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Article

Alternatives to the Use of Carbon Dioxide in Two Phases for the Improvement of Broiler Chickens' Welfare During Stunning

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Simple Summary: Stunning during the slaughter process consists of inducing unconsciousness in animals in order to prevent them any avoidable pain, distress, or suffering during bleeding and related operations. In an unconsciousness state, the animal is unable to perceive and respond to any external stimuli, including pain. Currently, the two main stunning methods used commercially in broiler chickens are electrical waterbath and carbon dioxide in two phases stunning. Although the latter is widely recommended over electrical waterbath stunning, it is still not exempt of risks for animal welfare. For instance, the induction to unconsciousness is not instantaneous and involves a transitional period during which aversion to carbon dioxide might occur. The gas inhaled by the birds activates chemoreceptors in the mucous membranes of the respiratory tract, which causes discomfort, pain and dyspnoea. The present study evaluates two different gas mixtures of carbon dioxide associated with nitrogen as alternatives to carbon dioxide in two phases.

Abstract: This study evaluated gas mixtures of carbon dioxide (CO_2) associated with nitrogen (N_2) as alternatives to the use of CO_2 in two phases to improve the welfare of broiler chickens at slaughter. Broilers were exposed to one of three treatments: 40C90C (1st phase: <40% CO_2 for 2 min; 2nd phase: >90% CO_2 and <2% O_2 for 2 min, n=92), 40C60N (40% CO_2, 60% N_2 and <2% O_2 for 4 min, n=79), or 20C80N (20% CO_2, 80% N_2 and <2% O_2 for 4 min, n=72). The time to onset of loss of consciousness (LOC) and death was assessed by brain activity (EEG) and behaviour. Behaviour also allowed characterisation of the aversive response to the treatments. Exposure to 40C60N and 20C80N induced LOC faster (19 s [14 – 30 s] and 21 s [16 – 37 s], respectively) but also with less inter-individual variability compared to 40C90C (53 s [26 – 156 s], $P < 0.001$) and birds took less time to die (40C60N: 64 s [43 – 108 s]; 20C80N: 70 s [45 – 88 s]; 40C90C: 177 s [89 – 212 s], $P < 0.001$). The 40C90C birds not only experienced more aversion during induction to LOC but are at risk of remaining conscious when the CO2 concentration is increased in the 2nd phase. From an animal welfare point of view, 40C60N provided the highest welfare, followed by 20C80N and 40C90C.

Keywords: controlled atmosphere stunning; gas stunning; carbon dioxide; inert gases; nitrogen; unconsciousness; death; aversion; broiler chicken

1. Introduction

Pre-slaughter stunning is mandatory in the European Union [1] and many other countries [2]. It consists of inducing unconsciousness in animals in order to prevent them any avoidable pain, distress, or suffering during bleeding and related operations. In an unconsciousness state, the animal is unable to perceive and respond any external stimuli, including pain [3]. To protect animal welfare at slaughter, it is essential to induce an effective stunning so that the animals do not regain consciousness before brain death due to bleeding.

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 stunning method is of great concern regarding the bird welfare [4]. For this reason, CAS emerged as an alternative stunning method to WBS [5]. 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 [6].

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 [7,8]. Most slaughterhouses with CAS 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 [8].

Commercial equipment for stunning poultry 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 [4] and enter the system on a conveyor belt. In tunnels and pits, the system is pre-filled with gas 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 concentrations. 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 and removed upon completion of the stunning cycle [8].

The physiological principle during induction of unconsciousness using CO_2 is to induce acidosis and neuronal depression [9]. However, prior to LOC, CO_2 activates chemoreceptors on mucous membranes of respiratory tract in poultry, which may induce discomfort, pain and breathlessness as shown by behaviours indicative of aversion [10]. The degree of aversion depends on the CO_2 concentration. The higher the concentration, the greater the aversion, but the more rapid the LOC [11]. 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 while animals are unconscious [4].

As an alternative to CO_2 in two phases, the exposure to inert gases (e.g., nitrogen N_2; argon Ar) are expected to reduce aversion. Inert gases are colourless, odourless, tasteless, and non-irritative and therefore, they are imperceptible to birds. In addition, inert gases displace oxygen (O_2) from the atmospheric air, and this ensures that the birds lose consciousness by anoxia [3] which it is not perceived by the birds [12] due to lack of chemoreceptors on air ways to inert gases. In this sense, inhalation of inert gases is expected not to cause aversive reactions [13,14]. When birds enter a chamber filled with inert gases, their behaviour does not differ from when they were breathing atmospheric air [13], they do not withdraw [11] and they barely show behavioural signs of distress [10,15]. Nevertheless, when birds are unconscious, they perform severe convulsion expressed as wing flapping [10,13], which may cause self-inflicted injuries (wing fractures) or injuries and pain to the other birds that have not yet lost consciousness [5,16]. However, it is not entirely clear whether the onset of wing flapping are reflexive reactions occurring after the bird loses consciousness or whether the birds are still conscious trying to escape from such modified atmosphere [16,17].

EFSA pointed out that research-evaluating stunning methods require well-controlled studies under laboratory conditions to characterise the animals' responses to the stunning method (i.e., onset of unconsciousness and death, magnitude of aversion) [18]. Measuring electrical brain activity by means of electroencephalography (EEG) is the most accurate method to assess the onset and duration of unconsciousness and time to death [18] and therefore, behaviours that occur before LOC could indicate aversion while during LOC could be related to convulsions [18]. Nevertheless, this approach is scarce in scientific literature.

The aim of this study was to assess the use of different gas mixtures of CO_2 and N_2 as potential alternatives to the use of CO_2 in two phases for the improvement of animal welfare during the stunning of broiler chickens. To this end, we aimed to correlate EEG and behavioural recordings to determine the time to onset of unconsciousness and death and to characterize the gas aversion response.

2. Materials and Methods

2.1. Experimental Design and Facilities

A total of two hundred ninety-three 39-day-old mixed-sex Ross 308 broiler chickens were transported from a commercial farm to the experimental facilities of the Institute of Research and Technology for Agriculture and Food (IRTA) in Monells (Spain). Birds were weighted (2.42 ± 0.18 kg) and individually identified with numbered leg bands before being allocated randomly to the different treatments. On arrival and after checking their health status and appropriate locomotor behaviour, birds were distributed randomly into seven adjacent lairage pens of 2 m × 1.8 m (35 broiler 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 pens served as a lairage before slaughter but were not associated to any specific treatment.

