# Remote monitoring of electroencephalogram, electrocardiogram, and behavior during controlled atmosphere stunning in broilers: Implications for welfare

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**ABSTRACT** This study examined the welfare implications of euthanizing broilers with 3 gas mixtures relevant to the commercial application of controlled atmosphere stunning (CAS). Birds were implanted/equipped with electrodes to measure brain activity (electroencephalogram, EEG) and heart rate. These signals were recorded using a purpose-built telemetry-logging system, small enough to be worn by each bird in a spandex backpack. The birds were euthanized in a scaled-down CAS apparatus consisting of a conveyor belt passing through 2 compartments. Three gas environments were applied (8 birds per treatment): 1) anoxia ($N_2$ with <2% residual $O_2$, in both compartments), 2) hypercapnic anoxia ($N_2$ with 30% $CO_2$ and <2% residual $O_2$, in both compartments), and 3) a 2-phase approach with a hypercapnic hyperoxygenated anesthetic phase (40% $CO_2$, 30% $O_2$, and 30% $N_2$, in the first compartment, 80 s) followed by a second euthanasia phase (80% $CO_2$ in air, in the second compartment). All 3 CAS approaches effectively achieved nonrecovery states, and time to loss of consciousness for each bird was determined by visual determination of isoelectric EEG and by calculation of the correlation dimension of the EEG. Hypercapnic anoxia resulted in rapid unconsciousness and death; both anoxic treatments were associated with early onset prolonged wing flapping and sustained tonic convulsions as displayed in the electrophysiological recordings. These responses were seen in the period when consciousness remained a possibility. Hypercapnic hyperoxygenation (the 2-phase approach) was associated with respiratory disruption, but this treatment eliminated initial clonic convulsions in the stunning process, and tonic convulsions were not seen. These results suggest that the presence of $O_2$ in the first stage of CAS is associated with an absence of potentially distressing behavioral responses. The respiratory discomfort associated with hypercapnic hyperoxygenation is an issue. We propose that this may be compensated by a more gradual induction to unconsciousness, which eliminates the impact of other potentially negative experiences.

**Key words:** broiler, euthanasia, controlled atmosphere stunning, welfare, electroencephalogram

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## INTRODUCTION

Slaughter methods for animals are acceptable when they result in minimal signs of agitation and distress during the period that animals have some degree of perception and consciousness. Humane slaughter must guarantee an elimination of conscious experience, as rapidly and painlessly as possible, so that killing can be accomplished when animals are totally unconscious. Controlled atmosphere (gas) stunning (**CAS**) has the potential to improve welfare of poultry at slaughter by eliminating the stress associated with live bird shackling, and CAS also has the potential to ensure that that every bird is effectively stunned.

In previous experiments, a 2-phase stunning method with potential welfare advantages has been examined in rats and chickens in the laboratory (Coenen et al., 1995, 2000; McKeegan et al., 2007b). This method employs a hyperoxygenated hypercapnic induction or anesthesia phase (associated with a relatively smooth induction of unconsciousness), followed by a more severely hypercapnic and hypoxic euthanasia phase, applied once the animals are unconscious. It is known that anesthesia and unconsciousness occur quickly with $CO_2$ concentrations of minimally 15 to 20% (Kohler


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et al., 1999), and a mixture of 40% $CO_2$, 30% $O_2$, and 30% $N_2$ has been used in the anesthesia phase, lasting 1 min. In principle, this 2-phase approach is comparable to gradual-fill $CO_2$ euthanasia method in which air is slowly replaced by $CO_2$, recently described by Niel and Weary (1991).

Previous work in the laboratory by us and others (e.g., Raj et al., 1991, 1992; McKeegan et al., 2007b) provided extensive data relating to physiological and behavioral responses to various CAS mixtures (including the 2-phase approach) but had some limitations. The birds were stunned individually and in some experiments were restrained – conditions that are not relevant to commercial stunning, in which the birds are freely moving and are exposed to additional stressors such as a moving conveyor belt and the presence of other birds. The recent development of purpose-built telemetry-logging units (Lowe et al., 2007), small enough to be worn by each bird in a spandex backpack, has allowed detailed measurement of key physiological parameters in these CAS environments. This approach facilitates welfare assessment by the examination of electrocardiogram (**ECG**) and respiratory responses in chickens passing through commercial gas stunning systems (Abeyesinghe et al., 2007; McKeegan et al., 2007a). The current work represents the first study in which the electroencephalogram (**EEG**), a crucial measure to determine time to loss of consciousness, has been measured in freely moving animals during commercially relevant CAS.

The aim of this study was to compare gas mixtures representing different CAS methodologies. The 2-phase method with a mixture of $N_2$, $CO_2$, and $O_2$, was compared with a 1-phase method based on $N_2$ anoxia, or hypercapnic anoxia using $N_2$ with $CO_2$. The implications for welfare associated with each approach were investigated, with particular focus on whether potentially negative experiences were minimized in the period that consciousness could not fully be excluded (i.e., in the period that the EEG was not fully isoelectric or showed low complexity, reminiscent of deep anesthesia). Birds were instrumented with electrodes to measure EEG and heart rate before being exposed to the chosen gas mixtures in a scaled-down but commercially relevant CAS apparatus (the mini-system, see also Abeyesinghe et al., 2007). It was anticipated that subsequent analysis of EEG, ECG, and behavioral responses would allow inferences to be drawn about time to loss of consciousness and potential welfare insults experienced by the birds during the euthanasia process.

## MATERIALS AND METHODS

### Subjects and Husbandry

The experiments were performed on 30 commercially reared broiler chickens (*Gallus domesticus*), with appropriate ethical approval from the Animal Experimental Committee of the Radboud University Nijmegen under number KUNDEC 2004-85. The birds were sourced from a commercial rearing unit and brought to Radboud University Nijmegen at 24 d of age. They were housed in a floor pen with wood shavings litter and ad libitum access to standard broiler food and water. An intermittent lighting schedule (lights on between 0100 and 0330, 0500 and 0730, 0900 and 1130, 1300 and 1530, 1700 and 1930, and 2100 and 2330) was applied.

