Effects of light on responses to Low Atmospheric Pressure Stunning in Broilers

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Short title: Effects of light on broiler responses to Low Atmospheric Pressure Stunning

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Abstract

1. Low Atmospheric Pressure Stunning (LAPS) is a novel approach to poultry stunning involving the application of gradual decompression lasting 280s according to a prescribed pressure curve.
2. The aim of this study was to determine how behavioural, electroencephalogram (EEG) and electrocardiogram (ECG) responses to LAPS are influenced by illumination of the decompression chamber. A secondary aim was to examine responses to the decompression chamber without LAPS being applied, as such a 'sham' control has been absent in previous studies.
3. A two by two factorial design was employed, with LAPS/light, LAPS/dark, sham/light and sham/dark treatments (N=20 per treatment). Broilers were exposed to each treatment in pairs, in each of which one bird was instrumented for recording EEG and ECG. Illumination was applied at 500 lux and in sham treatments birds were identically handled but remained undisturbed in the LAPS chamber without decompression for 280s.
4. Birds which underwent the sham treatment exhibited behaviours which were also observed in LAPS (e.g. sitting) while those exposed to LAPS exhibited hypoxia related behaviours (e.g. ataxia, loss of posture). Behavioural latencies and durations were increased in the sham treatments, since the whole cycle time was available (in LAPS; birds were motionless by 186s).
5. Within the sham treatments, illumination increased active behaviour and darkness induced sleep, but slow-wave EEG was seen in both. The pattern of EEG response to LAPS (steep reduction in median frequency in the first 60s and increased total power) was similar, irrespective of illumination, though birds in darkness had shorter latencies to loss of consciousness and isoelectric EEG. Cardiac responses to LAPS (pronounced bradycardia) closely matched those reported previously and were not affected by illumination.

6. The effects of LAPS/sham treatment primarily reflected the presence/absence of hypoxia, while illumination affected activity/sleep levels in sham treated birds and slowed time to unconsciousness in birds undergoing LAPS. Therefore it is recommended that LAPS be conducted in darkness for poultry.

Keywords

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

Introduction

Low atmospheric pressure stunning (LAPS) is a novel approach to pre-slaughter stunning of chickens in which birds are rendered unconscious by exposure to progressive hypobaric hypoxia. Similarly to controlled atmosphere stunning (CAS) sytems (which utilise exposure to hypoxic and/or hypercapnic gas mixtures (Coenen *et al.,* 2009; McKeegan *et al.,* 2007a, 2007b; Raj *et al.,* 1991; Vizzier-Thaxton *et al.,* 2010)), LAPS irreversibly stuns poultry in their transport crates, thus avoiding poor welfare associated with live shackling (Sparrey and Kettlewell, 1994; Gentle and Tilston, 2000) and ensuring all birds are stunned before neck cutting. 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.

The welfare consequences of LAPS have been recently reported in a series of studies. McKeegan et al (2013) recorded the electroencephalogram (EEG) and electrocardiogram (ECG) responses of broilers undergoing LAPS with results indicating a gradual loss of consciousness (highly significant increases in total power, decreases in mean frequency and progressive increases in slow wave (delta) activity). Mackie and McKeegan (2016) carried out a detailed study of the behavioural responses to LAPS and observed a consistent sequence: ataxia, loss of posture, clonic and tonic convulsions and leg paddling, as well as

mandibulation, headshaking and open bill breathing in a proportion of birds. These responses are similar to those seen with hypoxic (normobaric) gas exposure (e.g. Abeysinghe *et al.,* 2007; Gerritzen *et al.,* 2000; McKeegan *et al.,* 2011) suggesting they relate to changing oxygen availability rather than atmospheric pressure. In the first study to collect behavioural, EEG and ECG data in the same individuals, Martin *et al.,* (submitted b) found corroboration between behavioural, EEG and cardiac indicators of loss of consciousness and provided a time to unconsciousness estimate of around 60 s. However, it was noted that 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 (Martin *et al.,* submitted b).

In both previous studies examining EEG responses to LAPS, it was noted that slow wave EEG patterns are seen early in the LAPS process, before behavioural evidence of loss of consciousness such as ataxia and loss of posture (McKeegan *et al.,* 2013; Martin *et al.,* submitted b). This is almost certainly due to the fact that it is completely dark in the sealed LAPS chamber, and similar changes in EEG characteristics induced by darkness in apparently conscious birds have been reported previously (Ookawa and Gotoh, 1965; Gentle and Richardson, 1972). Thus, conducting LAPS in darkness (as it is done commercially) introduces a confounding factor affecting the interpretation of EEG responses. Thus, the primary aim of this study was to determine how behavioural, EEG and ECG responses to LAPS are influenced by illumination of the decompression chamber. A secondary aim was to provide data on responses to exposure to the decompression chamber without LAPS being applied, as such a control has been absent in previous studies. EFSA (2013) recommend the measurement of behavioural and physiological responses to control or 'sham' operations of stunning, to aid the determination of whether a stunning intervention is considered to induce pain, distress and suffering before the onset of unconsciousness and insensibility. To examine these issues, a two by two factorial design was employed, with LAPS/dark, LAPS/light, SHAM/dark, and SHAM/light treatments. Broiler

chickens were exposed to each treatment in pairs, in each of which one bird was instrumented for recording of EEG and ECG. As before (Martin *et al.,* submitted b), we applied a range of methods to interpret EEG responses in relation to loss of consciousness including spectral analysis (Delmore and Mckeig, 2004; Gibson *et al.,* 2009; Johnson *et al.,* 2005; Tonner, 2006; Verhoeven *et al.,* 2014) and determination of latencies to validated thresholds for different clinical states of consciousness (Sandercock *et al.,* 2014; Martin, 2015; Martin *et al.,* 2016).

Methods

Subjects and husbandry

Eighty 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).

LAPS process

The LAPS system was developed by Technocatch LLC in Mississippi, USA and the pressure curves applied by the process are patented (Cheek & Cattarazzi, 2010). 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) was fitted into the chamber to observe the birds. The LAPS cycle takes exactly 280 s 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 ~250Torr (~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 (Mackie and McKeegan, 2016; Martin *et al.,* submitted b), slightly different pressure reduction curves must be applied to achieve the same hypoxic effect under different ambient conditions. A range of pressure curves based on temperature setting are created automatically by a computer programme to control the level of oxygen available to the birds. According to ambient temperature, one of six possible temperature settings was applied in this study (setting 4, applied between 5 and 12 ºC). Ambient temperature and humidity were recorded for each LAPS cycle and means were 11.6 ± 0.3 °C and 51.8 ± 1.8%, respectively. In the afternoon of day 1 of the trials, ambient temperature unexpectedly rose beyond the upper limit of the setting 4 range to 16.7 °C,

however the system was overridden to ensure all runs received the setting 4 pressure curve. This overriding affected 5/40 LAPS runs, and the actual ambient temperature at the time of each run was included in statistical analysis (see below). LAPS is normally carried out in darkness, but in these trials, according to treatment, lighting was provided by six 17 W LED lights (Osram Sylvania Ultra LED), arranged in three pairs, at the front and either side of the LAPS chamber. These were positioned at the middle point of the side and end walls. The level of illumination at bird head height (12.5 cm above module tier where birds were placed) was 500 lux, as measured with a calibrated illuminance meter (Solar Light SL-3101).

EEG electrode implantation

At 40-41 days of age, 40 broilers underwent surgery to implant EEG electrodes under general anaesthesia, induced and maintained with Sevoflurane (Sevoflo, Abbott Drug). At the start of surgery, Carprofen (8mg/kg, administered SC, Rimadyl, Pfizer Animal Health, NY) analgesic was administered to provide post-operative pain relief. The EEG implantation approach has been described previously (e.g. McKeegan *et al.,* 2011; Martin, 2015). Briefly, the EEG was recorded by two 0.35mm diameter Teflon insulated silver electrodes connected to a socket (DIN, RS components), 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 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.

