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DL.3.2.7. REPORT OF THE ASSESSMENT OF WELFARE DURING THE EXPOSURE TO ALTERNATIVE MIXTURES TO CARBON DIOXIDE IN TWO PHASES IN BROILER CHICKENS.

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1. Introduction

Pre-slaughter stunning is mandatory in the European Union (Council Regulations EC No 1099/2009). It consists of inducing unconsciousness in animals in order to prevent any avoidable pain, distress or suffering during killing and related operations. To protect animal welfare at the time of killing, it is essential to induce an effective stunning (i.e., animals do not regain consciousness before brain death due to bleeding) without avoidable pain and fear during the induction of the stunning.

Currently, the two main stunning methods used commercially in broiler chickens are electrical waterbath stunning (WBS) and controlled atmosphere stunning (CAS). Since WBS implies live bird shackling, pre-stun shocks may occur and the stunning is not always effective, this is of great concern regarding the welfare of this stunning method (EFSA, 2019). For this reason, CAS emerged as an alternative stunning method to WBS (Berg & Raj, 2015). It consists in exposing large numbers of broiler chickens, to modified atmosphere environments (e.g. carbon dioxide in two phases) or by reducing the atmosphere pressure (LAPS), which induce a gradual loss of consciousness (LOC) and if the duration of the exposure is long enough, it causes death. However, CAS methods are not exempt of risks for animal welfare. For instance, the induction to unconsciousness is not instantaneous and involves a transitional period during which negative welfare outcomes may occur (Verhoeven et al., 2015).

Although WBS is the most common method used in the European Union, the number of slaughterhouses with CAS has dramatically increased during the last years (European Commission, 2013; EURCAW-Poultry-SFA, 2022). Most slaughterhouses use carbon dioxide (CO_2) in two phases, while very few commercial slaughterhouses use CO_2 associated with inert gases and none of them use neither inert gases nor LAPS yet.

The LOC results from the intra and extracellular acidosis in the brain due to CO_2 exposure (Martoft et al., 2002). However, prior to loss of consciousness, acidosis in mucous membranes activates nociceptors in poultry which may induce discomfort and pain, as shown by behaviours indicative of aversion (Gent et al., 2020). The degree of aversion depends on the carbon dioxide concentration. Higher concentrations of carbon dioxide, although inducing a more rapid induction of anaesthesia, are more aversive to pigs. To reduce the aversion during the induction, stunning with CO_2 in two phases is carried out. It consists in exposing broiler chickens to an initial concentration of up to 40% of CO_2 until LOC occurs. Thereafter, the CO_2 concentration is increased in the second phase inducing a deeper state of unconsciousness and then death (EFSA, 2019).

Commercial equipment for stunning with CO_2 differs in design, either being tunnels, pits or closed cabinets. In tunnels and pits, birds enter the system in their transport crates, or they are uncrated by tilting the container (EFSA, 2019) and enter the system on a conveyor belt. In tunnels and pits, the system is pre-filled with the different concentrations of CO_2 and birds enter continuously at one end of the system, and while they are conveyed to the opposite end, they are exposed to different gas concentration. In closed cabinets, birds enter the system in their transport crates as one batch at a time. Once the birds have been loaded within the system, the gas is then added in two (or more) phases into the cabinet and removed upon completion of the stunning cycle (EURCAW-Poultry-SFA, 2022).

As an alternative to CO_2 in two phases, the exposure to inert gases, such as nitrogen (N_2) or argon (Ar) are presumed to reduce aversion. Inert gases are colourless, odourless and tasteless, and therefore, their inhalation does not cause aversive reactions after initial exposure, as they are imperceptible to birds (Raj, 1996; Webster and Fletcher, 2004). In addition, inert gases displace oxygen from the atmospheric air and this ensures that the birds lose consciousness by anoxia (EFSA, 2019). The progressive reduction of oxygen (O_2) in the atmospheric air is not perceived by the birds (Velarde & Raj, 2016). When birds enter a chamber filled with inert gases the behaviour does not differ from when they were breathing atmospheric air (Webster and Fletcher, 2004), they do not withdraw (McKeegan et al., 2006) and they barely show behavioural signs of distress (Wooley and Gentle, 1988; Gent *et al*., 2000). On the other hand, after LOC, birds perform severe convulsion expressed as wing flapping (Webster and Fletcher, 2004; Gent et al., 2020), which may cause self-inflicted injuries (wing fractures) or injuries and pain to the other birds that have not yet lost consciousness (McKeegan *et al.*, 2007; Berg and Raj, 2015). However, it is not entirely clear whether the convulsions are reflexive reactions occurring after the bird loses consciousness or whether the birds are still conscious trying to escape from such modified atmosphere (McKeegan *et al.*, 2007a; Coenen *et al.*, 2009; Shields and Raj, 2010).

Brain activity measured through electroencephalography (EEG) is the most accurate method to assess the onset and duration of unconsciousness and time to death. Determining these times can shed light on what behaviours are performed involuntarily when unconscious and what behaviours are a conscious response to the gases (aversive behaviours). Since EEG can only be performed under laboratory conditions, correlation between neurological measures and behavioural indicators are required to characterise the goodness of the method and identify what indicator can be used as an approach for unconsciousness and death in slaughterhouse conditions. Nevertheless, this approach is scarce in scientific literature.

It is reported that CO_2 has an anaesthetic effect when inhaled (Kohler et al., 1998) and when combined with inert gases, these potential adverse behaviours have a shorter duration (Gent et al., 2020). In addition, it is presumed that the time to onset to LOC is shorter when CO_2 associated with inert gases and can reduce the prevalence and severity of aversive behaviours compared to the use of CO_2 in two phases. Additionally, we want to verify if such gas mixture can reduce the time to LOC. However, faster onset of unconsciousness in gas mixtures of CO_2 with inert gases in a dip-lift system offers presumably relatively better welfare.