The study was carried out at the experimental slaughterhouse of IRTA, located next to the lairage pens. It was 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 descended until the base of a pit at a depth of 290 cm. The pit was 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 cm 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 exposed to AIR (atmospheric air, n= 100) serving as control. These birds were allocated in equal number to one of the three experimental treatments. Therefore, from d 2 to d 5, broiler chickens were stunned with one of the following gas treatments: 40C90C (CO_2 in two phases: the 1st phase with <40% CO_2 by volume in air for 2 min followed by a 2nd phase with >90% CO_2 for 2 min, n=92), 40C60N (gas mixture of 40% CO2 and 60% N2 with less than 2% of residual 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). Each day included two sessions: from 0800 to 1200h and from 1500 to 1900h, with treatments alternating per session to avoid potential bias (Table 1).

Each session consisted of 8 to 11 cycles (dips into the pit). In each cycle, four broiler chickens were placed in the lift and exposed to the treatment. In one of the four chickens, the EEG was assessed while the other three were used for behavioural assessment. The bird used for brain activity assessment was placed on the lift separated from the three other conspecifics by a transparent methacrylate wall with a floor area of 48 cm × 112 cm (0.53 m^2). The separation was intended to prevent EEG signal interference from other birds. The three birds used for behavioural activity were placed in the lift with a floor area of 144 cm × 112 cm (1.6 m^2).

The exposure time was considered from when the lift started to descend into the pit until the lift arrived at original position. The duration of exposure to each experimental treatment was determined from pre-trials aimed at assuring death in all animals at the end of the process. Gases used were pure CO_2 and premixed mixtures of CO_2 with N_2 (Freshline gases® for food use, Carburos Metálicos, Barcelona, ES).

Table 1. Schedule of the gas stunning treatments applied along the 5-d experimental period to broiler chickens.

Session	day 1	day 2	day 3	day 4	day 5
0800 to 1200h	-	40C90C	40C60N	20C80N	40C90C
1500 to 1900h	AIR	40C60N	20C60N	40C90C	-

AIR: atmospheric air (n=100); 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=108); 40C60N: a gas mixture of 40% CO_2 and

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60% N_2 with less than 2% of O_2 for 4 min (n=95) and 20C80N: a gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min (n=90).

2.2. Gas Concentration Assessment

The pit was gas-filled before entering the birds, and CO_2 and O_2 concentrations were continuously monitored before, during and after each cycle in all treatments. Gas concentrations were measured with a mobile sounding line every 10 cm vertically from bottom to top of the pit. It allows measures of CO_2 and O_2 concentration at different depth.

In 40C90C, for the first phase, the height with the CO_2 concentration of closest to 40% but without exceeding this concentration was measured, as distance varied throughout the cycles. Then the distance from the top of the pit was registered and the lift descended until reaching such depth (53.1 ± 15.7 cm) to ensure that animals will be exposed to the target concentration. During the second phase in 40C90C, 40C60N and 20C80N the sounding line monitored the gas concentration at 30 cm from the bottom of the pit in order to monitor the gas concentrations at the level of the chicken's head.

The gas mixtures 40C60N and 20C80N were provided with a CO_2 concentration variation of ±4%. In 40C60N the CO_2 concentration at the bottom of the pit was the closest to 40% (36.3 ± 1.1%) being always below to 40%, and O_2 below 2% (1.6 ± 0.3%). In 20C80N the CO_2 concentration was close to 20% (18.0 ± 0.3%) and O_2 below 2% (1.9 ± 0.3%). At the end of each cycle, gas was added in the pit when needed whereas 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 for electrical brain activity assessment through electroencephalography (EEG). Chickens were distributed in 40C90C (n=16), 40C60N (n=16) and 20C80N (n=18) treatment groups.

Each bird was prepared immediately before to be submitted to exposure to the treatment. Firstly, the bird was wrapped with a textile mesh to restrain wings, body, and legs and leaving head and neck exposed in order to minimize movement during EEG recording. Secondly, the chicken´s neck was restrained gently (Figure 1). 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% subcutaneously injected with an insulin needle and syringe in regions where EEG electrodes were to be placed. Once the skin was desensitized, 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. [19].

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. The 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 the ground electrode was inserted in the neck in caudal direction (Figure 1). Electrodes were secured in position with surgical tape (Durapore, 3M, Maplewood, MN, USA). Inter-electrode impedance was checked to be between 1.2 to 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).

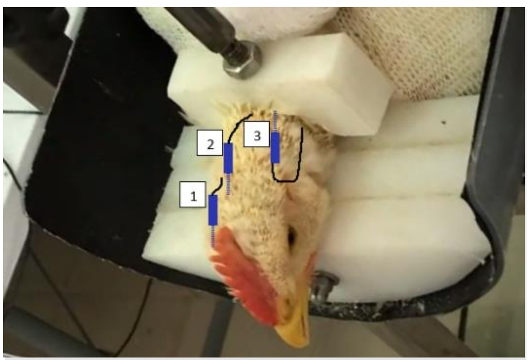

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Figure 1. Representation of needle electrodes position for brain activity assessment through electroencephalography in feather shaved broiler chickens being 1) active electrode, 2) reference electrode and 3) ground electrode.

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 analogue signals were digitalized (1 kHz) with a 4/20 PowerLab (ADInstruments Ltd, Sydney, AU) converter and recorded using a laptop for offline analyses. Pretreatment EEG signals were collected for 90 s when the bird was on the floor of the lift with the other three birds prior to descent into the pit to obtain the normal EEG data (i.e., baseline) to compare with post treatment results.

EEG recordings were monitored, saved and pre-processed using LabChart 8 Pro (v.8.1.21, AD Instruments, DE).

Spectral analysis of EEG recordings was used for detecting waveform changes that indicate the onset of unconsciousness. Spectral variables, including total power (Ptot), median frequency (F50), and spectral edge frequency (F95) were computed from the EEG data. Ptot represents the overall area under the power spectrum curve, F50 corresponds to the median frequency of the power spectrum curve, and F95 indicates the frequency at which 95% of the power spectrum curve is located [20]. On the other hand, the brain's electrical activity recorded in the EEG were categorized into different frequency bands: Delta (<4 Hz), Theta (4 to 8 Hz), Alpha (8 to 13 Hz), Beta (13 to 32 Hz), and Gamma (31 to 200 Hz). The F50 and Delta frequency contribution were utilized to estimate the loss of consciousness (LOC), while Ptot was employed to estimate death on spectral analysis.

2.4. Behavioural Assessment

Broiler chicken´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) aimed at recording the birds for subsequent behaviour assessment. Therefore, two of them were placed inside the chamber on each of the laterals and one in the central part of the lift ceiling. Video cameras were connected to a digital image recorder (Network video recorder DHI-NVR4108-8P-4KS2/L, Zhejiang Dahua Vision Technology CO. LTD., CN). Vocalisations were recorded with a digital voice recorder (Olympus VN-712PC, Olympus imaging Corp, JP). Subsequently, the video records and audios were synchronized.

