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Physiological and behavioural responses of broilers to controlled atmosphere stunning: implications for welfare

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Abstract

Controlled atmosphere (gas) stunning (CAS) has the potential to improve the welfare of poultry at slaughter but there is a lack of consensus about which gas mixtures are most humane. The aim of this study was to evaluate the welfare consequences of different gas stunning approaches. Individual broilers were exposed to gas mixtures capable of inducing unconsciousness and euthanasia while their behavioural, cardiac, respiratory and neurophysiological responses were measured simultaneously. The approaches investigated included anoxia (N_2 or Ar with < 2% residual O_2), hypercapnic anoxia (30% CO_2 in Ar, 40% CO_2 in N_2) and a biphasic method (40% CO_2, 30% O_2, 30% N_2 for 60 s followed by 80% CO_2 in air). Evaluation of the welfare implications of each approach centred on the likelihood of them inducing negative states or experiences during the conscious phase. Hypercapnic mixtures were associated with strong respiratory responses, while anoxic mixtures induced vigorous wing flapping. Electroencephalogram analysis using the correlation dimension (a non-linear measure of complexity) suggested that anoxic wing flapping occurred during periods in which a form of consciousness could not be excluded. Hypercapnic hyperoxygenation (biphasic approach) exacerbated respiratory responses but eliminated the possibility of vigorous behavioural responses occurring during a conscious phase. The relative importance of respiratory discomfort versus the potential to induce significant distress due to convulsive wing flapping and associated trauma is a matter for debate. We argue that respiratory discomfort is unpleasant but may be preferable to the risk of vigorous wing flapping and associated injury while conscious in poultry during CAS.

Keywords: *animal welfare, broilers, carbon dioxide, euthanasia, gas stunning, slaughter*

Introduction

A central requirement for the humane slaughter of food animals, including poultry, is effective stunning prior to exsanguination. The purpose of stunning is to induce unconsciousness and insensibility and the stunning method itself should not cause avoidable pain, suffering and distress. The Department for Environment, Food and Rural Affairs (Defra) monitors the commercial slaughter of livestock in the UK under the Welfare of Animals (Slaughter or Killing) Regulations (WASK) 1995 HMSO (1995).

In the UK, the most common method of pre-slaughter stunning for poultry is immersion of the head in an electrified water bath. When applied correctly, the resulting electrocution induces immediate prolonged unconsciousness and cardiac arrest, thereby meeting the requirements that the birds are rendered insensible and do not recover consciousness before the neck cut (Raj & Tserveni-Gousi 2000). However, electrical stunning is associated with a number of welfare insults such as inversion and live shackling (Gentle & Tilston 2000), pre-stun shocks if the birds' wings touch the water before their heads (Raj & Tserveni-Gousi 2000), inadequate stunning or birds missing the water bath completely. Thus, this approach may compromise welfare prior to stunning and does not always guarantee effective stunning.

Controlled atmosphere (gas) stunning (CAS) involves immersion or transfer of birds into an appropriate gas environment to induce unconsciousness prior to neck cutting. This approach has the potential to improve welfare of poultry at slaughter by eliminating the stress associated with live bird shackling; instead birds may be stunned in their transport crates or freestanding on a conveyor belt and are shackled post-stunning. CAS also has the potential to ensure effective stunning of every animal. For CAS to provide welfare benefits, it is essential that birds do not regain

consciousness before neck cutting and, for this reason, all current commercial CAS installations apply methods which achieve non-recovery states (ie they stun-kill). It is also necessary that any gas mixture(s) applied minimise pain, suffering and distress during the induction of unconsciousness. This is particularly relevant for CAS since the narcotic effects of the gas atmosphere applied are not instantaneous.

Currently, commercial poultry plants in the UK and continental Europe apply various CAS approaches; anoxia (nitrogen [N_2] or argon [Ar] with less than 3% residual oxygen [O_2]), hypercapnic anoxia (N_2 or Ar with the addition of 25–30% carbon dioxide [CO_2], < 2% residual oxygen) or a biphasic approach where an anaesthetic phase (40% CO_2/30% O_2/30% N_2) is applied for 1 minute followed by application of a second phase (~ 80% CO_2 in air, 2 mins) to prevent recovery. In the UK, under WASK, only nitrogen/argon anoxia with or without inclusion of up to 30% CO_2 is permitted (HMSO 1995).

Although the potential welfare benefits of CAS are well recognised, the use of gas mixtures containing CO_2 in particular raises concerns as exposure to this gas above certain levels is known to be nociceptive in humans, eliciting painful sensations at inhaled concentrations of between 40 and 55% (Anton *et al* 1992). In addition, in birds as in mammals, inhalation of CO_2 activates both central and peripheral chemoreceptors to evoke a potent respiratory response. In particular, the discharge rate of intra-pulmonary chemoreceptors (vagal afferents unique to birds and reptiles [Milsom *et al* 2004]) is inversely related to inhaled CO_2 concentration and the suppression of their activity during hypercapnic exposure results in prolonged inspiration. These responses are immediate (and thus occur while birds are conscious) and are an understandable source of welfare concern about CAS mixtures containing CO_2.

In a related study, we recently examined the immediate behavioural responses to gas mixtures currently or potentially used in CAS, focusing particularly on the potency of different CO_2 concentrations in eliciting behaviour thought to indicate aversion (McKeegan *et al* 2006). In accordance with previous studies, we concluded that CO_2 has aversive properties, particularly when inhaled at high concentrations. However, our findings suggest that aversion to CO_2 is mild to moderate (it interrupted feeding but did not cause withdrawal from the vicinity of the gas outlet in the majority of birds) and we stressed that the aversiveness of initial gas exposure must be viewed in relation to the severity and duration of subsequent events to achieve optimum welfare during CAS.

Euthanasia methods are only acceptable when they result in minimal signs of agitation and distress in the period that some degree of consciousness cannot be excluded. The induction of unconsciousness with CAS is not immediate, and thus this period extends beyond initial exposure, incorporating behavioural and physiological changes induced by the gas mixture inhaled by the bird. We aimed to evaluate the welfare consequences of different CAS approaches and our choice of gas mixtures was informed by the results of immediate aversion tests (McKeegan *et al* 2006) and current commercial practice. A purpose-built apparatus was used to expose individual birds to gas mixtures capable of inducing unconsciousness and euthanasia while their behavioural, cardiac, respiratory and neurophysiological responses were measured simultaneously. Our interpretation of the EEG was aided by the pilot application of a novel analysis technique, the correlation dimension, enabling us to relate the timing of potential welfare insults to the likelihood of consciousness on an individual basis. The design of the gas delivery system allowed pre-stunning baselines to be recorded in individual, restrained birds, before a rapid changeover to the test gas mixture. Unlike previous studies, this approach eliminated the need for bird handling at the time of entry to the gas, thereby allowing continuous measurement of behaviour and physiological variables without disturbance during early induction. Also, in contrast to previous studies of the same nature, we paid considerable attention to accurate gas control and took steps to measure the gas atmosphere experienced by the birds during CAS.

Materials and methods

Subjects and husbandry

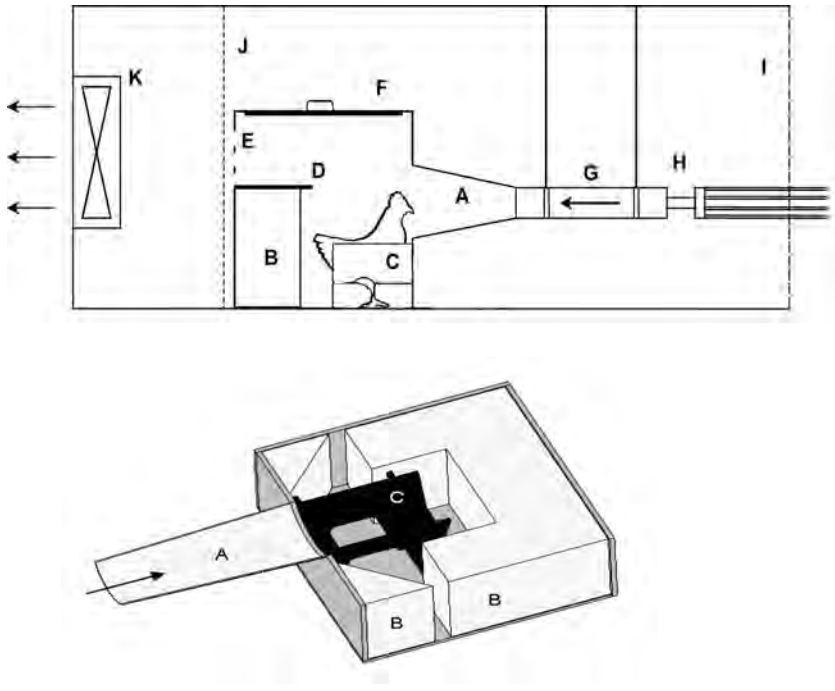
The experiments were carried out on 36 broiler chickens and were performed with the approval of the Roslin Institute Ethical Committee and with authorisation from the UK Home Office through both Project and Personal Licences. The birds had been reared from day old in floor pens with wood shavings litter and *ad libitum* access to food and water. The photoperiod was 14 h light, 10 h dark. The ambient temperature was maintained at approximately 22°C by controlled ventilation and heating for the duration of the experiment and supplementary heat was provided by dull emitter heaters for the first 3 weeks of life. The birds were 28 days old when implanted with EEG electrodes (see below) and were experimentally euthanised 6 days later.

Experimental apparatus

The subjects were exposed to selected gas mixtures inside a purpose-built box, placed inside a larger apparatus previously used to examine the behavioural responses of chickens to various gases and utilising the same gas delivery system (McKeegan *et al* 2005, 2006). The whole apparatus was situated in a well-ventilated, dimly lit room. The large, outer box was clear Perspex (233 × 90 × 90 cm; length × breadth × height; Figure 1) and was electrically screened with wire mesh (aperture diameter 10 mm) to minimise interference during physiological recordings.