The aim of this study was to assess the neurological and behavioural responses of broiler chickens when exposed to CO_2 in two phases, 40% CO_2 and 60% N_2 and 20% CO_2 and 80% nitrogen in a pre-filled pit system in order to evaluate the stun efficiency, the time to onset of unconsciousness and death and the potential aversive behaviours that may occur during the induction to unconsciousness.

2. Methods

2.1. Experimental design and facilities

A total of two hundred forty-three 39-day-old mixed-sex Ross 308 broiler chickens were obtained from a commercial farm and transported to the experimental facilities of the Institute of Research and Technology for Agriculture and Food (IRTA) in Monells (Spain). Birds were selected, weighted (2.42 ± 0.18 kg) and identified with numbered leg bands. On arrival and after checking their health status and appropriate locomotor behaviour, birds were distributed into seven adjacent lairage pens of 2 m × 1.8 m (35 broilers chickens per pen; stocking density of 23.5 kg/m^2. Each pen was provided with litter material (wood shavings) and feed and water *ad libitum* throughout the experiment.

The study was carried out at the experimental slaughterhouse of IRTA, located next to the lairage pens. It is equipped with a Dip-lift XL G2 gas stunning system (Butina Aps, Copenhagen, DK) that contained a lift (240 cm × 111 cm × 100 cm) with perforated floor. The lift descends until the base of a pit at a depth of 290 cm. The pit is pre-filled with gas mixtures through an inlet valve placed at the bottom of the pit. CO_2 and O_2 concentration were measured through a portable infrared single beam sensor for CO_2 and electrochemical sensor for O_2 (Dansensor® CheckPoint 3 O_2/CO_2, MOCON Europe A/S, DK) using one fixed sounding line placed at a depth of 260 m and another mobile sounding line to check CO_2 concentrations at different depths.

The experimental period lasted 5 days. On the first day, broiler chickens were submitted to AIR (atmospheric air, n= 92) serving as control. The animals exposed to AIR were re-used for the three experimental treatments. Therefore, from day 2 to day 5, broiler chickens were stunned with one of the following gas mixtures treatments: 40C90C (CO_2 in two phases: the first phase with <40% CO_2 by volume in air for 2 min and the second phase with >90% CO_2 and less than 2% of residual O_2 for 2 min, n=92), 40C60N (gas mixture of 40% CO_2 and 60% N_2 with less than 2% of residual oxygen O_2 for 4 min, n=79) and 20C80N (gas mixture of 20% CO_2 and 80% N_2 with less than 2% of residual O_2 for 4 min, n=72). The duration of exposure to each experimental treatment was determined from pre-trials based giving a on the time the broiler chickens lost consciousness. Gas used were pure CO_2 and mixtures of CO_2 with N_2 (Freshline gases® for food use, Carburos Metálicos, Barcelona, ES). The duration of exposure to each experimental treatment was determined from previous trials giving a time margin based on the time at which broilers chickens lost consciousness. Each day included two sessions, from 09.00 to 13.00h in the morning and from 15.00 to 19.00h in the afternoon, with treatments alternating per session to avoid potential bias. Each session consisted of 8 to 11 cycles (dips into the pit). In each cycle, four broiler chickens were stunned and sacrificed at a time and at the end of each session, the remaining gas in the stunning system was emptied and refilled with the next gas mixture.

Of these four chickens per cycle, one of them was used for brain activity assessment while three of them were used for behavioural assessment. Birds used for brain activity assessment were placed on the lift separated from the other conspecifics by a transparent methacrylate wall with a floor area of 48 cm × 112 cm (0.53 m^2). Birds used for behavioural activity were individually caught in upright position and moved from the lairage pens to the lift having a floor area of 144 cm × 112 cm (1.6 m^2).

2.2. Gas concentration assessment

CO_2 concentrations were measured in 40C90C with a mobile sounding line marked every 10 cm and introduced from the top of the pit to find the concentration of CO_2 closest to 40% without exceeding this concentration. Then the distance from the top of the pit was registered and the lift descended until reaching such deep. CO_2 and O_2 concentrations were monitored in 40C60N and 20C80N. The gas mixture was considered adequate in 40C60C when the CO_2 concentration was close to 40% and O_2 below 2% and in 20C80N when CO_2 concentration was close to 20% and O_2 below 2%. At the end of each treatment, gas contained in the stunning system was emptied so it could be refilled with the following gas mixture.

2.3. Brain activity assessment

Fifty broiler chickens were randomly selected to register their brain activity through electroencephalography (EEG) being distributed in 40C90 (n=16), 40C60N (n=16) and 20C80N (n=18). Each bird was prepared immediately before to be submitted to exposure to the treatment in order to spare as much stress as possible. Firstly, the bird was wrap with a mesh to restrain wings, body, and legs and leaving head and neck exposed. Secondly, the chicken was restrained gently by their neck. Then, head and neck feathers were shaved with an electric shaver and a gauze pad with topical alcohol was rubbed on the bare skin before subcutaneous administration of the local anaesthesia on the head and neck. Local anaesthesia consisted in 0.1 mL of lidocaine 2% was subcutaneously injected with an insulin needle and syringe on the same place where it was planned to put the electrodes. Once anesthetized, three 24-gauge stainless steel subdermal needle electrodes (Neuroline Subdermal, Ambu Inc., Glen Burnie, MD, USA) were placed on the head as described in Gibson et al. (2018). Briefly, the active electrode was inserted at ≈6 mm right of midline and ≈3 mm cranial from the end of the comb over the right optic lobe; reference electrode was inserted over the right rostral aspect of the forebrain ≈6 mm right of midline and ≈20 mm caudal from the end of comb; and ground electrode inserted in the neck toward the head (Figure 1). Electrodes were secured in position with surgical tape (Durapore, 3M, Maplewood, MN, USA). Inter-electrode impedance was checked to be lower than 2.0 kΩ (MkIII Checktrode, UFI, Morro Bay, CA, USA) and electrode leads were further secured with a loose band of surgical auto-fixing tape around the neck (Coeban, 3M).

