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Behavioural, brain and cardiac responses to hypobaric hypoxia in broiler chickens

Jessica E Martin[1,2,4]*, Karen Christensen[3], Yvonne Vizzier-Thaxton[3], Malcolm Mitchell[4] and Dorothy E F McKeegan[2]

[1] The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, UK

[2] Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, G61 1QH, UK

[3] Centre of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701

[4] Animal Behaviour and Welfare, Animal and Veterinary Science Research Group, SRUC, Roslin Institute Building, Easter Bush, Edinburgh, EH25 9RG, UK

Short title

Responses to hypobaric hypoxia in broilers

* Corresponding author at: The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, UK,

Jessica.Martin@ed.ac.uk

Abstract

A novel approach to pre-slaughter stunning of chickens has been developed in which birds are rendered unconscious by progressive hypobaric hypoxia. Termed Low Atmospheric Pressure Stunning (LAPS), this approach involves application of gradual decompression lasting 280 s according to a prescribed curve. We examined responses to LAPS by recording behaviour, electroencephalogram (EEG) and electrocardiogram (ECG) in individual male chickens, and interpreted these with regards to the welfare impact of the process. We also examined the effect of two temperature adjusted pressure curves on these responses. Broiler chickens were exposed to LAPS in 30 triplets (16 and 14 triplets assigned to each pressure curve). In each triplet, one bird was instrumented for recording of EEG and ECG while the behaviour of all three birds was observed. Birds showed a consistent sequence of behaviours during LAPS (ataxia, loss of posture, clonic convulsions and motionless) which were observed in all birds. Leg paddling, tonic convulsions, slow wing flapping, mandibulation, head shaking, open bill breathing, deep inhalation, jumping and vocalisation were observed in a proportion of birds. Spectral analysis of EEG responses at 2 s intervals throughout LAPS revealed progressive decreases in median frequency at the same time as corresponding progressive increases in total power, followed later by decreases in total power as all birds exhibited isoelectric EEG and died. There was a very pronounced increase in total power at 50-60 s into the LAPS cycle, which corresponded to dominance of the signal by high amplitude slow waves, indicating loss of consciousness. Slow wave EEG was seen early in the LAPS process, before behavioural evidence of loss of consciousness such as ataxia and loss of posture, almost certainly due to the fact that it was completely dark in the LAPS chamber. ECG recordings showed a pronounced bradycardia (starting on average 49.6 s into LAPS), often associated with arrhythmia, until around 60 s into LAPS when heart rate levelled off. There was a good correlation between behavioural, EEG and cardiac measures in relation to loss of consciousness which collectively provide a loss of consciousness estimate of around 60 s. There were some effects of temperature

adjusted pressure curves on behavioural latencies and ECG responses, but in general responses were consistent and very similar to those reported in previous research on controlled atmosphere stunning with inert gases. The results suggest that the process is humane (slaughter without avoidable fear, anxiety, pain, suffering and distress). In particular, the maintenance of slow wave EEG patterns in the early part of LAPS (while birds are still conscious) is strongly suggestive that LAPS is non-aversive, since we would expect this to be interrupted by pain or discomfort.

Highlights

- A novel stunning system uses hypobaric hypoxia to render poultry unconscious
- We measured electroencephalogram, electrocardiogram and behavioural responses in male chickens undergoing this process
- Birds exhibited bradycardia and a consistent series of behaviours, similar to responses induced by normobaric hypoxia
- Progressive decreases in the median frequency of the EEG occurred at the alongside increases in total power, indicating loss of consciousness
- We suggest that this approach to stunning is equivalent in welfare terms to stunning with inert gases.

Keywords

Hypobaric hypoxia; low atmosphere pressure stunning; behaviour; electroencephalogram; electrocardiogram; animal welfare.

1. Introduction

Chicken production for meat continues to grow globally, and since more than 58 billion broilers are killed annually for food production [13], determining appropriate methodology to

achieve their humane death is highly desirable. A novel approach to pre-slaughter stunning of chickens has been developed in which birds are rendered unconscious by progressive hypobaric hypoxia. Termed Low Atmospheric Pressure Stunning (LAPS), this approach shares many of the welfare advantages of controlled atmosphere stunning (CAS) sytems, which irreversibly stun poultry by exposure to hypoxic and/or hypercapnic gas mixtures [6, 47, 53]. These systems ensure that all birds are reliably stunned and they do not require the unloading and shackling of birds while conscious, a source of significant stress and pain [17, 49]. The LAPS system has been given 'no objection' status by both the United States Department for Agriculture in 2010 and the Canadian Food Inspection Agency in 2013 and is in routine commercial use at a poultry processing plant in Arkansas. As temperature rises, water vapour pressure increases and the amount of oxygen available in the air decreases [54]. The LAPS system uses a computer programme to adjust the pressure curves for different ambient temperature to achieve the same hypoxic effect (described in [5]). Mackie and McKeegan (2016) found slight differences in the behavioural responses of chickens to LAPS at different temperatures despite these pressure curve corrections (for example birds killed at colder temperatures took slightly longer to exhibit ataxia than at warmer temperatures) [30].

Previous scientific reports of LAPS relate to its development [44], some behavioural and corticosterone responses, meat quality and pathology [2, 52]. More recently, a series of studies have specifically examined the welfare consequences of LAPS by examining in detail the behavioural and physiological responses it elicits. McKeegan et al (2013) recorded electroencephalogram (EEG) and electrocardiogram (ECG) responses of broilers undergoing LAPS and showed that the process was associated with changes in the EEG pattern in the form of highly significant increases in total power, decreases in mean frequency and progressive increases in slow wave (delta) activity, indicating a gradual loss of consciousness [35]. Encouragingly, there was no evidence of heart rate elevation in the

conscious period which may be expected if the birds are stressed by the procedure. A detailed study of the behavioural responses of broilers undergoing LAPS reported a consistent sequence of actions: ataxia, loss of posture, clonic and tonic convulsions and leg paddling [30]. Mandibulation, headshaking and open bill breathing were observed in a proportion of birds, and since these behaviours are also routinely seen in response to hypoxic (normobaric) gas exposure [1, 18, 37] they could be considered to be responses to reduced oxygen availability rather than atmospheric pressure reduction per se.

These studies provide evidence suggesting that LAPS could meet the criteria of EU Regulation Number 1099/2009, in particular allowing slaughter to be performed without avoidable fear, anxiety, pain, suffering and distress. However, no studies have been carried out in which EEG and behaviour have been recorded in the same individual and the timings for loss of posture (a behavioural indicator for loss of consciousness, [9, 10]) have not been consistent between studies. McKeegan et al (2013), studying birds who were exposed to LAPS at high ambient temperatures (35-40°C), suggested a time to loss of consciousness of 40 s based on spectral analysis of EEG recordings [35]. Mackie and McKeegan (2016) using only behavioural indicators, reported an average loss of posture timing of 80 s, at a temperature range of (7-20 $^{\circ}$C) [30]. While time to loss of posture may be affected by a range of factors including ambient temperature, there is a need for brain and behavioural measures in the same bird to allow a more robust assessment of the welfare impact of the process and corroborate indicators of loss of consciousness [51].

The primary aim of this study was to comprehensively examine responses to LAPS by recording behaviour, EEG and ECG in individual broiler chickens, and interpret these with regard to the welfare of birds undergoing the process. A secondary aim was to examine the effects of temperature and temperature adjusted pressure curve on these responses, to

determine if the LAPS system is compensating adequately for temperature and humidity effects on oxygen availability. Broiler chickens were exposed to LAPS in triplets, in each of which one bird was instrumented for recording of EEG and ECG. The behaviour of all three birds was observed. In this way, we ensured good visibility of birds for observations while controlling for social isolation which might otherwise affect the behaviour of birds killed individually, while maximising the size of the behavioural data set. To interpret EEG responses in relation to loss of consciousness, we applied a range of methods including spectral analysis which has been recently widely adopted [7, 20, 25, 50, 51]. In particular, we determined latencies to recently validated species-specific thresholds for different clinical states of consciousness [32, 33, 47].

2. Methods

2.1 Subjects and husbandry

Ninety Cobb 500 male broiler chickens (*Gallus gallus domesticus)* from the female breeder line were sourced from a commercial hatchery and housed at the University of Arkansas poultry facilities within a larger single flock split into three groups, reared in three identical environmental chambers (measuring 3.05 X 3.05 m, approximately 100 birds per pen resulted in a stocking density of ~30 kg/m^2). The birds were wing tagged at four weeks of age. Single-pass ventilation was maintained at a constant rate of 6 m^3/min in all chambers and the photoperiod was 23L:1D for d 1 to 4, and 16L:8D thereafter. Chambers were equipped with clean pine shavings litter, 2 rows of nipple waterers, and 2 hanging feeders and birds had *ad libitum* access to feed (standard commercial starter and grower diet) and water. Birds and environmental controls were checked twice daily by trained staff. The experiments were performed following the EU Directive on the Protection of Animals used for Scientific Purposes (EU 2010/63) and ARRIVE protocol and were specifically authorized

by the University of Arkansas Institutional Animal Care and Use Committee (Protocol 15031).