The smaller, inner box (50 × 50 × 50 cm; length × breadth × height, [total volume 0.125 m^3]) was also constructed of clear Perspex to maximise visibility, and was connected to the gas delivery system via a conical Perspex tube (diameter 10 cm widening to 15 cm; Figure 1, A). A series of closed Perspex boxes were fitted inside the inner box, reducing the available internal volume by approximately 50% (Figure 1, B). A removable Perspex cradle (Figure 1, C) could be secured in the box and provided partial restraint (it supported the bird's weight and birds were free to move their head, neck and wings but the legs

Figure 1



Upper figure shows diagrammatical representation of the experimental apparatus as viewed from the side, showing outer and inner boxes. A: conical connection tube; B: closed Perspex boxes to reduce internal volume; C: cradle restraint; D: shelf for recording apparatus; E: apertures to allow gas flow; F: inner box lid; G: gas delivery tube; H: humidifier; I: mesh wall (12 mm aperture); J: mesh wall (5 mm aperture); K: extractor fan. Lower figure shows three dimensional 'cut-away' diagram showing the internal structure of the inner box as viewed from above, labels match those above. For clarity, note that some of the closed Perspex boxes shown below (labelled B), are not shown above. In both diagrams, arrows indicate the direction of gas flow through the apparatus.

were secured [loosely tied] preventing gross ambulatory movement). The cradle was positioned in the box opposite the point of gas delivery so that birds could not move their head out of the gas flow over the range of normal movement (Figure 1, C). The system was designed to allow a constant flow of the desired gas mixture over the bird, so that a very rapid changeover from air to test gas could be achieved. A shelf on which to place the three amplifiers required to record the physiological signals (see below) was incorporated in the inner box, above and behind the bird's position (Figure 1, D). Opposite the gas delivery point were a series of small apertures to allow gas to escape, and these were also utilised to route leads from the amplifiers to outside the apparatus (Figure 1, E). Birds were placed in the inner box via a lid at the top (Figure 1, F).

Gas delivery system

The gas delivery system had been used previously to examine the behavioural responses of chickens to various gases (McKeegan *et al* 2005, 2006), providing short pulses of gas in a constant air stream without a change in flow rate. In this experiment it was used to provide a prolonged application of the chosen gas treatment, to allow euthanasia.

The system has been described previously (McKeegan *et al* 2005, 2006) and was used here with minor modifications. The gas delivery system operates three mass flow controllers to generate two flows, a clean airflow and a mixed airflow containing the stimulus gas. Between gas applications (eg during baseline recording), an adjustable proportion of the clean airflow was allowed to flow through the delivery tube at a rate (95 l min^{-1}, 0.15 m s^{-1}). The remainder of the clean air and the air/stimulus gas mix was removed using a vacuum pump. Activation of a 3-way switching valve caused an identical airflow of preset stimulus gas mix to flow from the delivery tube, followed by the clean airflow being reinstated after the gas application. The onset and duration of gas application was controlled manually. A humidifier (Perma Pure, New Jersey, USA) was fitted between the gas delivery tube and the stainless steel connector block containing the switching valve (Figure 1, H). High purity water heated to 30ºC was circulated through the humidifier at a flow rate of 11 ml min^{-1} to achieve a consistent 60% relative humidity in the gases delivered.

The air source was a stationary oil-free compressor (Atlas Copco SF2, Hemel Hempstead, UK), and air from it passed through a filter (0.01 μm) to remove fine mist and particulates before entering the gas delivery system via mass flow controllers. The source of gases used for stimulation were cylinders of CO_2 (cylinder fitted with a vaporiser [Sirocco, Messer, Sulzbach, Germany]), argon, nitrogen or a premix of 40% CO_2, 30% O_2 and 30% N_2, all stored at room temperature. The gas delivery system was capable of producing accurate mixes of two gases (eg CO_2 in N_2) but not three, necessitating the use of a three gas pre-mix (see above). For the biphasic treatment, a manual valve was used to switch the gas input to the appropriate mass flow controller from the phase 1 premix (40% CO_2, 30% O_2 and 30% N_2) to a cylinder of CO_2 and the settings were rapidly adjusted to deliver 80% CO_2 in air.

Before the experiments began, the delivery system was calibrated with a fast flame ionisation detector (Cambustion, Cambridge, UK) using methane in synthetic air as the stimulus gas. The rise time of stimulus delivery to 95% of full scale was typically 7 s although 50% of full scale was achieved much more rapidly (1–1.5 s). This delay was taken into account in physiological and behavioural measurements. Calibration measurements with methane confirmed that while in the cradle, a full gas stimulus would be inhaled by the bird at all positions of the head within the possible movement range. To further confirm that desired gas concentrations were achieved in the vicinity of the bird's head and to determine the temporal profile of gas exposure, measurements were made with a combination carbon dioxide/oxygen analyser (Dansensor Combi-check, Anatrol Ltd, Cheshire, UK). The sampling tube of this device was attached to the EEG lead just above the birds head. To determine the baseline capabilities of the gas delivery system, this check was carried out initially using a 'dummy' bird (model bird of equivalent size and shape) for all treatments and then for each treatment during a minimum of two euthanasia trials.

Implantation of EEG electrodes

At 28 days of age, the birds underwent surgery to implant EEG recording electrodes. The procedure was carried out under general ketamine-xylazine anaesthesia; 15 mg kg^{-1} ketamine (Vetalar, Pfizer, UK) followed by supplementation with xylazine 2 mg kg^{-1} (Rompun, Bayer, Germany), both administered intramuscularly. After positioning of the anaesthetised bird in a head-holder, an incision was made in the skin overlying the cranium. The implant consisted of two 0.35 mm diameter Teflon insulated silver electrodes connected to a socket (DIN, RS components). The electrodes were placed on the dura through holes drilled in the cranium (dental drill), one each on the dorsal surfaces of the right and left telencephalon at their approximate rostro-caudal and medio-lateral midpoints. An indifferent electrode placed between the cranium and the overlying tissue under the comb was also connected to the socket. The EEG implant was cemented to the skull with dental cement and the surrounding skin was sutured. To allow full recovery from surgery and ensure stable EEG recordings, birds were allowed to recover for 6 days before undergoing any further experimental procedure.

ECG and respiration sensors

Immediately before experimental euthanasia, birds already fitted with permanent EEG electrodes (see above) were also fitted with ECG electrodes. These were commercially available, disposable self-adhesive ECG electrodes with press-stud electrical connections (Blue Sensor, Ambu Ltd, St Ives, UK) which were bonded to cleaned skin overlying the *pectoralis* muscle either side of the sternum. Cyanoacrylate tissue adhesive (Vetbond 3M, St Paul, MN, USA) was applied to the ECG electrodes before placement on the skin to improve bonding and ensure that the electrodes did not detach before or during euthanasia.

Each bird was also fitted with a reusable Lycra harness which incorporated the respiration sensor. Abdominal movement related to respiration was recorded using an adapted piezoelectric paediatric respiratory effort transducer (Grass Telefactor, Model F-RCTP, Grass Technologies, West Warwick, USA). The transducer was held in place in the appropriate position (encircling the abdomen, ventral to the cloaca) by the harness which was secured using Velcro fastenings. The sensor produced a clear sinusoidal output related to respiration when the bird was sitting at rest. The harness also contained appropriate leads which were connected to the two ECG electrodes.

Physiological recordings

The physiological signals were amplified using three battery powered pre-amplifiers (two P15, [Grass Technologies, West Warwick, USA]; one Dam 50, [WPI, Stevenage, UK]) which were placed on a shelf in the inner box, above and behind the birds' position. Analogue outputs from the amplifiers were fed from the inner and outer Perspex boxes to an A/D interface (1401 plus; Cambridge Electronic Design, Cambridge, UK). An analogue output from the gas delivery system (signalling test onset) was also connected to the interface. The digitised signals were viewed and stored in separate channels in a data acquisition and analysis programme (Spike 2, Cambridge Electronic Design, Cambridge, UK) on a PC (Genie P3 500, Viglen, St Albans, UK).

Behavioural recordings

The behavioural responses of birds during the experiments were observed using low light sensitive video cameras (Panasonic low light WV-BP310/B, Panasonic, Bracknell, UK) arranged outside the Perspex box in three different positions: directly above the subject, in front of and to the right of the subject and behind/to the left of the subject. A fourth (colour) video camera (Colour Sony CCD-IRIS, Sony, Basingstoke, UK) was positioned facing the control unit of the gas delivery system. The four cameras fed a quad unit (Panasonic colour quad system WJ-450, Bracknell, UK) connected to a video recorder and monitor (Panasonic TL350, Bracknell, UK). This set-up allowed simultaneous recording of stimulus onset indication and the behaviour of the subject observed from three angles.

Experimental procedure

Individual birds fitted with EEG and ECG electrodes and the Lycra harness containing the respiration monitor (see above) were placed in a specially designed Perspex 'cradle'. The bird in the cradle was then placed in the inner box of the apparatus and the cradle was secured in place. A lead with a plug (DIN, RS components) fitting the socket of the EEG implant was attached and connected to a pre-amplifier (see above). Leads from the ECG and respiration sensors were also connected to the appropriate pre-amplifiers.

The inner and outer box lids were closed and the bird was allowed a few minutes to acclimatise in the presence of airflow through the apparatus. During this time the existence of clear physiological signals (EEG, ECG and

Table 1 Nominal composition and number of birds tested for each gas treatment applied.

CAS treatment	Composition	n
Argon anoxia	100% Ar (< 2% residual O_2)	4
Nitrogen anoxia	100% N_2 (< 2% residual O_2)	8
Hypercapnic argon anoxia	30% CO_2 in Ar	8
Hypercapnic nitrogen anoxia	40% CO_2 in N_2	8
Biphasic	40% CO_2 and 30% CO_2 in N_2 (60 s) followed by 80% CO_2 in air (120 s)	8

respiration) was verified. Sometimes it was necessary to make an adjustment to a pre-amp setting to ensure a good signal before continuing. The images produced by the cameras were examined and focus adjusted if necessary.

After all checks were complete, the settings of the gas delivery system were primed to deliver the required gas mixture. A baseline period of 1 minute was initiated with all signals and behaviour recorded during continuing air delivery by the apparatus. A switch to the euthanasia treatment gas was then triggered, its onset visible both on the video footage (light on the control box of the system) and on the physiological traces via a voltage shift marker on a dedicated input channel. Gas delivery and all recordings continued until unequivocal signs of death were seen (combination of cessation of all respiratory movements and isoelectric EEG signal).