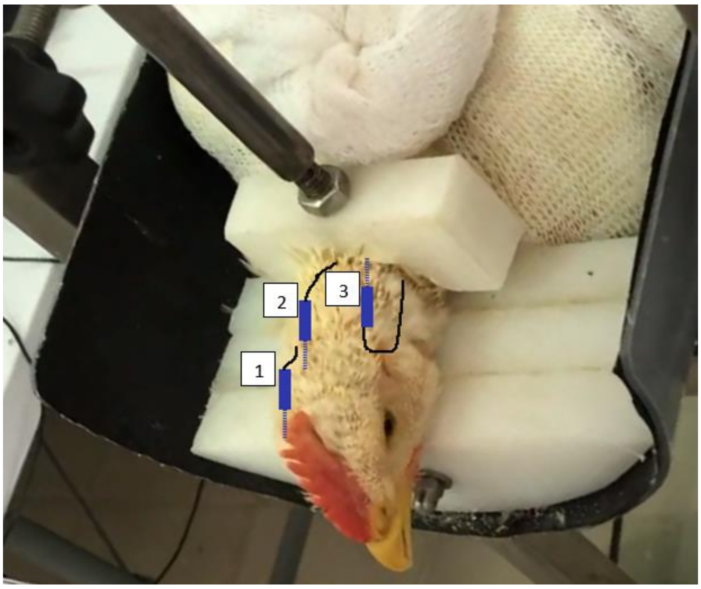


Figure 1. Representation of needle electrodes position for brain activity assessment through electroencephalography in broiler chickens being 1) active electrode; 2) reference electrode and 3) ground electrode.

The time between the end of the baseline and the beginning of gas exposure was recorded to allow the synchronization between the videos and the EEGs records. EEG signals were amplified and filtered with an analogue filter (dual Bio Amp, ADInstruments Ltd., Sydney, AU) with low and high pass filters of 100 and 0.1 Hz, respectively. The signals were digitalised (1 kHz) with a 4/20 PowerLab (ADInstruments Ltd, Sydney, AU) digital to analogue converter and recorded on a personal laptop for offline analysis.

Baseline EEG was recorded during 90 s when the bird was on the floor of the lift with three other birds before the descend. After the baseline recording, the lift descended into the pit. The elapsed time was marked on the EEG trace and the time until lift descended to the desired depth was timed in order to synchronise the video and EEGs recordings. All recording times were synchronised with the time the lift with the broiler chickens started to descend into the pit.

To reduce potential bias during visual and spectral analysis of EEGs, brain activity assessment was performed by an experienced EEG analyst blinded to the experimental treatments. EEG recordings were monitored, saved and pre-processed using LabChart 8 Pro (v.8.1.21, AD Instruments, GE). Movement artefacts found in the recorded waveforms caused by rhythmic breathing, blinking and head shaking were excluded from subsequent analysis. EEG recordings were also filtered to remove noise interference (band pass: 0.1 to 30 Hz).

Representative 1 s epochs free of interference were selected from EEG trace for spectral analysis at individual level (Gibson et al., 2018). Three out of the 50 EEG traces were discarded, one trace from 40C90C due to interference from eyelid movement, preventing the selection of several 1 s epochs on the baseline and two other traces, one from 40C60N and the other from 20C80N, due to disconnection from the EEG equipment during the exposure to the treatment. Hence, spectral analysis was performed on 47 traces (40C90C: n=15; 40C60N: n=15; 20C80N: n=17). Spectral analysis on the filtered recording (band pass: 0.1 to 30 Hz) was set: fast Fourier transform 1K; data window Hamming and window overlap 50%, including the zero frequency. Median frequency (F50, Hz) was calculated from each 1 s epochs and classified according to gas exposure time. Decrease of F50 values in 50% in comparison to baseline was correlated to clinical signs of loss of consciousness (EFSA, 2017). The total power (Ptot, nV^2) decrease below 10% of the baseline power was correlated to brain death (EFSA, 2017).

2.4. Behavioural assessment

Broiler´s behaviour during exposure to the gases was recorded with three video cameras (IP Camera DH-IPC-HDW2231TP-ZS-S2, Zhejiang Dahua Vision Technology CO. LTD., CN) placed inside the chamber on each of the long sides (2) and at the top of the lift (1). Cameras aimed at recording a clear overview of the birds from all sides. Video cameras were connected to digital image recorder (Network video recorder DHI-NVR4108-8P-4KS2/L, Zhejiang Dahua Vision Technology CO. LTD., CN). For better identification throughout the behavioural assessment, before placing the chicken in the lift, each broiler chicken was spray-painted with a specific colour (green, red, or blue) on both the dorsal and ventral parts of the body to identify individual behaviour. Files were saved for subsequent analysis.

Ethogram used to assess the behaviour of broiler chickens during stunning process is shown in Table 1. Behavioural annotation was performed by a blind researcher to the experimental treatments using the software Boris (Behavioral Observation Research Interactive Software) v.7.13.8 8 (Friard and Gamba, 2016)

Table 1. Ethogram used to assess the behaviour of broiler chickens submitted to different experimental gas stunning treatments.

Behaviour	Description	Reference
Deep inhalation	Wide open-mouth breathing with neck extension	Gent et al. (2020)
Gasping	Open and close mouth without neck extension and reduced frequency	Gent et al. (2020)
Head shaking	Rapid side-to-side movement of the head, which occurred whilst the animal was standing, walking or sitting	Gent et al. (2020)
Jumping	Any vertical movement from a plantar stance, resulting in both feet leaving contact with the floor	
Loss of posture	Unable to regain/maintain a controlled posture. It was defined by cessation of standing with the head resting against either the floor or wall of the gas stunning system	Gent et al. (2020)
Ataxia	Uncoordinated walking with exaggerated lateral movement or as the use of wing when standing to maintain posture	Gent et al. (2020)
Leg paddling	Involuntary, usually alternating, leg movements in the air or towards the ground depending on the body position of the bird. It can also be determined by an alternating upwards and downwards movement of the body if bird is lying sternal	Martin et al. (2016)
Motionless	Limp carcass with the bird being completely still including the cessation of visible breathing movements. It reflects complete and irreversible loss of muscle tone	EFSA (2017)

Wing flapping	Bouts of fast, short flapping, rapid movement of the wings in a motion similar to attempted flight	Gerritzen et al. (2013), Gent et al. (2020)

The behaviours that occur before the loss of posture (LOP) are considered to be aversive reactions to gas exposure (e.g., pain, distress, fear). This is because LOP is the behavioural indicator of onset of unconsciousness (Benson et al., 2012). Therefore, any behaviour that occur after LOP is not considered aversive since it is presumed that the animal is not able to feel any pain, distress or fear but rather behaviours resulting from convulsions. On the other hand, motionless is the indicator of death (Gerritzen et al., 2004).