To facilitate the track on visual monitoring during the behavioural assessment, each chicken was spray-marked with a specific colour (green, red or blue) on both the dorsal and ventral parts of the body before being placed on the lift.

Manual behavioural observations were recorded retrospectively using BORIS (Behavioural Observation Research Interactive Software) v.7.13.8 [24] by a researcher blinded to the experimental treatments based on the ethogram shown in Table 2. Behaviour was assessed continuously at individual level, in which the focal birds was observed for 4 min (i.e., from the time the lift started to descend into the pit until the end of gas exposure).

Table 2. Ethogram used to assess the behaviour of broiler chickens submitted to different experimental gas stunning treatments.

Behaviour	Description	Reference
Loss of posture	Cessation of standing with the head resting against either the floor or wall of the gas stunning system.	[10,25]
Motionless	Limp carcass with the bird being completely still including the cessation of visible breathing movements.	[23]
Sitting	Legs underneath the body cavity and wings relaxed against body wall.	[10]
Standing	Standing with the body fully or partly lifted off of the ground.	[26]

Walking	Moving forward to explore the area.	[27]
Ataxia	Uncoordinated walking with exaggerated lateral movement or as the use of wing when standing to maintain posture.	[10]
Deep inhalation	Wide open-mouth breathing with neck extension.	[10]
Gasping	Open and close mouth without neck extension and reduced frequency.	[10]
Head shaking	Rapid side-to-side movement of the head, which occurred whilst the animal was standing, walking or sitting.	[10]
Jumping	Any vertical movement from a plantar stance, resulting in both feet leaving contact with the floor.	[10]
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.	[28]
Wing flapping	Bouts of fast, short flapping, rapid movement of the wings in a motion similar to attempted flight.	[10,29]
High pitch vocalisations	Single or repeated short and loud shrieking (screaming)	[4]

Loss of posture (LOP) was considered a behavioural indicator of onset of unconsciousness [10,25,30,31], therefore, behaviours appearing before LOP may be related to aversion (e.g., pain, distress, breathlessness) as the birds were still conscious during gas exposure while behaviours appearing after LOP were related to convulsions or any other involuntary movements. For this reason, behaviours were separated and annotated into two different groups: those occurring before and after LOP. Motionless was considered the behavioural indicator of death [23,26,29].

2.5. Data Pre-Processing and Statistical Analyses

EEG recordings were pre-processed using LabChart 8 Pro (v.8.1.21, AD Instruments, DE). First, EEG recordings were digitally filtered to remove noise interference (band pass: 1 to 30 Hz). Then, epochs of 1 s from baseline and during the 4 min of gas exposure were selected for spectral analysis. Data was set at: 1K Fast Fourier transformed, Hamming windowed, 50% window overlap and the zero-frequency removed. For each bird, the following spectral data variables were calculated from each epoch: total power (Ptot, μV^2), spectral median frequency (F50, Hz) and contribution from Delta frequency (1-4 Hz) to Ptot (%). The median value from the baseline was calculated for all variables at individual level. Data generated was exported and analyzed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA). Then, F50 values under 4 Hz on the baseline were removed in order to discard low frequency artefacts caused by bird movements.

Onset to LOC was calculated as the mean time that F50 decreased below 50% [23] and Delta frequency increased above 65% in comparison to baseline value in four consecutive epochs. Brain death was determined by spectral analysis when Ptot decreased 90% in comparison to baseline values in four consecutive epochs [23] and visually when trace was isoelectric, representing an almost flat line with very low power [32]. Spectral and visual analyses have been well-established in previous studies, allowed for a more accurate estimation of the time to death and its subsequent relation with behaviour.

Time to LOC and brain death fulfilled the normality and homoscedasticity conditions. Therefore, means were compared by analysis of variance (ANOVA).

Behavioural Assessment

Data pre-processing, statistical analyses and plots were performed using R software v.4.1.0. [33]. For all the statistical analyses, significance was declared at $P < 0.05$.

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Behavioural measurements analysis comprised the time to onset of LOP and motionless, the proportion of broilers that performed the rest of the behaviours listed in the ethogram, as well as the number of events and total duration of each both before and after LOP per treatment.

The proportion of broilers that performed a certain behaviour between treatments was compared by means of Pearson's Chi-squared test. The time to onset and last, the number of events and the total duration of the different behaviours did not follow a normal distribution and therefore, Kruskal-Wallis rank sum test was used to compare the medians between treatments and the p-values were adjusted with the Benjamini-Hochberg method for multiple comparisons.

Variables with normal distribution are presented as mean ± standard deviation otherwise as median [min - max].

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 residual 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 mean value was below 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 in 40C60N compared to 20C80N.

3.2. Brain Activity Assessment

Brain activity during the gas stunning procedure was recorded through EEG in 50 broiler chickens (40C90C: n=16; 40C60N: n=16 and 20C80N: n=18). Twenty-seven out of these 50 EEG traces were discarded: one trace due to interference from eyelid movement, preventing the selection of several 1 s epochs on the baseline; two traces, due to disconnection from the EEG equipment during the exposure to the treatment and 24 traces due to recording issues. Hence, EEG analysis was performed on the 23 remaining traces (40C90C: n=0; 40CN60: n=14; 20C80N: n=9).

The time to onset of LOC and brain death according to the experimental treatment is summarized in Table 3. The time to onset of LOC did not differ significantly between 40C60N and 20C80N. However, the time to brain death was similar between the two treatments when EEG traces were analysed visually but not when the spectral were calculated.

Table 3. Time to onset of loss of consciousness and death determined by electroencephalography of broiler chickens submitted to different experimental gas stunning treatments.

	Treatments			
Item	40C90C	40C60N	20C80N	P-value
Loss of consciousness, s	NA	25.7 ± 7.0	20.7 ± 6.6	0.144
Death (spectral), s	NA	65.8 ± 14.1	122 ± 53.2	0.048
Death (isoelectric), s	NA	69.8 ± 11.9	66.3 ± 8.1	0.456

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=0); 40C60N: a gas mixture of 40% CO_2 and 60% nitrogen (N_2) with less than 2% of O_2 for 4 min (n=14) and 20C80N: a gas mixture of 20% CO_2 and 80% N_2 with less than 2% of O_2 for 4 min (n=9). NA means not available.