### Implantation of EEG Electrodes

At 28 d of age, the 30 birds underwent surgery to implant EEG recording electrodes under general xylazine-ketamine anesthesia [xylazine (Rompun, Bayer, Leverkusen, Germany) given as a 20 mg/kg i.m. injection, supplemented with 2 mg/kg of ketamine (Vetalar, Pfizer, Surrey, UK)]. The EEG was recorded by two 0.35-mm diameter Teflon-insulated silver electrodes (World Precision Instruments, Stevenage, UK) connected to a socket (Deutsches Institut für Normung (Berlin, Germany), RS Components, Northants, UK). The electrodes were placed on the dura through holes drilled in the skull, one each on the dorsal surfaces of the right and left telencephalon at their approximate rostrocaudal and mediolateral midpoints. An indifferent electrode placed between the skull and the overlying tissue under the comb was also connected to the socket. The EEG implant was cemented to the skull with dental cement (DuraLay, Reliance Dental Co., Worth, IL), and the surrounding skin was sutured. Birds were allowed to recover for 7 d before the experimental procedure. Once recovered, the birds were transported to the research facilities of Stork Food Systems in Boxmeer, the Netherlands, where the experiment took place.

### CAS System

The birds were exposed to the CAS euthanasia treatments in a pilot-scale stunning system (the mini-system), with a capacity of 10% of normal industrial processing speed (Hoen and Lankhaar, 1999). This mini-system consists of a conveyor belt traveling (with a speed of 0.045 m/sec) through 2 compartments, in which the gas atmosphere can be separately controlled. This allows the application of a single-phase approach, when both compartments contain the same gas mixture, or a 2-phase approach, consisting of an anesthetic phase in the first compartment, followed by a euthanasia phase in the second compartment. The intended time in the first compartment is 60 s (length of this compartment is 275 cm) and 120 s in the second compartment (length of this compartment is 625 cm). However, due to bird movements on the belt, the timings in the first compartment varied: in practice, the birds remained in this compartment for approximately 80 s (range 56 to 101). Nonimplanted birds of the same age and size were placed in the system immediately before and after the experimental birds so that groups of 3 birds traveled

though the system. This was done to mimic the group-stunning conditions of commercial practice.

The 3 gas treatments applied were as follows: 1) $N_2$ – $N_2$ anoxia (less than 2% residual $O_2$), 2) $N_2CO_2$ – $N_2$ with 30% $CO_2$, and 3) a 2-phase treatment, consisting of 40% $CO_2$ and 30% $O_2$ in $N_2$ (anesthesia phase), followed by 80% $CO_2$ in air (euthanasia phase). The 2 anoxic treatments used the same gas in both compartments; in the 2-phase treatment, the gases differed and the compartments were separated by a curtain to prevent mixing of the gases. The experiments were carried out over 2 d and the order of application of each gas treatment was randomized. Each treatment was applied to 10 birds. In each group, the data for 2 birds were lost, due to the loss of either the EEG or the ECG electrodes, or both. The final number of birds per treatment was 8. One bird (102) lost its ECG electrodes in the first compartment and could not be included in the ECG analysis but could be included in the EEG analysis, whereas another (218) lost ECG electrodes only on entering the second compartment and was included in the full analysis.

## ECG Electrodes and Telemetry-Logging System

Directly before being placed in the stunning system, birds, already equipped with permanent EEG electrodes, were fitted with ECG electrodes (Blue Sensor, Ambu, St Ives, UK). These were commercially available disposable self-adhesive ECG electrodes, with press-stud electrical connections, which were adhered to cleaned skin overlying the pectoralis muscle on either side of the sternum. A harmless cyanoacrylate tissue adhesive (Vetbond, 3M, Bracknell, UK) was applied to the ECG electrodes before placement on the skin to improve bonding and prevent the electrodes from detaching before or during the euthanasia process. Each bird was also fitted with a reusable spandex harness, which was secured using hook-and-loop fastenings behind the head of the bird and incorporated a pocket containing the telemetry-logging system.

The telemetry-logging system was custom built to allow simultaneous capture of physiological waveforms on 2 channels, each at a sampling rate of 1,200 samples/sec with an 8-bit resolution. The system consisted of 2 primary components: the telemetry-logging unit itself, mounted on the bird in the spandex harness, and a base station unit, which was connected to a laptop computer via a serial (com) link. Custom-written software on the laptop computer controlled the base station. Communication between the telemetry-logging unit and the base station was via a bidirectional digital radio link, operating in the industrial-scientific-medical band at 433 MHz (Lowe et al., 2007). The system had 2 modes of operation. In the telemetry mode, bursts of waveforms of typically 5-s duration could be requested from the telemetry-logging unit via the radio link, the waveforms being displayed on the laptop computer screen for examination. If necessary, the gain of the amplifiers on the telemetry-logging unit could be changed at this time, by radio commands sent to the unit. A radio command could be sent to the telemetry-logging unit to switch it into the logging mode. The unit would then start to record both ECG and EEG waveforms simultaneously, at 1,200 samples/sec, into internal memory (see Figure 1 for example of logged data). The recording time could be specified in the radio command, with a maximum limit of approximately 7 min due to memory constraints. At the end of the recording period, the telemetry-logging unit was connected to the laptop computer, and the stored waveforms were downloaded to the computer.