2.5. Statistical analyses

Data pre-processing, statistical analyses and plots were performed using R software v.4.1.0. (R Core Team, 2021). For all the statistical analyses, significance was declared at $P < 0.05$. Behavioural measurements analysis comprised the time to onset, the number of events, the duration and the proportion of broilers that performed the behaviours in a treatment. Since the time to onset of the different behaviours analysed did not follow a normal distribution, Kruskal-Wallis rank sum test was used to compare the medians between treatments and the p-values that were adjusted with the Benjamini-Hochberg method. The number of times that each broiler chicken performed a certain behaviour was computed and the occurrence between treatments was compared by means of Pearson's Chi-squared test. The duration of behaviours did not follow a normal distribution, so it was transformed to logarithm prior to apply an analysis of variance with post-hoc Tukey HSD. On the other hand, the proportion of broilers that experienced each of the aversive behaviours per treatment was calculated and proportions between treatments were compared using Chi-squared test. EEG data for each broiler was calculated and display as percentage change in the F50 and Ptot from baseline values (Gibson et al., 2018).

3. Results

3.1. Gas concentration assessment

Broilers chickens submitted to 40C90C were exposed to CO_2 concentration below 40% by volume in atmospheric air on the first phase in all cycles (38.1 ± 0.1%). On the second phase CO_2 was kept higher than 90% (92.2 ± 0.6%) and O_2 lower than 2% by volume (1.0 ± 0.1%). On the other hand, broilers chickens submitted to gas mixtures of CO_2 with inert gases were exposed to CO_2 concentrations at 36.3 ± 1.1% in 40C60N and 18.0 ± 0.3% in 20C80N, while O_2 was below or close to 2% by volume during the 4 min of exposure (40C60N: 1.6 ± 0.3%; 20C80N: 1.9 ± 0.3%). However, the anoxic atmosphere (<2% of O_2) was steadier through time of exposure in 40C60N compared to 20C80N.

3.2. Brain activity assessment

Brain activity during the gas stunning procedure was recorded by EEG in 50 broiler chickens (40C90: n=16; 40C60N: n=16 and 20C80N: n=18). However, 22 records were not suitable for statistical analysis due to flat trace during procedure that did not allow to observe a clear shift on EEG trace. Hence, statistical analyses were performed on 28 remaining EEG traces: 5 EEGs (40C90C), 14 EEGs (40CN60) and 9 EEGs (20C80N). The mean time to onset to LOC, considered when the mean of F50 was reduced by 50% from the baseline (time 0), it was established at 30 s in 40C60N and 29 s 20C80N. However, the time to LOC according to the reduction of F50 could not be assessed in 40C90C by EEG due to high number artefacts caused by birds' movements. The relationship between LOC determinate for the decrease of F50 and LOP is shown in Figure 2.

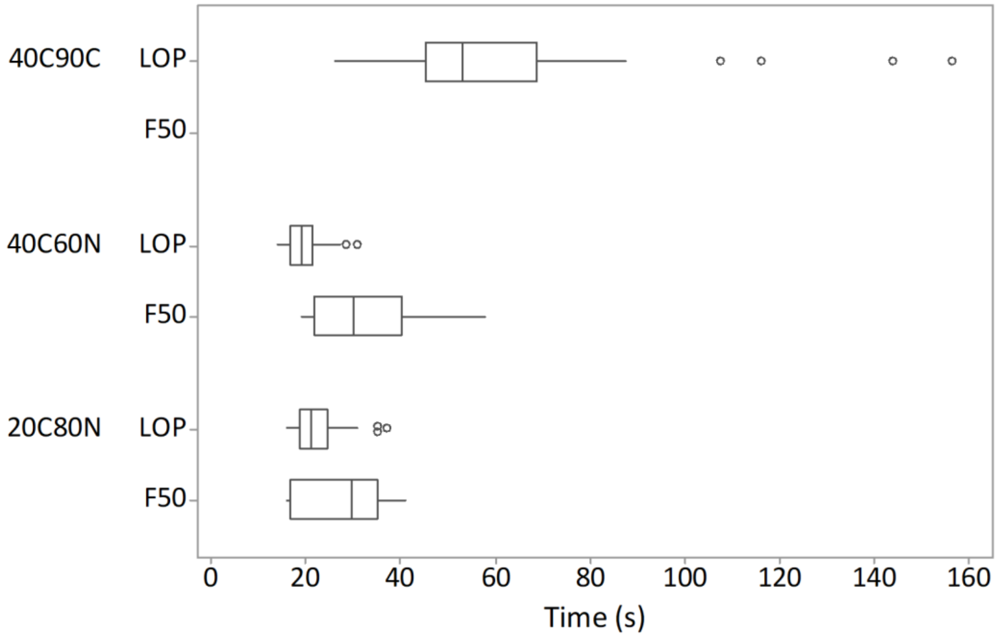


Figure 2. Boxplot of time of onset to observed loss of posture (LOP) and reduction on median frequency (F50) loss of consciousness (LOC) after exposure to different gas stunning mixtures in broiler chickens. 40C90C: CO_2 in two phases being the first phase with <40% CO_2 by volume in air during 2 min and second phase with >90% CO_2 during 2 min. 40C60N: gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min and 20C80N: gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min.