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3.3. Behavioural Assessment

3.3.1. Behavioural Assessment of Loss of Posture and Motionless

The time to onset of LOP and motionless according to the three experimental treatments are summarized in Table 4. Both times were significantly greater in 40C90C in contrast to 40C60N and 20C80N ($P < 0.001$) and the time in 20C80N was also greater compared to 40C60N ($P < 0.01$). It is noteworthy that the range of time to LOP and motionless were wider in 40C90C 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 (after 2 min) and therefore, they were still conscious when the lift descended to a higher CO_2 concentration during the second phase. Furthermore, the latest appearance of motionless was in all cases before the end of the exposure (240 s), indicating that all birds were dead before the end of the process.

Table 4. Time to onset of loss of posture and motionless of broiler chickens submitted to different experimental gas stunning treatments.

Behaviour	Treatment			
	40C90C	40C60N	20C80N	P-value
Loss of posture	53.2 [26.1 - 156.5] [a]	19.0 [14.0 - 30.8] [c]	21.0 [15.8 - 37.0] [b]	<0.001
Motionless	176.9 [89.2 - 212.7] [a]	64.2 [43.0 - 107.8] [c]	69.5 [45.2 - 88.5] [b]	<0.001

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). Different superscripts on median values mean significant difference between treatments ($P < 0.05$).

3.3.2. Behavioural Assessment before Loss of Posture

The proportion of birds performing the behaviours and the number of events per bird before LOP according to the experimental treatment is shown in Table 5. Sitting, standing, walking and head shaking were behaviours that broiler chickens performed either in AIR or in all experimental gas treatments before LOP. However, in AIR, all birds were sat (100%) and only some stood (30%) and very few walked (4%) while birds that inhaled any of the three gas treatments tested, the proportion of standing and walking significantly increased compared to those in AIR ($P < 0.05$). In AIR, two out of 100 broilers showed head shaking once or twice but the proportion of birds performing head shaking in AIR differed significantly from the experimental gas treatments (2% vs 100%; $P < 0.001$). All broiler chickens exposed to AIR exhibited no signs of ataxia, deep inhalation, gasping, jumping, wing flapping, or HPV.

Table 5. Proportion of broiler chickens that showed the different behaviours and the number of events per individual expressed as median [min - max], when inhaling the experimental gas stunning treatments tested before losing posture.

	Proportion, %				**Number events/bird**			
Behaviour	**40C90C**	**40C60N**	**20C80N**	***P*-value**	**40C90C**	**40C60N**	**20C80N**	***P*-value**
Sitting	98.7	92.1	92.6	0.951	2 [1 - 6] [a]	1 [1 - 3] [b]	2 [1 - 4] [ab]	< 0.001
Standing	93.4	81.0	88.9	0.848	2 [1 - 6] [a]	1 [1 - 3] [b]	1 [1 - 5] [b]	0.004
Walking	59.2	39.7	51.9	0.413	2 [1 - 7] [a]	1 [1 - 3] [b]	1 [1 - 3] [b]	0.007
Head shaking	100	100	100	1.000	5 [1 - 13]	5 [1 - 9]	5 [1 - 9]	0.072
Ataxia	82.9	87.3	88.8	0.297	1 [1 - 4]	1 [1 - 2]	1 [1 - 5]	0.061
Deep inhalation	100	100	100	1.000	9 [2 - 21] [a]	4 [1 - 9] [c]	5 [1 - 9] [b]	< 0.001

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Gasping	26.3	0.0	0.0	-	1.5 [1 - 10]	0 [0 - 0]	0 [0 - 0]	-
Jumping	6.6	4.8	9.3	0.664	1 [1 - 1]	1 [1 - 1]	1 [1 - 1]	-
Wing flapping	26.3 [c]	55.5 [b]	79.6 [a]	0.001	1 [1 - 4]	1 [1 - 2]	1 [1- 3]	0.709
High pitch vocalisations	55.3	68.3	98.1	0.101	3 [0 - 12] [a]	1 [0 - 6] [b]	4 [0 - 10] [a]	< 0.001

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). Different superscripts in proportions and number of events mean significant differences between 40C90C, 40C60N and 20C80N ($P < 0.05$).

When considering only gas treatments (i.e., leaving AIR aside), head shacking and deep inhalation were observed in all birds while gasping was only performed by some birds in 40C90C but never in 40C60N and 20C80N. All other behaviours assessed were observed in some birds to a greater or lesser extent. In any case, the proportion of birds performing sitting, standing, walking, head shaking, ataxia, deep inhalation, jumping and HPV was similar between treatments ($P > 0.05$). The proportion of broiler chickens showing wing flapping was dramatically increased in 20C80N compared to 40C60N and 40C90C ($P < 0.01$), and in 40C60N compared to 40C90C treatment ($P < 0.01$). When comparing the number of events per behaviour, the number of deep inhalation events was dramatically reduced in 40C60N and 20C80N compared to 40C90C ($P < 0.001$) but still 40C60N with lower number of events than 20C80N ($P < 0.001$) while gasping did not occur neither in 40C60N nor 20C80N but in 40C90C. Birds exposed to 40C60N also exhibited less sitting, standing, walking, and HPV events compared to 40C90C ($P < 0.01$). On the other hand, 20C80N exhibited less standing, walking, deep inhalation and gasping ($P < 0.01$) but similar sitting, wing flapping and HPV compared to 40C90C (P > 0,05). Both 40C60N and 20C80N showed a tendency towards a lower number of events of head shaking and ataxia compared to 40C90C ($P < 0.10$).

The duration of the behaviours performed before LOP according to the experimental treatment is shown in Table 6. Broiler chickens exposed to either 40C60N or 20C80N spent significantly less time sitting and standing compared to 40C90C ($P < 0.001$). Ataxia was significantly shorter in 40C60N compared to 20C80N ($P = 0.009$) and 40C90C ($P < 0.001$) while 20C80N was also shorter than 40C90C ($P < 0.027$). The duration of wing flapping did not differ among the gas treatments ($P > 0.05$).

Table 6. Time spent per broiler chicken in different behaviours before loss of posture expressed as median [minimum, maximum] when submitted to different experimental gas stunning treatments.

	Treatment			
Behaviour	**40C90C**	**40C60N**	**20C80N**	**P-value**
Sitting, s	32.5 [3.5 - 135.0] [a]	7.4 [0.5 - 19.5] [b]	5.7 [0.5 - 22.8] [b]	< 0.001
Standing, s	13.1 [2.8 - 37.9] [a]	10.2 [0.5 - 22.2] [b]	10.2 [0.0 - 18.2] [b]	< 0.001
Walking, s	4.3 [0.7 - 11.8] [a]	3.8 [0.7 - 10.5] [ab]	2.1 [0.7 - 9.3] [b]	0.012
Ataxia, s	4.8 [0.7 - 19.7] [a]	3.0 [0.3 - 7.1] [c]	4.0 [0.5 - 8.2] [b]	< 0.001
Wing flapping, s	1.2 [0.2 - 5.8]	1.4 [0.2 - 6.2]	1.5 [0.2 - 6.5]	0.177

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). Different superscripts mean significantly different time spent per behaviour between the three gas stunning treatments ($P < 0.05$).