## Experimental Procedure

Once the electrodes and harness had been fitted to the bird, leads from the telemetry-logging system were connected to the ECG and EEG electrodes, and the bird was allowed to rest in a sitting position on the bench. During this time, the existence of clear ECG and EEG waveforms was verified using the telemetry capability of the system, and, if necessary, the amplifier gains were adjusted. After all checks were completed, the telemetry-logging unit was switched into logging mode and a stopwatch was started. The ECG and EEG signals were logged simultaneously for a total time of 5 min that incorporated the 1-min baseline period, during which the bird was sitting but alert on the bench. Logging continued as the bird was transferred to the start box of the stunning system, and as it traveled through the system for the remainder of the recording period. The harness was retrieved from the bird when it exited the system, and the logged data were downloaded and stored onto the laptop computer. The behavior of the bird wearing the harness was recorded using a hand-held camcorder (Sony Digital Video Camera DCR-TRV20E, Sony Corporation, Tokyo, Japan) during the baseline and as far as possible while in the stunning system.

## Analysis

The downloaded waveform sample data were converted to a comma-separated-variable format, allowing import into a commercially available data acquisition and analysis program. Visual inspection of the EEG and ECG traces was used to determine normal, abnormal, and isoelectric EEG activity, and heart rate was determined. Duration and number of artifacts both from EEG and ECG traces were visually determined and measured, as well as the time to isoelectricity (**IE**, a flat EEG trace) and the time to death (EEG IE and a heart rate lower than 180 beats per minute; Coenen et al., 2000). Electrophysiological variables were analyzed using a 1-way ANOVA, followed by post hoc *t*-

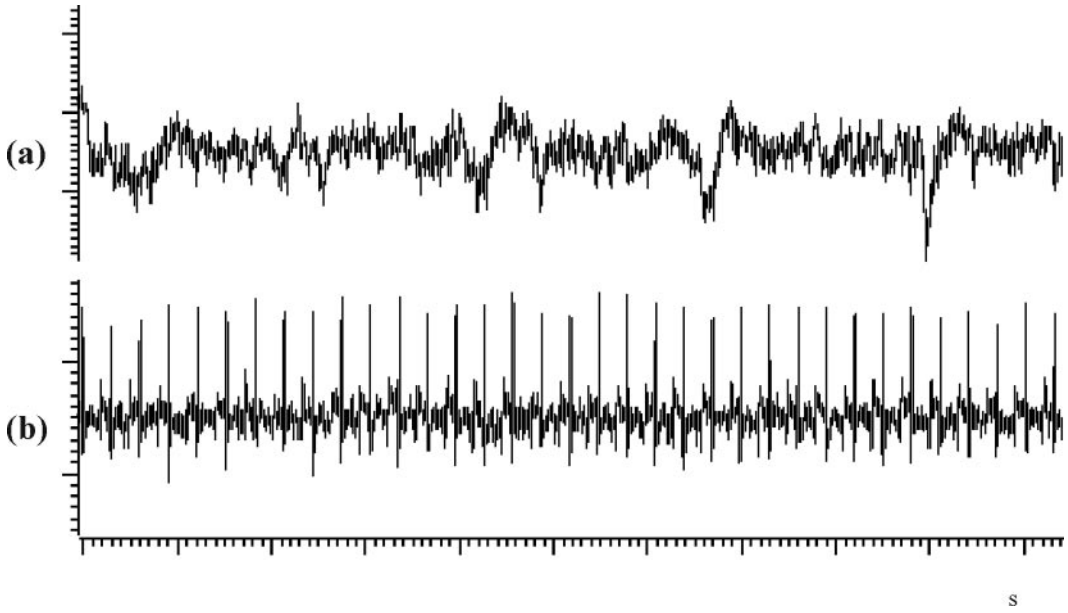


**Figure 1.** Example trace from 1 bird showing simultaneous baseline measurements of (a) electroencephalogram and (b) electrocardiogram.

tests (Bonferroni corrected) to determine differences between treatments.

Videotapes of the behavioral responses of each bird were analyzed noting a range of activities (see Table 1 for behavioral categories and definitions). Continuous observations assigned these descriptions of behavior at 1-s intervals during stunning. These raw data were further analyzed to produce counts of the number, duration, and timing of bouts of some behaviors. Behavioral variables did not meet the assumptions required by parametric analysis and so were analyzed using the Kruskal-Wallis test to compare medians. Post hoc Mann-Whitney U tests were applied as appropriate to determine differences between treatments. Behavioral observations were also used to identify movement artifacts in the EEG traces such as walking, attempts to regain balance, loss of posture, and wing flapping.

## *Correlation Dimension*

Correlation dimension, a measure of complexity (van den Broek et al., 2000, 2005), was determined on the EEG recordings made during stunning. After downsampling the EEG signal 250 Hz, the correlation dimension (**CD**) was computed using nonoverlapping EEG epochs of 5-s duration (1,250 samples) of the entire trace. Only artifact-free epochs were used. Isoelectric EEG signals at the end of the euthanasia process present a problem, because the noise of this signal has an infinite correlation dimension. To decrease the influence of the noise, a pure sinus, having a CD of 1, was added to the signal. When the EEG is completely isoelectric, the CD approaches a value of 2 (1 for the straight isoelectric EEG and 1 for the sinus). For the reason that absolute values of CD vary between individuals, changes in CD were expressed relative to baseline as a percentage (McKeegan et al., 2007b).

To estimate the effects of the 3 different gas treatments on the decline of the CD in time, a 1-phase exponential decay equation was fitted to the data (GraphPad Prism 4.03, GraphPad Software, La Jolla, CA): $Y = (\text{start} - \text{plateau}) \times \exp[-K \times (X) + \text{plateau}]$, where X = time; Y = CD; Y starts at start and declines to plateau; $\ln 2/k$ = the time needed to reach the one-half of the maximum decline. Each replicate was considered as an individual point. An F-test was used to determine whether the individual parameter estimates (start, plateau, and k) were statistically distinguishable between

**Table 1.** Descriptions of the recorded behaviors

| Behavior | Description |
|---|---|
| Headshake | Rapid shaking or lateral movement of the head |
| Mandibulation | Repetitive tasting movement with bill |
| Respiratory disruption | Deep open bill breathing with prolonged inspiration or prolonged open bill gaping, or both, combined with apparent apnea or difficulty inhaling |
| Loss of posture | Loss of balance or posture, or both |
| Wing flapping | A bout of continuous, rapid wing flapping |
| Wings rigid | Wings held rigid, sometimes accompanied by twitching resulting from muscular spasms |
| Leg paddling | Apparently involuntary leg movement |
| Motionless | No discernable body movements |
| Out of view | Focal bird not visible |

the 3 groups. Post hoc testing compared the treatment groups.