Five separate gas treatments were applied (Table 1), N_2 anoxia, Ar anoxia, 40% CO_2 in N_2, 30% CO_2 in Ar (hypercapnic anoxia) and a biphasic process, consisting of 40% CO_2 and 30% O_2 in N_2 (1 minute, anaesthesia phase, hypercapnic hyperoxygenation) followed by 80% CO_2 in air (euthanasia phase, hypercapnic hypoxia). In practice, oxygen was eliminated rapidly but not instantaneously in the anoxia treatments (see Results). The order of application of each gas treatment was randomised as far as possible. These mixtures were chosen to represent each of the main CAS methodologies used commercially (anoxia, hypercapnic anoxia and hypercapnic hyperoxygenation) and to allow several meaningful comparisons: the effect of anoxia, different levels of CO_2 in anoxia and CO_2 in the presence or absence of supplemented oxygen. Each treatment was applied to 8 birds, except Ar anoxia which was applied to 4 birds.

Analysis

A combination of automated and manual Spike 2 analysis techniques were used to produce dedicated event channels representing heart beats per minute (1 s bins) and breaths per minute from the raw traces during baseline and euthanasia. Where clear waveforms were present, heart rate was measured every 5 s. Visual inspection of the traces was used to examine respiratory disturbances (increase in amplitude and decrease in rate in respiration trace with a characteristic waveform) and where possible the cycle length and amplitude of these responses were measured.

Residual maximum likelihood (REML) analysis was carried out on heart rate data to examine treatment and time effects during three time phases: baseline (60 s), initial gas exposure (12 separate time points 65–120 s) and post 120 s. There was evidence of autocorrelation between measures at adjacent time points and this was taken into account in the analysis.

Inspection of the traces also determined the timing of onset of different types of EEG activity. In a pilot application of the technique, the EEG traces of a subset of birds (2 per treatment) were subjected to correlation dimension (CD) analysis. This analysis provides a non-linear (fractal) measure of signal complexity (for algorithm details see van den Broek *et al* 2005) and was used to return a value for non-overlapping 5 s epochs of the trace throughout euthanasia. Only artefact-free epochs were used. The mean correlation dimension over a 30 s portion of baseline was calculated for the same individuals to provide a basis for comparison. Isoelectric EEG signals at the end of the euthanasia process presented a problem since 'noise' theoretically has an infinite correlation dimension. To reduce the influence of noise on the correlation dimension calculation, the addition of a sinusoidal signal with amplitude related to the baseline signal (10 Hz and amplitude 2 × standard deviation of the EEG signal calculated from the first 27 seconds) was carried out. A pure sinusoidal signal has a correlation dimension equal to one. The combination of the EEG signal and sinusoid raises the correlation dimension to values higher than one. When the EEG is isoelectric the signal is equivalent to a sinusoid plus a small amount of noise, with a CD approaching a value of one. To take this into account and because baseline CD values vary between individuals, changes were expressed relative to the baseline as a percentage.

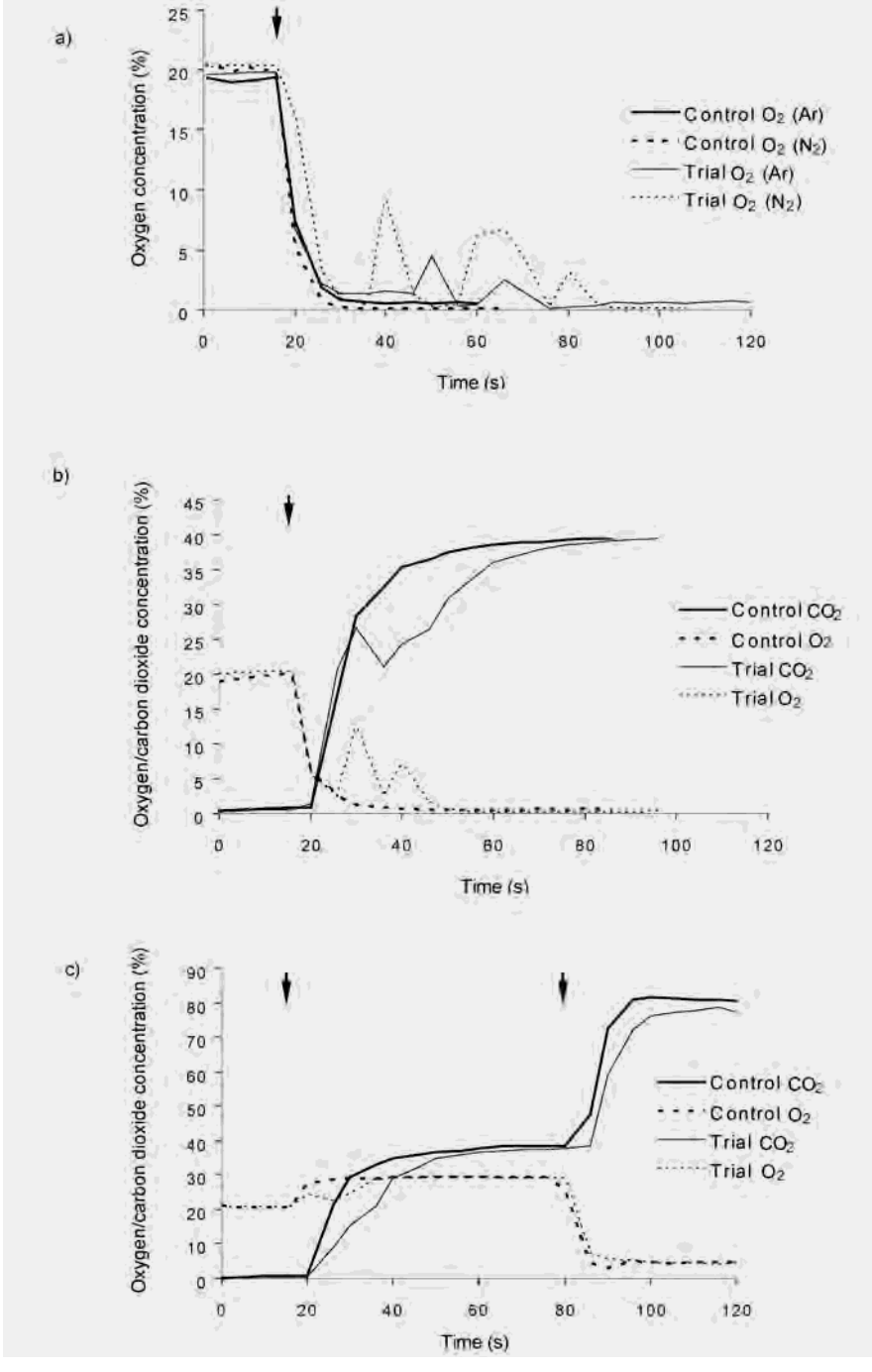
Videotapes of each bird's behavioural responses were analysed noting a range of activities (see Table 2 for behaviour categories and definitions). Continuous observations assigned these descriptions of behaviour at 1 s intervals from gas onset until unequivocal signs of death were seen. These raw data were further analysed to produce counts of the number, duration and timing of bouts of some behaviours.

Behavioural variables were analysed using one-way ANOVA. The data in some categories (head shakes, strug-

Table 2 Description of the recorded behaviours.

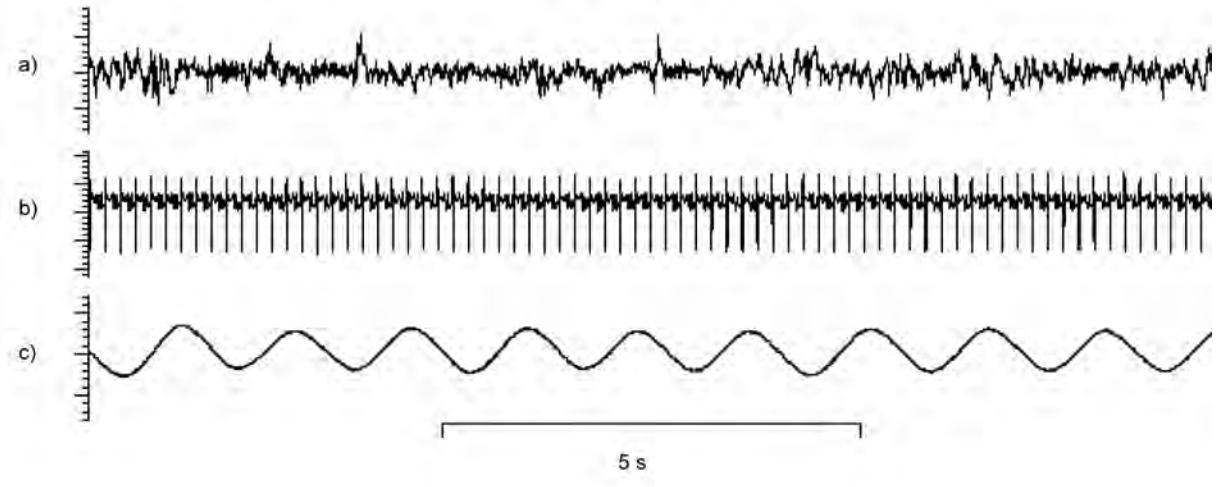
Behaviour	Description
Headshake/flick	Rapid shaking or lateral movement of the head.
Mandibulation	A bout of mandibular movements involving rapid bill opening and closing.
Respiratory disruption	One or more deep open bill breaths with prolonged inspiration and/or prolonged open bill gaping combined with apparent apnoea or difficulty inhaling.
Struggling	A struggling/escape response consisting of purposeful forward and upward movement with or without wing flaps.
Wing flapping	A prolonged (> 2 s) bout of continuous, rapid wing flapping.
Twitching	Visible muscular spasms causing body movement.

Figure 2



Graphs showing the concentrations of oxygen and carbon dioxide at the position of the bird's head during gas application in control conditions (model bird in apparatus) and in an example trial (euthanasia) with anoxia (Ar and N_2; a]), hypercapnic anoxia (40% CO_2 in N_2; b]) and the biphasic approach (c). Arrows indicate onset time of gas delivery, additional arrow on graph c) indicates onset of the second phase.