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 their treatment in pairs where one bird was implanted and instrumented to record EEG and ECG; behavioural observations were carried out on both birds. The trials were carried out over two days (40 runs/pairs per day) at 44-45 days of age (mean bodyweight 2.96 ± 0.41 kg). Four treatments were applied in a 2 x 2 factorial design: LAPS/light, LAPS/dark, SHAM/light, SHAM/dark (20 pairs per treatment). The pair treatment order was generated using a Graeco Latin square to balance day (Martin and Bateson, 2007), treatment and source pen for EEG implanted birds. 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 2-8 hrs before each run, dependent on the pair order. Birds implanted with EEG electrodes were brought to the LAPS apparatus from their recovery pens in individual cardboard pet carriers. Immediately before each 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 (McKeegan *et al.,* 2011) with cyanoacrylate tissue adhesive (Vetbond, 3M). Birds were then fitted with a reusable 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, capable of logging simultaneous EEG and ECG signals and described elsewhere (Lowe *et al.,* 2007; McKeegan *et al.,* 2011, Sandercock *et al.,* 2014). 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). 'Behaviour only' birds were removed from their transport crates and weighed. Both 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 pair 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. The chamber lights were on or off at bird placement depending on allocated treatment. 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 6.43 Kg/m^2 based on average bird weight of 2.96 Kg), in order to minimise damage to the birds when convulsing and reduce the risk of birds from disappearing from camera view during the trial. 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. The LAPS cycle then started, or in the sham treatment birds remained undisturbed in the chamber for an identical period (280 s). A compressor required to operate the LAPS chamber was running during both LAPS and sham trials. However, during LAPS, additional noise associated with the vacuum pump and pressure valve would have been experienced by LAPS treated birds. 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) to allow detailed behavioural observations to be conducted later. Continuous recordings from 5 s prior to the start of the run to 5 s after the end of the cycle period were obtained for each pair. On completion of the run, birds were removed from the chamber if exposed to LAPS, reflexes were immediately assessed (e.g. presence of rhythmic breathing, nictitating membrane) to confirm death.

Behavioural observations

252 An ethogram developed in previous behavioural work on LAPS (Mackie & McKeegan, 2016;
253 Martin *et al.,* submitted a, b) was used (Table 1). The behaviour of each bird was recorded
254 using The Noldus Observer XT 11.0 programme by a single observer. Blinding to treatment
255 was not possible as it could be seen on the video recording whether the lights were on or
256 not; it was not clear if LAPS was on until about 40 s into the cycle when birds began to show
257 signs of ataxia. Behavioural variables measured included latencies, counts, total durations,
258 bout durations and bout counts; see Table 1 for specific measures for each behaviour. Birds
259 which went out of sight for more than 10% of the total observation time (280 s) were
260 excluded from the data set. Data was exported from Observer to Microsoft Excel 2010.
261
262 *EEG and ECG analysis*
263 The logged data files were uploaded into a data acquisition and analysis program (Spike 2
264 Version 4.2, Cambridge Electronic Design). Analysis consisted of examining consecutive
265 artefact-free 2 s excerpts from the EEG signals during baseline and throughout the LAPS
266 process (280 s). Visual inspection was used to eliminate severe movement artefacts which
267 rendered the signal meaningless, while epochs that were apparently affected by electrical
268 noise interference were subject to post hoc 'filtering' using the data interpolation technique
269 described by Martin, 2015; Martin *et al.,* 2016. The EEG was analysed by producing power
270 spectra of each 2 s epoch using a fast Fourier transform algorithm (1024, Hanning window,
271 resolution 0.976 Hz bins). We also determined the latency for the signal to have a total
272 power equal to 10% of baseline (Raj *et al.,* 1991; Raj, 2006). The onset of isoelectric EEG
273 signal was determined in two ways, by visual interpretation and by identification of validated
274 spectral characteristics (PTOT less than 170 mv and F50 greater than 22 Hz) (Sandercock
275 *et al.,* 2014; Martin *et al.,* 2016; and Martin, 2015). Two spectral variables were calculated
276 with coded Genstat programs: total power (PTOT), defined as the total area under the power
277 spectrum curve (Murrell and Johnson, 2006) and median frequency (F50), the frequency
278 below which 50% of the EEG power resides (Tonner, 2006). Latency variables to
279 unconsciousness were defined as time for F50 < 12.7 Hz (non-responsive state) and <

6.8Hz (general anaesthetic (GA) plane) (Martin, 2015, Sandercock *et al.,* 2014). 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 6^{th} baseline value on an individual bird basis.

Statistical Analysis

All data were summarised in Microsoft Excel (2010) spread sheets and analysed using Genstat (14^{th} Edition). Statistical significance was based on F statistics and $P<0.05$ significance level. Summary graphs and statistics were produced at bird and treatment level. Statistical comparisons 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 pair number as random effects. All fixed effects were treated as factors and all interactions between factors were included in maximal models. All models included LAPS/sham treatment, light/dark treatment and whether the bird was implanted as fixed effects and bird weight, ambient temperature, ambient humidity, and feed withdrawal time as covariates. It was necessary to group behavioural data for analysis dependent on treatment (LAPS/sham) due to the majority of behaviours not being exhibited when birds did not undergo LAPS. The complete data set was analysed for some behaviours shown in all treatments (notice, standing, sitting, headshake, mandibulation, vigilance, vocalisations). Spearman correlations were used to determine directional associations between temperature and humidity (ambient and within chamber) and behavioural measures.

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) were carried 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 2s epochs); latencies for the signal to have a total power equal to 10% of baseline; and finally latencies to isoelectric (PTOT less than 170mv and F50 greater than 22 Hz). These spectral variable thresholds were never reached in sham treatment groups, therefore as with behavioural observations data were split into subsets for modelling of other effects. The ECG data was analysed by carrying out GLMMs (Poisson distribution) or LLMs (normal distribution), dependent on the data distributions for each heart rate interval, including the six baseline intervals and latencies to bradycardia. Latencies to bradycardia and bpm <100 were never reached in sham treatment groups, therefore as before subsets of data were analysed. Paired t-tests were used to do comparisons within treatment groups at individual bird level to compare heart rate at specific time points.

Results

None of the birds exposed to LAPS showed any signs of life at the end of the cycle (absence of rhythmic breathing, absence of corneal or palpebral reflex (EFSA 2013)). A total of 5/80 birds went out of sight at some point during behavioural observations, but only 2 birds went out of sight for an extensive period of time (1 bird each in dark/sham and light/sham). Based on exclusion criteria (>50% observation time out of sight), these birds were removed from analysis to avoid bias. The mean time out of sight was 117.1 ± 66.0 s.

Behavioural responses

A consistent sequence of behaviours was observed during LAPS: ataxia, loss of posture, clonic/tonic convulsions and motionless. Seven behaviours were seen in all birds which underwent LAPS (clonic convulsions, sitting, lying, ataxia, loss of posture, vigilance and motionless). Other behaviours (standing, leg paddling, tonic convulsions, loss of jaw tone, slow wing flapping, mandibulation, headshaking, open bill breathing, deep inhalation, jumping and vocalisation) were observed in a proportion of birds as shown in Table 2. Birds which underwent the sham treatment exhibited standing, slow wing flapping, vigilance, mandibulation, headshakes, vocalisations, sitting, pecking and panting behaviours (Table 2). Pecking (2 birds) and panting (1 bird) were seen only in the light/sham treatment, and vocalisations were exhibited by six birds (three in each of the LAPS/light and sham/light treatments). EEG implantation had no effect on behaviour.