The order in which each of the behaviours appears for the first time before LOP is shown in Figure 2. As can be observed, there is a pattern in which the first behaviours displayed in response to gas treatments are head shaking or deep inhalation. Next, the birds begin to walk, vocalize, become

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ataxic, and eventually jump and flap their wings. Gasping is only observed in 40C90C, and it is the latest behaviour observed before LOP.

3.3.3. Behavioural Assessment after Loss of Posture

The proportion of birds performing the behaviours and the number of events per bird after LOP according to the experimental treatment is shown in Table 7. After LOP, observed behaviours were gasping, jumping, leg paddling, wing flapping, and HPV. Leg paddling was the only observed behaviours that occurred in a similar proportion and in a similar number of events in all treatments ($P < 0.05$). A lower proportion of birds performed gasping with 40C60N and 20C80N, along with a lower number of gasps per animal, compared to 40C90Cs ($P < 0.001$). The proportion of birds showing wing flapping was similar between treatments ($P > 0.05$). However, broilers in 40C90C had a tendency of performing less wing flapping events compared to 40C60N ($P = 0.058$) and both 40C90C and 40C60N performing significantly less events of wing flapping than 20C80N ($P < 0.001$). In addition, 40C90C was the treatment in which broilers chickens performed wing flapping for the least amount of time ($P = 0.05$). The proportion of birds that vocalized was similar between treatments ($P > 0.05$) although birds in 40C90C and 40C60N vocalized less times than 20C80N ($P < 0.05$). In proportion, jumping was the least observed behaviour in all treatments. However, the lowest proportion of animals jumping was at 40C90C and 40C60N compared to 20C80N ($P < 0.01$). In all treatments, birds that jumped did so a similar number of times ($P > 0.05$).

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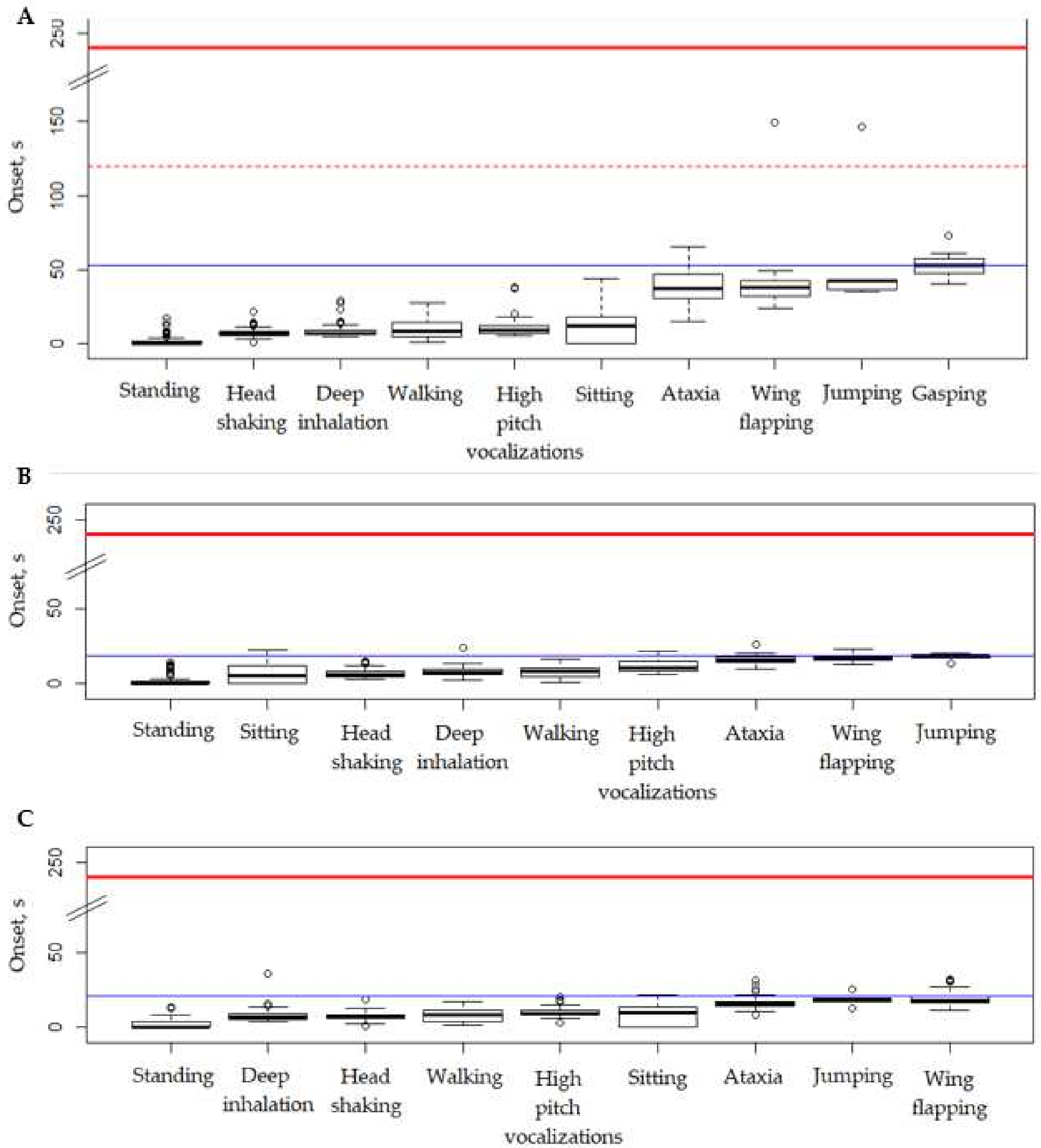

Figure 2. Boxplot of time to onset of the different behaviours before loss of posture in sequence after exposure to different gas stunning mixtures in broiler chickens. Blue line represents median time to loss of posture, dotted red line means the second phase on 40C90C treatment, red line end of time 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_2 with less than 2% of O_2 for 4 min (n=54).

Table 7. Proportion, number of events/bird and duration of behaviours observed in broiler chickens exposed to different experimental gas stunning treatments after losing posture.