Figure 3



Example trace from one bird showing simultaneous baseline measurements of electroencephhalogram (a), electrocardiogram (b) and respiration (c). Small divisions on the y-axis are 0.02 volts for (a) and (b) and 0.01 volts for (c). Scale bar is 5 s.

gling bouts and flapping bout duration) did not meet the assumptions required by parametric analysis and so were analysed using the Kruskal-Wallis test to compare medians. *Post hoc* Tukey pairwise comparisons or Mann-Whitney *U* tests were applied as appropriate to determine differences between treatments.

Results

Gas control

Measurements of oxygen and carbon dioxide at the position of the bird's head are shown in Figure 2. Baseline measurements in the presence of a model bird showed that the system was capable of producing a rapid, accurate changeover from air to the desired gas treatment. Oxygen levels fell very rapidly during the anoxia treatments (Figure 2 [a]), with some evidence that argon was slightly more effective at displacing oxygen during trials with live birds. However, the target of < 2% oxygen was not consistently achieved and transient increases in oxygen levels later in the process were due to air mixing as a result of vigorous bird movement (common during the anoxia treatments). Importantly, these occurred after oxygen levels had fallen below 3% and did not seem to disrupt induction, since the time to death was reasonably consistent within treatment. Our measurements demonstrated that the system effectively achieved carbon dioxide delivery and oxygen elimination in the hypercapnic anoxia treatments, again with some deflections due to mixing as a result of bird movement (Figure 2 [b]). It is worth noting that the measurements of carbon dioxide are conservative, since there was an unavoidable delay in the sensor technology used to measure this gas (approximately 10 seconds, manufacturer's data). Deflections during euthanasia were less pronounced with the 2 phase application, as vigorous bird movement was less common during this treatment (Figure 2 [c]).

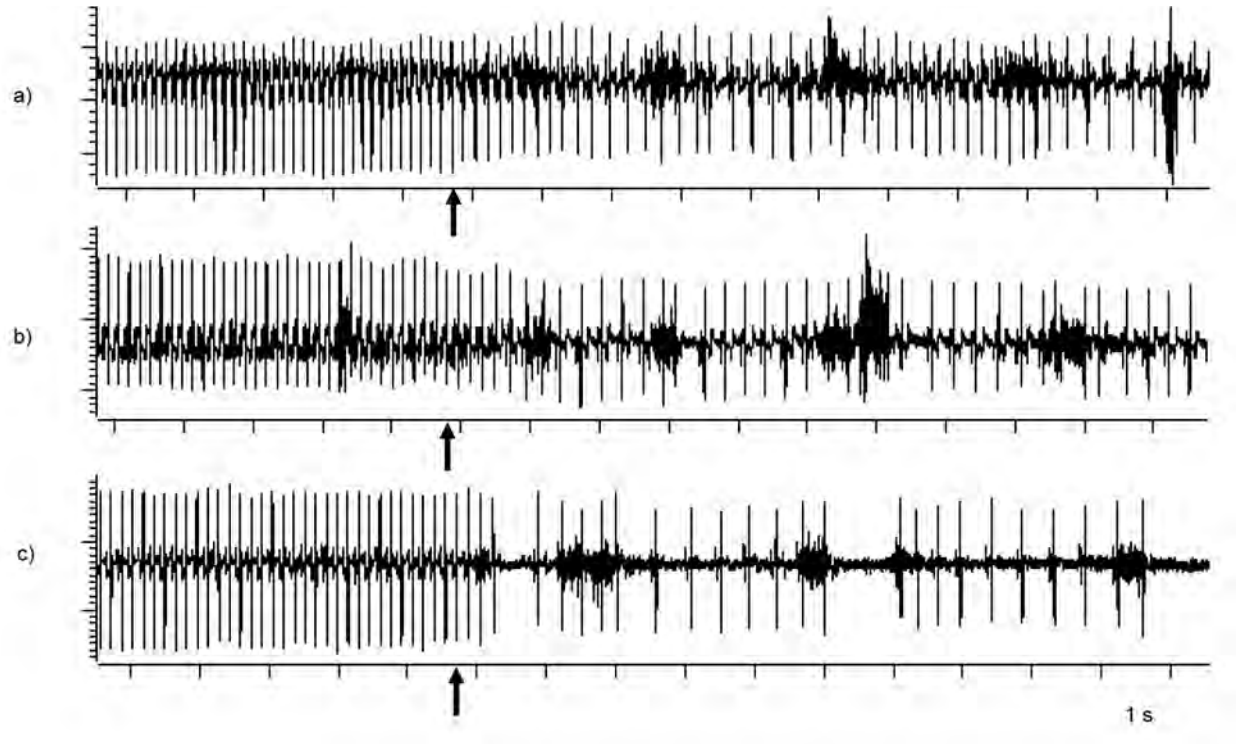
ECG

Clear and detailed ECG waveforms were obtained from all birds during the baseline (Figure 3). During euthanasia the ECG waveform was regularly obscured, sometimes for extended periods. This was due to interference from electromyogram activity from the pectoral muscles and movement artefacts arising from wing flapping and/or prolonged clonic muscle contraction. Behavioural observations confirmed the synchronisation of wing flapping and obscured areas of the trace. Where possible, clean ECG waveforms allowed the determination of heart rate during the baseline and throughout euthanasia.

Exposure to hypercapnic gas mixtures induced an immediate, rapid and pronounced bradycardia, often accompanied by obvious arrhythmia (Figure 4). A decline in heart rate and arrhythmia was apparent in all gas treatments but was most rapid and pronounced with hypercapnic mixtures (see Figure 5 showing heart rate changes with time in individuals and also the 'average' response). REML analysis revealed a significant interaction between the effects of treatment and time (Wald statistic = 300.53, 48 df; $P < 0.001$). There was no significant difference between baseline values for each treatment and the biggest treatment effects were seen in the first 30 s of gas exposure.

Generally, the trend of a large initial reduction in heart rate was followed by varying degrees of recovery and or/stabilisation before a final, non-recoverable decline. An extended period of stabilisation (at a reduced heart rate indicative of an anaesthetised state) was visible in birds treated with the biphasic approach (Figure 5, [e]) reflecting their maintenance on an 'anaesthetic only' gas mixture (40% CO_2, 30% O_2, 30% N_2) for 60 s before application of the final euthanasia phase. A regular ECG waveform of some kind was always present at the end of recording regardless of gas

Figure 4



Example traces from three different birds showing characteristic ECG activity (bradycardia and arrhythmia) during induction with hypercapnic gas mixtures: 30% CO_2 in Ar (a); 40% CO_2 in N2 (b) and 40% CO_2, 30% O_2 and 30% N_2 (c). Arrows indicate the time of gas changeover. Small divisions on the y-axes are 0.01 volts, x-axes divisions are 1 s.

treatment, although at this point the heart rate was always substantially reduced (Figure 5) and abnormal ECG waveforms were usually apparent.

Respiration sensor

Clear sinusoidal waveforms representing respiratory activity were obtained for all birds during the baseline period (Figure 3). Because the piezoelectric sensor around the bird's abdomen acted as a sensitive movement sensor, during euthanasia substantial body movements such as wing flapping and struggling overrode the respiration signal. Responses to hypercapnic mixtures were characterised behaviourally as deep, open bill breathing with prolonged inspiration and this type of breathing was sometimes recognisable on the respiration trace as characteristically shaped waveforms (Figure 6). These could only be validly measured (ie calibrated to the same scale as the baseline) if they occurred before substantial body movement which might have moved the physical position of the sensor. Because many of the birds struggled during early gas exposure (see below), unequivocal data of this type was recorded for only four individuals, two receiving the N_2CO_2 treatment and two receiving the biphasic treatment. Measurements of amplitude and duration of such signals in relation to baseline (for the same individual) revealed a mean increase in amplitude by a factor of 2.8 (± 0.4) for N_2CO_2 and 13.3 (± 2.7) for biphasic and an increase in the duration of the breathing cycle by a factor of 1.9 (± 0.1) for N_2CO_2 and 4.9 (± 1.5) for biphasic. While there was too little data to allow statistical comparison of treatments here, visible respiratory disturbances were also noted and analysed from behavioural recordings (see below).

As mentioned above, the respiration sensor acted as a body movement sensor and examination of the traces revealed large, consistent, regularly spaced deflections temporally correlated with bouts of wing flapping on the video recordings. These represented individual wing movement cycles and thus a maximum wing flapping rate was calculated for each bird by counting the greatest number of regular deflections over a 1 s period in any flapping bout.

EEG

Baseline EEG activity consisted of low amplitude, high frequency activity reflecting the birds' alert state (Figure 3). During euthanasia a series of distinct and consistent changes in the appearance of the EEG were apparent. At times the EEG trace contained extremely large amplitude deflections and these artefacts corresponded to wing flapping or other substantial body movements. Portions of the EEG traces during euthanasia were assigned to one of 4 phases with particular characteristics (Figure 7) where phase 1 was activity identical to the baseline. Phase 2 varied

according to treatment, in anoxia (N_2 and Ar) it appeared as a portion of high amplitude, low frequency activity (Figure 7, [a]) often preceded by and sometimes interspersed with a high frequency but reduced amplitude signal. In hypercapnic treatments the appearance of phase 2 activity was high frequency but reduced amplitude signal compared to the baseline. Phase 3 in all treatments was a greatly suppressed EEG but containing some activity including occasional spiking, and phase 4 was isoelectric (appearance of residual low-level noise indicating lack of EEG activity).

Across treatments, there was no significant difference in the timing of the onset of phase 2 ($P = 0.065$, one-way ANOVA; mean (± SE) for each gas Ar 10.2 ± 1.7 s; N_2 14.7 ± 1.1 s; $ArCO_2$ 14.7 ± 2.3 s; N_2CO_2 7.8 ± 1.4 s; biphase 11.2 ± 1.4 s but there was a significant effect of treatment on the duration of phase 2, with the onset of phase 3 significantly later in anoxia treatments ($P < 0.001$, one-way ANOVA, mean (± SE) for each gas Ar 55.0 ± 10.8 s; N_2 45.0 ± 3.2 s; $ArCO_2$ 26.0 ± 1.9 s; N_2CO_2 23.2 ± 4.6 s; biphase 29.5 ± 1.9 s; Figure 8). The cessation of EEG activity (onset of phase 4) was also affected by treatment ($P < 0.001$, one-way ANOVA, mean (± SE) for each gas Ar 74.7 ± 12.9 s; N_2 76.7 ± 4.4 s; $ArCO_2$ 70.7 ± 6.5 s; N_2CO_2 67.8 ± 4.6 s; biphase 120.7 ± 3.7 s; Figure 8) and occurred significantly later in birds receiving the biphasic treatment.