## RESULTS

After placing the birds in the start box of the minisystem, they entered the first compartment and moved on the conveyor belt for an intended time of 1 min. The actual time in this first compartment was variable, because the birds moved forward or backward on the belt. The time variation in the first compartment was in the range from 56 − 101 s with an average of 80 s [$N_2$ group: 88.0 ± 10.3 (range 73 to 102), $N_2CO_2$ group: 74.6 ± 11.1 (range 57 to 91), 2-phase group: 82.8 ± 8.5 (range 71 to 99)]. All of the chickens became immobile in the first compartment, so there was little variation in travel time through the second compartment (120 s). Upon entering the second compartment, the immobile birds fell a few centimeters down due to a small height difference between the 2 compartments. This drop induced a clear movement artifact in both the EEG and ECG recordings. All birds left the system immobile and in a nonrecovery state, although many still exhibited a slow heart beat. Figure 2 shows the raw EEG and ECG recordings of all birds of the 3 treatments [group 1 ($N_2$), group 2 ($N_2CO_2$), and group 3 (2-phase)] including some baseline (45 s) and the first part of the euthanasia process (up to 105 s).

### Artifacts in EEG and ECG Recordings

Figure 2 shows the occurrence of many striking artifacts, expressed as high amplitude deflections in the EEG and, in particular, in the ECG recordings. Looking first at the EEG, artifacts started immediately after placing the birds in the system. These were caused by physical movements of the birds, such as struggling, wing flaps, and clonic convulsions, which were verified by comparing the EEG traces with behavioral recordings. Movement artifacts occurred over the entire duration of the first compartment, but their number and length strongly diminished with time. Movement artifacts were rare or absent in the second compartment. The total artifact duration and number in the first compartment were measured, and these data are presented in Table 2. These data show that both the total duration and number of artifacts were least in the 2-phase group, where the gas mixture in the first compartment was hyperoxygenated.

The artifacts visible in the ECG recordings provided further information (Figure 2 and Table 2). Here, the duration of artifacts was much longer than in the EEG traces, except in the 2-phase treatment. Statistics show that the latter treatment differed significantly from the other 2 groups in the extent of EEG artifacts, and it was also apparent that ECG artifacts observed almost completely coincided with those seen in the EEG recordings, produced by obvious movements of the birds. However, ECG artifacts seen in birds receiving the anoxic treatments only partly coincided with the EEG artifacts: in general, they lasted much longer and were characterized by prolonged artifacts as opposed to several short disturbances. Behavioral observations indicated that these prolonged ECG artifacts coincided with convulsive activity often followed by a distinctive posture in which the wings were held rigidly forward. This is thought to indicate tonic convulsion and in particular sustained contraction of the pectoralis muscle. Thus, the artifacts in the ECG recordings were composed of movement artifacts and artifacts caused by tonic convulsions.

An indication of the contribution of the tonic convulsions to the total artifact was determined by measuring the difference in duration between the ECG artifacts (movement artifacts and tonic convulsions) and the EEG artifacts (movement only), see Table 2. The results show that anoxia ($N_2$ and $N_2CO_2$) induced considerable tonic convulsions, whereas this response was seen only once with the 2-phase approach.

### Unconsciousness and Death

Unconsciousness is defined as the point where the EEG shows an isoelectric pattern, whereas we defined death as the point when birds showed an isoelectric EEG pattern with nonreversible properties, and this is always so when heart rate is extremely low (in chickens less then 180 beats per minute; Coenen et al., 2000). Assessment of time to IE was challenging given the artifacts in the EEG recording and was carried out by an experienced EEG analyst, who was blind to the experimental treatments. Onset of isoelecticity for each bird is shown by arrows in Figure 2. Data and statistics (Table 1) indicated no significant differences between groups, although the $N_2CO_2$ group tended to have the shortest time to IE and the time to death was significantly shorter for the $N_2CO_2$ group.

### CD of the EEG

The correlation dimension, as a measure of EEG complexity, correlates well with the level of vigilance of animals and humans (Coenen, 1998; Kobayashi et al., 2000; van den Broek et al., 2000). Therefore, the CD of the EEG is a useful measure to examine loss of consciousness during the euthanasia process. The baseline values of the CD were 5.85 ± 0.23 (mean and SEM; n = 3 groups of 8 birds, 6 epochs). This baseline value is considered as 100%, and all subsequent values are expressed relative to it. Data and best fit curves from the beginning of the euthanasia procedure to 120 s (approximately halfway through the second compartment) are given in Figure 3. The F-test shows that the parameters start (0 s) and plateau (120 s) do not differ between the 3 groups. Therefore, the fit procedure is constrained, and these parameters are shared between the groups. The k value differs between the groups [$F_{(2,597)} = 18.53$]. Post hoc analysis (comparing the groups 2 by

2) shows that the k best fit value of the $N_2CO_2$ group [k = 0.0038 (0.008)]/s] differs from the other 2 groups ($P < 0.05$) and that the k best fit value of the groups $N_2$ [k = 0.0021 (0.003)/s] and 2-phase [k = 0.0016 (0.002)/s] do not differ statistically (in parentheses, the approximate SE is given).