		Treatment			
Item	**Behaviour**	**40C90C**	**40C60N**	**20C80N**	**P-value**
Proportion, %	Gasping	56.6 [a]	22.2 [b]	25.9 [b]	0.009
	Jumping	7.9 [a]	10.5 [a]	25.0 [b]	0.003
	Leg paddling	98.7	100	100	0.998

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	Wing flapping	69.7	96.8	98.1	0.320
	High pitch vocalisations	85.5	84.1	98.1	0.818
Events/bird, n	Gasping	3 [1 - 16] [a]	1 [1 - 3] [b]	1 [1 - 3] [b]	< 0.001
	Jumping	1 [1 - 2]	1 [1 - 3]	1 [1 - 3]	0.250
	Leg paddling	4 [1 - 12]	4 [1 - 8]	5 [2 - 8]	0.187
	Wing flapping	2 [1 - 7] [b]	2 [1 - 7] [b]	3 [1 - 7] [a]	< 0.001
	High pitch vocalisations	2 [0 - 10] [b]	2 [0 - 9] [b]	3 [0 - 6] [a]	0.012
Duration, s	Leg paddling	2.5 [0.2 - 22]	2 [0.2 - 30.8]	2 [0.2 - 36.2]	0.220
	Wing flapping	3.6 [0.2 - 11.2] [b]	5 [1 - 12.0] [a]	5.5 [1 - 8.8] [a]	0.050

40C90C: CO_2 in two phases being the 1st phase with <40% CO_2 by volume in air during 2 min and 2nd 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). Different superscripts in the same column mean significant difference between treatments ($P < 0.05$).

3.3.3. Behavioural Assessment after Loss of Posture

The time it takes for F50 reduction matched with the time of statistical dispersion until LOP. In 40C60N treatment, the F50 values remained below the 50% of baseline during all gas exposure period. In contrast, the F50 values of 20C80N treatment exhibited an increase after LOP and remained elevated until the end of the gas exposure period. LOP serves as a reliable indicator of the initial loss of consciousness in broiler chickens exposed to gas mixtures. Behaviours observed before LOP, except for sitting, standing, and walking, were interpreted as aversive responses of the birds to the gas exposure, suggesting possible experiences of pain, respiratory distress, or fear. On the other hand, behaviours occurring after LOP were not considered aversive, as it is presumed that the birds are unconscious.

During the gas exposure, there was a 65% increase in the contribution of Delta frequency compared to the baseline. In the EEGs of birds exposed to 20C80N, the contribution of Delta frequencies initially increased but then decreased after 75 seconds of gas exposure. In the 20C80N treatment, there was an increase in the contribution of Beta frequencies after LOP. The decrease in Delta frequencies and increase in Beta frequencies observed in the 20C80N treatment are not biologically relevant, as they corresponded to the times of the isoelectric pattern. The contribution of Gamma frequencies, which is associated with background noise, remained below 2%. The 65% increase in the contribution of Delta frequency coincided with the time of statistical dispersion until LOP in both the 40C60N and 20C80N treatments.

As expected, the baseline power spectrum in both treatments demonstrates a greater dominance of frequencies above 4 Hz, suggesting a state of consciousness. In contrast, the isoelectric power spectrum in the 40C60N treatment exhibited no power, suggesting permanent unconsciousness or brain death. In the case of the 20C80N treatment, the power spectrum during the isoelectric pattern had minimal contribution at 25 Hz. These results explain the increased of both F50 values and Beta frequencies observed during the isoelectric pattern in the 20C80N treatment. Hence, the power spectrum confirmed the non-biological relevance of this increase on 20C80N treatment.

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 of animals at the time of killing according to each gas treatment [1].

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4.2. Brain Activity Assessment

The purpose of stunning is to induce a temporary or permanent disruption of brain function, rendering the animal unconscious and insensitive to pain. In contrast to electrical and mechanical stunning methods, gas stunning does not immediately result in the loss of consciousness. Instead, there is a prolonged delay between initiation and the onset of unconsciousness, known as the induction phase. Results demonstrate that exposure to 40C60N or 20C80N gas mixtures in a pre-filled pit led to faster onset of LOC compared to 40C90C.

The time elapsed until the onset of LOC was determined using EEG spectral variables. Specifically, onset of LOC was identified when the F50 frequency decreased below 50% and the Delta frequency increased above 65% of the baseline. It is important to highlight that the chosen threshold values in this study differ from those employed in previous research. For example, in broiler chickens exposed to LAPS, LOC was determined when the F50 dropped below 7 Hz compared to baseline values of 20 Hz [26] or when the F50 was reduced below 75% compared to baseline [34]. The different thresholds used in our study compared to LAPS are attributed to the type of electrodes used. In our study, subcutaneous electrodes were used, while other authors [26] utilized implanted electrodes placed through the skull, resulting in outputs on a different scale. Although subcutaneous electrodes provide lower-quality EEG recordings compared to implanted electrodes, they are less invasive for the animals as they do not require surgery or recovery time prior to the experiment. Furthermore, results from the contribution of Gamma frequency to Ptot remained at an average of 0.1%, indicating an irrelevant interference from background noise [23]. This confirms the quality of the EEG recordings and the reliability of the obtained results in determining the time elapsed until the onset of LOC and death.

General anaesthesia serves as an ideal model for understanding the EEG waveform alterations during the transition from a conscious to an unconscious state [21]. During general anaesthesia stages, there is a predominance of Delta and Theta frequency bands [21]. F50 is particularly sensitive to changes in lower frequencies, while F95 is more responsive to shifts toward higher frequencies [21]. Reductions in Ptot and F50 are well-established indicators correlated with clinical signs of loss of consciousness and anaesthesia in animals [22,23].

In the study conducted by Sandercock et al. [32], the reduction of F50 was observed during inhalational anaesthesia using a face mask with a Sevoflurane vaporizer at a concentration of 8% in hens and turkeys. So far, there are no studies that have reported a specific threshold for Delta frequency contribution. Previous reports have described the analysis of Delta frequency on EEG as a visual change in the EEG trace, without an objective threshold [19,35]. Therefore, the decrease below 50% of F50 and the increase above 65% of Delta frequency contribution can be considered potential indicators of LOC during EEG recording in broiler chickens exposed to gas stunning.

The time elapsed until onset of LOC found in 40C60N and 20C80N was similar to those reported in previous studies [16,17]. McKeegan et al. [16] reported, LOC in broiler chickens exposed to 40C60N was observed to occur at 23 ± 4 s, compared to 24.2 s [15 - 38 s] with 40C60N and 18.8 s [15 - 32 s] with 20C80N in the present study. This slight difference may be due to the use of a pre-filled pit with gas instead of a closed cabined (not pre-filled but flushed) used in McKeegan [16] and, therefore, the reduction in available O_2 was reported to be not instantaneous and thus, there was a slight delay in reaching the desired modified atmosphere. Coenen et al. [17] reported LOC in broiler chickens at 34 ± 12 s determined by visual analysis on EEG waveform when the trace was isoelectric, during gas exposure using a tunnel system where the combination of 30% CO_2 with 70% N_2 [17]. Hence, the time to LOC can be different with similar conditions depending on the criteria used to assess EEG activity. The visual interpretation of EEGs can be subjective, and various studies have employed different waveform patterns to determine the LOC, such as suppressed, isoelectric, high amplitude low frequency (HALF), and transitional states [17,19,36]. In addition to visual analysis, spectral analysis provides a quantitative approach for estimating LOC by generating numerical results. The utilization of numerical variables derived from EEGs has gained attention in similar experimental studies in recent years [21,23,37,38]. This complementary approach enhances the accuracy and reliability of assessing LOC. This study identified F50 and Delta frequency contribution as promising spectral variables for determining LOC in broiler chickens exposed to gas mixtures.