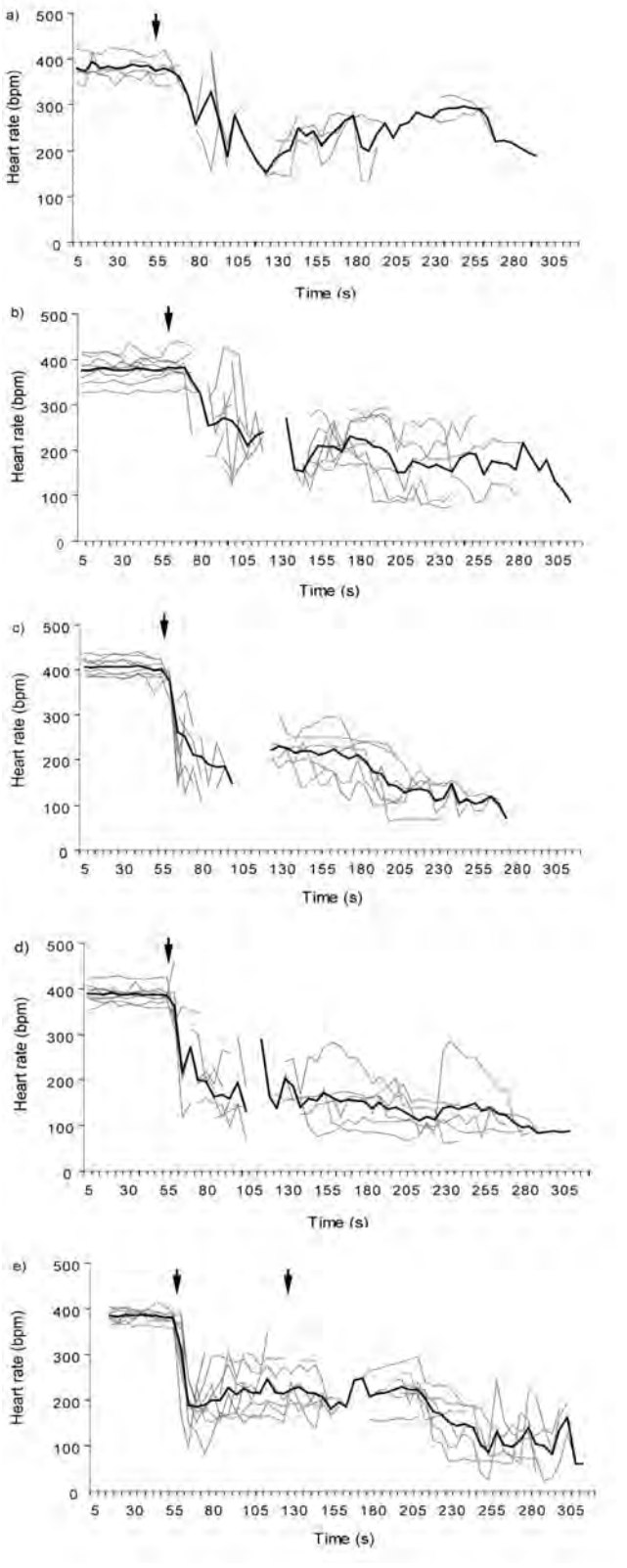
Behaviour

Headshaking was exhibited by a proportion of birds (19) irrespective of treatment and although its occurrence was not significantly different between gas mixtures it was most common in the N_2CO_2 and biphasic treatments (6 out of 8 birds) and rarest in the $ArCO_2$ treatment (1 out of 8 birds). The behaviour was observed as an initial response to gas delivery, and the number of headshakes performed by individuals ranged from 1–6. The highest number of headshakes was observed in response to the N_2 anoxia, N_2CO_2 and biphasic treatments ($P = 0.04$, Kruskal-Wallis; Table 3) which did not significantly differ from each other.

Mandibulation, a distinctive bout of rapid bill movements, was also observed during early gas exposure in a proportion of birds (17), but only in those receiving the hypercapnic treatments. The proportion of birds exhibiting the behaviour or the number of bouts of mandibulation they exhibited (1–3) did not significantly differ between the $ArCO_2$, N_2CO_2 and biphasic treatments.

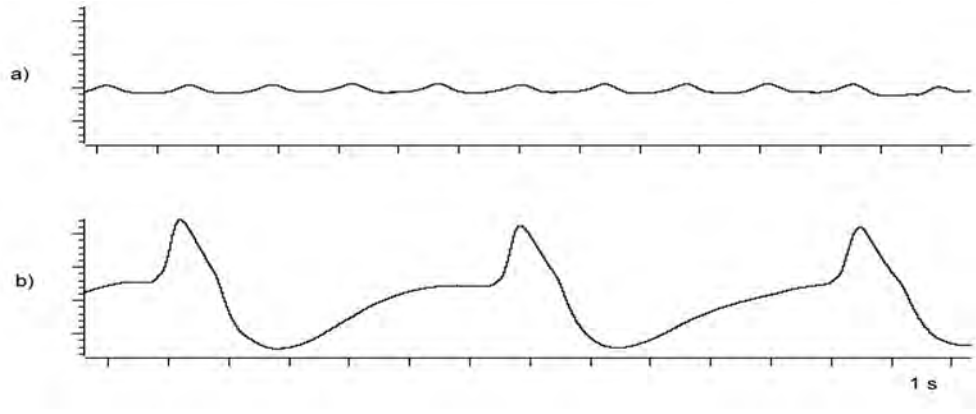
All the gas mixtures applied induced some type of respiratory disruption but its nature was qualitatively different between hypercapnic and anoxic treatments. Hypercapnic mixtures induced an immediate 'deep breathing' response consisting of visibly increased inhalation depth and duration. Anoxia had no immediate effect on respiration, but a characteristic open bill gaping and periods of apparent apnoea (cessation of breathing) were sometimes observed later in the euthanasia process. While it is acknowledged that these responses differ in their physiological basis and possible welfare consequences, to allow direct comparison between treatments and because hypercapnic anoxic treat-

Figure 5



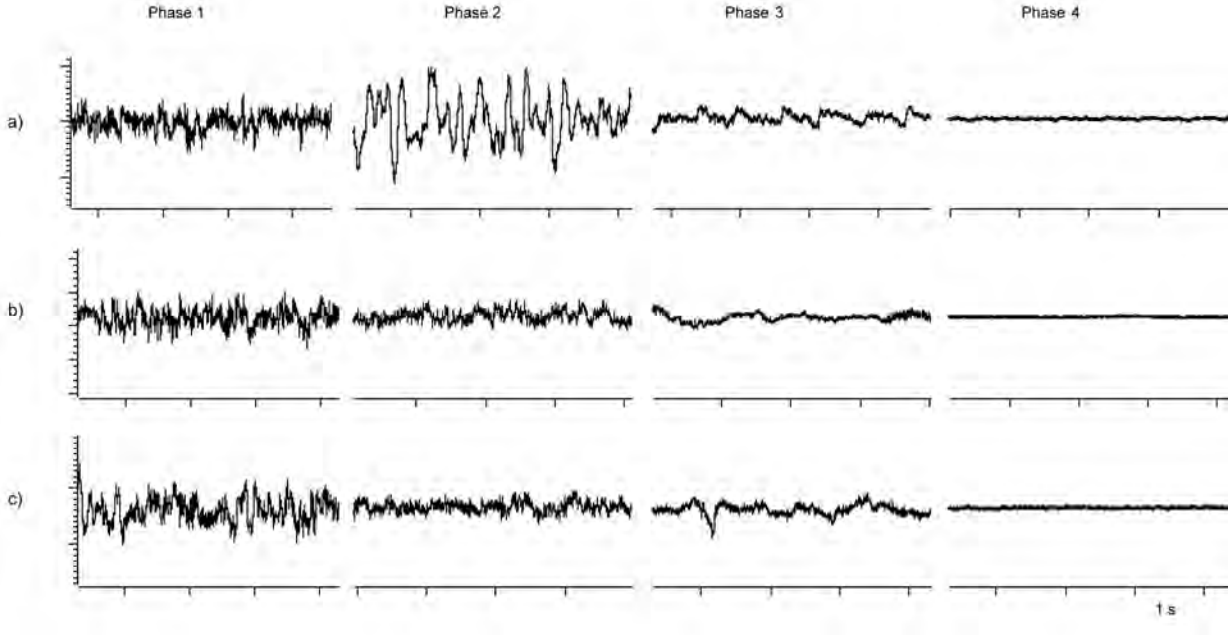
Individual and mean heart rate changes at 5 s intervals in response to each gas treatment: Ar anoxia (a); N_2 anoxia (b); 30% CO_2 in Ar (c); 40% CO_2 in N_2 (d) and biphasic (e). 60 s of baseline heart pre-gas delivery is shown, arrows indicate onset time of gas delivery. Additional arrow on (e) indicates the onset of the second phase. On each graph thicker lines represent the mean calculated with the data vailable at each time point.

Figure 6



Example traces showing the appearance of deep breathing on the respiration trace as indicated by increased amplitude and decreased frequency. A baseline breathing trace (a) is shown above a trace from the same bird deep breathing in response to a hypercapnic gas mixture containing 40% CO_2, 30% O_2 and 30% N_2 (b). The y-axes scales are identical, small divisions represent 0.1 volts. X-axes divisions are 1 s.

Figure 7



Example segments of EEG trace illustrating the typical appearance of the four EEG phases in each treatment type: anoxia ([a], N_2 treatment); hypercapnic anoxia ([b], N_2CO_2 treatment) and biphase (c). X-axes divisions are 1 s, small divisions on the y-axes are 0.01 volts for (a) and (c) and 0.02 volts for (b).

ments could have induced both responses, they were analysed as a single behavioural category, 'respiratory disruption'. There was a highly significant effect of treatment on respiratory disruption with mixtures containing CO_2 eliciting the greatest number, and N_2 anoxia eliciting least disruption (one-way ANOVA, $P < 0.001$; Table 3). Although not significantly greater than the other hypercapnic treatments, numerically most respiratory disruption was associated with the hyperoxygenated phase one of the biphasic treatment.