Studies examining CD values in vigilant and anaesthetised animals and humans reveal a reduction in CD to 60% of the baseline value when they are deeply unconscious (van den Broek et al., 2000; Widman et al., 2000; McKeegan et al., 2007b). The time taken to reach 60% of the baseline values (and approximate SE) is for the $N_2CO_2$ group 25.8 ± 8.9 s, for the $N_2$ group 47.4 ± 12.0 s, and for the 2-phase group 61.7 ± 13.8 s.

## Observations of Behavior

Regardless of treatment, there were brief periods when the instrumented birds were not visible, because they were obscured either by other birds or by the stunning equipment. This was recorded as out of view and

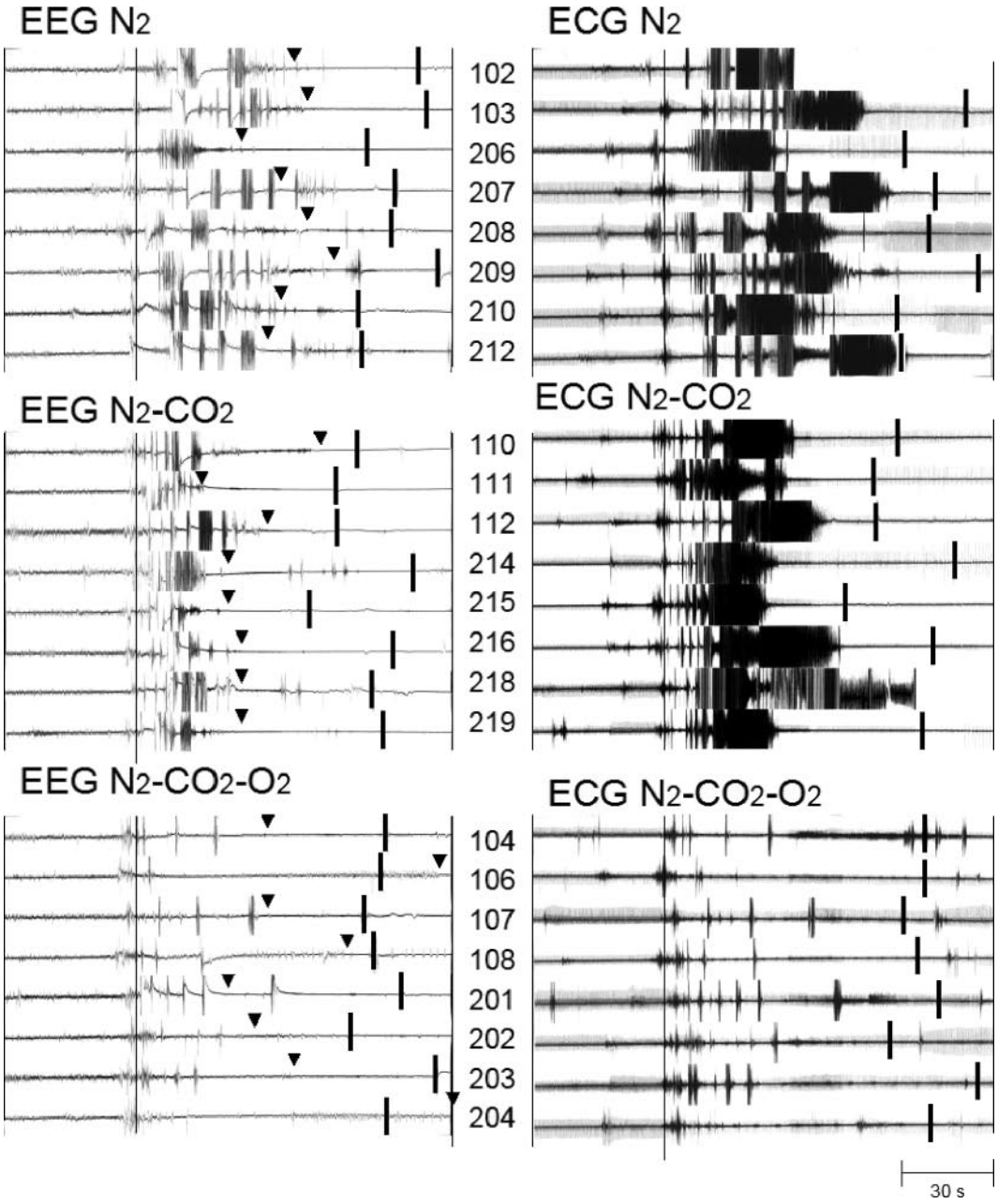


**Figure 2.** Electroencephalogram (EEG) recordings (left) and electrocardiogram (ECG) recordings (right) of chickens placed in the 3 treatment conditions ($N_2$ – $N_2$-$CO_2$ – 2-phase or $N_2$-$CO_2$-$O_2$). The recordings are synchronized when birds enter the first compartment, as indicated by a continuous vertical line, representing approximately 45 s after the beginning of the recording (the baseline EEG and ECG). In the EEG recordings, the onset of isoelectricity is indicated by an arrow. The drop artifact (the transition between the first and second compartment) can be seen in variable positions in the right-central part of the recordings and is highlighted with a black vertical bar.