Comparisons of the LAPS and sham treatment were limited to behaviours which were performed in both treatments. Analysis of latencies to slow wing flapping and pecking was not possible due to their rarity. All latencies were affected by LAPS/sham treatment, longer latencies in sham treated birds compared those exposed to LAPS (Table 3). In the sham treatment, behavioural latencies were spread across the entire 280 s cycle time, while LAPS birds were motionless in a mean time of 145s (Table 4). Light/dark treatment had no effect on latencies of any behaviour shown in both LAPS and sham treatments, except for standing (Table 3), where birds in the light had shorter latencies compared to birds in the dark in the sham treatment, but there was no difference when exposed to LAPS. There was a significant interaction between LAPS/sham and light treatments on the latencies to mandibulation (longest latency in sham/light) and standing behaviours (shortest latency in LAPS/dark). Shortest latencies to stand were seen in LAPS/dark and longest in sham/dark. Birds which underwent LAPS showed shorter bout durations of sitting and longer bouts of vigilance while birds in dark treatments had longer bout durations of sitting, and shorter bouts of vigilance and standing. The same relationships were seen for mean total durations for these behaviours (Table 3). Mean bout duration and total duration of standing was

affected by an interaction between treatments, with durations shorter in LAPS birds, and within these groups, shorter durations in the dark (Table 3). LAPS treatment affected the frequency (counts) of sitting, vigilance, headshakes, standing, and slow wing flapping, with all behaviours being performed more times in sham conditions (Table 3), apart from headshaking and slow wing flapping, where the opposite was seen (although note that only two sham birds showed slow wing flapping). Illumination had an effect on the frequency of sitting, vigilance and standing, with all behaviours performed more frequently in the light. Numbers of vigilance bouts were affected by an interaction between laps treatment and lighting, with the highest frequency seen in sham/light and lowest in laps/light.

Bird weight and feed withdrawal time had no effect on latencies, bout duration or total durations of behaviours shared across LAPS and sham treatments. Temperature and humidity had sporadic significant effects on behavioural latencies for mandibulation, standing and headshaking, however spearman's correlations showed that there were no significant associations. Both temperature ($F_{(1,70)}$=78.27, P<0.001) and humidity ($F_{(1,70)}$=33.89, P<0.001) affected bout duration of standing, with a negative correlation between temperature and mean bout duration (r=-0.525, P=0.006), but a positive correlation between humidity and mean bout duration (r=0.404, P=0.040). Temperature ($F_{(1,70)}$=51.27, P<0.001) and humidity ($F_{(1,70)}$=12.85, P<0.001) also affected total duration of standing, however there were no significant correlations. Weight, feed withdrawal time, temperature, and humidity had no effects on behavioural frequencies.

Comparing the wider range of behaviours exhibited during LAPS, illumination had no effect on the majority of behavioural latencies, with effects only on standing and deep inhalation. Latencies to stand in light and dark treatments were numerically very similar (17.0 s and 17.7 s, Table 4), but the range was much wider for birds in the light. Birds undergoing LAPS in the dark had longer latencies to deep inhalation (Table 4). Vigilance was shown almost immediately to the onset of LAPS, irrespective of light treatment. There was no effect of

388 illumination on latencies of key indicator behaviours associated with loss of consciousness
389 (ataxia, loss of posture, loss of jaw tone and onset of convulsions). In darkness, birds had
390 increased bout duration, total duration and frequency of bouts of sitting (Table 5). The
391 opposite effect was seen for durations of standing, performed more by birds in the light
392 treatment, as was vigilance. Illumination also increased total durations of leg paddling and
393 clonic convulsions. Light or dark conditions had no effect on the counts of jumping,
394 mandibulation, vocalisation, headshaking, deep inhalation and pecking (Table 6).
395
396 Bird weight had an effect on the latency to deep inhalation ($F_{(1,35)}$=14.75, *P*<0.001),
397 headshaking ($F_{(1,35)}$=7.05, *P*=0.012) and jumping ($F_{(1,35)}$=12.45, *P*<0.001). Latency to
398 jumping and deep inhalation were negatively correlated with weight (r = -0.395, *P* = 0.050
399 and r=-0.618, *P*=0.024 respectively). No significant correlation was found for latency to
400 headshaking. Latencies to sit ($F_{(1,35)}$=7.73, *P*=0.009), slow wing flap ($F_{(1,35)}$=4.85, *P*=0.035)
401 stand ($F_{(1,35)}$=51.03, *P*<0.001) and tonic convulsions ($F_{(1,35)}$=5.04, *P*=0.031) were affected by
402 feed withdrawal time, but correlation analysis, showed no significant correlations except for
403 sitting, which was positively correlated (r=0.451, *P*=0.004). Bird weight affected bout and
404 total durations for leg paddling (bout $F_{(1,35)}$=3.32, *P*=0.008; total $F_{(1,35)}$=11.97, *P*=0.001), tonic
405 convulsions (bout $F_{(1,35)}$=10.53, *P*=0.003; total $F_{(1,35)}$=30.60, *P*=0.001) and open-bill breathing
406 (bout $F_{(1,35)}$=25.56, *P*<0.001; total $F_{(1,35)}$=21.59, *P*=0.001), which were all negatively
407 correlated with bird weight (r=-0.186—0.512, *P*=0.004-0.045). Numbers of tonic convulsions
408 were also related to bird weight ($F_{(1,35)}$=12.07, *P*=0.001), with a significant negative
409 correlation (r=-0.522, *P*=0.001).
410
411 *EEG responses*
412 High quality EEG signals were recorded for 33 birds, 28 of these traces provided data for the
413 first 150 s of LAPS (equivalent to time to motionless in LAPS birds). EEG characteristics in
414 terms of temporal changes in median frequency and total power in response to each

415 treatment are shown in Figure 1 (A: sham/dark and B: sham/light) and Figure 2 (A:
416 LAPS/dark and B: LAPS/light). Figure 3 shows a representative series of EEG trace
417 excerpts from birds undergoing LAPS/light and LAPS/dark treatments. In all treatments,
418 during baseline the EEG signal was characterised by high median frequency (20-25 Hz) and
419 low total power, as expected for conscious birds. Birds exposed to the sham treatments
420 exhibited regularly fluctuating median frequencies relating to transitions between waking and
421 apparent drowsy/sleep states. In the sham/dark treatment, birds showed general downward
422 trend in F50, a higher proportion of slow waves and higher total power than sham/light
423 (Figure 1). Of the first 82 two-second epochs (equivalent to time to motionless in LAPS
424 birds), the mean F50 of birds exposed to sham/dark reflected a non-responsive state (F50 <
425 12.7 Hz) for 3 time points (3.75%), were in the sedation range (F50 <14 Hz) for 16 time
426 points on average. The average F50 of sham/light birds never entered this range, but some
427 individuals showed both F50 <12.7 Hz and F50 <6.8 Hz at certain time points (see below).
428 In birds undergoing LAPS, a steep reduction in F50 and consequential increase in total
429 power was observed between 0-50 s (most pronounced in the dark treatment), followed by a
430 continuing, shallower trend from 50-70 s. Comparisons across groups revealed no effects of
431 LAPS, illumination or their interactions on visually assessed latency to presence of slow-
432 wave EEG signal (Table 7). Time to reach F50 <6.8 Hz was reduced in birds exposed to
433 LAPS and darkness, with a significant interaction where sham/dark birds had the shortest
434 latency. Sham/light birds rarely reached this state (9/20 birds, and then only for single
435 epochs). Within LAPS treatments, illumination delayed the onset of unconsciousness (GA
436 plane) by approximately 15 s, a significant difference. Time to reach a non-responsive state
437 (F50 <12.7 Hz) was not affected by LAPS or LAPS/illumination interaction, but had shorter
438 latencies in the dark. Within LAPS, birds in the dark had shorter latencies to reach a non-
439 responsive state (F50 <12.7 Hz) than birds in light ($F_{(1,16)}$=8.90, *P*=0.010). Comparisons of
440 latencies indicating brain inactivity were only carried out within the LAPS treatment, as no
441 birds in the sham treatments exhibited these states. There were too few birds to do
442 statistical comparisons for PTOT <10% of baseline, however numerically latencies were