During euthanasia birds exhibited struggling and wing flapping regardless of treatment (Table 4). All the birds struggled except four (2 out 4 during Ar anoxia and 2 out of 8 during biphasic did not struggle). The number of struggling bouts exhibited (up to 5) was not affected by treatment (Table 4). The onset of wing flapping varied between treatments (Kruskal-Wallis, $P < 0.001$), occurring earliest in Ar and $ArCO_2$ anoxia (median onset 16 and 21 s respectively; Table 4), slightly later in N_2 and N_2CO_2 anoxia (median onset 40 and 28 s, respectively) and latest in biphasic

Table 3 Mean and median values for numbers of headshakes and respiratory disruptions, twitching duration and time to death for each treatment, with results of one-way ANOVA or Kruskal-Wallis analysis.

CAS treatment	Median number headshakes	Mean respiratory disruptions	Mean duration twitching (s)	Mean time to death (s)
Ar anoxia	0.50[ab]	2.25[a]	11.5	107.50[ab]
N_2 anoxia	1.00[b]	1.29[b]	7.71	94.25[a]
$ArCO_2$	0[a]	8.75[ac]	5.63	82.50[a]
$N2CO_2$	1.00[b]	13.00[ac]	5.43	80.71[a]
Biphasic	1.00[b]	15.87[c]	3.75	129.25[b]
ANOVA/Kruskal-Wallis	$P = 0.04$	$P < 0.001$	ns	$P < 0.001$

Values in the same column with different supercripts are significantly different.

Table 4 Mean and median values for struggling and wing-flapping behaviour by treatment with results of one-way ANOVA or Kruskal-Wallis analysis.

CAS treatment	Median bouts of struggling	Mean bouts of wing flapping	Median bouts of wing flapping (s)	Mean duration wing flapping (s)	Median wing flapping onset (s)
Ar anoxia	1.00	5.25[a]	3.60[a]	17.50[a]	16.00[a]
N_2 anoxia	2.00	4.71[ab]	3.40[a]	15.71[b]	40.00[b]
$ArCO_2$	3.00	5.88[a]	1.84[b]	11.00[b]	21.00[a]
N_2CO_2	4.00	6.71[a]	1.86[b]	12.71[b]	28.00[ab]
Biphasic	1.00	3.88[b]	2.09[b]	8.25[c]	90.50[c]
ANOVA/Kruskal-Wallis	ns	$P = 0.035$	$P = 0.009$	$P < 0.001$	$P < 0.001$

Values in the same column with different supercripts are significantly different.

(median onset 90.5 s). The number of wing flapping bouts, bout duration and total wing flapping duration were all related to gas treatment, although maximum wing flapping rate did not differ (overall mean 8.9 ± 2.9 flaps s^{-1}). Numbers of bouts of wing flapping were greatest in anoxic treatments and fewer during application of the biphasic approach (one-way ANOVA, $P = 0.035$). Wing flapping bout duration was significantly shorter for hypercapnic mixes compared to the Ar and N_2 anoxia treatments (Kruskal-Wallis, $P = 0.009$). Total wing flapping duration was greatest in Ar anoxia, least in biphasic and intermediate in the other treatments (one-way ANOVA, $P < 0.001$).

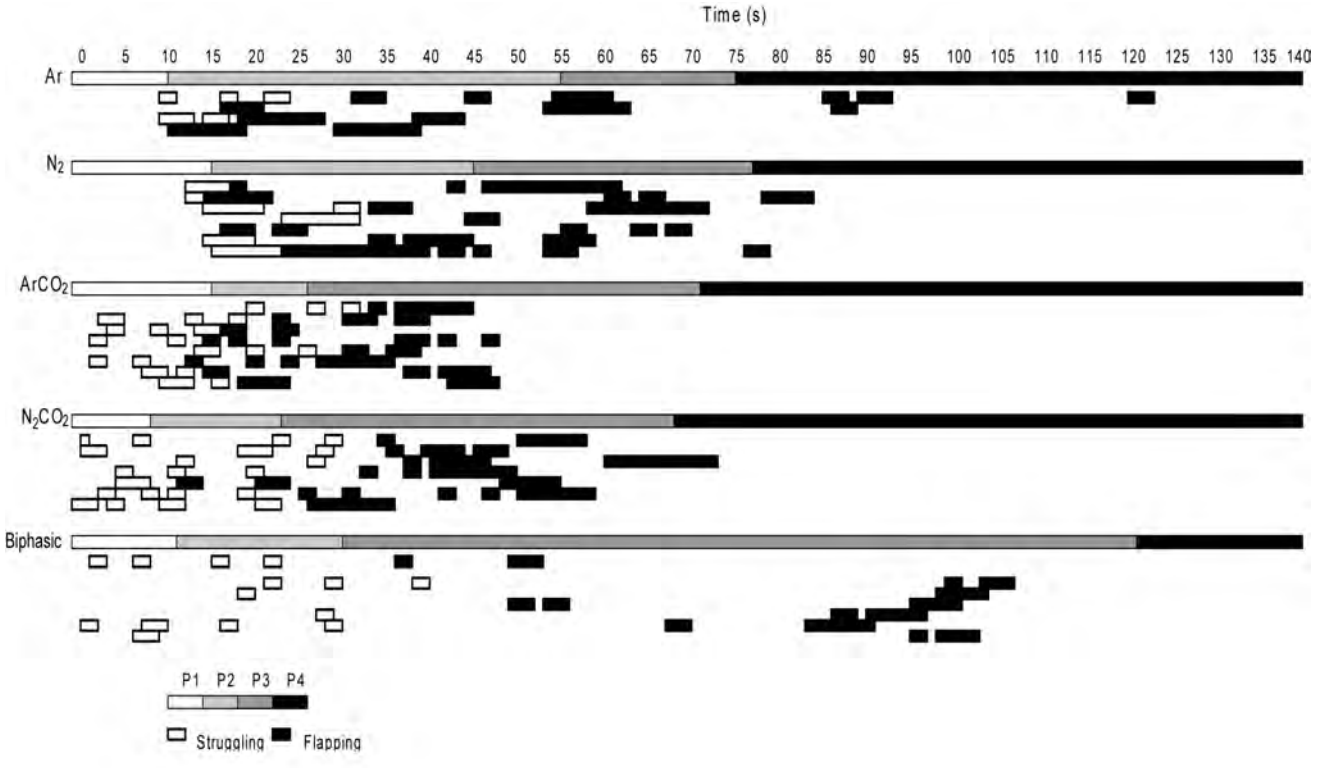
Twitching in the form of visible muscular spasms was observed near the end of the euthanasia process with all gas treatments and its duration was not significantly related to gas mixture (Table 3).

Behaviour in relation to EEG and correlation dimension measurements

To ascertain the extent to which vigorous behavioural responses might negatively impact on welfare, the timing of bouts of struggling and wing flapping were plotted against mean EEG phase timings (Figure 8). In some birds treated with hypercapnic mixtures, struggling was observed immediately or soon after gas onset, while in Ar and N_2 anoxia treatments there tended to be a short delay before any struggling was observed. On average, wing flapping onset for Ar anoxia, N_2 and $ArCO_2$ treatments occurred in EEG phase 2 (Figure 8) but not until early in phase 3 for N_2CO_2 and late in phase 3 for biphasic. Thus in anoxic treatments Ar, N_2 and $ArCO_2$ wing flapping was observed in periods when the EEG was not substantially suppressed or isoelectric.

Preliminary studies examining correlation dimension values in alert or deeply anaesthetised hens allowed 'calibration' of CD measurements and revealed a reduction in CD to 60% of the baseline value in deeply unconscious birds, a similar change to that seen in humans. Mean absolute baseline values in the current experiment were 6.78 ± 0.76. CD measurements during euthanasia showed a gradual reduction in CD values reflecting changes in the complexity of the EEG. Figure 9 shows example CD measurements during euthanasia for 5 individual birds, each receiving different CAS treatments. While EEG data was not available during wing flapping and substantial body movement, there was evidence that immediately before, after or between bouts of wing flapping during anoxia treatments, CD values exceeded 60% of baseline values on some occasions (Figure 9[b], [c] and [d]). CD measurements never exceeded 60% immediately preceding or following wing flapping during the biphasic method because this behaviour was reduced or absent during the first part of the stunning process.

Figure 8



Struggling and wing flapping during euthanasia in relation to EEG phase. Mean EEG phase (phase 1 – P1, phase 2 – P2, phase 3 – P3 and phase 4 – P4) timings are represented by the shaded bar at the top of each series, labelled with gas treatment. Below each, bars correspond to the timing and duration of each behaviour for individual birds receiving the treatment, with each horizontal line representing one bird (only birds with complete EEG and behavioural records were included: n = 8 for all treatments except N_2 and N_2CO_2, where n = 7 and Ar, where N = 4).

Time to death

Death was defined as the cessation of all respiratory movement and an isoelectric EEG trace. Presence of an isoelectric EEG normally preceded cessation of respiratory movement. All the gas mixtures tested achieved non-recovery states and the time this took was related to treatment (one-way ANOVA, $P < 0.001$; Table 3). Hypercapnic anoxia achieved faster euthanasia compared to anoxia (Table 3), while the biphasic approach was significantly slower than the anoxic treatments, with euthanasia taking 40–50 s longer due to the anaesthetic phase.