2.2 LAPS process

The LAPS system was developed by Technocatch LLC in Mississippi, USA and the pressure curves applied by the process are patented [5]. The LAPS chamber, it's monitoring and control systems used in the current study is a scaled down research unit, but is otherwise identical to those used commercially except for manual door operation. The chamber is cylindrical (2.2 m in length and 1.8 m in diameter) and is designed to accommodate a reduced scale transport module (153 cm x 121 cm x 102 cm, three tiers each 23 cm height). The required decompression curve is automatically applied and controlled by a computer and once started, can only be stopped in the case of an emergency. An infra-red camera (130^{o} camera with 18 infra-red illuminators, Model #RVS-507, RVS Systems, USA) was fitted into the chamber to observe the birds. The LAPS cycle takes exactly 280 seconds and consists of two phases, in the first of which the vacuum chamber pressure is reduced from atmospheric pressure to an absolute vacuum pressure of ~250 Torr (~33 kPa) in ~67 s. In the second phase, a sliding gate valve is partially closed gradually reducing the effective pumping speed by 'choke flow', to a minimum chamber pressure of ~150Torr (~20 kPa). The rate of reduction of chamber pressure in the second phase is varied in relation to starting ambient temperature and barometric pressure. The reduction in total pressure results in a reduced oxygen partial pressure. At the end of the second phase at 280 s the chamber is returned to atmospheric pressure using a baffled air inlet, prior to the door opening and the exit of the transport module. Because cold air is denser and therefore contains more oxygen than warm air and birds have been shown to respond differently to LAPS at different temperatures [30], slightly different pressure reduction curves must be applied to achieve the same hypobaric effect under different ambient conditions. A range of pressure curves based

on temperature setting are created automatically by a computer programme to control the extraction of O_2 from the environment. According to ambient temperature, two of six possible temperature settings were applied in this study (settings 3 (applied between 13 and 18 ºC), and 4 (applied between 5 and 12 ºC)). The pressure curves of all temperature settings are identical in the first phase of LAP and all the curves converge on a final pressure of 20.7 kPa. Ambient temperature and humidity were recorded for each LAPS cycle and means were 16.0 ± 0.3 °C and 63.8 ± 0.5%, respectively. The aim was to apply each temperature setting to 15 replicates, but changes in ambient temperate resulted in 16 replicates for setting 3 and 14 replicates for setting 4).

2.3 EEG electrode implantation

At 34-35 days of age, 30 broilers underwent surgery to implant EEG electrodes under general anaesthesia, induced and maintained with sevoflurane (Sevoflo, Abbott Drug, USA). At the start of surgery, carprofen (8mg/kg, administered SC, Rimadyl, Pfizer Animal Health, NY, USA) analgesic was administered to provide post-operative pain relief. The EEG implantation approach has been described previously [e.g. 32, 38, 47]. Briefly, the EEG was recorded by two 0.35 mm diameter Teflon insulated silver electrodes (World Precision Instruments Ltd., Hertfordshire, UK) connected to a socket (DIN, RS components Ltd., Corby, UK), placed on the dura through small holes drilled in the skull, one on each of the dorsal surfaces of the right and left telencephalon at their approximate rostro-caudal and medio-lateral midpoints. An indifferent electrode was placed between the skull and the overlying tissue under the comb. The EEG implant was secured to the skull with dental cement (Duralay, Dental Directory Ltd., Witham, UK) and the surrounding skin was closed with sutures. After recovery from the anaesthetic, birds were individually housed in recovery pens (equipped with wood shavings litter, and food and water) and were closely monitored.

Birds had visual and auditory contact with their neighbours and were allowed to recover for 4 days before undergoing LAPS.

2.4 Experimental Procedure

The experimental birds were randomly selected from the flock by a random number generator (Microsoft Excel 2010) based on wing tag number. The birds underwent LAPS in triplets where one bird was implanted and instrumented to record EEG and ECG; behavioural observations were carried out on all birds. The 30 triplets were exposed to LAPS over two days (day 1 = 15 triplets; day 2 = 15 triplets) at 38-39 days of age (mean bodyweight 2.36 ± 0.38 kg). The triplet treatment order was generated by a Graeco latin square design to balance day, temperature treatment and source pen for EEG implanted birds. A further latin square used to allocate individual birds to each triplet. To mimic commercial transport and lairage conditions, non-implanted 'behaviour only' birds were removed from the flock and held in poultry transport crates (97 x 58 x 27 cm, maximum 8 birds per crate) for between 2-6 hrs before LAPS, dependent on the triplet kill order. Birds implanted with EEG electrodes were brought to the LAPS apparatus from their recovery pens in individual cardboard pet carriers. Immediately before each LAPS run, the EEG implanted bird was fitted with instrumentation. Commercially available disposable self-adhesive EKG electrodes (Blue Sensor, Ambu Ltd, Henry Schein Medical, London, UK), with press-stud electrical connections, were adhered to cleaned skin overlying the pectoralis muscle either side of the sternum [38] with cyanoacrylate tissue adhesive (Vetbond, 3M^{TM}, Agri-Med, Maryland Heights, USA). Birds were then fitted with a reusable custom made Lycra harness which was secured using velcro fastenings behind the bird's head and incorporated a pocket positioned on the bird's back which contained a telemetry/logging device (custom made), capable of logging simultaneous EEG and ECG signals and described elsewhere [29, 38, 47]. Briefly, the logging units were battery powered, and each was small enough to be worn by a bird in a Lycra backpack, thus requiring no trailing leads. Two 'physiological waveform' input channels were provided and were used to record ECG

and EEG (sampling frequency 1000 Hz). Logging was triggered and stopped with an external switch and logged data were recorded onto industry-standard 'micro-SD' memory cards (SanDisk 32GB, Maplin Electronics Ltd. Rotherham, UK). Two identical loggers were alternated. The logger harness was additionally secured to the birds with elastic bandage (Vetrap, 3M^{TM}, Agri-Med, Maryland Heights, USA). 'Behaviour only' birds were removed from their transport crates and weighed. The wing tip feathers of one non-instrumented bird per triplet were marked with a black permanent marker (Sharpie® Magnum chisel tip, Staples, UK) to allow better visualisation of individuals during behavioural observations. Marking was randomly allocated by wing tag number, irrespective of treatment. All three birds were then housed in cardboard pet carriers (28 x 35 x 46 cm) until transferred into the LAPS chamber by hand. Signal logging was triggered in the instrumented bird and a 2 min period of baseline EEG and ECG recording commenced during which the bird was replaced in its pet carrier.

Each triplet of birds was placed in the top right tier (1.53 x 1.21 x 0.23 m) of the container within the LAPS chamber. Soft polystyrene dividers were used to position the birds at the front of the tier (available space 0.76 x 1.21 x 0.23 m, resulting in a stocking density of 7.78 Kg/m^2 based on average bird weight of 2.36 Kg), in order to minimise damage to the birds when convulsing and reduce the risk of birds from disappearing from camera view during the LAPS cycle. Once the birds had been placed in the tier, further 2 minute period of baseline data was collected, after which the chamber door was closed and sealed and the LAPS cycle started. During the trials, the birds were watched in real time on a monitor to check for unexpected behaviour. Video footage was recorded on a digital video recorder (Datavideo M# DN300, Datavideo, USA) to allow detailed behavioural observations to be conducted later. Continuous recordings from 5 s prior to the start of LAPS to 5 s after the end of the cycle were obtained for each triplet. On completion of the LAPS cycle, the birds were removed from the chamber and reflexes were immediately assessed (e.g. presence of rhythmic breathing, nictitating membrane) to confirm death.

2.5 Behavioural observations

An ethogram developed in previous behavioural work on LAPS [30] was used (Table 1). The behaviour of each bird was recorded using The Noldus Observer XT 11.0 (Noldus Information Technology, The Netherlands) programme by a single observer who was blinded to run number and temperature setting. Behavioural variables measured included latencies, counts, total durations, bout durations and bout counts; see Table 1 for specific measures for each behaviour. Birds which went out of sight for more than 10% of the total observation time (280 s) were excluded from the data set. Data was exported from Observer to Microsoft Excel 2010.