**Table 2.** Artifact number and duration for each treatment[1]

| Item[2] | n | Treatment | | | $F_{(2,19)}$ | *P*-value | *t*-test |
|---|---|---|---|---|---|---|---|
| | | $N_2$ (1) | $N_2CO_2$ (2) | 2-phase (3) | | | |
| EEG artifacts | | | | | | | |
| Duration (s) | 8 | $9.0 \pm 2.3^a$ | $5.1 \pm 1.9^b$ | $2.0 \pm 1.8^c$ | 23.2 | >0.001 | 1 > 2 > 3 |
| Number | 8 | $6.7 \pm 1.9^a$ | $5.6 \pm 1.0^b$ | $2.8 \pm 1.7^c$ | 12.0 | >0.001 | 1, 2 > 3 |
| ECG artifacts | | | | | | | |
| Duration (s) | 7 | $24.3 \pm 3.1^a$ | $21.7 \pm 4.7^b$ | $2.5 \pm 1.5^c$ | 99.3 | >0.001 | 1, 2 > 3 |
| Number | 7 | $8.0 \pm 3.7^a$ | $7.1 \pm 1.7^b$ | $3.5 \pm 2.0^c$ | 6.7 | >0.01 | 1, 2 > 3 |
| Artifact duration difference | | | | | | | |
| ECG − EEG | 7 | $15.3 \pm 2.9^a$ | $16.6 \pm 5.1^b$ | $0.8 \pm 1.0^c$ | 52.7 | >0.001 | 1, 2 > 3 |
| Time to | | | | | | | |
| Isoelectricity (s) | 8 | $48.9 \pm 10.4^a$ | $34.3 \pm 12.1^b$ | $57.9 \pm 30.6^c$ | 2.5 | 0.11 | |
| Death (s) | 7 | $194.3 \pm 59.5^a$ | $124.1 \pm 62.1^b$ | $215.0 \pm 37.4^a$ | 5.8 | 0.01 | 2 < 1, 3 |

[a–c]Values in the same row with different superscripts are significantly different.

[1]Mean (±SE) artifact number and duration (s) for each treatment. Time to unconsciousness and death are also indicated (see text).

[2]EEG = electroencephalogram; ECG = electrocardiogram.

analyzed like the other variables. Time out of view was significantly greater for the 2-phase treatment ($P < 0.001$, Table 3), primarily because this treatment necessitated the fitting of a curtain to separate the different gas atmospheres in each phase. This curtain obscured the camera view of the conveyor belt around the vicinity of the transition between first and second compartments.

Tables 3 and 4 provide a summary of the behavioral responses of the birds to each treatment. Headshaking was exhibited in all treatments by similar proportions of birds (6 out of 8 in $N_2$ and 2-phase, 5 out of 8 for $N_2CO_2$). The number of headshakes performed by individuals ranged from 1 to 4 and was also not significantly affected by treatment. Mandibulation, a distinctive tasting response, was also exhibited and its frequency

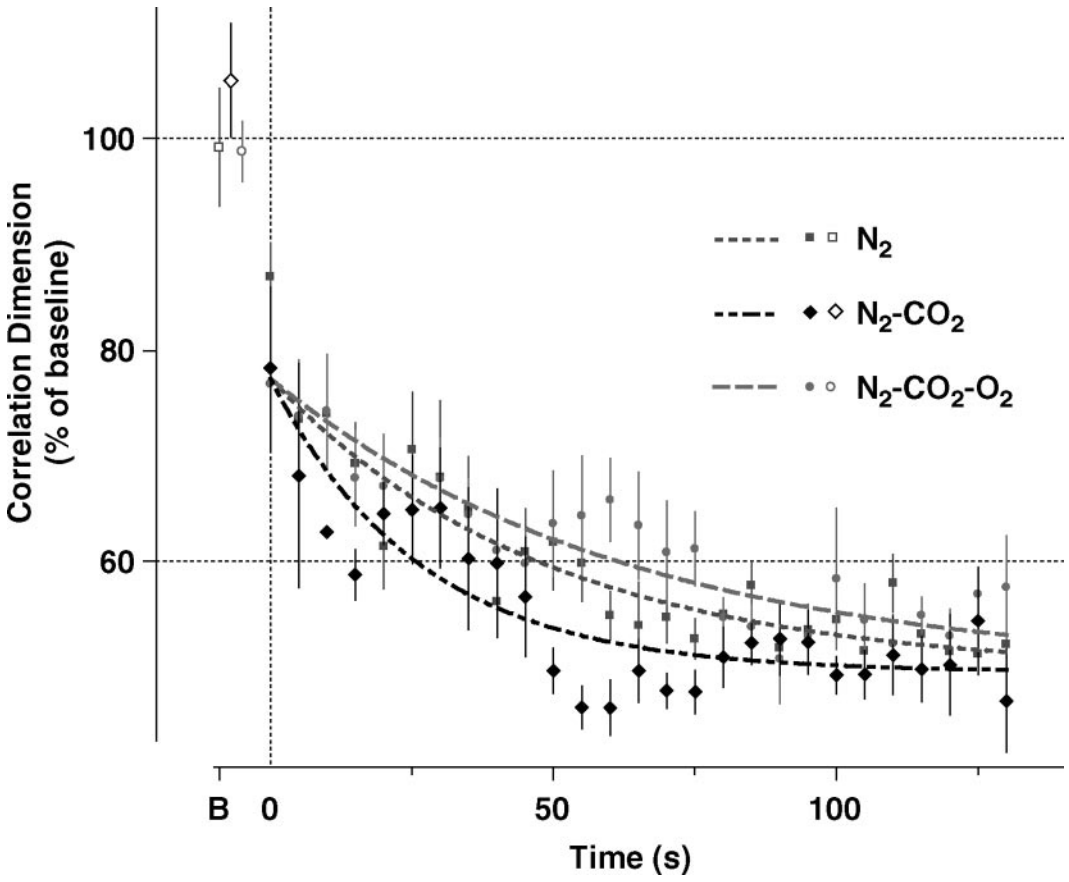


**Figure 3.** Correlation dimension of the electroencephalogram (mean and SEM) of the 3 treatments: in the baseline and in a 120-s period after placing the birds in the system. A 60% reduction related to baseline is taken as an indication of unconsciousness (see text). The fit procedure is described in the text.