The identification of brain death was estimated by spectral analysis when Ptot was reduced by 90% from baseline values, the observation of isolectrical trace and the observation of motionless in unrestrained broilers. The onset to brain death can be observed on Figure 3, for treatment 40C90C the values from spectral analysis were not considered due to low quality records on such treatment.

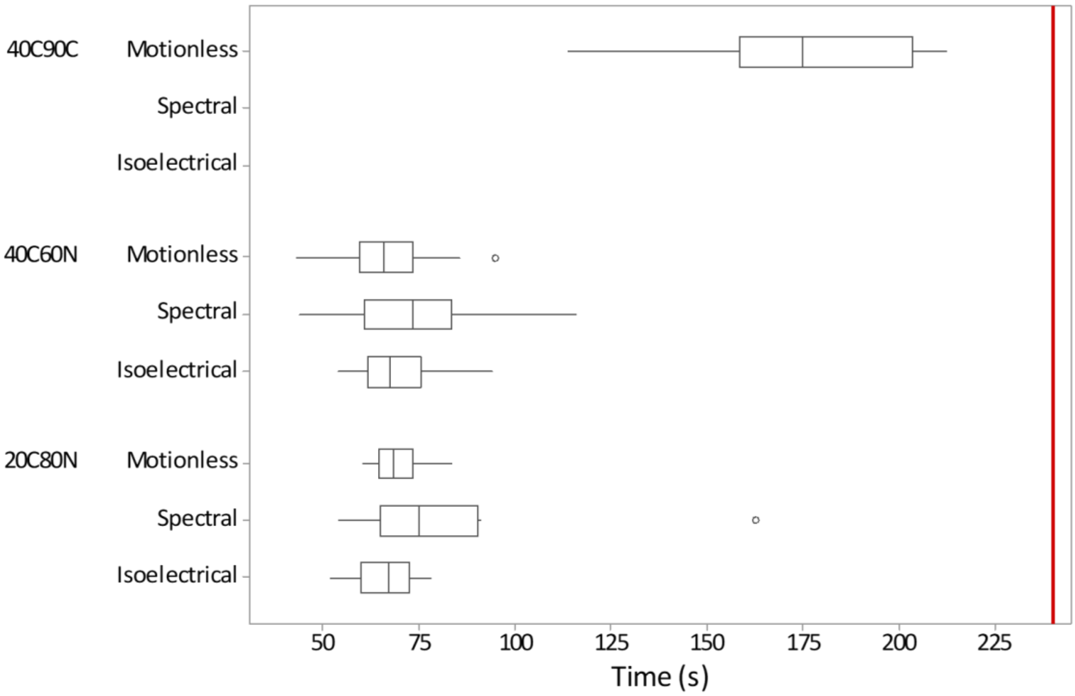


Figure 3. Boxplot of time of onset to brain death after exposure to different gas stunning mixtures in broiler chickens. 40C90C: CO_2 in two phases being the first phase with <40% $CO_2$by volume in air during 2 min and second phase with >90% CO_2 during 2 min. 40C60N: gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min and 20C80N: gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min. The red line means the end of the gas exposure.

3.3. Behavioural assessment

Broiler chickens exposed to AIR did not show neither ataxia, deep inhalation, wing flapping, LOP, motionless, gasping, jumping or leg paddling. Nevertheless, two out of 92 broilers showed head shaking once or twice.

The time to onset of LOP and motionless according to the three experimental treatments are summarized in Table 2. Both times were significantly greater in 40C90C in contrast to 40C60N and 20C80N ($P < 0.001$) and also the time in 20C80N was greater compared to 40C60N ($P < 0.01$). It is noteworthy that the range of time to LOP and motionless in 40C90C was wider than 40C60N and 20C80N, whereas 40C60N and 20C80N broiler chickens showed very low variability. In addition, two 40C90C broiler chickens lost posture at 144 and 156 s and therefore, they were still conscious when the lift descended to a higher CO_2 concentration during the second phase. In addition, the latest appearance of motionless was at 212.7 s (40C90C), 107.8 s (40C60N) and 88.5 s (20C80N) during gas exposure and thus, only 27 s (40C90C), 132 s (40C60N) and 151s (20C80N) before the end of the exposure (240 s). This indicates that all birds were dead before the end of the process.

Table 2. Detailed summary statistics on the time to onset of loss of posture and motionless of broiler chickens submitted to different experimental gas stunning treatments.

	Time to onset of loss of posture during gas exposure, s			Time to onset of motionless during gas exposure, s		
	40C90C	40C60N	20C80N	40C90C	40C60N	20C80N
Min.	26.1	14.0	15.8	89.2	43.0	45.2

1st Quartile	45.4	17.0	18.7	153.3	59.1	64.9
Median	53.2	19.0	21.0	176.9	64.2	69.5
Mean	59.2	19.6	22.3	168.8	66.1	70.4
3rd Quartile	68.4	21.2	24.5	184.5	71.0	75.8
Max.	156.5	30.8	37	212.7	107.8	88.5

40C90C: CO_2 in two phases being the first phase with <40% CO_2 by volume in air during 2 min and second phase with >90% CO_2 during 2 min (n=76); 40C60N: a gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min (n=63) and 20C80N: a gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min (n=54).