2.6 EEG and ECG analysis

The logged data files were uploaded into a data acquisition and analysis program (Spike 2 Version 4.2, Cambridge Electronic Design, Cambridge, UK). Analysis consisted of examining consecutive artefact-free 2 s excerpts from the EEG signals during baseline and throughout the LAPS process (280 s). Visual inspection was used to eliminate severe movement artefacts which rendered the signal meaningless, while epochs that were apparently affected by electrical noise interference were subject to post hoc 'filtering' using the data interpolation technique described in [32, 33]. The EEG was analysed by producing power spectra of each 2 s epoch using a fast Fourier transform algorithm (1024, Hanning window, resolution 0.976 Hz bins). We also determined the latency for the signal to have a total power equal to 10% of baseline [46, 47]. The onset of isoelectric EEG signal was determined in two ways, by visual interpretation and by identification of validated spectral characteristics (PTOT less than 170mv and F50 greater than 22 Hz) as described in [32, 33, 47]. Two spectral variables were calculated with coded Genstat (Genstat, 14th Edition, Rothamsted Research, UK) programs: total power (PTOT), defined as the total area under the power spectrum curve [41] and median frequency (F50), the frequency below which 50% of the EEG power resides [49]. Latency variables to unconsciousness were defined as time

for F50 <12.7 Hz (non-responsive state) and <6.8Hz (general anaesthetic plane) [32, 33, 47]. In Spike 2, clean ECG signal was used to determine heart rate (bpm derived from the number of QRS complexes in a 5 s epoch) at 6 baseline time points before LAPS (3 outside chamber, 3 inside chamber with door open) and every 5 s during the LAPS cycle. Latency to bradycardia was generated for each bird, defined as a 30% reduction in heart rate compared to the 6th baseline value on an individual bird basis.

2.7 Statistical Analysis

All data were summarised in Microsoft Excel (2010) spread sheets and analysed using Genstat (14th Edition). Statistical significance was based on F statistics and 5% threshold level (i.e. *p* value <0.05). Summary graphs and statistics were produced at bird level.

2.7.1 Behaviour

Statistical comparisons of behavioural variables were conducted via Generalised Linear Mixed Models (GLMM) (Poisson distribution) or Linear Mixed Models (LLM) (normal distribution) dependent on the data distributions for each variable. Data transformations were attempted when necessary via Logarithm function. All models included bird ID and triplet number as random effects. All fixed effects were treated as factors and all interactions between factors were included in maximal models. All models included treatment, triplet order, and marked bird as fixed effects and bird weight, ambient temperature, ambient humidity as covariates. Correlations between variables and fixed effects were performed as Pearson's Correlations for parametric data, and Spearman's Rank Correlations for non-transformable non parametric data.

2.7.2 EEG

Summary statistics and graphs were produced at bird level, while statistical comparisons focussed on estimated means and differences between means. GLMMs (Poisson distribution) or LLMs (normal distribution) dependent on the data distributions for latency

variables to unconsciousness (F50 <12.7 Hz (non-responsive state); and <6.8 Hz (general anaesthetic plane); latencies to visual inspection characteristics (presence of slow-wave and 3 consecutive isoelectric 2 s epochs); latencies for the signal to have a total power equal to 10% of baseline; and finally latencies to isoelectric (PTOT less than 170 mv and F50 greater than 22 Hz). All models included bird ID and triplet number as random effects. All fixed effects were treated as factors and all interactions between factors were included in maximal models. All models included treatment, triplet order, and marked bird as fixed effects and bird weight, ambient temperature, ambient humidity as covariates.

2.7.3 ECG

GLMMs (Poisson distribution) or LLMs (normal distribution) were carried out, dependent on the data distributions for each heart rate interval, including the six baseline intervals and latencies to bradycardia. All models included bird ID and triplet number as random effects. All fixed effects were treated as factors and all interactions between factors were included in maximal models. All models included treatment, triplet order, and marked bird as fixed effects and bird weight, ambient temperature, ambient humidity as covariates.

2.7.4 Behaviour, ECG and EEG associations

A Pearson's correlation matrix was produced to examine associations between latencies to key behavioural responses (latency to ataxia, loss of posture, loss of jaw tone and motionless), EEG (latency to slow wave based on visual inspection, latency to isoelectric EEG based on visual inspection and spectral characteristics, latency to PTOT <10% of baseline, latency to F50 <7 Hz and F50 < 12 Hz) and ECG (latency to bradycardia) events during LAPS.

3. Results

None of the birds showed any signs of life at the end of the LAPS cycle (i.e. absence of rhythmic breathing, absence of corneal or palpebral reflex [10]). A total of 45/90 birds went

out of sight at some point during behavioural observations, but only 12 birds went out of sight for an extensive period of time (by temperature setting treatment: 3 = 5; and 4 = 7). Based on exclusion criteria (> 50% of observation time out of sight), 10 birds were removed from analysis to avoid bias. The mean time out of sight was 103.4 ± 20.0 s at setting 3 and 71.2 ± 16.2 s at setting 4.

3.1 Behavioural responses

A consistent sequence of behaviours was observed during LAPS: ataxia, loss of posture, clonic/tonic convulsions and motionless. Clonic convulsions, sitting, lying, ataxia, loss of posture, loss of jaw tone, vigilance and motionless were observed in all birds. Standing, leg paddling, tonic convulsions, slow wing flapping, mandibulation, head shaking, open bill breathing, deep inhalation, jumping and vocalisation were observed in a proportion of birds as outlined in Table 2 (data pooled across treatments). The birds did not perform escape behaviour, pecking or panting.

Mean behavioural latencies at each temperature setting are shown in Table 3. Vigilance was shown almost immediately by all birds at the onset of the LAPS cycle, and was not affected by temperature setting. Next, some birds showed mandibulation and headshaking, followed by ataxia, the latency of which was shorter at temperature setting 4 (mean latency 37.9 s compared to 43.3 s). Jumping, slow wing flapping and loss of posture were the next behaviours to be shown, with latencies to jumping (mean latency 46.9 s vs 60.8 s) and loss of posture (mean latency 57.5 s vs 62.3 s) shorter at temperature setting 4 (Table 3). Clonic convulsions, open bill breathing, deep inhalation, vocalising and loss of jaw tone and were seen in some birds, on average between 70-100 s into LAPS, and loss of jaw tone and open bill breathing happened significantly sooner at temperature setting 4 (Table 3). Finally, tonic convulsions, leg paddling and motionlessness were observed after 100 s into LAPS on average, with time to motionless quicker at setting 4. To illustrate the extent of individual variation in key behaviours related to loss of consciousness, Figure 1 shows scatter plots of

latency to ataxia, loss of posture, loss of jaw tone and onset of wing flapping: these all illustrate minimal variation across individual birds (the split of treatments in each panel is indicative of temperature treatment and killing day).

Table 4 shows bout duration, bout frequency and total duration of relevant behaviours. These were mostly unaffected by treatment, except for total durations of sitting and vigilance, which were reduced at temperature setting 4. The frequency of bouts of open bill breathing was greater at temperature setting 4. Counts of jumping, mandibulation, peeping, head shaking and deep inhalation were unaffected by treatment (Table 5). Vocalisations were observed in 10 birds, and these took the form of repeated sounds resembling short peeps [40]. One bird exhibited multiple vocalisations, starting 74 s into the LAPS cycle, after mean time to loss of posture. In fact, nine of the 10 birds vocalised after loss posture and of those that had EEG implants (3 birds), all showed slow-wave activity in the trace and FFT variables showed F50 <12.7 Hz. One bird vocalised prior to loss of posture (latency 9 s; 2 consecutive vocals recorded).

Fixed effects were not significant in the great majority of cases, but there were significant positive correlations between bird weight and time to loss of jaw tone ($p < 0.001$), deep inhalation ($p = 0.014$) and head shaking ($p = 0.020$). Implanted status had an effect on leg paddling ($p = 0.014$), jumping ($p = 0.014$) and open bill breathing ($p = 0.014$), where implanted birds showed more of these behaviours, probably because the logger and harness affected their balance and when they did lose posture, they always ended up lying ventrally (this was common but non-implanted birds occasionally came to rest dorsally as well as on their sides). The harness and elastic bandage may also have affected thermoregulation increasing the likelihood of open bill breathing. Food withdrawal time affected loss of jaw tone ($p = 0.037$), motionless ($p = 0.022$), leg paddling ($p = 0.004$), jumping ($p = 0.050$), and tonic convulsions ($p = 0.016$), which may reflect differences in energy availability.