**Table 3.** Behavioral responses[1]

| CAS treatment[2] | Headshakes (median number, range) | Respiratory disruptions (median number, range) | Time to loss of posture [median (s), range (s)] | Total time motionless [median (s), range (s)] | Total time out of view [median (s), range (s)] |
|---|---|---|---|---|---|
| $N_2$ | 1.5 | 0.5[a] | 11.5[a] | 110.5[a] | 24.0[a] |
| | 0 to 4 | 0 to 3 | 7 to 18 | 88 to 128 | 10 to 39 |
| $N_2CO_2$ | 1.0 | 2.0[a] | 7.5[b] | 123.5[b] | 17.5[a] |
| | 0 to 2 | 0 to 4 | 4 to 10 | 109 to 135 | 14 to 42 |
| 2-phase | 1.5 | 6.5[b] | 23.0[a] | 58.5[c] | 72.5[b] |
| | 0 to 2 | 4 to 11 | 9 to 33 | 37 to 86 | 57 to 104 |
| Kruskal-Wallis | NS | $P < 0.001$ | $P = 0.003$ | $P < 0.001$ | $P < 0.001$ |

[a–c]Values in the same column with different superscripts are significantly different.

[1]Median values for numbers of headshakes and respiratory disruptions, time to loss of posture, total time motionless, and total time out of view for each treatment, with results of Kruskal-Wallis analysis.

[2]Controlled atmosphere (gas) stunning.

and number (0 to 2 bouts) were unaffected by treatment. Respiratory disruption, either in the form of deep open bill breathing or bill gaping combined with apparent apnea, was observed in response to all 3 treatments. Respiratory disruptions were seen to a greater extent in the hypercapnic treatments and were particularly prevalent with the 2-phase treatment (median number 6.5 compared with 0.5 and 2.0, $P < 0.001$, Table 3). Time to loss of posture was also significantly affected by treatment, with a more rapid loss of posture associated with the $N_2CO_2$ treatment (median 7.5 s compared with 11.5 or 23.0 s, $P = 0.003$, Table 3). The total time motionless was greatest for the $N_2CO_2$ treatment, intermediate for the $N_2$ treatment, and least for the 2-phase treatment (median 123.5, 110.5, and 58.5 s, respectively, $P < 0.001$, Table 3).

Wing flapping was exhibited by every bird at some point in the euthanasia process, but timings of the onset, number, and length of bouts varied according to treatment. The onset of wing flapping was significantly later and occurred over a much greater range in the 2-phase treatment (median onset 23.5 s compared with 12.0 and 10.0 s, $P < 0.001$, Table 4). The number of wing flapping bouts was significantly greater with $N_2$ anoxia (median number 5.0 compared with 3.0 or 1.5, $P = 0.029$, Table 4), whereas the flapping bout duration was shortest with the 2-phase treatment (median 1.3 s compared with 2.9 or 3.3 s, $P = 0.001$, Table 4). Total wing flapping duration was greatest with $N_2$, intermediate with $N_2CO_2$, and least with the 2-phase treatment (median duration 13.5, 9.5, and 2.0 s respectively, $P < 0.001$, Table 4). A distinctive posture in which the bird, normally lying on its back, held its wings rigid up over the sternum (associated with a distinctive artifact on the ECG trace) was observed in all treatments (though by only 1 bird in the 2-phase group). The onset of this behavior was earliest in the $N_2CO_2$ treatment (median 20 s compared with 32.5 s, $P = 0.019$, Table 4).

## DISCUSSION

This is the first study in which EEG, cardiac, and behavioral responses have been simultaneously measured in freely moving birds being exposed to a commercially

**Table 4.** Wing flapping responses[1]

| CAS treatment[2] | Wing flapping onset [median (s), range (s)] | Wing flapping bouts (median number, range) | Wing flapping bout duration [median (s), range (s)] | Wing flapping total duration [median (s), range (s)] | Wings rigid onset [median (s), range (s)] |
|---|---|---|---|---|---|
| $N_2$ | 12.0[a] | 5.0[a] | 2.9[a] | 13.5[a] | 32.5[a] |
| | 9 to 15 | 1 to 8 | 1.9 to 9.0 | 9 to 21 | 18 to 45 |
| $N_2CO_2$ | 10.0[a] | 3.0[ab] | 3.3[a] | 9.5[b] | 20.0[b] |
| | 7 to 13 | 1 to 5 | 2.0 to 9.0 | 7 to 13 | 16 to 23 |
| 2-phase | 23.5[b] | 1.5[b] | 1.3[b] | 2.0[c] | 143[3] |
| | 13 to 129 | 1 to 4 | 1.0 to 2.0 | 1 to 7 | — |
| Kruskal-Wallis | $P < 0.001$ | $P = 0.029$ | $P = 0.001$ | $P < 0.001$ | $P = 0.019$ |

[a–c]Values in the same column with different superscripts are significantly different.

[1]Median values for wing flapping onset, number of wing flapping bouts, wing flapping bout duration, and total duration of wing flapping for each treatment, with results of Kruskal-Wallis analysis.

[2]Controlled atmosphere (gas) stunning.

[3]Only 1 bird in this group exhibited this behavior, so no range is shown and Mann-Whitney U analysis could not be carried out.

relevant euthanasia process. The data generated therefore allow a realistic welfare assessment of the 3 CAS approaches examined.