443 shorter in the dark compared to birds in the light. Illumination increased latencies to
444 spectrally determined isoelectric EEG by 10 s, on average (Table 7). Bird weight affected
445 latency to F50 <12.7 Hz ($F_{(1,25)}$=4.21, *P*=0.046), with heavier birds showing longer latencies
446 (r = 0.342, *P* = 0.048). Feed withdrawal, temperature and humidity had no effects on EEG
447 variables.
448
449 *Cardiac responses*
450 Clear ECG waveforms were obtained from all birds during baseline, but ECG traces for 8
451 birds were lost after transfer to the module and the onset of LAPS. Throughout recording,
452 ECG waveforms were sometimes obscured due to electromyogram activity arising from the
453 pectoral muscles or movement artefacts. Figure 4 shows mean heart rate before and during
454 LAPS or sham treatment based on available data at each time point. In all cases, birds
455 exhibited elevated heart rates following handling for instrumentation (mean 385bpm) and
456 there was no evidence of initial heart rate decrease during undisturbed baseline (*P*=0.061-
457 0.783, N=29; first to last baseline point comparison). The initial heart rate of birds was
458 affected by illumination; in LAPS treatments light birds had a lower heart rate than those in
459 the dark, however in sham treatments this trend was reversed (Figure 4). Birds undergoing
460 LAPS showed pronounced bradycardia and arrhythmia from around 30 s continuing until 60
461 s when heart rate levelled off. The mean latency to bradycardia in LAPS birds was 45.7±2.5
462 s. Latency to bradycardia not affected by light treatment (dark: 42,5 ± 1.9 s; light: 49.3 ± 4.8
463 s) feed withdrawal time, bird weight or humidity. However the internal temperature of the
464 chamber did have a marginal significant effect on time to bradycardia ($F_{(1,18)}$=4.75, *P*=0.048),
465 but there was no significant correlation. At the end of the LAPS process, mean heart rate
466 was low (dark: 126±18 bpm; light: 160±15 bpm) at which time there was also evidence of
467 heart failure, recognisable as strong arrhythmia, very low and fluctuating amplitudes and
468 fibrillation. Bradycardia and arrhythmia were absent in the sham treatments. There was a
469 significant decrease in heart rate between the average baseline (374.6±5.2 bpm) of

individual birds and the end of the cycle (332.1±4.9 bpm) (Paired T-Test: T = 7.08, *P* <0.001) irrespective of light treatment (Balanced ANOVA $F_{(1,14)}$=0.10, *P*=0.760).

Discussion

The results of this experiment provide important data controlling for the effects of illumination and exposure to the decompression chamber without LAPS. In particular, they inform our interpretation of EEG indicators of loss of consciousness in the absence of the confounding effects of total darkness. Only some behaviour categories were shared between LAPS and sham treatments, since many behavioural patterns associated with LAPS relate to loss of consciousness and death by anoxia. Analysis of these in relation to treatment revealed that in general, behavioural latencies and durations were increased in the sham treatments, primarily because the whole 280 s cycle time was available, whereas in LAPS, birds were losing posture at about 55 s and becoming motionless at 145 s. Vigilance, headshaking and mandibulation were observed during LAPS and sham treatments; unsurprisingly vigilance was increased in light treatments. It has been suggested that headshaking indicates that that the bird is in a less preferred environment (Nicol, 2011) and it has also been associated with disorientation, discomfort, respiratory distress (Webster and Fletcher, 2001) or contexts demanding increased attention (such as the presentation of novel or disturbing stimuli (Hughes, 1983). The fact that this behaviour was seen in sham treatments suggests that some of the headshaking seen during LAPS is due to the placement of the birds in a novel environment. However, headshaking was increased by LAPS (both in terms of frequency and number of birds exhibiting the behaviour), which probably relates to increased noise levels in the chamber (caused by the vacuum pump and valve) as well as the likelihood that birds are aware of atmospheric pressure reduction and/or reducing oxygen concentration while conscious. The maximum number of headshakes seen during LAPS was 5, which is equivalent to exposure to controlled atmosphere stunning with inert gases (e.g. McKeegan *et al*., 2007a, 2007b). Open bill breathing and deep inhalation were only seen during LAPS

and relate to hypoxia (Mackie and McKeegan, 2016), as confirmed by studies on controlled atmosphere stunning (McKeegan *et al.,* 2011; McKeegan *et al.,* 2007b; Gerritzen *et al.,* 2004; Abeysinghe *et al.,* 2007).

Within the sham treatments, illumination induced active behaviour (shorter latency to stand, more time standing, less time sitting, more vigilance) and exploratory pecking was seen only in the sham/light treatment. In the sham/dark treatment, birds spent a 277 s sitting on average, and EEG data revealed fluctuating and regularly reduced median frequencies suggesting that the birds were drowsy or sleeping for a significant proportion of the time with F50 showing a general downward trend. Such slow wave EEG activity was also seen in the sham/light treatment, but this was less pronounced, less frequent and had shorter duration than in sham/dark. While low light intensity is well known to induce slow wave EEG activity and sleep in birds (Gentle and Richards, 1972; Ookawa and Gotch, 1965; Gentle, 1975, 1976), the presence of intermittent sleep-like EEG patterns in the illuminated sham treatment mays reflect fatigue following handling (Sparrey and Kettlewell, 1993; Knowles and Broom, 1990). A significant heart rate decrease during the cycle was apparent in sham treated birds, suggesting continuing recovery from the stress of handling, irrespective of light treatment.

Within LAPS treatments, illumination had no effect on latencies to behavioural indicators of loss of consciousness (ataxia, loss of posture, loss of jaw tone and onset of convulsions), confirming that these are primarily related to oxygen availability. Light/dark treatment did increase latencies to standing and deep inhalation and total durations of leg paddling and clonic convulsions; the reasons for these effects are unclear. In general, the consistent pattering and timing of behaviours in response to LAPS are in close agreement with previous reports (Mackie and McKeegan, 2016; Martin *et al.,* submitted a, b).

The regular appearance of slow wave EEG in the sham/dark treatment explains the results of previous studies of LAPS carried out in darkness where low median frequencies accompany apparently conscious states (McKeegan *et al.,* 2013; Martin *et al.,* submitted b). Effects of illumination were apparent in the EEG responses of birds undergoing LAPS. While the overall EEG response to LAPS (steep reduction in F50 in the first 60 s and increased total power) was similar with and without illumination, birds exposed to LAPS in the dark had shorter latencies to reach a non-responsive state (F50 <12.7 Hz) and GA plane (F50 <6.8 Hz) and their total power was higher throughout induction to unconsciousness. A shorter time to isoelectric EEG (reduced by 10 s, as defined by spectral parameters) was also observed in darkness. Thus, in light conditions, slow wave EEG is induced by hypoxia, while in the dark, it is induced by both hypoxia and the absence of light stimulation, decreasing time to unconsciousness by approximately 15 s. Previously, we suggested that the presence of slow wave EEG patterns in conscious birds in the early part of LAPS suggests an absence of negative stimulation which would evoke a desynchronization of the EEG (e.g. Gentle, 1975). This notion is supported by the current study where the same patterns were seen and where slightly increased desynchronisation was related to the presence of light stimulation.

The initial heart rate of birds was affected by illumination treatment, however the direction of this difference was not consistent between LAPS and sham treatments, making its basis difficult to determine. As reported previously for LAPS (McKeegan *et al.,* 2013; Martin *et al.,* submitted b) and anoxic CAS (Butler, 1967; McKeegan *et al.,* 2007a, 2007b; McKeegan *et al.,* 2011; Raj, 2006), pronounced bradycardia and arrhythmia was apparent from 30 – 60 s when heart rate levelled off. Latency to bradycardia was not affected by light treatment, suggesting that these responses are primarily due to hypoxia in the early part of the LAPS cycle.

Collectively, these results add to a growing body of evidence that behavioural and EEG responses to LAPS are consistent and indicative of a process that is largely equivalent to controlled atmosphere stunning with anoxic gases. As would be expected, the effects of LAPS/sham treatment primarily related to the presence or absence of hypoxia. Illumination affected activity/sleep levels in sham treated birds and slightly slowed time to loss of consciousness in birds undergoing LAPS. The data lead to the recommendation that LAPS is carried out in darkness, as is currently the case commercially.

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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	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 numbers of birds exhibiting each behaviour (yes, N=20), and missing data due to birds being out of sight in each treatment.