3.2 EEG responses

High quality EEG signals were recorded for 24 birds, 22 of these traces represented the entire duration of LAPS. The overall pattern of EEG response to LAPS in terms of changes in total power and median frequency is shown in Figure 2. During baseline, the EEG signal was characterised by high median frequency (20-25 Hz) and low total power, as expected for conscious birds. Once LAPS began, a steep reduction in F50 was observed between 0-30 s, followed by a continuing, shallower reduction between 30-60 s. Figure 2 also shows the mean F50 in relation to three previously validated thresholds – sedation (<14 Hz [32, 47]), non-responsive to toe pinch after rapid anaesthetic 'knock down' (<12 Hz [32]) and surgical plane of general anaesthesia (<7 Hz [32, 47]). The results suggest that after approximately 16 s, the EEG characteristics of the birds correspond to a state associated with sedation, and after approximately 30 s are in a brain state showing characteristics of unconsciousness. These changes in median frequency happened at the same time as corresponding progressive increases in total power, followed later by decreases in total power as all birds exhibited isoelectric EEG and died. There was a very pronounced increase in total power at 50-60 s into the LAPS cycle, which corresponded to dominance of the signal by high amplitude slow waves. Later in the LAPS cycle, the mean pattern became less reliable as progressively less data was available as birds were excluded due to isoelectric signal or signal loss due to movement artefact. Figure 3 shows the typical appearance of the EEG signal at different points in the LAPS cycle (data from Bird 304). Baseline EEG waveforms consisted of low amplitude, high frequency activity, reflecting the birds' alert state. There were changes in the visual characteristics of the EEG from 10 s onwards, with increasing evidence of slow wave activity, which eventually dominated the signal (e.g. Figure 3 LAPS 40). Later in the cycle (e.g. Figure 3 LAPS 100), a suppressed EEG signal was evident and at the end of the LAPS cycle, the EEG was always isoelectric (e.g. Figure 3 LAPS 200).

Table 6 shows mean latencies to key events in the EEG trace, as determined by visual inspection of the trace or by spectral characteristics. There was generally good agreement between timings generated by visual inspection and spectral properties. These parameters were mostly unaffected by temperature setting, except latency to isoelectric signal based on visual inspection, which had a reduced latency at setting 4 (Table 6). Latency to total power less than 10% of baseline was similar to latency to isoelectric EEG but was only achieved in 10 birds because of very clean, low power baseline signals relative to background electrical noise in isoelectric signals. There was no significant bird weight or other fixed effects on EEG responses, apart from food withdrawal time, which had an effect on latency to F50 <7 Hz ($F_{(1,24)}$=13.10, p = 0.001); and latency to F50 <12 Hz ($F_{(1,24)}$=7.38, p = 0.012), with positive correlations, but neither were significant (r = 0.271-0.371, p = 0.055-0.200).

3.3 Cardiac responses

Clear ECG waveforms were obtained from all birds during baseline, but ECG traces for 5 birds were lost after transfer to the module and the onset of LAPS. Throughout recording, ECG waveforms were sometimes obscured due to electromyogram activity arising from the pectoral muscles or movement artefacts. Figure 4 shows mean heart rate before, during and after LAPS based on available data at each time point and temperature setting. In all cases, the birds exhibited increased heart rates following handling for instrumentation (mean 420 bpm) and there was some evidence of initial heart rate decrease during undisturbed baseline (to mean 375 bpm) (p = 0.042-0.802, N = 25; first to last baseline point comparison) with 17 birds (68%) showing a significant initial decrease in heart rate during undisturbed baseline. The baseline heart rate of birds killed at temperature setting 4 (applied at colder temperatures) was higher than those killed at setting 3, and this difference persisted in the first 30 s of LAPS. At around 25 s into LAPS, pronounced bradycardia began, often associated with arrhythmia, until 60 s into LAPS when heart rate levelled off. The latency to bradycardia was significantly affected by temperature setting, with setting 4 being associated

with a shorter latency (52.5 ± 4.5 s at setting 3; 46.7 ± 2.2 s at setting 4; $F_{(1,23)}$= 0.51, p = 0.021). There was no evidence of heart rate increase during LAPS. Differences between temperature setting were not apparent during the steep decrease in heart rate (Figure 3), but were present later when in general birds exposed to LAPS at temperature setting 3 had higher heart rates than those exposed to setting 4 (opposite to the baseline difference). The traces shown in Figure 5 illustrate bradyarrhythmia (data from bird 332) and the appearance of a typical ECG trace at different points in the LAPS cycle (data from bird 383). During LAPS, prolonged and irregular inter beat intervals were obvious (Figure 5A). At the end of the LAPS process, mean heart rate was low (150 ± 4 bpm) at which time there was also evidence of heart failure, recognisable as strong arrhythmia, very low and fluctuating amplitudes and fibrillation (Figure 5B). There was no significant bird weight or other fixed effects on ECG responses.

3.4 Relationship between responses

Figure 6 illustrates the temporal relationships between the mean latencies to key behavioural events (mandibulation, ataxia, head shaking, loss of posture, loss of jaw tone, and motionless), mean onset of spectral EEG thresholds and mean time to bradycardia. Clearly, EEG characteristics indicating potential unconsciousness (F50 values in the non-responsive and GA-plane) are apparent before loss of posture, and at a time when behavioural evidence suggests that the birds are conscious, and this probably relates to the fact that the birds are in complete darkness, which stimulates slow wave EEG [16, 17]. However, as noted above, very large increases in total power (associated with domination of slow wave activity) are not seen until around 60 s into LAPS, which matches well to observed loss of posture. Also, around this time, the steep phase of heart rate reduction levels off, resembling that of an anaesthetised bird. The correlation matrix revealed a number of significant associations. Time to loss of posture was positively correlated with latency to F50 <7 Hz (r = 0.459; p = 0.036) and latency to F50 <12 Hz (r = 0.441; p = 0.046). Unsurprisingly, latency to slow wave EEG based on visual inspection was strongly

associated with time to F50 <7 Hz (r = 0.598; *p* = 0.002), as was time to F50 <7 Hz with F50 <12 Hz (r = 0.592; *p* = 0.002). Latency to headshake was negatively associated with time to bradycardia (r = -0.851; *p* = 0.015), and latency to isoelectric EEG based on visual inspection was positively associated with time to open bill breathing (r = 0.605; *p* = 0.013).

4. Discussion

This study is the first to comprehensively describe the responses of chickens to hypobaric stunning where high quality physiological signals and detailed behavioural responses have been recorded in the same individuals. This approach allowed us to relate the timing of key behavioural events with EEG measures of loss of consciousness. A consistent series of behavioural responses was observed; ataxia, loss of posture, clonic and tonic convulsions, leg paddling and becoming motionless. Vigilance was seen early in the process, probably as a response to the sounds associated with pump activation. This pattern has been reported previously both in response to LAPS [30] and controlled atmosphere stunning with inert gases [e.g. 1, 6, 19, 36, 37, 45, 46]. Similarly, head shaking, mandibulation and behaviours relating to altered respiration (open bill breathing, deep inhalation) are seen in response to hypoxia in both hypobaric and normobaric conditions and their welfare implications have been discussed elsewhere [30, 37]. The parallels observed between LAPS and inert gas induced anoxia such as CAS suggest that these responses during LAPS are related to reduced oxygen availability rather than changes in atmospheric pressure; EFSA (2004) concluded that normobaric hypoxia induced with argon or nitrogen is not aversive to poultry [9].

Ten out of 90 birds exhibited vocalisations during LAPS. Potential vocalisations were observed in a small number of birds in a previous LAPS study, but the nature of the sounds generated by birds indicated that they were caused by involuntary movement of air through the body during convulsions [Martin et al, unpublished]. In the current study, nine of the 10

birds vocalised after loss posture and of those that had EEG implants showed slow-wave activity, however repeated vocalisations resembling 'peeping' were apparent. Marx et al (2001) describes vocal responses to isolation in chicks and distinguishes between distress calls (characterised by declining frequency and high energy) and short peeps (characterised by declining frequency, low energy and short duration) [34]. Thus peeping-type vocalisations may signal social or isolation distress, but have also been claimed to be a common pattern in the regular vocalisation of chicks in non-isolation situations [31]. The occurrence of vocalisations during LAPS raises questions about the conscious state of the birds and also the welfare implications of the process. In general, vocalisation after stunning is thought to indicate consciousness and possibly distress [51], though vocalisations have been reported in pigs during stunning after loss of posture [22] and in dogs during anaesthesia when apparently unconscious [e.g. 3]. Peek et al., (1975) reported that the same nerves and muscles were active during both vocalisation and respiration in chickens there was strong neural interaction between these systems during stimulation of the respiratory system with carbon dioxide [43]. The respiratory effects of hypoxia therefore could possibly be responsible for the vocalisations observed, especially since their frequency and timing exactly matched abdominal respiratory movements; this may explain the presence of this response in apparently unconscious birds. The welfare relevance of these vocalisations is difficult to assess, but vocalisations occurring following loss of posture in a subset birds does not fit with the idea that they are caused by negative sensations as a direct consequence of undergoing LAPS – which we would expect to affect all birds and be exhibited in the conscious phase. Recently, a study subjecting birds to LAPS with and without administration of an opioid analgesic (butorphanol) showed that head-shaking and vocalisation were not reduced with provision of analgesic agent [Martin et al, unpublished].