Permanent unconsciousness or brain death was assessed using EEG through visual analysis and spectral analysis. The average time for reaching irreversible loss of brain function and brain stem death was estimated to be 69.8 ± 11.9 s in the 40C60N mixture and 66.3 ± 8.1 s in the 20C80N mixture. The high variability observed in 20C80N for death, as indicated by spectral analysis, may be attributed to signal degradation. In such cases, visualizing the isoelectric pattern could provide a more accurate assessment. Both visual and spectral analysis methods demonstrated the irreversible cessation of brain activity and brain stem function [19,21,23,32]. In similar studies in broiler chickens exposed at a gas stunning in an experimental chamber using 40% CO_2 and 60% N_2 by flushing, the time to death estimated by visual analysis of EEG was 67.8 ± 4.6 s [16].

Raj et al. [39] reported a slightly different onset time of isoelectric EEG at 58 ± 2.3 s in broiler chickens exposed to a gas mixture (30% CO_2 + 60% Argon + 10% Air) in a pre-filled box, which may be attributed to the time it takes for the lift to descend into the pit (23 s) and reach the target gas concentration. In contrast, McKeegan et al. [16] visually observed the onset of death at 80.7 s through the isoelectric pattern on EEG and the absence of motion in broiler chickens exposed to 40% CO_2 and 60% N_2 in a gas-flushed closed cabin. If the exposure time to the gas is insufficient to induce brain death and the animals can breathe atmospheric air, they may quickly regain consciousness [12]. Results suggested that a four-minute exposure to 40C60N and 20C80N gas mixtures induced permanent unconsciousness in all birds, eliminating the possibility of regaining consciousness. The integration of visual and spectral analysis of EEGs proved to be a reliable method for accurately estimating brain death in broiler chickens exposed to gas stunning.

The present study was intended to record EEG traces from 50 broiler chickens. However, 28 out of 50 (56%) EEGs records were unsuitable for analyses due to loss of electrodes during gas exposure or non-readable EEG activity. This decrease of sample size is common when EEG is performed. Previous studies reported a loss of readable EEGs in 9–71% of animals [35,40,41]. Unfortunately, in this study, no EEG traces were suitable for analyses in the 40C90C treatment, and the reasons for this systematic loss remain unknown. Therefore, it is important to consider refining the methodology in future studies to improve the quality of EEG recordings. In human and animal anaesthesia monitoring, high-quality EEG recordings are essential, and the use of different drugs to induce insensibility to pain, loss of consciousness, and loss of muscle tone can result in higher-quality EEGs [42]. In this experimental set-up, using general anaesthesia prevents identifying the transition from consciousness to unconsciousness, rendering it unfeasible. Measures such as minimizing recording errors, reducing background electrical noise, and optimizing electrode placement on the bird's head may contribute to improving the quality of EEG records.

4.3. Behavioural Assessment

4.3.1. Behavioural Assessment of Loss of Posture and Motionless

Broiler chickens differed in the elapsed time to LOP and motionless according to the experimental gas treatment. In particular, exposure to 40C60N or 20C80N not only drastically reduced the time until birds lost posture and became motionless, but there was also much less inter-individual variability in time to LOP compared to 40C90C. The high inter-individual variability in time to LOP in 40C90C represents a serious welfare risk if they still remain conscious when the first phase time has expired and the certain birds are therefore exposed to more than 40% CO_2 during the second phase while conscious, as was the case in the present study. It is known that inhalation of concentrations above 40% CO_2 in conscious chickens generates a very painful mucosal stimulus [15,46]. The difference in time to LOP and motionless of 40C90C compared to 40C60N and 20C80N is explained by the anoxic environment in 40C60N and 20C80N since the lower the O_2 concentration, in hypercapnic conditions, the faster is the stunning method [43].

4.3.2. Behavioural Assessment before Loss of Posture

Since LOP is the behavioural indicator of the onset of unconsciousness [10,25,30,44], all those behaviours observed before LOP occur in birds that are conscious and therefore may be potential indicators of pain, distress or dyspnoea caused by inhalation of the gas or gas mixture. In order to discern whether the descent into the pit *per se* caused aversive behaviours in broiler chickens and caused confusion in the results, first, the behaviour of the birds in the gas stunning equipment was assessed by breathing atmospheric air (AIR) and then, when subjected to experimental treatments. In AIR, most chickens remained sit while to some stand, few walked and very few broiler chickens exhibited head shaking once or twice while descending the pit. Although these behaviours were also observed in the other three experimental treatments, these behaviours themselves were also considered indicators of aversion to gas treatments. On the one hand, the proportion of animals performing sitting and walking was higher in the gas mixtures compared to AIR, indicating an increase on locomotor behaviours and perhaps related to fear. The cause of head shaking observed in only two birds in the AIR group remains unclear. It is possible that these birds are sensitive to new stimuli [11, 45], like the descent of the lift, or that they are attempting to self-activate after a period of rest [46]. The almost minimal proportion of animals exhibiting head shaking in the AIR treatment, along with the absence of other considered aversive behaviours (i.e., deep inhalation, gasping, wing flapping, jumping, HPV, ataxia, leg paddling) suggests that descending the pit did not induce aversion in broiler chickens, unlike what was observed in the three experimental treatments.

Comparing the three experimental gas treatments, it was observed that both 40C60N and 20C80N showed fewer transitions between locomotor behaviours (sitting, standing and walking) and of shorter duration than 40C90C indicating lower stress and fear response to gas treatment. Head shaking and deep inhalation was performed at least once in all animals from all treatments, but gasping was only observed in some birds exposed to 40C90C. Head shaking, deep inhalation and gasping reflect dyspnoea and breathlessness "air hunger" reducing welfare during gas stunning [23]. Head shaking and deep inhalation were the first aversive behaviours displayed during the stunning process. While head shaking is associated to unpleasant stimulus caused by the activation of chemoreceptors sensitive to CO_2 on respiratory tract, deep inhalation and gasping are associated to hyperventilation during CO_2 stunning, however, these responses have also been observed with the inhalation of inert gases alone, such as argon [43] and are related to respiratory distress [25]. When it comes to the number of events per behaviour, the lower occurrence of head shaking and deep inhalation events at 40C60N and 20C80N compared to 40C90C could be related to a more rapid induction of LOP and, therefore, birds remain less time conscious in the gas stunning equipment, so the likelihood of repeating aversive behaviours is lower.