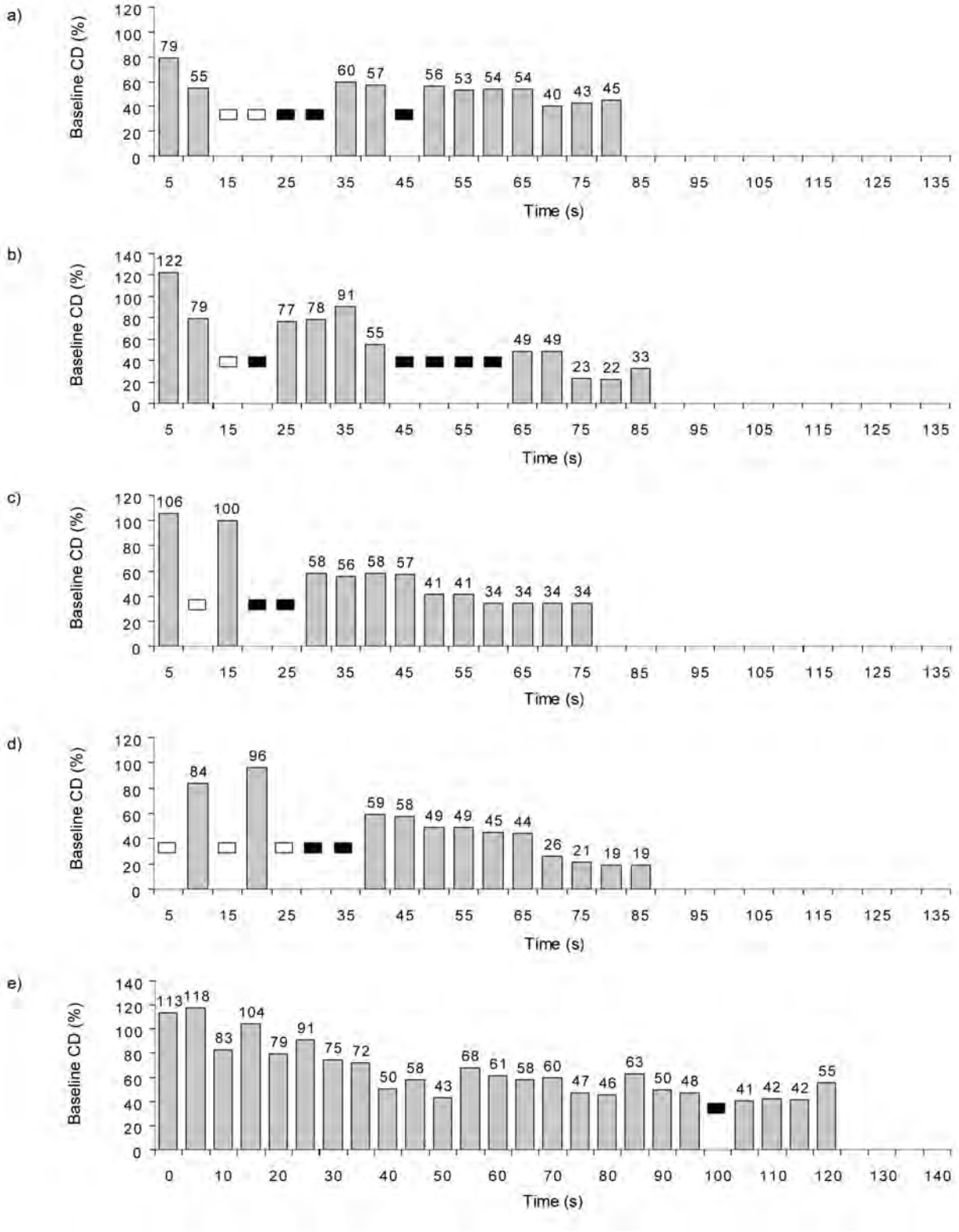
Discussion

This is the first study to combine simultaneous measurements of EEG, respiration, ECG and behaviour to assess welfare during CAS in poultry. Discussion of the welfare implications of each CAS approach must attempt to interpret the behavioural and physiological responses observed in the context of whether they occur during the conscious phase and their likelihood of inducing negative states or experiences such as pain, fear and distress.

Gas control

The 'changeover' method employed by our gas delivery system was designed to allow accurate monitoring during induction without extraneous disruption such as bird handling. However, air movements during subsequent wing flapping did cause transient fluctuations because the flow-through system meant that the whole box was not immediately filled with the treatment gas. It should be noted that immersion approaches (introducing the subject to a pre-filled chamber) also suffer from transient changes as air is introduced during the lid opening and via the plumage and similar fluctuations also occur in commercial systems. We believe that our approach of obtaining *in-situ* baseline and stable recordings by not moving the bird at the time of initial gas exposure is preferable to immersion because it produces high quality

Figure 9



Histograms showing relative EEG correlation dimension (CD) values for non-overlapping 5 s epochs during euthanasia of five individual birds, each with a different treatment: a) Ar anoxia; b) N_2 anoxia, c) $ArCO_2$; d) N_2CO_2 and e) biphasic. Values above each bar indicate percentages and smaller floating bars represent EEG data loss associated with struggling (open bars) or wing flapping (black bars). The zero time point is treatment gas onset.

behavioural and physiological records of early induction. The use of partial restraint in the study was unavoidable due to the need to protect recording equipment.

Cardiac responses

Although clear baseline ECG records were obtained for all the birds, substantial bird movement and especially wing flapping during euthanasia caused the ECG record to be obscured by electromyogram activity, despite restraint. This was an unavoidable limitation of measuring the ECG from electrode positions overlying the pectoral muscles. Nevertheless, sufficient ECG data was generated to determine that the heart rate response over time was related to treatment. Significant and sudden bradycardia and arrhythmia were evident with all the CAS mixtures applied and thus these responses were not solely related to anoxia or hypercapnia, with all treatments exerting a powerful and immediate effect on the cardiovascular system. Although specific receptors responding to inert gases are not present, avian arterial chemoreceptors sense hypoxia and similar asphyxic bradyarrhythmias have been observed in birds and mammals (Bamford & Jones 1976; Tomori *et al* 1997; Campen *et al* 2004). During hypercapnia, bradycardia was particularly profound and was similar whether oxygen was present or not suggesting that the presence of CO_2 may have overridden anoxic cardiac reflexes.

Because we can be certain that the most profound effects on heart rate occurred in the period where birds were likely to be conscious (within the first 30 s of exposure), the welfare implications of these cardiac responses centre on the likelihood of them inducing unpleasant sensations, pain or distress. In humans, palpitations and other sensations are associated with bradycardia and arrhythmia but ultimately, we have no way of knowing if the birds experienced an unpleasant sensation as a result of cardiac changes during the early part of the euthanasia process. This lack of knowledge about subjective cardiac sensation and the fact that all the gas mixtures induced bradyarrhythmias limits the extent to which this measure can be used as a criteria for choosing between gases on the basis of welfare.

Respiratory responses

The activation and perception of respiratory distress is one of the most controversial areas in the debate over which CAS mixture is most welfare friendly. Birds possess intrapulmonary chemoreceptors (IPCs), specialised vagal afferents located in or near the gas exchange surface which respond extremely rapidly to fluctuations in inhaled CO_2 (Hempleman & Posner 2004; Milsom *et al* 2004). IPCs are stimulated by low PCO_2 and inhibited by high PCO_2 (Hempleman & Posner 2004), and under hypercapnic conditions, greatly reduced or absent IPC discharge results in prolonged inspiration, increased tidal volume and decreased breathing frequency (Banzett & Burger 1977; Tallman & Grodins 1982; Milsom *et al* 2004). In birds (as in mammals), pulmonary stretch receptors, arterial chemoreceptors and brain stem central respiratory neurones are also present, detecting changes in tidal volume, PCO_2 and PO_2 and playing an important role in ventilatory control (Milsom *et al* 2004).

Respiration monitoring has not previously been attempted during CAS in poultry and effective measurement proved to be challenging. Avian lungs are non-compliant, flow-through structures, ventilated by separate air sacs which occupy much of the available abdominal and thoracic volume. Thus, respiratory movements are manifested to the greatest extent in the abdominal region and our experiments showed that the position of the piezoelectric sensor around the abdomen was crucial for effective measurement. With appropriate placement excellent baseline data could be obtained but the reliability of the necessarily sensitive sensor was affected by substantial body movement. This meant that respiratory effects in anoxic treatments were particularly difficult to measure since respiratory disruption appeared to occur during and after periods of wing flapping. Nevertheless, clear traces during early induction for a small number of individuals provided a record of hypercapnic respiratory disruption and an indication of the magnitude of amplitude (tidal volume) and breath cycle duration change for two gas treatments. This limited data suggests approximate increases in amplitude ranging from approximately three-fold (N_2CO_2) to over ten-fold (hypercapnic hyperoxygenation, biphasic) with accompanying increased breathing cycle duration. An approximate five-fold increase in tidal volume (measured by plethysmographic pressure changes) was reported in chickens inhaling 10% CO_2 in N_2 (Hambolu *et al* 1985). Comparison of this value with the current findings suggests that increasing CO_2 concentration in anoxic mixtures does not increase the extent of ventilation in terms of tidal volume, but it appears that hyperoxygenation of equivalent CO_2 concentrations (40% in both mixtures) does increase hyperventilation.

Although to a significantly lesser extent, some respiratory disruption was also observed with the inert anoxic treatments, usually taking the form of gaping and apparent apnoea. Tomori *et al* (1997) reported that anoxia (N_2 inhalation in a model of acute respiratory failure) in cats evoked hyperventilation progressing into apnoea. It is possible that an earlier hyperventilatory phase during inert anoxia was masked here by vigorous behavioural responses which interfered with both respiratory measurements and detailed behavioural observations.

Because of their immediate and ongoing nature, the extent to which respiratory responses (particularly to hypercapnia) constitute a negative experience is an important welfare issue. While comparison with human experience will always be of limited validity, hypercapnic stimulations in humans are known to induce unpleasant sensations variously described as dyspnoea (breathing discomfort or difficulty breathing), breathlessness and 'air hunger' (Banzett *et al* 1996). For this reason hypercapnia has been described as 'mildly to moderately anxiogenic' to humans and has been used to induce a reliable stress response (Kaye *et al* 2004). However, species differences are demonstrated by a study in pigs which failed to find a plasma cortisol response to high

concentration CO_2 inhalation (Forslid & Augustinsson 1988). Danneman *et al* (1997) asked human volunteers to rate the discomfort of breathing 50–100% CO_2 in oxygen. Increasing concentrations were judged as progressively more noxious, and at 50% CO_2 (the minimum level tested and most comparable to the current study), all four ratings of 'not at all unpleasant', 'unpleasant', 'uncomfortable' and 'painful' were reported. While there are many reports of dyspnoea following hypercapnic stimulation, recent human data suggest that hypoxia also elicits air hunger and that hypoxia and hypercapnia are equipotent in generating air hunger (Moosavi *et al* 2003). Obviously these results cannot be directly extrapolated to birds, but this human data does lend weight to our observations that both anoxia and hypercapnic CAS approaches elicit respiratory disruption.