There were some effects of temperature setting on behavioural variables, with latencies generally decreased at setting 4, applied when temperatures are colder (setting 3 was applied between 13 and 18 ºC, and setting 4 was applied between 5 and 12 ºC). This does

not agree with previous findings where behavioural latencies increased at temperature settings applied at colder temperatures [30], possibly due to the fact that cold air has greater density and lower humidity (with consequently increased oxygen availability). The differences between the studies may be due to the exact temperature and pressure curve applied at the time of LAPS (each setting relates to a range) and resulting under or overcompensation. They may also relate to the underlying physiological state of the birds when they underwent LAPS in relation to food withdrawal, metabolic status, previous activity, presence of any sub-clinical pathology and body temperature. In Mackie and McKeegan (2016), food withdrawal time was standardised at 8 hrs, but in the current trial, because ambient conditions (and thus temperature setting) and time of day were related, these effects are confounded [30]. The baseline heart rate of birds killed at temperature setting 4 was higher than those killed at setting 3 – these birds were killed earlier in the day and underwent a holding period in crate in cold conditions. The effect of temperature on heart rate is well established; thermoregulatory effort increases heart rate and oxygen consumption [21, 51] and there is evidence that modern broilers have compromised thermoregulatory capacity compared to laying hens [28]. These factors suggest that responses to LAPS will be related to ambient temperature in a non-linear way, since thermoregulatory effort and potential alterations in metabolic rate and respiratory control outside the thermally neutral zone will be altered in both hot and cold conditions. This could explain the apparent paradox in the current results of decreased behavioural latencies in cold conditions, despite increased air density, and is supported by the baseline heart rate data. Substantial thermoregulatory effort and associated changes in acid-base and electrolyte status [24] in hot conditions may also explain reduced estimates of time to loss of consciousness (as measured by EEG) in earlier work [35]. Effects of temperature setting on EEG responses were much less apparent, with only one variable (time to isoelectric EEG) affected, being reduced at the colder setting.

The observed cardiac responses to LAPS closely resemble those reported previously [35]. In the same way as earlier work [35], heart rate responses were necessarily judged against an elevated baseline, related to handling for instrumentation (though we attempted to minimise this by allowing a longer 'rest' period than previously). The baseline figures reported are in line with those reported elsewhere for broilers, though the range is wide (e.g. 258 bpm, [26]; 328 bpm, [54]; 387 bpm, [27]; 419 bpm [8]). Stress associated with handling is a relevant consideration in any slaughter system, as slaughter processes routinely occur following manual catching and transportation, though handling related stress is minimized in the commercial LAPS system by stunning the birds in their transport containers. In the current study, relatively rapid recovery from increased heart rate induced by manual handling and instrumentation was apparent, with 68% of birds showing a significant decrease in heart rate during undisturbed baseline, and thus was generally not maximal as LAPS began. Heart rate did not increase during LAPS and consistently decreased, as would be expected as a response to hypoxia [37, 55]. Bradycardia and arrhythmia was particularly pronounced observed after 49 s, and after 60 s the heart rate stabilised showing only a slight downward trend until the end of LAPS. Slow exposure to hypoxia has been associated with increased heart rate responses in laying hens [4], and exposure to altitude in man also causes tachycardia [53]. The bradycardia observed here in broilers may relate to their compromised cardiovascular physiology [28] but in any case is consistent with exposure to relatively rapid hypoxia, as also seen during controlled atmosphere stunning in broilers [45, 36-38].

As reported previously [35], it is striking that slow wave EEG is seen early in the LAPS process, before behavioural evidence of loss of consciousness such as ataxia and loss of posture. This is almost certainly due to the fact that it is completely dark in the sealed LAPS chamber. The presence of slow wave EEG induced by darkness in apparently conscious birds has been reported previously [16, 42] and Gentle (1975, 1976) used this phenomenon in hens to detect gustatory responses [14, 15]. Oral stimulation of hens with bitter tasting

quinine caused desynchronisation of the EEG resembling 'waking' from sleep [14]. In light of this, the ongoing presence of slow wave EEG patterns in the early part of LAPS suggests an absence of stimulation as may be caused by pain or discomfort which would evoke a desynchronization of the EEG. Superimposed on the effects of darkness, the EEG response to LAPS was characterised by progressive domination of slow wave activity, with total power peaking at a time coinciding with loss of posture, a widely recognised proxy for loss of consciousness [10, 19, 39]. This shared latency, at around 60 s into LAPS, also matches the onset of prolonged reduced heart rates associated with unconsciousness [47]. Therefore, the effect of darkness on EEG pattern notwithstanding, there was a good correlation between behavioural, EEG and cardiac measures in relation to loss of consciousness in this study. Together, they provide a loss of consciousness estimate of around 60 s [30], which is not identical to values reported previously. More conservative measures of loss of consciousness would be onset of clonic convulsions [11, 12, 26, 51], or time for the EEG signal to have less than 10% of the total power of baseline [45, 46]. On this basis, the data suggest that mean estimates of potential consciousness during LAPS are 75 s and 114 s, respectively.

As this experiment and previous work has shown, individual bird variability, ambient temperature and humidity conditions, as well as the particular decompression curve applied all affect the timings of responses during the LAPS process [30, 35]. From a welfare perspective, the pattern of events and experience of the birds before loss of consciousness are more important than the exact timings of events. The results of this and earlier studies demonstrate that responses to LAPS are consistent across trials and temperature settings, adding to the growing literature which suggests that the process may meet the criteria of allowing slaughter to be performed without avoidable fear, anxiety, pain, suffering and distress [10]. In particular, the evidence provided by this study regarding the maintenance of slow wave EEG patterns in the early part of LAPS (while birds are still conscious) is strongly suggestive that LAPS is non-aversive.

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Figure 1 Scatter plots showing the variation between birds in latency to key behaviours associated with loss of conciseness: wing flapping (top left); loss of posture (top right); ataxia (bottom left); and loss of jaw tone (bottom right). N per individual behaviour is shown in each panel.

Figure 2 Changes in mean (± SE) F50 and PTOT during LAPS (onset 0 s) to 150 s (mean time to motionless based on behavioural observations). Baseline points refer to signal collected prior to LAPS (3 outside chamber, 3 inside chamber). The coloured horizontal lines correspond to mean F50 previously recorded in chickens during sedation (<14 Hz [50], non-responsive to toe pinch after rapid anaesthetic 'knock down' (<12 Hz [33, 36]) and surgical plane of general anaesthesia (<7 Hz [33, 36, 50]). N=24. Missing values indicate that epochs were excluded from analysis due to noise interference rendering too few data points available (<3 birds). Mean values constructed with less than 6 data points are highlighted.

Figure 3 A representative series of EEG trace excerpts (each 5 s duration, data from Bird 304) illustrating the typical appearance of the EEG at 12 time points (baseline, LAPS on, +10, +20, +30, +40, +50, +60, +100, +140, +200 s and LAPS off). Y-axis units are microvolts, x-axis units (large tick marks) are seconds.

Figure 4 Mean (± SE) heart rate (bpm) every 5 s for the throughput the LAPS cycle at each temperature setting. The six baseline points refer to signal collected prior to LAPS (3 outside chamber, 3 inside chamber). N=13 in temperature setting 3; N=12 in temperature setting 4. Asterisks indicate significant differences between temperature settings.

Figure 5 Example traces showing characteristic ECG activity. A: 6 s trace excerpt showing bradycardia and arrhythmia (arrow indicates onset, 52 s into LAPS, Broiler 332); x-axis large rick marks are 0.5 s. B: 1 s trace excerpts showing characteristic appearance of the ECG at different points of the LAPS cycle (a) 1-2 s (awake/conscious); (b) 58-59 s (unconscious); (c) 162-163 s (isoelectric EEG); and (d) 278-279 s (end of LAPS cycle, isoelectric EEG); x-axis large rick marks are 1 s.

Figure 6 Diagram showing the relationship in time between mean latencies to key behavioural events (mandibulation, ataxia, head shaking, loss of posture (LOP), loss of jaw tone, and motionless) and mean EEG characteristics during the first 160 s of LAPS. The mean latency to bradycardia (reduction in heart of 30 % compared to baseline) is also shown.

