. Part I: neurobiological mechanisms underlying stunning and killing. Meat. Sci. 118:133–146.

Turner, P. V., H. Kloeze, A. Dam, D. Ward, N. Leung, E. E. L. Brown, A. Whiteman, M. E. Chiappetta, and D. B. Hunter. 2012. Mass depopulation of laying hens in whole barns with liquid carbon dioxide: evaluation of welfare impact. Poult. Sci. 91:1558–1568.