	LAPS				SHAM			
	Dark		Light		Dark		Light	
Behaviour	**Yes**	**Missing data**	**Yes**	**Missing data**	**Yes**	**Missing data**	**Yes**	**Missing data**
Standing	2	0	12	0	2	1	10	1
Leg paddling	16	0	12	0	0	1	0	1
Clonic convulsions	20	0	20	0	0	1	0	1
Tonic convulsions	17	3	13	0	0	1	0	1
Slow-wing flapping	12	0	9	0	0	1	2	1
Vigilance	20	0	20	0	19	1	19	1
Mandibulation	12	0	12	0	6	1	9	1
Head shaking	5	0	11	0	3	1	4	1
Open-bill breathing	18	0	13	0	0	1	0	1
Deep inhalation	8	0	5	0	0	1	0	1
Jump	11	0	14	0	0	1	0	1
Vocalisation	0	0	3	0	0	1	3	1
Sitting	20	0	20	0	19	1	19	1
Lying	19	1	20	0	0	1	0	1
Motionless	20	0	20	0	0	1	0	1
Loss of jaw tone	17	3	17	3	0	1	0	1
Ataxia	19	0	20	0	0	1	0	1
LOP	20	0	20	0	0	1	0	1
Escape	0	0	0	0	0	1	0	1
Peck	0	0	0	0	0	1	2	1
Panting	0	0	0	0	0	1	1	1

Table 3 Summary statistics (mean ±SE) of latencies, bout duration, total duration and frequency of behaviours exhibited in both LAPS and sham conditions, and statistical differences (F statistic and P value) dependent on LAPS and light treatment and their interaction. Significant *P* values (< 0.05) are in bold type.

	Behaviour	LAPS		SHAM		*LAPS/sham*		*Light/dark*		*LAPS/sham* light/dark*	
		Dark	Light	Dark	Light						
		Mean ±SE	Mean ±SE	Mean ±SE	Mean ±SE	*F*	*P*	*F*	*P*	*F*	*P*
Latency	Sitting	0.4 ± 0.1	0.5 ± 0.1	2.1± 1.3	1.6 ± 0.5	14.40	**<0.001**	2.21	0.142	1.63	0.206
	Vigilance	1.1 ± 0.1	1.1 ± 0.2	10.7± 1.6	7.7 ± 1.7	58.72	**<0.001**	2.92	0.092	0.00	0.963
	Mandibulation	20.1 ± 2.3	24.2 ± 2.9	49.8 ±13.3	55.9 ± 15.4	114.92	**<0.001**	0.56	0.458	4.71	**0.033**
	Headshake	28.7 ± 7.5	33.9 ± 4.7	69.5 ± 29.0	151.6 ± 43.7	587.46	**<0.001**	0.46	0.498	3.93	0.051
	Standing	17.0 ± 2.2	17.7 ± 4.7	107.2 ± 83.9	88.9 ± 25.8	118.76	**<0.001**	255.9	**<0.001**	25.25	**0.001**
	Slow WF*	57.6 ± 1.5	55.1 ± 1.9	-	119.0 ± 80.0	-	-	-	-	-	-
	Peck*	-	-	-	42.0 ± 3.9	-	-	-	-	-	-
Bout duration	Sitting	56.0 ± 5.4	39.6 ± 3.5	266.5 ± 11.1	166.5 ± 19.2	227.37	**<0.001**	31.49	**<0.001**	0.55	0.462
	Vigilance	19.5 ± 2.6	29.9 ± 1.8	7.7 ± 1.3	13.9 ± 1.1	66.59	**<0.001**	24.82	**<0.001**	0.07	0.797
	Standing	3.3 ± 0.9	12.3 ± 3.3	5.1 ± 3.5	11.4 ± 2.4	0.06	0.802	52.99	**<0.001**	5.49	**0.022**
	Slow WF*	3.1 ± 0.4	3.1 ± 1.2	-	1.3 ± 1.3	-	-	-	-	-	-
Total duration	Sitting	58.6 ± 5.3	43.3 ± 3.4	277.2 ± 1.3	268.5 ± 3.0	123.97	**<0.001**	4.86	**0.031**	0.55	0.462
	Vigilance	20.5 ± 2.3	30.9 ± 1.7	40.9 ± 8.0	52.6 ± 3.3	28.56	**<0.001**	5.60	**0.018**	0.07	0.797
	Standing	3.3 ± 0.9	12.3 ± 3.3	5.1 ± 3.5	19.8 ± 3.9	22.38	**<0.001**	120.9	**<0.001**	5.49	**0.022**
	Slow WF*	4.0 ± 0.5	3.1 ± 1.2	-	1.3 ± 0.1	-	-	-	-	-	-
Bout frequency	Sitting	1.0 ± 0.0	1.3 ± 0.1	1.2 ± 0.2	2.4 ± 0.4	17.01	**<0.001**	17.10	<**0.001**	1.85	0.178
	Vigilance	1.2 ± 0.1	1.1 ± 0.1	2.0 ± 0.3	3.8 ± 0.4	81.37	**<0.001**	5.09	**0.027**	4.50	**0.037**
	Mandibulation	1.8 ± 0.4	1.5 ± 0.4	0.9 ± 0.4	1.5 ± 0.4	0.50	0.481	0.08	0.776	0.03	0.858
	Headshake	0.5 ± 0.2	1.2 ± 0.3	0.2 ± 0.1	0.3 ± 0.2	7.34	**0.010**	3.41	0.069	0.00	0.966
	Standing	0.1 ± 0.1	0.7 ± 0.1	0.1 ± 0.1	1.5 ± 0.4	6.13	**0.016**	14.64	**<0.001**	0.46	0.501
	Slow WF	0.8 ± 0.2	0.5 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	10.39	**0.002**	3.84	0.054	0.03	0.856
	Pecking	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.02	0.879	0.01	0.933	0.00	0.955
	Vocalisation	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.4 ± 0.2	0.01	0.917	0.01	0.925	0.00	0.997

**No modelling possible due to too few observations*

Table 4 Summary statistics (mean, SE, min, max) of latencies to behaviours exhibited during LAPS, and statistical differences (F statistic and P value) dependent on light treatment and their interaction. Significant *P* values (< 0.05) are in bold type.

Behaviour	LAPS DARK				LAPS LIGHT				*F* statistic	*P* value
	Mean	SE	Min	Max	Mean	SE	Min	Max		
Sitting	0.4	0.1	0.1	1.2	0.5	0.1	0.1	2.2	0.16	0.692
Vigilance	1.1	0.1	0.3	2.0	1.1	0.2	0.3	2.8	0.82	0.370
Standing	17.0	2.2	14.9	19.2	17.7	4.7	2.6	50.5	14.47	**<0.001**
Mandibulation	20.1	2.3	4.1	32.0	24.2	2.9	6.8	39.2	0.69	0.412
Head shaking	28.7	7.5	10.9	47.0	33.9	4.7	6.3	54.6	2.14	0.153
Ataxia	39.5	13.4	29.1	48.0	38.3	1.26	26.1	45.8	0.09	0.770
Jump	49.0	1.8	38.2	56.9	47.3	1.7	35.4	55.0	3.71	0.063
LOP	54.7	1.3	40.6	62.4	55.9	1.19	40.1	61.4	1.37	0.250
Lying	56.6	1.4	42.2	67.2	55.6	1.6	41.7	68.2	0.24	0.623
Slow-wing flapping	57.6	1.5	49.5	69.0	55.1	1.9	46.8	65.0	4.12	0.051
Open-bill breathing	59.5	4.1	11.1	89.9	57.5	2.5	46.2	76.2	0.57	0.457
Clonic convulsions	63.8	1.4	52.9	77.4	60.1	1.37	41.4	71.41	3.65	0.065
Loss of jaw tone	76.3	1.8	65.5	91.4	77.9	1.7	64.6	96.2	0.11	0.747
Deep inhalation	86.1	4.0	71.3	100.6	64.0	3.9	52.3	72.2	137.00	**<0.001**
Leg paddling	92.1	3.7	58.9	129.5	91.8	4.05	61.4	118.2	1.00	0.325
Tonic convulsions	105.0	3.8	81.2	135.3	110.9	6.61	81.4	158.6	0.79	0.381
Motionless	145.2	3.3	116.3	171.2	142.8	4.8	103.8	186.7	0.00	0.964
Vocalisation*	-	-	-	-	50.7	20.2	11.4	78.2	-	-

**No modelling possible due to too few observations*

Table 5 Summary statistics (mean, SE, min, max) of bout durations, total duration and bout frequency of behaviours exhibited during LAPS, and statistical differences (F statistic and P value) dependent on light treatment and their interaction. Significant *P* values (< 0.05) are in bold type.