A crucial measure enabled by the measurement of the EEG is determination of the time from the beginning of the euthanasia process to the time that the animal is unconscious and no longer able to perceive pain or distress. We used 2 approaches to establish the time to loss of consciousness: 1) onset of an isoelectric EEG and 2) time taken for the CD to decrease to 60% of its baseline value. The first of these is noncontentious, because it is widely accepted that an isoelectric EEG is associated with unconsciousness. The use of the CD for this purpose is also established, with various previous studies demonstrating that deep anesthesia is associated with a CD below 60% of baseline values, in hens (Coenen et al., 2000; McKeegan et al., 2007b), rats (Coenen, 1998), as well as for humans (van den Broek, 2000). Isoelectricity and CD values corresponded reasonably well in the current experiment, although time to 60% was significantly different between groups, whereas IE showed only a tendency to be related to treatment. In the $N_2CO_2$ treatment, IE was achieved slightly later than 60% CD (IE: 40.3 ± 10.8 s; CD: 26 ± 8.9 s), and 33 s represents an average between the two. The $N_2$ group figures corresponded closely (IE: 48.9 ± 10.4 s; CD: 47.4 ± 12.1 s), and 48 s is the average. In the 2-phase treatment, IE was achieved before 60% CD (IE: 57.9 ± 30.6 s; CD: 61.7 ± 13.8 s, mean 60 s), but a reservation has to be made in the assessment of the time to unconsciousness, because 3 out of 8 birds (numbers 106, 108, and 204) exhibited a long-lasting aberrant spiking EEG pattern, which began shortly after the onset of IE. This spiking activity was reminiscent of epileptiform activity, which is usually associated with unconsciousness (Coenen, 1998). If the spiking activity is considered as such, the time to unconsciousness in the 2-phase treatment is shortened from 57.9 ± 30.6 s to 44.0 ± 13.3 s, and the mean drops to 50 s. Taking these averages, we can conclude that the time until unconsciousness is approximately 33 s for the $N_2CO_2$ group and approximately 50 s for the 2 other groups. The time to death in the $N_2CO_2$ group is also significantly shorter than in the $N_2$ and 2-phase groups. These results concur with our previous findings, which showed that adding the anesthetic $CO_2$ to $N_2$ hastens the time to unconsciousness and death, whereas adding $O_2$ to the mix of $N_2$ and $CO_2$ in an anesthetic phase slows down the euthanasia process (McKeegan et al., 2007b).

The presence of artifact in the EEG and ECG traces was an inevitable consequence of recording these signals in freely moving birds, but this loss of data also provided us with information. A striking difference in the appearance of the ECG artifacts was seen between the 2 anoxic groups and the 2-phase treatment. Examination of the traces and corroboration with behavioral data confirmed that the ECG artifacts were produced by 2 factors: first, substantial body movements, which caused corresponding artifacts on the EEG trace, and second, strong muscle spasms in immobile birds not associated with EEG trace disturbances. Thus, subtractions between EEG and ECG artifacts provided an indication of the extent of tonic convulsions and showed that these were extensive in the anoxic groups but practically nonexistent in the birds exposed to the 2-phase approach. With reference to the EEG and time to loss of consciousness data, it appears that these sustained muscle contractions overlap with a period before unconsciousness is reached and could result in negative experiences (for example, pain), which are a cause for welfare concern.

In agreement with previous studies (Abeyesinghe et al., 2007; McKeegan et al., 2007a,b), the behavioral data showed that the birds exposed to anoxic gas mixtures showed stronger behavioral responses in the form of wing flapping, and the onset of this response was earlier than with the 2-phase approach. Other behavioral responses such as headshaking and mandibulation did not differ between treatments. Onset of wing rigidity (corresponding to tonic convulsive artifacts on the ECG trace) was more rapid in the 2 anoxic groups, as was the time until birds became motionless. Thus, in anoxic treatments, there is evidence of potentially negative behavioral responses in the period that the EEG is not isoelectric. This gives rise to the possibility of distress caused by the behavioral responses themselves, or by pain caused by injury during wing flapping or disturbance or injury, or both, resulting from nearby birds exhibiting similar responses. It should also be noted that the time to loss of posture (which differed between treatments, being shortest in the $N_2CO_2$ group) was significantly less than the time to loss of consciousness as indicated by both electrophysiological methods (IE and CD), highlighting the limitations of this behavioral response as a reliable indicator of unconsciousness.

Behavioral observations showed that respiratory disruption (described as deep open bill breathing with prolonged inspiration or prolonged open bill gaping combined with apparent apnea or difficulty inhaling, or both) was most extensive with the 2-phase approach. This finding corresponds to previous work, which suggested that hypercapnic hyperoxygenation (as opposed to just the presence of $CO_2$) appears to exacerbate respiratory disruption (McKeegan et. al, 2007b). The potential welfare consequences of this type of respiratory discomfort have been discussed elsewhere (McKeegan et al., 2007b), and this important issue deserves further study. However, it is also worth pointing out that the anoxic mixtures also induced respiratory disruption, and the extent of this could have been underestimated due to the vigorous behavioral responses in the early part of the stunning process, which are associated with these treatments.

The aim of this experiment was to assess the welfare implications of CAS with different approaches: anoxia, hypercapnic anoxia, and a 2-phase approach. General agreement with previous findings demonstrates that the neurophysiological responses as seen in the labora-

tory are relevant to commercial practice. Although the results suggest that all the CAS approaches tested were effective in achieving nonrecovery states, the recommendation for which is the most welfare friendly centers on an interpretation of the pros and cons of the 3 approaches. Comparing the anoxic treatments ($N_2$ and $N_2CO_2$), hypercapnic anoxia resulted in a more rapid unconsciousness and death than $N_2$ anoxia alone. However, both were associated with early onset prolonged wing flapping and sustained tonic convulsions, which occurred in the time when consciousness remained a possibility. Comparing the 2 hypercapnic approaches ($N_2CO_2$ and 2-phase), hyperoxygenation was associated with respiratory disruption, but eliminated clonic convulsions in the early part of the stunning process and tonic convulsions were not seen. Hypercapnic anoxia had 2 advantages: respiratory disruption was decreased (though still present) and time to unconsciousness was shorter.

The most important issue for welfare is what the animal experiences, so what occurs during the time that the EEG is not isoelectric or has a CD greater than 60% of baseline (a lower value represents a state of unconsciousness) is crucial. These results suggest that presence of $O_2$ in the first stage of CAS is associated with an absence of potentially distressing behavioral responses when consciousness is a possibility. Although acknowledging that the respiratory discomfort associated with hypercapnic hyperoxygenation is an issue, we propose that this may be compensated by a more gradual induction to unconsciousness, which eliminates the risk of other potentially negative experiences.

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