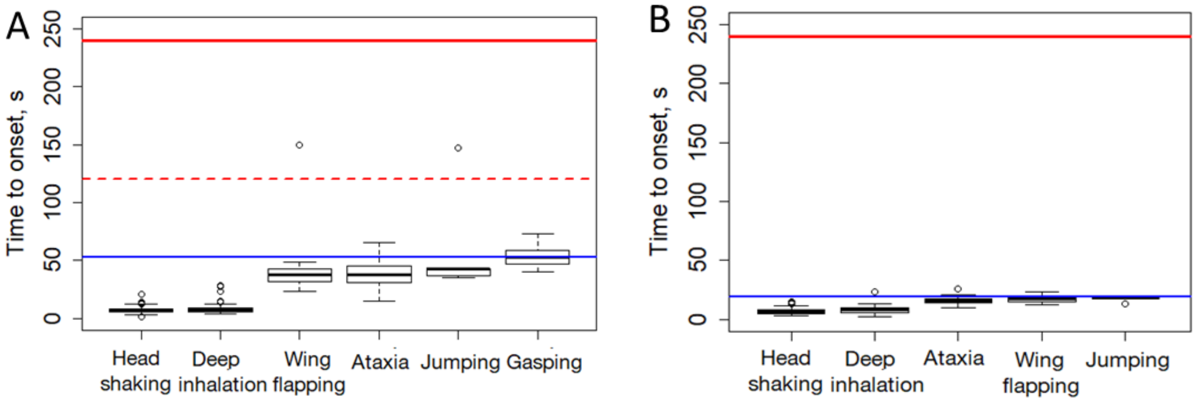
The number and proportion of broiler chickens that showed behaviours before LOP related to both respiratory and locomotor manifestations in each treatment are shown in Table 3. The experimental treatments caused head shaking and deep inhalation in all birds. Ataxia was observed in most of the animals with no statistical differences between treatments (P = 0.585). However, while 40C90C caused gasping in many animals, no bird gasped under 40C60N and 20C80N. Wing flapping was dramatically increased in 40C60N compared to 40C90 ($P < 0.01$), in 20C80N compared to 40C60N ($P < 0.01$) and in 20C80N compared to 40C90C ($P < 0.001$). Jumping behaviour was observed in few animals with no statistical differences between treatments ($P < 0.854$).

Table 3. Number of broilers that showed behaviours before loss of posture related to both respiratory and locomotor manifestations in broilers chickens submitted to different experimental gas stunning treatments. The number of birds affected report the number of birds showing the behaviour.

	Number of birds affected/ total number of observed birds			
Aversive behaviour	**40C90C**	**40C60N**	**20C80N**	***P*-value**
Head shaking	76/76	63/63	54/54	1.000
Deep inhalation	76/76	63/63	54/54	1.000
Ataxia	63/76	55/63	48/54	0.585
Gasping	20/76[b]	0/63[a]	0/54[a]	<0.001
Wing flapping	20/76[a]	35/63[b]	43/54[c]	<0.001
Jumping	5/76	5/63	5/54	0.852

40C90C: CO_2 in two phases being the first phase with <40% CO_2 by volume in air during 2 min and second phase with >90% CO_2during 2 min (n=76); 40C60N: a gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min (n=63) and 20C80N: a gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min (n=54). Different superscripts mean significantly different proportions between treatments ($P < 0.05$).

The range of the time to onset to the different behaviours compared to the onset of LOP is displayed in Figure 4. All behaviours appearing before the LOP are below the blue line and represent a welfare concern as the animal is still conscious during gas exposure.



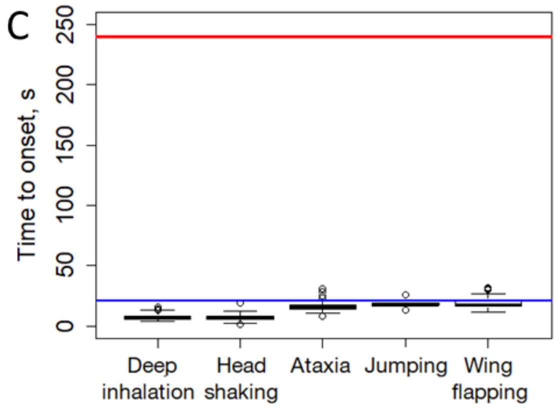


Figure 4. Boxplot of time of onset of the different aversive behaviours in sequence after exposure to different gas stunning mixtures in broiler chickens. Blue line represents median time to LOP, dotted red line means the second phase on 40C90C treatment, red line end of time of exposure. A) 40C90C: CO_2 in two phases being the first phase with <40% CO_2 by volume in air during 2 min and second phase with >90% CO_2 during 2 min (n=76). B) 40C60N: gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min (n=63) and C) 20C80N: gas mixture of 20% CO_2 and 80% N_2n with less than 2% of O_2 for 4 min (n=54). The red line means the end of the gas exposure.

Besides the time to onset of each behaviour, the number of events of each behaviour and duration was also assessed (Figure 5). In this regard, the number of episodes of head shacking per bird was similar between treatments (*P* = 0.350), deep inhalation was between two and three-fold times more frequent in 40C90C than in 40C60N and 20C80N (*P* < 0.001) and also in 20C80N compared to 40C60N (*P* < 0.01) while gasping only occurred in 40C90C. Concerning ataxia, the number of episodes was similar between 40C90C and 20C80N (*P* = 0.793) and both expressed in later time than 40C60N (*P* = 0.003), the number of episodes of wing flapping was similar between treatments (*P* = 0.223).