	Behaviour	LAPS DARK				LAPS LIGHT				*F*	*P*
		Mean	**SE**	**Min**	**Max**	**Mean**	**SE**	**Min**	**Max**		
Individual bout duration	Sitting	56.0	5.4	37.0	151.9	39.6	3.5	9.9	60.2	7.29	**0.011**
	Vigilance	19.5	2.6	4.9	40.1	29.9	1.8	17.9	42.7	7.13	**0.012**
	Standing	3.3	0.9	2.4	4.2	12.3	3.3	1.3	41.0	70.54	**<0.001**
	Ataxia	19.1	1.3	8.4	32.5	18.7	2.0	5.4	38.8	0.20	0.658
	Lying	79.9	3.3	45.7	100.2	91.3	4.5	55.9	133.2	2.00	0.166
	Slow-wing flapping	3.1	0.4	1.4	5.6	3.1	1.2	0.4	11.8	0.15	0.705
	Open-bill breathing	25.6	11.6	1.8	211.8	15.0	2.7	5.3	42.4	0.07	0.796
	Clonic convulsions	6.0	0.7	2.1	12.9	8.0	0.9	1.3	15.9	2.73	0.108
	Leg paddling	6.7	1.1	1.4	16.6	11.3	4.5	1.9	59.3	3.74	0.062
	Tonic convulsions	5.5	0.8	0.7	12.4	8.2	1.8	1.2	25.8	3.84	0.058
	Motionless	144.7	3.3	116.9	177.6	138.6	4.9	93.3	179.4	2.10	0.147
Total duration	Sitting	58.6	5.3	37.0	151.5	43.3	3.4	9.9	60.1	4.87	**0.027**
	Vigilance	20.5	2.3	4.9	40.1	30.9	1.7	17.9	42.7	10.72	**0.002**
	Standing	3.3	0.9	2.4	4.2	12.3	3.3	1.3	41.0	70.54	**<0.001**
	Ataxia	19.1	1.3	8.4	32.5	19.0	1.9	7.4	38.8	0.09	0.767
	Lying	82.3	2.7	61.5	100.2	91.3	4.5	55.9	133.2	1.78	0.182
	Slow-wing flapping	4.0	0.5	1.4	7.2	3.1	1.2	0.4	11.8	0.44	0.511
	Open-bill breathing	35.8	15.3	2.5	212.0	16.1	2.9	5.3	42.4	0.01	0.908
	Clonic convulsions	20.3	2.1	3.8	51.7	27.1	1.7	15.6	47.1	4.89	**0.034**
	Leg paddling	9.62	2.0	1.4	33.2	14.2	4.4	1.8	59.2	8.98	**0.005**
	Tonic convulsions	9.5	2.3	0.7	36.8	11.4	2.6	1.2	31.8	0.43	0.516
	Motionless	144.7	3.3	116.9	177.5	138.6	4.9	93.3	179.3	0.57	0.449
Frequency of bouts	Sitting	1.0	0.0	1.0	1.0	1.3	0.1	1.0	3.0	5.08	**0.031**
	Vigilance	1.2	0.1	1.0	2.0	1.1	0.1	1.0	2.0	0.16	0.692
	Standing	0.1	0.1	0.0	1.0	0.7	0.1	0.0	2.0	5.65	**0.023**
	Ataxia	1.0	0.1	0.0	1.0	1.0	0.0	1.0	1.0	2.80	0.103
	Lying	1.0	0.1	0.0	1.0	1.0	0.0	1.0	1.0	0.03	0.862
	Slow-wing flapping	0.8	0.2	0.0	3.0	0.5	0.1	0.0	1.0	3.44	0.072
	Open-bill breathing	1.1	0.2	0.0	3.0	0.7	0.1	0.0	2.0	1.50	0.229
	Clonic convulsions	2.8	0.3	1.0	6.0	2.8	0.3	1.0	5.0	0.01	0.907
	Leg paddling	1.2	0.2	0.0	2.0	0.8	0.2	0.0	2.0	0.75	0.392
	Tonic convulsions	1.3	0.2	0.0	4.0	0.9	0.2	0.0	3.0	0.01	0.905
	Motionless	1.0	0.1	0.0	1.0	1.0	0.0	1.0	1.0	0.01	0.989

Table 6 Summary statistics (mean, SE, min, max) of counts of behaviours exhibited in LAPS, and statistical differences (F statistic and P value) dependent on light treatment and their interaction.

Behaviour	DARK				LIGHT				*F* statistic	*P* value
	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.		
Jump	1.0	0.3	0.0	4.0	1.6	0.3	0.0	4.0	1.95	0.172
Mandibulation	1.8	0.4	0.0	5.0	1.5	0.4	0.0	5.0	0.12	0.736
Peeping	0.0	0.0	0.0	0.0	0.4	0.2	0.0	4.0	0.00	0.974
Head shake	0.5	0.2	0.0	3.0	1.2	0.3	0.0	5.0	0.43	0.512
Deep inhalation	0.7	0.2	0.0	3.0	0.4	0.2	0.0	2.0	0.83	0.368
Peck*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-

**No modelling possible due to too few observations*

Table 7 Summary statistics (mean, SE, minimum and maximum) of latencies to various EEG parameters according to treatment and statistical differences (F statistic and P value) dependent on LAPS and light treatment and their interaction. Significant *P* values (< 0.05) are in bold type.

		LAPS/DARK		LAPS/LIGHT		SHAM/DARK		SHAM/LIGHT		LAPS/SHAM		LIGHT/DARK		Interaction	
Measure (s)	**N**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	***F***	***P***	***F***	***P***	***F***	***P***
Slow-wave*	21	28.3	4.3	55.2	10.9	35.8	11.6	33.5	8.1	1.1	0.303	3.15	0.085	0.62	0.435
F50<6.8Hz	21	39.1	6.3	53.6	11.8	12.7	5.3	88.0	29.5	21.8	**<0.001**	63.55	**<0.001**	56.65	**<0.001**
F50<12.7Hz	31	27.1	4.9	40.3	5.8	20.4	6.7	37.0	8.4	0.36	0.554	7.04	**0.012**	1.55	0.222
PTOT <10% baseline	5	97.0	25.0	122.7	5.9	-	-	-	-	-	-	-	-	-	-
Isoelectric*	13	89.7	15.0	99.0	4.2	-	-	-	-	-	-	0.40	0.539	-	-
Isoelectric (spectral)[†]	10	91.6	12.3	101.6	6.1	-	-	-	-	-	-	6.25	**0.025**	-	-