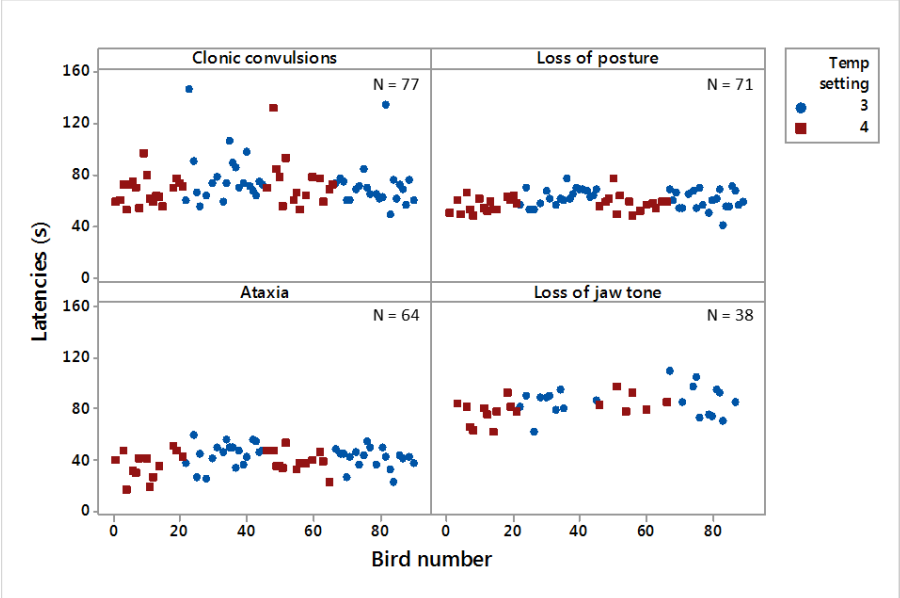

Fig. 1

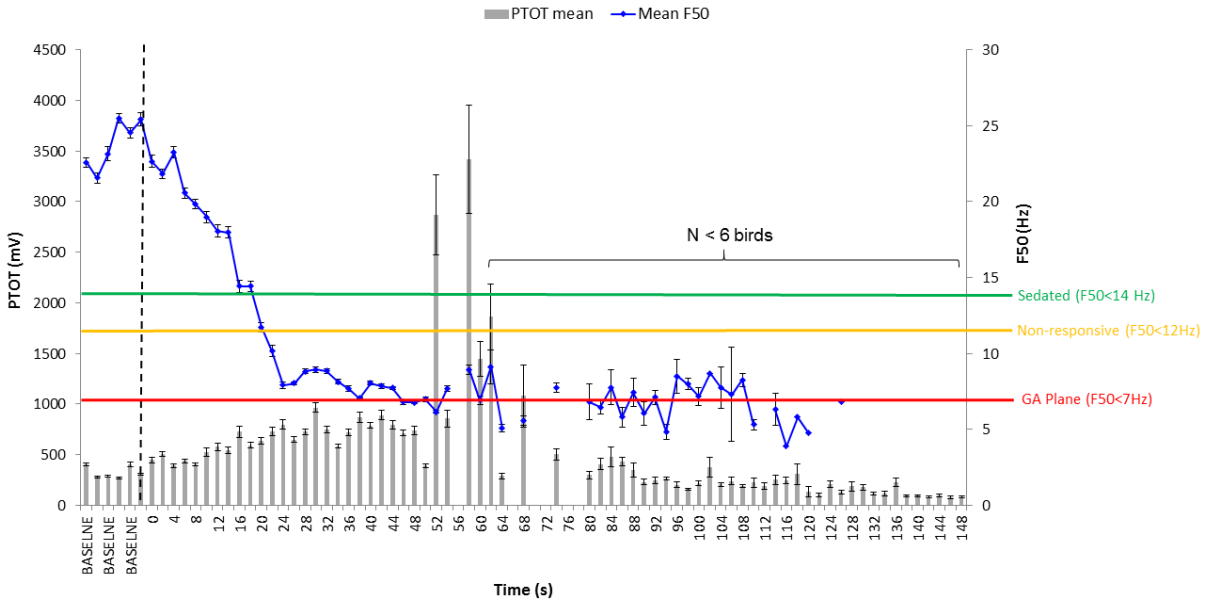

Fig. 2

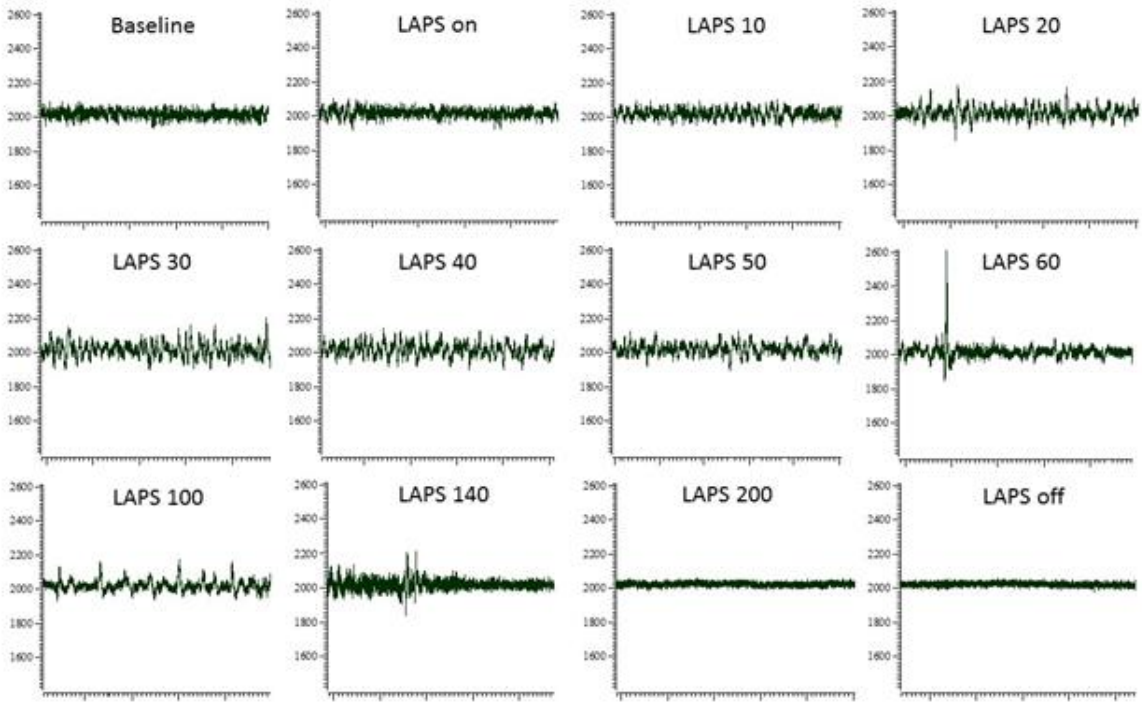

Fig. 3

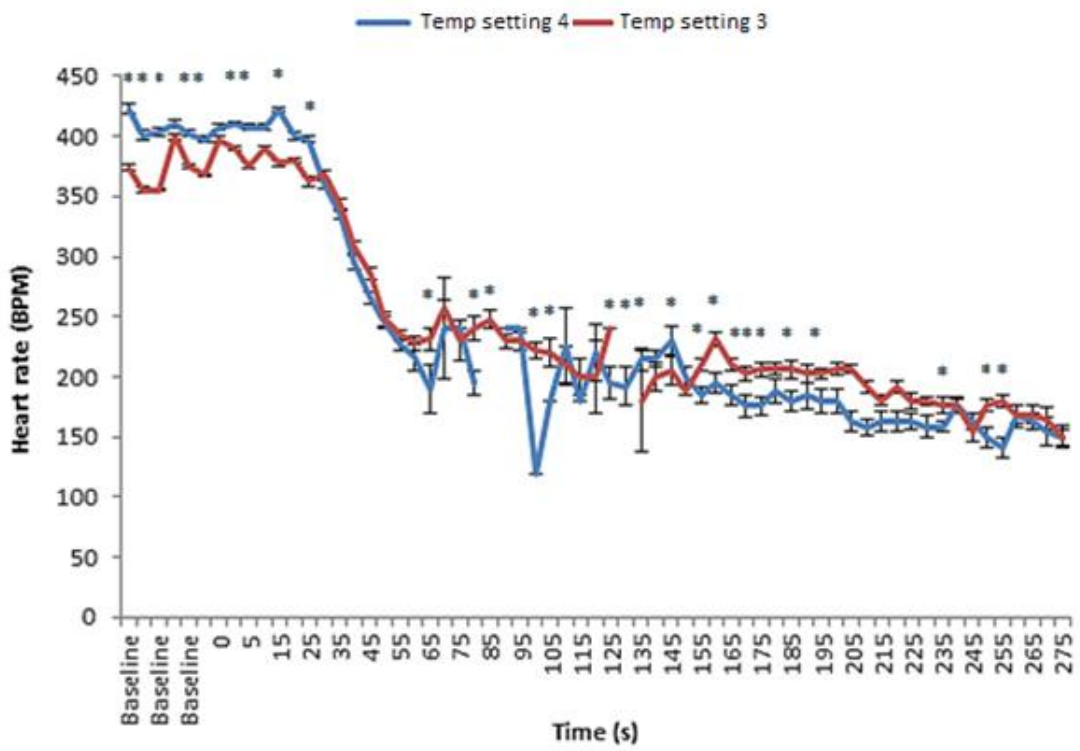

Fig. 4

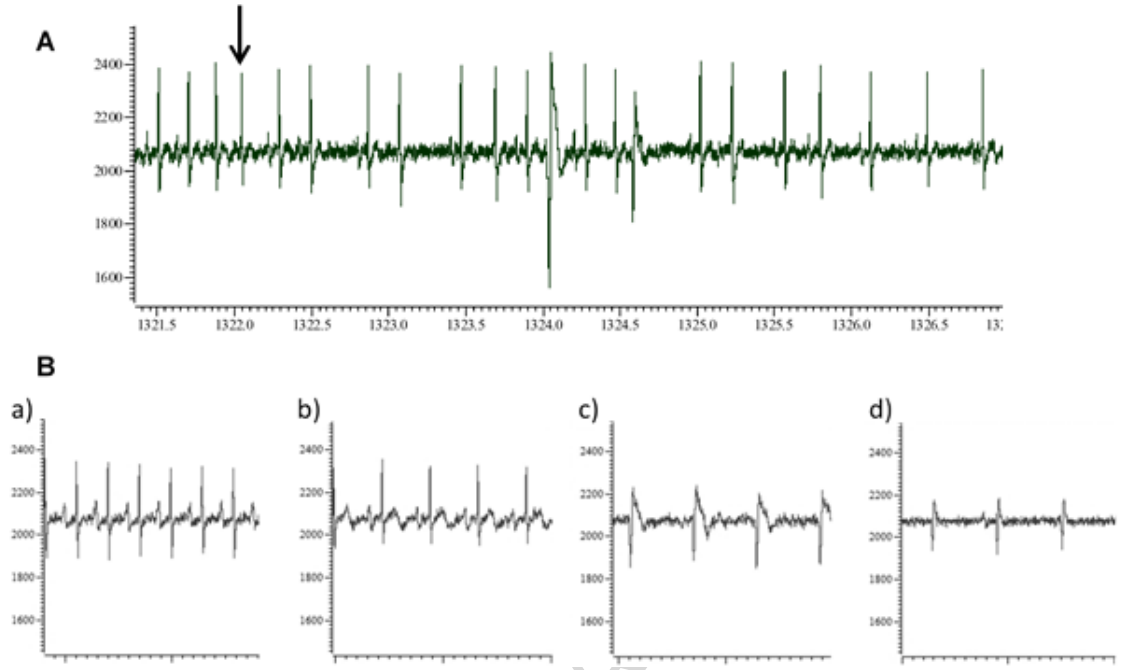

Fig. 5

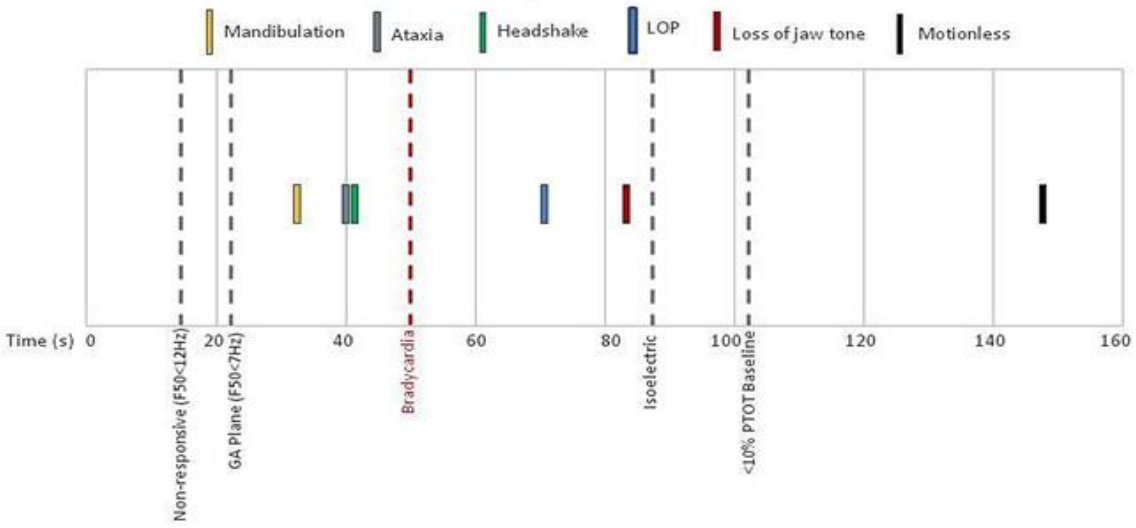

Fig. 6

Table 1 Ethogram showing behavioural latencies, counts and durations recorded

Behaviour	**Description**	**Measures**
Vigilance	Alert movements of the head, including 'Vigilance' as defined by Mackie and McKeegan (submitted).	Latency duration
Mandibulation	Repetitive and rapid opening and closing of the bill, not associated with inspiration or exhalation.	Counts Latency
Headshake	Rapid lateral head movement.	Counts Latency
Open bill breathing	Gentle rhythmic breathing with bill open, with or without neck extension.	Latency durations
Panting	Rapid rhythmic breathing with bill open with tongue extended	Latency durations
Deep inhalation	Deep non-rhythmic inspiration from the mouth may be accompanied by extension of the neck	Counts Latency
Ataxia	Apparent dizziness, staggering, swaying of body and/or head, attempts to stand/sit or flaps wings to try and regain balance.	Duration Latency
Loss of posture	Unable to regain/maintain a controlled posture.	Latency
Clonic convulsion	Rapid/vigorous movement of the wings, a new bout was defined as following a pause of at least one second.	Duration Latency
Tonic convulsion	Rigid posture accompanied by uncontrolled twitching (visible muscular spasms within the body). A new bout was defined as following a pause of at least one second.	Duration Latency
Slow wing flapping	One short burst or prolonged slow/moderate movement of the wings, occurring without any twitching of the body. A new bout was defined by a pause of one second.	Duration Latency
Leg paddling	Involuntary, usually alternating, leg movements in the air or towards the ground depending on the body position of the bird. Leg paddling can also be determined by an alternating upwards and downwards movement of the body if bird is lying sternal. A new bout was defined by a pause of one second.	Duration Latency