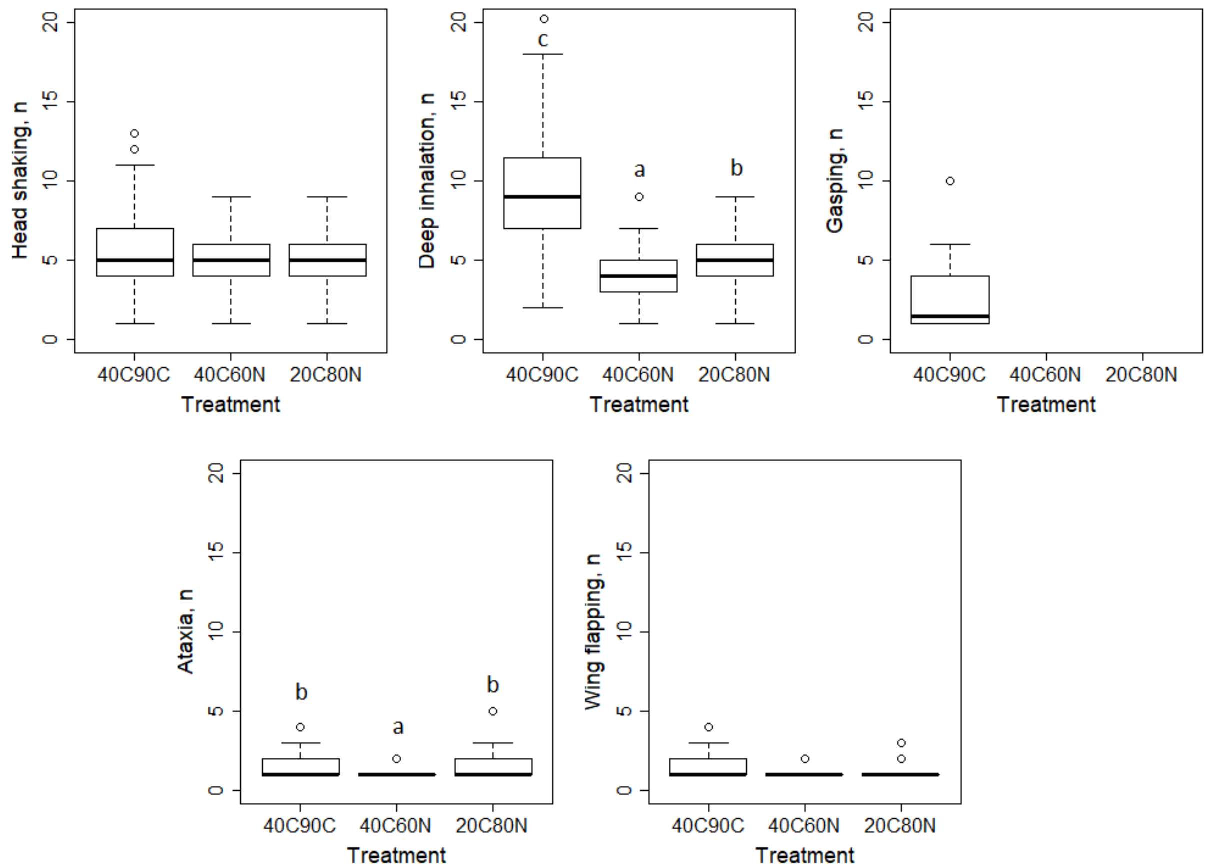


Figure 5. The boxplots describe the number of events that occur before the loss of posture (indicative of aversion) of broiler chickens submitted to different experimental gas stunning treatment being 40C90C: CO_2 in two phases being the first phase with <40% CO_2 by volume in air during 2 min and second phase with >90% CO_2 during 2 min (n=76); 40C60N: gas mixture of 40% CO_2 and 60% N_2 with less than 2% of O_2 for 4 min (n=63) and 20C80N: gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min (n=54).

The total duration of wing flapping before (escape attempts) and after LOP (convulsions) was similar between treatments (mean = 2.7 s; P = 0.641 before LOP and mean = 1.9 s; P = 0.287 after LOP). The behaviours occurred after LOP were wing flapping, leg paddling and gasping in all three experimental treatments.

4. Discussion

4.1. Gas concentration assessment

Concentration of both CO_2 and O_2 stayed stable during the cycles and within the limits required by the European Union legislation for the protection animals at the time of killing (Council Regulation, 2009) according to each gas treatment.

4.2. Brain activity assessment

The present study was designed for the recording of EEG traces from 50 broiler chickens randomly distributed among the three experimental treatments. However, at the end of the study, only 22 records were suitable for analysis. The loss of more than half of the intended sample size must be considered in future experiments since multiple studies also report loss of electrodes or non-readable EEG activity in 9–71% (Velarde et al., 2002; Gerritzen et al., 2004; Lambooij et al., 2006). In poultry, head shaking, deep inhalation, blinking and wing flapping interfere on EEG regarding selection of free epoch artefacts, even in the restrained animals. Such issues can compromise the number and quality of data for spectral analysis as it has been also reported before (McKeegan et al., 2007; Martin et al., 2019). In the present study, the main limitation was the low number of suitable EEG recordings in 40C90C and the selection of free artefact epochs. It was not possible to identify the shift from consciousness to unconsciousness state due to flat traces (i.e., high prevalence of low frequencies).

According to our results, the exposure to either 40C60N or 20C80N leads to onset of unconsciousness at approximately 32 s in a pre-filled pit. It should be noted that 32 s is the average time birds begin to lose consciousness as there is not an abrupt shift between consciousness to unconsciousness but gradual process. In previous studies, the reduction on F50 values was used to estimate the induction of unconsciousness in broilers submitted to anaesthesia, or LAPS (Sandercock et al., 2014; EFSA, 2017). In any case, this average time found in 40C60N and 20C80N is similar to those reported in previous literature (McKeegan et al., 2007a; Coenen et al., 2009). McKeegan et al. (2007a) pointed out that broilers exposed to 100% inert gases (Argon or Nitrogen) lasted two-fold times more than when exposed to a combination of 60% N_2 + 40% CO_2. Their results are similar to ours since LOC was observed to occur at around 23 s in the 60% N_2 + 40% CO_2 treatment. However, our method was slightly faster as we used a pre-loaded pit with a O_2 concentration lower than 2% all time. McKeegan's study was in a closed cabined (not pre-filled gas but flushed) and the reduction in available oxygen was reported to be not instantaneous. Similarly occurred in Coenen et al. (2009) when using a tunnel system where the combination of 70% N_2 + 30% CO_2 led to LOC in broilers at around 26 s and the treatment of 100% N_2 lasted two-fold times. The identification of brain death was estimated by spectral analysis. The animal is considered death when the EEG Ptot decreases below 10% from baseline values. Residual Ptot on spectral was used to estimated brain death in broilers submitted to LAPS (EFSA, 2017). The observation of isolectrical trace on EEG and the observation of motionless in unrestrained broilers are also indicators related to brain death (Gerritzen et al. 2004). However, it is known that normal brain activity may be restored in animals stunned with inert gases or CO_2 associated with inert gases (as in 40C60N and 20C80N) if O_2 is administered, or if they can breathe atmospheric air. Inevitably, the recovery of consciousness in these animals is rapid (Velarde & Raj, 2016) so death must be guaranteed at the end of the stunning/killing process.