Apart from reflex respiratory responses, it is possible that other negative sensations arise during hypercapnia due to activation of chemoreceptive airway afferents and recent electrophysiological studies of the responses of avian single trigeminal nociceptors confirm that CO_2 is a nociceptive stimulus (McKeegan 2004). Single nasal and oral afferent exhibited clear responses to stimulation with 100, 80 and 60% CO_2, but the responses to 50 and 40% CO_2 were equivocal and difficult to distinguish from ongoing spontaneous activity. The data therefore suggest a nociceptive threshold in the region of 50% which is in the same range as rat electrophysiological and human reported thresholds (Anton *et al* 1991, 1992; Peppel & Anton 1993).

The current data and previous studies support the conclusion that inhaling hypercapnic (and possibly also anoxic) CAS mixtures is likely to be an unpleasant and disconcerting experience for birds. At lower CO_2 concentrations (below 50%) this is more likely to be due to dyspnoea than airway or pulmonary pain. The relative 'importance' of dyspnoea to the animal is very difficult to assess, but in recent work examining aversion of broilers to acute stimulation with CO_2 (McKeegan *et al* 2006), birds were observed continuing to feed during and immediately after exhibiting deep breathing responses, a finding which argues against extreme anxiety or distress during this type of respiratory disruption.

Behavioural responses

Head shaking was exhibited by a proportion of birds at the onset of all the gas treatments suggesting that it was not a specific response to CO_2 and this is in accordance with the findings of previous work examining acute responses to similar gas mixtures (McKeegan *et al* 2006). The functional interpretation of head shaking and its contribution to welfare assessment remain unclear. While the proportion of birds head shaking was not affected by treatment, the number of head shakes exhibited was greatest in the N_2, N_2CO_2 and biphasic treatments. It seems likely that as previously suggested, the response is primarily related to novel or alerting stimuli (Hughes 1983; Dunnington *et al* 1984; Dunnington and Siegel 1986) which may equally apply to all gas mixtures. Mandibulation (a distinctive bout of bill movements) was seen only in response to delivery of hypercapnic gas mixtures and is thought to be related to gustatory and/or trigeminal stimulation (McKeegan *et al* 2005). The extent of mandibulation observed did not differ between the hypercapnic treatments suggesting that this response was unaffected by the level of CO_2 (30 vs 40%) or hyperoxygenation.

Struggling in the form of apparently purposeful escape attempts was seen in all treatments and in almost all individuals and obviously indicates aversion to the situation. The lack of significant difference between treatments in both numbers of bouts and proportion of birds exhibiting the behaviour suggests that either treatments were similarly initially aversive or that struggling arose from a condition common to all the trials, such as restraint.

Tonic and clonic convulsive activity (in the form of vigorous wing flapping and visible twitching respectively) were also observed during euthanasia with all gas mixtures, though the timing of onset of wing flapping varied considerably between treatments and to a lesser extent between individuals. Wing flapping began earliest with Ar anoxia and $ArCO_2$, significantly later with N_2 anoxia and N_2CO_2, and significantly later again with the biphasic treatment. Significant differences in the patterning of wing flapping were also observed in terms of bout number, length and total duration but not flap rate. Hypercapnic anoxic mixtures tended to induce higher numbers of short bouts of wing flapping, while the Ar and N_2 anoxia induced longer bouts. The biphasic treatment induced fewest bouts which had intermediate length. These results were reflected in total wing flapping duration which was highest for the anoxic treatments.

Time to loss of consciousness

In agreement with previous studies (Poole & Fletcher 1995; Webster & Fletcher 2001), our results show that anoxic gas mixtures elicit early onset, repeated and sometimes prolonged bouts of vigorous wing flapping. The welfare implication of this finding centres on whether birds experience any distress or fear during, between and/or after flapping bouts. The vigorousness of this behavioural response is such that injuries may be sustained through the bird's own wing flapping or that of neighbouring birds and this could have painful consequences. Thus, it is vital to attempt to determine time to loss of consciousness during the euthanasia process to examine whether the behavioural responses observed are occurring in a period where consciousness is a possibility. To avoid philosophical debate, it should be made clear that consciousness is meant here in terms of the ability to experience sensations. We would expect conscious animals to maintain normal posture, exhibit species-appropriate spontaneous behaviour and reactivity to environmental stimuli, whereas unconscious animals adopt an immobile behavioural state and are unresponsive to internal nor external stimuli. Intermediate states are also possible and 'level of consciousness' indicates the degree of reactivity of a subject, and is a continuum varying in relation to the level of vigilance during sleeping and waking states (Coenen 1998).

Previously, in studies of poultry stunning, measures such as loss of posture, eye closure and responses to painful

external stimuli such as comb pinching have been used as indicators of loss of consciousness but their reliability is uncertain. It is conceivable that there may be a delay between loss of posture and loss of sensation, and that depth of unconsciousness varies at the time of collapse. In humans, loss of posture with partially preserved consciousness has been reported in studies where transient cerebral hypoxia was induced (Lempert *et al* 1994). In the current study, comparison of EEG with time to loss of posture could not be investigated because the nature of the restraint used supported the bird's posture. To test the reactivity of the CNS during stunning, somatosensory evoked potentials (SEPs) have also been used via electrical stimulation of the radial nerve (eg Raj *et al* 1992). However, a major limitation of this approach is that to obtain a single SEP, numerous electrical stimuli must be applied and the accompanying responses averaged. This restricts the technique's ability to detect the very rapid changes which are characteristic of the euthanasia process.

The electroencephalogram (EEG) is an electrically measurable signal that reflects the overall activity of the CNS and exhibits clear changes in its characteristics during different states of vigilance. Although the EEG could not be measured during wing flapping in our experiments because of movement artefacts, the available records showed clear, consistent and treatment dependant changes during the euthanasia process. It is thought that anoxic convulsions result from the release of subcortical motor centres from suppression by higher centres and this is associated with the implication that their existence may be used as an indicator of loss of consciousness (Raj *et al* 1998; Raj & Tserveni-Gousi 2000). However, in the current experiment there were many instances of substantial EEG activity before, between and after wing flapping bouts and, in previous studies in poultry, anoxic convulsions have been reported to start before SEPs are lost (hens: Raj *et al* 1991; broilers: Raj *et al* 1998). Similarly, Coenen *et al* (2000) suggested that anoxic gas mixtures induced agitation "during the period when consciousness cannot be fully excluded". During hypercapnic anoxia, it is likely that consciousness is lost more quickly due to the anaesthetic effects of CO_2 which act through a reduction in brain pH (Martoft *et al* 2003) and loss of SEPs have been reported to precede onset of convulsions in both hens (Raj *et al* 1992) and broilers (Raj *et al* 1998) with these gas mixtures.

Correlation dimension (CD) analysis of the electroencephalogram (EEG) is a relatively new technique that has been customised to measure depth of anaesthesia in human clinical studies (van den Broek 2003, 2005). The application of a non-linear (fractal) time series analysis provides a measure of complexity reflecting the number of subprocesses contributing to the EEG signal (van den Broek 2003). The small amplitude, high frequency 'awake' EEG is more complex than the large amplitude, low frequency 'sleep' EEG and thus high CD values are related to high vigilance states and alertness while low values are related to low vigilance states, sleep and unconsciousness. Such nonlinear methods have shown their ability to excel over traditional spectral techniques and these properties have led to the use of CD analysis in studies of anaesthesia (Widman *et al* 2000; van den Broek *et al* 2000), sleep (Pradhan *et al* 1995; Kobayashi *et al* 2000) and cognitive load (Lamberts *et al* 2000). This is the first time CD has been applied to the study of euthanasia and the first time it has been applied to the avian EEG. Before the euthanasia study began the technique was 'calibrated' by calculating CD values of representative EEG signals from alert and deeply anaesthetised chickens. These revealed a reduction in CD to 60% of the baseline value in unconscious birds, a similar relative change to that seen in humans undergoing anaesthetic induction.

CD measurements were calculated for 5 s epochs of clean EEG signal during euthanasia and expressed in relation to individual baselines. These were interpreted in relation to the premise that when EEG correlation dimension values are high relative to the baseline (> 60%), some form of consciousness cannot be excluded. The findings revealed high relative CD values regularly observed in close temporal proximity to wing flapping during anoxia. In some cases CD values exceeding 90% of baseline values were observed between wing flapping bouts. These findings add weight to initial visual observations of EEG traces which indicated that wing flapping bouts were sometimes interspersed with portions of EEG that were not substantially suppressed or isoelectric. In contrast, although the use of hypercapnic hyperoxygenation (as an anaesthetic phase) extended time to loss of consciousness, wing flapping was greatly reduced or completely absent during the early part of the stunning process when CD values exceed 60%.

Conclusions and animal welfare implications

In this paper we have attempted to objectively examine each possible source of poor welfare during CAS and assess its contribution to the whole euthanasia experience. While the results suggest that all the CAS approaches tested have potentially negative aspects, the recommendation for which is the most welfare friendly centres on the interpretation of behavioural responses to CO_2 (particularly respiratory responses) and the interpretation of state of consciousness during vigorous wing flapping. Our simultaneous measures of behaviour and EEG provide evidence that anoxic CAS is associated with vigorous behavioural responses in a period when consciousness cannot be excluded. The biphasic method appears to eliminate this possibility, but is associated with increased respiratory disruption and a longer induction. Respiratory concerns also apply to hypercapnic anoxic mixtures, and indeed these mixtures may induce a combination of unpleasant respiratory sensations as well as the risk of convulsions being experienced. In addition, there is at least some evidence that anoxic CAS may induce dyspnoeic sensations, and that hypercapnic respiratory reflexes may not be as aversive as previously suggested (chickens sometimes continued eating while exhibiting these respiratory signs; McKeegan *et al* 2006). Comparing the relative importance of respiratory discomfort versus potentially negative behavioural responses inevitably involves a value judgement as to which is deemed a more

significant issue for the animal. We would argue that respiratory discomfort may be a 'price worth paying' to eliminate the risk of experiencing vigorous wing flapping and associated injury while conscious in poultry during controlled atmosphere stunning.

Acknowledgements

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