Although presence of HPV suggests fear and pain, it is worth noting that any previous study on gas stunning in broiler chickens assessed vocalisations [15,16,29,47]. In the present study, the proportion of broiler chickens that vocalized was higher in 40C60N and 20C80N than in 40C90C. Nevertheless, 40C60N had less events of HPV compared to either 40C90C or 20C80N. Based on the sequence of the observed onset of behaviours it seems that broiler chickens vocalized just before becoming ataxic in all gas treatments but kept vocalizing along the stunning process. After ataxia, some birds flapped their wing or very few jumped. The proportion of chickens that flapped their wings before LOP varied depending on the experimental treatment. Nitrogen-containing gas mixtures caused a higher proportion of birds to flap their wings before losing posture than 40C90C. The higher the nitrogen concentration in the gas mixture, the higher the proportion of birds showing wing flapping. This could be due to the fact that anoxic environments lead to an increase of wing flapping although CO_2 has an anaesthetic effect on birds and therefore, when higher CO_2 concentration and anoxic environment is combined (as in 40C60N compared to 20C80N) can result in a calmer induction to unconsciousness. The occurrence of wing flapping in the gas stunning equipment is a welfare concern since it may cause injuries and pain to the other birds that have not yet lost consciousness [5,16].

In addition, in 40C60N or 20C80N there is no risk that birds inhale CO_2 concentrations above 40% as it can occur in birds that are still conscious when the 2nd phase of 40C90C starts. Therefore, in

40C60N or 20C80N experience of severe pain in mucosa is prevented. Taking all into consideration, it seems that the fastest induction to unconsciousness was achieved with 40C60N while being the least aversive gas mixture.

4.3.3. Behavioural Assessment after Loss of Posture

Since LOP was the behavioural indicator of onset of unconsciousness, behaviours after LOP do not represent a welfare concern (e.g., pain, distress, breathlessness) as the bird is unconscious. Behaviours observed may be related to convulsions or any other involuntary movements rather than aversive behaviours [23,35].

Gasping and HPV were performed after LOP in all gas treatments indicating that consciousness is not required for its performance. Convulsions in broiler chickens exposed to the three experimental treatments were expressed as leg paddling and wing flapping.

HPV after LOP are presumed to be a consequence of air movement through the syrinx caused by convulsions or other body movements. The number of HPV after LOP was higher in the 20C80N treatment, which is consistent with the higher number of wing flapping events observed in this treatment. Similarly, as occurred before LOP, anoxic environments also lead to an increase of wing flapping after LOP but the higher the CO_2 concentration, the lower the convulsions expressed as wing flapping due to the anaesthetic effect of CO_2 on birds. The occurrence of wing flapping in the gas stunning equipment may be a welfare concern since it may cause injuries and pain to the other birds that have not yet lost consciousness [5,16]. This finding is consistent with a study by Gent et al. [10], which reported a longer duration of wing flapping after LOP in broiler chickens exposed to nitrogen (19.7 s, on average) compared to CO_2 (7.1 s, on average) using an experimental chamber by gas flushing. Further research could investigate the effect of different gas mixtures on meat quality in broiler chickens subjected to stunning.

4.4. Relationship between Brain Activity and Behavioural Assessment

Correlating EEG activity with behavioural observations in unrestrained animals is crucial for interpreting behavioural indicators in slaughterhouse conditions where EEG recording is not feasible. The elapsed time until LOC determined through EEG spectral analyses matched with the time of LOP in 40C60N and 20C80N, confirming that LOP can be utilized as a proxy for the onset of LOC in broiler chickens, as previously reported Gerritzen et al. [35], Benson et al. [30], LAPS [23]. On the other hand, the time elapsed until isoelectric patterns on EEG (i.e., death) matched with the onset of motionless in broiler chickens. Hence, the LOP and motionless are reliable animal-based indicators of LOC and death, respectively.

LOP is defined as the point when a broiler is unable to regain/maintain controlled posture and neck tension. Using LOP as a behavioural indicator of LOC appears to be an accurate ABI for monitoring unconsciousness in broiler chickens exposed to gas mixtures in slaughterhouses. Moreover, European regulation (1099/2009) on animal welfare during slaughter prohibit the use of CO_2 concentrations exceeding 40% for poultry, with concentrations above this threshold only permitted for already unconscious birds [1]. Aversive behaviours such as head shaking, deep inhalation, ataxia, gasping, jumping, wing flapping, and vocalisations observed prior to LOP have negative consequences on animal welfare [23]. The utilization of two-phase CO_2 stunning in broiler chickens introduces significant variability in the time required for LOP to occur, posing a risk of exposing conscious animals to concentrations above 40% that can induce pain.

Our findings indicate a correlation between the observation of motionless behaviour in broiler chickens exposed to gas mixtures and the identification of isoelectric patterns on EEG. The occurrence of motionless coincided with the presence of isoelectric patterns, suggesting a potential relationship between the two factors. Results in this study suggests that the assessment of motionless behaviour in field conditions can be a reliable indicator for estimating brain death in broiler chickens exposed to gas stunning.

The observed elevation in F50, Beta frequencies contribution after LOC in the 20C80N treatment can be attributed to signal degradation in the EEG recordings. This is supported by the minimal power observed during the isoelectric pattern, which is indicative of brain death [43]. Hence, the changes observed in F50 and Beta frequency during a trace with very low power (<90% of baseline) are not biologically significant.

The use of LOP and motionless as animal-based indicators of loss of conscious and death, respectively, can be extended to other poultry species to assess the state of consciousness at the time of killing.

5. Conclusions

The exposure of broiler chickens to 40C90C, 40C60N or 20C80N does not induce immediate unconsciousness. Regardless of the gas mixture tested, all broiler chickens experience aversion during the induction of loss of consciousness. The exposure to 40C60N and 20C80N not only decrease dramatically the time to induction to LOC and death but also with less variability in the elapsed time between individuals compared to 40C90C. The 40C90C birds not only experienced more aversion during induction to LOC but are also at risk of remaining conscious when the CO_2 concentration is increased in the 2nd phase. On animal welfare grounds, 40C60N provided better welfare that the others, followed by 20C80N and 40C90C. Further research is required to explore alternative gases or gas mixtures that can minimize or eliminate aversive responses during the induction of unconsciousness to improve broiler chicken welfare during stunning.

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