**Based on visual inspection*

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

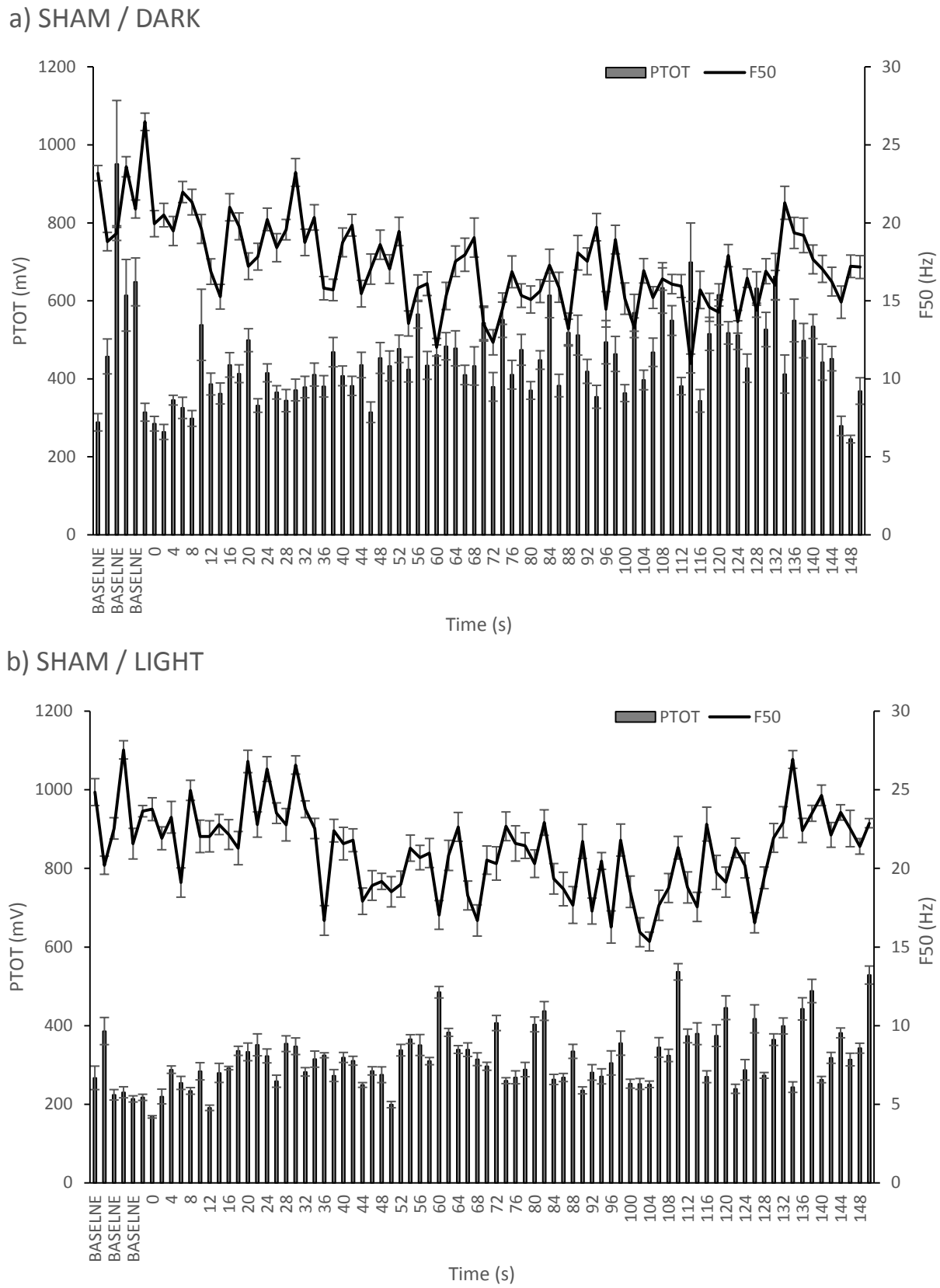

Figure 1 Changes in mean (± SE) F50 and PTOT for consecutive 2 s epochs during sham treatment in dark (a) or light (b) conditions (onset 0s) to 150s (mean time to motionless in LAPS). Baseline points refer to signal collected prior to LAPS (3 outside chamber, 3 inside chamber). N=19 birds.

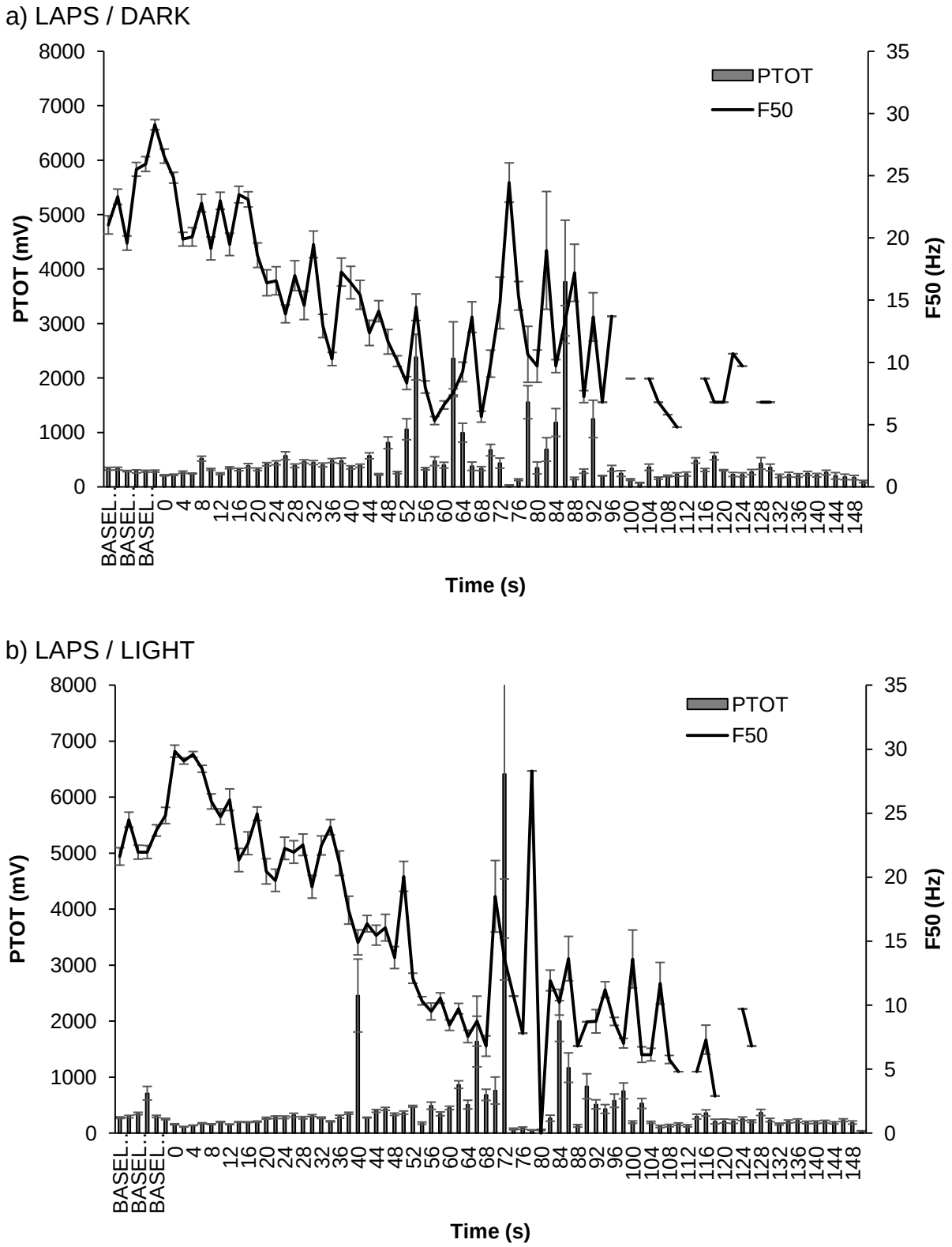

Figure 2 Changes in mean (± SE) F50 and PTOT for consecutive 2 s epochs during LAPS treatment in dark (a) or light (b) conditions (onset 0s) to 150s (mean time to motionless in LAPS). Baseline points refer to signal collected prior to LAPS (3 outside chamber, 3 inside chamber). N= 17 birds. To allow both graphs to be plotted on the same Y axis range, a single PTOT outlier was removed in LAPS/LIGHT treatment at 72 s (Bird 408: 53816.46 mV). Missing values indicate that epochs were excluded from analysis due to noise interference rendering too few data points available (<3 birds) or because the EEG had become isoelectric.