4.3. Behavioural assessment

Two out of 92 broiler chickens from AIR treatment performed head shaking once or twice while descending the pit. Head shaking may occur as a response to a new stimulus (descent of the lift) or as an effort to self-activate after resting (Webster and Fletcher, 2001). Hence, the low prevalence of head shaking and the absence of occurrence of other behaviours suggests that descending the pit did not cause aversion to broiler chickens as it occurs in the three experimental treatments tested.

In the present study, the onset to LOC measured through the EEG spectral analysis was set at an average of 30 s for 40C60N and 29 s for 20C80N exposure while LOP was observed to occur at a median of 19 – 21 s. This will confirm that LOP can be used as a proxy of LOC in broiler chickens as it was previously reported by Gerritzen et al. (2004) and Benson et al. (2012). In this sense, 40C90C broiler chickens have probably lost consciousness at an average of 59 s as this was their mean of LOP. Motionless is considered the behavioural indicator of death (EFSA, 2019). Notably, the broilers exposed to 40C90C took longer to LOP, had much greater inter-individual variability and took longer to die (motionless range: 123 s), than the broilers exposed to 40C60N (range: 64 s) and 20C80N (range: 43 s). The high inter-individual variability in the time to LOP in 40C90C is of severe welfare risk if birds remain conscious when exposed to higher CO_2 concentrations during the second phase due to the painful stimulus caused by CO_2 when inhaled at concentrations above 40% (McKeegan *et al.* 2006; Gerritzen *et al*., 2007).

Behaviours that occurred before the LOP can be associated with conscious animals and therefore reflexive reactions of aversion in all gas treatments. On the contrary, behaviour that occur after LOP is not considered aversive since it is presumed that the animal is unconscious and not able to feel pain, distress, or suffering (Gerritzen et al., 2004; EFSA, 2019). Before LOP, head shacking and deep inhalation are behaviours related to pain and respiratory distress whereas ataxia, wing flapping and jumping are behaviours related to tentative to maintain posture.

Behavioural assessment showed that the treatments assessed in the present study induce a certain degree of aversion. The proportion of broilers showing head shacking, deep inhalation, ataxia and jumps was similar between the three experimental treatments. All birds from all treatments showed head shaking and deep inhalation, and almost all, ataxia. Gasping was only observed in some birds exposed to 40C90C before LOP. Furthermore, the higher the nitrogen concentration in the CO_2 associated with inert gases mixtures, the higher the proportion of broilers showing wing flapping before LOP. Jumps were observed in very few broilers. The occurrence of aversion behaviours is explained for the activation of intrapulmonary chemoreceptors that are acutely sensitive to CO_2 and cause unpleasant sensation to broilers when inhaled (Webster and Fletcher, 2004; Raj *et al*., 2006). However, in this study the CO_2 concentrations on 40C60N, 20C80N and first phase of 40C90C remained below 40% by volume in air, according to European regulation.

The sequence of behaviours showed from the onset of the stunning process to LOP seems to be relevant for the animal welfare assessment. Thus, head shaking and deep inhalation were exhibited at the beginning of three gas treatments. Head shaking as indicator of aversion to CO_2 remained unclear as it has been also associated not with aversion, but with novel or alerting stimuli since it has also been observed to occur in anoxic atmospheres (100% N_2) in a similar number of events per bird compared to hypercapnic anoxia atmosphere (40% CO_2 and 60% N_2) (McKeegan et al., 2006, 2007a). However, although head shaking equally applied to all gas mixtures, the present study demonstrated that under AIR treatment, head shaking was performed in very few occasions so this behavioural indicator should undoubtedly be considered also a reaction to unpleasant experience to gas exposure in birds indicative of aversion. On the other hand, sequence of behaviours during exposure to the gas treatments showed that the onset of wing flapping occurred before the onset of ataxia in 40C90C while wing flapping occurred after ataxia in 40C60N and 20C80N. This seems to indicate that wing flapping in 40C90C is an attempt to escape and therefore an aversive reaction to the modified atmosphere. In contrast, in 40C60N and 20C80N, wing flapping may indicate an attempt to maintain posture before losing consciousness.

Once broiler chickens lost consciousness, they perform gasping, leg paddling and wing flapping. Gasping after LOP can be interpretated like last tentative to breath due to anoxia and hypercapnia atmosphere (Jacobs et al, 2019). Paddling and wing flapping are uncontrolled muscular movements associated to convulsions (Gerritzen et al., 2000). Wing flapping after LOP had a similar duration in all treatments but qualitatively, the intensity of these convulsions was higher in 20C80N than in the other treatments. This is of welfare concern since it may cause injuries and pain to the other birds that have not yet lost consciousness (McKeegan et al., 2007; Berg and Raj, 2015).

5. Conclusions

The exposure of broiler chickens to CO_2 in two phases or CO_2 with inert gases does not induce immediate unconsciousness. Regardless of the gas mixture tested, all broiler chickens experience aversion during the induction and before loss of consciousness.

The time to lose consciousness is on average around 60 s in CO_2 in two phases and around 29-30 s in CO_2 with inert gases. Combinations of CO_2 with inert gases induce unconsciousness and death considerably faster but also with less variability between individuals compared to CO_2 in two phases. The high variability in the time to lose consciousness when exposed to CO_2 in two phases leads to the risk of broilers to be conscious when the CO_2 concentration increases above 40% during the second phase. This may increase the severity in pain and respiratory distress in these conscious broilers.

From the result of this experiment, the best compromise regarding animal welfare and efficiency is the exposure in a pre-filled chamber to the gas mixture of 40% CO_2 + 60% N_2 in an anoxic atmosphere (<2 % O_2 residual), during at least four minutes to guarantee the death in all broilers.

When exposure to CO_2 in two phases is used, it is recommended to verify that all birds are unconscious before increasing the CO_2 above 40%.

It would be interesting to explore other gas mixtures with anoxic gases to try to decrease the aversion during the induction phase.

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