Loss of jaw tone	Bill open for more than 2s without deep breathing and/or neck extension.	Latency
Jump	Explosive upwards movement from a sitting/lying position during ataxia.	Counts
Escape	Rapid locomotor behaviours in an apparently conscious attempt to exit the situation	Counts
Peck	Moving head backwards and forwards in a pecking motion.	Counts
Vocalising	Any audible vocal produced by the focal bird (e.g. alarm call or peeping).	Counts Latency
Motionless	No discernible body or breathing movements.	Latency
Sitting	Legs underneath the body cavity and wings relaxed against body wall.	Duration
Standing	Standing with the body fully or partly lifted off of the ground.	Duration
Lying	Lying once posture is lost and not perceived to be purposefully controlling posture.	Duration
Out of sight	Bird was completely out of view.	Duration

Table 2 Frequency table showing the proportions of birds which were observed performing (yes), not performing (no) each behaviour, or were not recorded (missing data) due to being out of sight. Data pooled across different temperature treatments.

Behaviour	Frequency				
	Yes	No	Missing data	Total	Perform percentage (%)
Standing	6	62	22	68	8.8
Leg paddling	46	30	16	76	60.5
Clonic convulsions	78	0	12	78	100.0
Tonic convulsions	55	23	12	78	70.5
Slow-wing flapping	32	36	22	68	47.1
Vigilance	66	0	24	66	100.0
Mandibulation	43	36	12	79	54.4
Head shaking	42	37	12	79	53.2
Open-bill breathing	50	28	12	78	64.1
Deep inhalation	26	52	12	78	33.3
Jump	30	48	12	78	38.5
Vocalisation	10	68	12	78	12.8
Sitting	68	0	22	68	100.0
Lying	78	0	12	78	100.0
Motionless	77	0	19	77	100.0
loss of jaw tone	38	0	52	38	100.0
ataxia	64	0	26	64	100.0
LOP	71	0	19	71	100.0

Table 3 Summary statistics (mean, SE, minimum and maximum) of latencies to perform behaviours during LAPS and statistical differences (F statistic and P value) dependent on temperature setting treatment. Significant *P* values (< 0.05) are underlined.