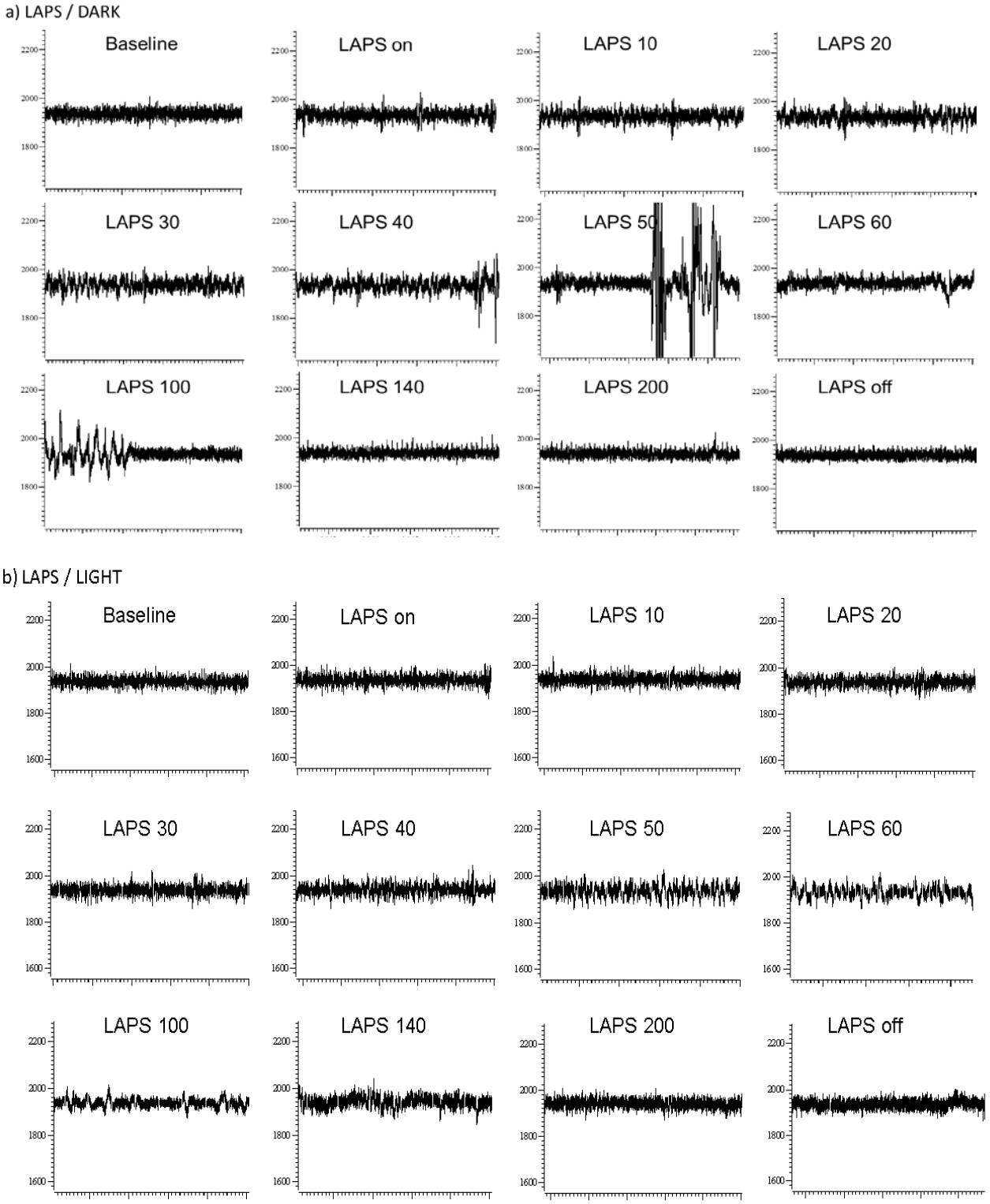

Figure 3 A representative series of EEG trace excerpts (each 5s duration, data from Bird 347 (LAPS/dark (a) and Bird 446 LAPS/light (b)) illustrating the typical appearance of the EEG at 12 time points (baseline, LAPS on, +10, +20, +30, +40, +50, +60, +100, +140, +200s and LAPS off). Y-axis units are microvolts, x-axis units (large tick marks) are seconds.

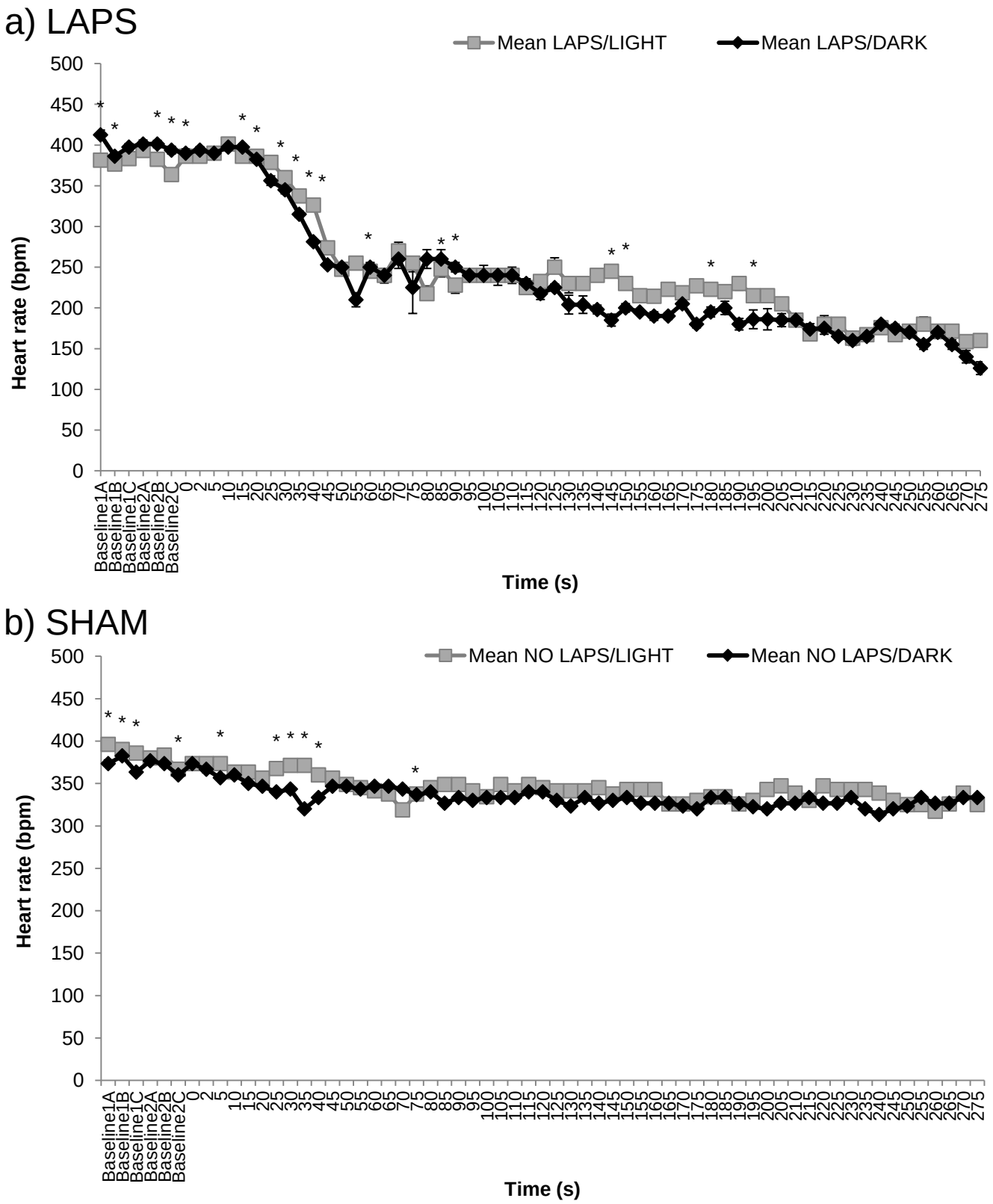

Figure 4 Mean (± SE) heart rate (bpm) at 5 s intervals throughout LAPS/sham treatment cycles at in light (orange) or dark (blue) treatments. The six baseline points (prior to 0 s) refer to signal collected prior to LAPS (3 outside chamber (1A-C), 3 inside chamber (2A-C)). N=17 for LAPS 3; N= 19 for sham. Asterisks indicate significant differences between light treatments.