Behaviour latency (s)	N	Temp Setting 3				Temp Setting 4				*F*	*P*
		Mean	SE	Min.	Max.	Mean	SE	Min.	Max.		
Vigilance	64	2.42	0.7	0.2	26.63	2.89	0.4	0.3	9.24	0.33	0.565
Mandibulation	78	35.2	4	5.5	72.3	29.5	3.2	4.7	64.2	0.01	0.934
Ataxia	54	43.3	1.4	23.2	59.5	37.9	1.8	16.8	54.1	5.3	0.027
Head shake	41	47.4	4.2	1.3	81.1	47.2	4.2	6.1	70.4	2.34	0.131
Slow wing-flapping	32	58.9	2.1	49.2	71.4	61.3	3.4	42.4	70.3	1.2	0.259
Jump	30	60.8	1.9	47.4	78.3	46.9	5.8	0	66.6	31.44	<0.001
Loss of posture	71	62.3	1.1	40.9	78.1	57.5	1.2	48.5	78.2	11.9	0.001
Open-bill breathing	51	72.3	4.1	45	154.4	64.2	2.8	47.5	101.4	6.19	0.015
Clonic convulsions	77	74.8	2.8	50.3	147	71	2.6	53.1	131.9	1.3	0.259
Vocalisation *	10	84.4	3.7	75	95.2	66	21.8	9.7	115.4	-	-
Loss of jaw tone	39	86.4	2.5	62.3	110.4	80.1	2.3	62.8	97.5	7.06	0.01
Deep inhalation	26	95.3	6.3	50.6	157.5	80.7	9	9.8	113.9	3.15	0.08
Leg paddling	44	107.8	6.3	58.6	169.7	103.6	4.2	59.5	133.6	1.3	0.259
Tonic convulsions	55	124.5	4.3	83.7	182.8	115.7	4.5	75	163	2.6	0.112
Motionless	71	153.9	2.6	121.7	191.7	145	2	108.7	166.4	6.05	0.016

** No modelling possible for latencies for vocalisations (too few observations (N=10)).*

Table 4 Summary statistics (mean, SE, minimum and maximum) of total durations, bout durations and bout frequencies of behaviour during LAPS and statistical differences (F statistic and P value) dependent on temperature setting treatment. Significant *P* values (< 0.05) are underlined.

Measure	Behaviour	N	Temp Setting 3				Temp Setting 4				*F*	*P*
			Mean	SE	Min	Max	Mean	SE	Min	Max		
Total duration (combined bouts) (s)	Ataxia	66	30.7	2.6	10.2	92.4	32.0	2.9	0.5	84.1	0.16	0.694
	Leg paddling	44	9.9	1.2	0.1	19.1	12.0	1.3	1.3	23.2	0.66	0.419
	Clonic convulsions	77	25.3	3.5	3.5	146.9	25.9	1.7	6.0	46.2	0.05	0.818
	Tonic convulsions	55	15.1	4.5	1.8	137.9	14.7	5.7	1.0	148.8	0.48	0.489
	Slow wing-flapping	32	3.1	0.7	0.5	6.9	3.3	0.8	0.5	6.1	0.03	0.854
	Sitting	66	60.5	1.9	13.4	78.3	54.6	2.8	1.6	76.4	6.50	0.013
	Standing	6	1.2	0.6	0.0	20.5	2.4	1.9	0.0	51.2	-	-
	Lying	78	73.6	4.8	1.7	119.6	73.6	4.0	16.0	111.8	0.03	0.872
	Open-bill breathing	51	30.1	7.7	1.0	227.6	24.4	8.4	3.2	195.4	0.33	0.573
	Vigilance	64	38.2	1.9	5.8	58.2	32.5	2.4	5.7	53.5	4.71	0.034
Bout duration (s)	Leg paddling	44	70.6	5.2	1.7	119.6	66.9	4.5	16.0	111.8	0.15	0.697
	Clonic convulsions	77	10.4	1.2	3.4	45.8	9.7	0.7	3.4	21.9	0.23	0.639
	Tonic convulsions	55	9.0	2.2	1.8	69.0	10.8	5.8	1.0	148.8	2.86	0.095
	Slow wing-flapping	32	0.7	0.1	0.5	4.1	0.9	0.2	0.5	6.1	0.11	0.677
	Sitting	66	57.3	2.7	13.4	76.3	53.5	2.9	1.6	76.1	2.13	0.149
	Standing	6	1.2	0.6	0.0	20.5	2.4	1.9	0.0	51.2	-	-
	Lying	78	70.6	5.2	1.7	119.6	66.9	4.5	16.0	111.8	0.19	0.660
	Open-bill breathing	51	29.4	7.8	1.0	227.6	20.5	8.5	3.2	195.4	0.90	0.351
	Vigilance	64	38.2	1.9	5.8	58.2	32.2	2.6	5.7	53.5	3.92	0.054
Frequency of bouts	Leg paddling	44	1.3	0.1	1.0	3.0	1.5	0.2	1.0	5.0	2.27	0.137
	Clonic convulsions	77	2.6	0.2	1.0	5.0	2.9	0.2	1.0	6.0	1.91	0.172
	Tonic convulsions	55	1.5	0.1	1.0	3.0	1.8	0.2	1.0	4.0	1.98	0.164
	Slow wing-flapping	32	1.1	0.1	1.0	3.0	1.3	0.1	1.0	4.0	0.94	0.878
	Sitting	66	1.1	0.1	1.0	2.0	1.0	0.0	1.0	2.0	2.46	0.122
	Standing	66	0.1	0.0	0.0	1.0	0.1	0.1	0.0	1.0	-	-
	Lying	78	1.1	0.1	1.0	3.0	1.2	0.1	1.0	2.0	0.12	0.736
	Open-bill breathing	52	1.1	0.1	1.0	2.0	1.4	0.1	1.0	2.0	18.71	0.001
	Vigilance	64	1.0	0.0	1.0	1.0	1.0	0.0	1.0	2.0	2.03	0.156

No individual bout duration data is available for ataxia, as ataxia only occurred in one single bout, therefore descriptive statistics listed under total duration.

Table 5 Summary statistics (mean, SE, minimum and maximum) of total counts of behaviour during LAPS and statistical differences (F statistic and P value) dependent on temperature setting treatment. Significant *P* values (< 0.05) are underlined.

Behaviour	N	Temp Setting 3				Temp Setting 4				F	P
		Mean	SE	Min.	Max.	Mean	SE	Min.	Max.		
Jump	30	0.6	0.1	0.0	4.0	1.0	0.1	0.0	4.0	1.56	0.223
Mandibulation	78	1.0	0.2	0.0	4.0	1.6	0.4	0.0	8.0	0.38	0.543
Vocalisation	78	0.8	0.4	0.0	11.0	0.8	0.5	0.0	18.0	1.73	0.193
Head shake	41	0.7	0.1	0.0	3.0	1.3	0.3	0.0	6.0	2.62	0.173
Deep inhalation	26	0.5	0.6	0.0	8.0	1.23	0.6	0.0	8.0	1.60	0.216

Table 6 Summary statistics (mean, SE, minimum and maximum) of latencies (s) to various EEG parameters during LAPS and statistical differences (F statistic and P value) dependent on temperature setting treatment. Significant *P* values (< 0.05) are underlined.

EEG latencies (s)	**N**	**Temp Setting 3**				**Temp Setting 4**				***F***	***P***
		Mean	**SE**	**Min.**	**Max.**	**Mean**	**SE**	**Min.**	**Max.**		
Slow-wave (visual inspection)	23	29.33	3.48	12	46	23.67	2.01	16	38	1.7	0.206
GA Plane (F50< 6.8Hz)	24	24.50	3.03	10	42	19.17	2.22	6	30	0.79	0.383
Non-responsive (F50< 12.7Hz)	24	14.83	3.23	14	42	13.00	1.62	12	24	0.56	0.463
PTOT <10% of baseline	10	93.60	11.5	74	132	114.00	11.8	68	136	-	-
Isoelectric (visual inspection)	23	94.83	8.08	78	144	77.45	6.93	50	120	5.16	0.033
Isoelectric (spectral)*	21	87.00	9.28	84	138	87.82	7.27	82	122	0.62	0.25

**Isoelectric EEG based on spectral characteristics was defined as PTOT<170mv and F50>22Hz.*