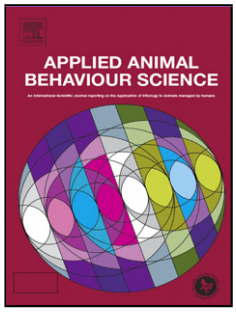
Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning



Title: Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning

Author: Jessica E. Martin Karen Christensen Yvonne Vizzier-Thaxton Dorothy E.F. McKeegan

PII: S0168-1591(16)30134-4
DOI: http://dx.doi.org/doi:10.1016/j.applanim.2016.05.007
Reference: APPLAN 4262

To appear in: *APPLAN*

Received date: 19-1-2016
Revised date: 24-4-2016
Accepted date: 1-5-2016

Please cite this article as: Martin, Jessica E., Christensen, Karen, Vizzier-Thaxton, Yvonne, McKeegan, Dorothy E.F., Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning.Applied Animal Behaviour Science http://dx.doi.org/10.1016/j.applanim.2016.05.007

Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning

Jessica E Martin[a,b]*, Karen Christensen[c], Yvonne Vizzier-Thaxton[c], Dorothy E F McKeegan[b]

[a] *The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, United Kingdom*

[b] *Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, G61 1QH, United Kingdom*

[c] *Centre of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, United States*

* Corresponding author at: The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, United Kingdom. Tel: +44 131 6506062. Email address: Jessica.Martin@ed.ac.uk (J. Martin).

Highlights

- A novel stunning system uses hypobaric hypoxia to render poultry unconscious.
- We investigated whether an opioid analgesic affected behavioural responses to this process.
- Evidence for pain was limited and observed responses relate primarily to hypoxia.
- This approach appears to be equivalent in welfare terms to stunning with inert gases.
- These findings contribute to a wider welfare assessment of low atmospheric pressure stunning.

Short title: Effects of pain relief on hypobaric hypoxia in chickens

Abstract

Worldwide, more than 50 billion chickens are killed annually for food production so their welfare at slaughter is an important concern. Low Atmospheric Pressure Stunning (LAPS) is a novel approach to pre-slaughter stunning of poultry in which birds are rendered unconscious by gradually reducing oxygen tension in the atmosphere to achieve a progressive anoxia (hypobaric hypoxia). Advantages of this approach over electrical stunning are that birds are not shackled while conscious and all birds are reliably and irreversibly stunned. However, concerns remain that birds undergoing LAPS could experience discomfort or pain. Here we investigated whether subjecting birds to LAPS with and without administration of an opioid analgesic (butorphanol) affected behavioural responses. A blocking design was used in which pairs of birds receiving either analgesic or sham treatment were allocated to three types (analgesic/analgesic, analgesic/sham, or sham/sham). In line with previous studies, birds showed a consistent sequence of behaviours during LAPS: ataxia, loss of posture, clonic/tonic convulsions, leg paddling and motionless. Overall, administration of butorphanol had no effect on the range and patterning of behavioural responses during LAPS, but there were some differences in behaviour latencies, counts and durations. For example, latencies to ataxia, mandibulation and deep inhalation were delayed by analgesic treatment, however the duration of ataxia and other behaviours related to loss of consciousness were unaffected. Fewer birds receiving analgesia showed jumping and slow wing flapping behaviour compared to controls, which suggests these may be pain related. These behaviours after the onset of ataxia and the results may reflect a smoother induction to unconsciousness in analgised birds. Collectively, the results do not provide convincing evidence that birds undergoing LAPS are experiencing pain. While there were effects of analgesia on some aspects of behaviour, these could be

explained by potential sedative, dysphoric and physiological side effects of butorphanol. The behavioural responses to LAPS appear to be primarily related to exposure to anoxia rather than hypobaric conditions, and thus in terms of welfare, this stunning method may be equivalent to controlled atmosphere stunning with inert gases.

Keywords: Hypobaric hypoxia, low atmosphere pressure stunning, pain, animal welfare, humane slaughter, broiler

1. Introduction

Low Atmospheric Pressure Stunning (LAPS) is a novel approach to pre-slaughter stunning of poultry in which birds are rendered unconscious by gradually reducing air pressure and thus oxygen tension to achieve a progressive hypobaric hypoxia. LAPS shares many of the welfare advantages of controlled atmosphere stunning (CAS) systems, which use exposure to hypoxic and/or hypercapnic gas mixtures, reliably and irreversibly stunning birds in their transport crates (Vizzier-Thaxton et al., 2010; Johnson, 2013). A major benefit of CAS systems and the LAPS system is that they avoid the considerable stress and pain of shackling of conscious birds (Gentle and Tilston, 2000) and 100% of the chickens are rendered insensible before shackling and bleeding. By contrast, electrical stunning is associated with various welfare issues such as shackling of conscious birds, pre-stun shocks and the risk of inadequate stunning (Raj, 2006). LAPS is in routine commercial use at a poultry processing plant in Arkansas, having been given ‘no objection’ status by both the United States Department for Agriculture (USDA) in 2010 and the Canadian Food Inspection Agency in 2013. While there has been much research to determine humane gas mixtures for CAS (e.g. McKeegan et al., 2007; Johnson, 2013; Joseph et al., 2013), less is known about the welfare impact of LAPS.

Previous work investigating the induction of unconsciousness in hypoxic gas environments (Woolley and Gentle 1988; Raj et al., 1991) suggests that the approach has promise, and the

gradual nature of LAPS avoids obvious concerns related to the welfare consequences of rapid decompression (Close et al., 1996; AVMA 2013). Previously, Purswell et al., (2007) identified process variables for a suitable decompression and some aspects of behaviour, corticosterone responses, meat quality and pathology have been investigated (Battula et al., 2008; Vizzier-Thaxton et al., 2010). Electroencephalogram (EEG) and electrocardiogram (ECG) responses of broilers undergoing LAPS were reported by McKeegan et al. (2013), where the process was associated with changes in the EEG pattern (highly significant increases in total power, decreases in median frequency and progressive increases in slow wave activity), indicating a gradual loss of consciousness. Recently, a detailed behavioural study described the responses of broilers undergoing LAPS and reported a consistent sequence of behaviours: ataxia, loss of posture, clonic and tonic convulsions and leg paddling (Mackie and McKeegan, 2016). Additional responses were observed in a proportion of birds such as mandibulation (repetitive and rapid opening and closing of the bill, 32% of birds), headshaking (76% of birds) and open bill breathing (74% of birds). Based on loss of posture (on average at 84 s), the data suggest that birds are in a conscious state for longer during LAPS than in controlled atmosphere stunning with inert gases (McKeegan et al., 2007a; Abeyesinghe et al., 2007),other behavioural responses are equivalent. Given that headshaking, mandibulation and open bill breathing are all seen during exposure to anoxic gases (normobaric hypoxia) and LAPS (hypobaric hypoxia), it is difficult to conclude whether they are a response to hypoxia or decompression, or both. Concerns remain that some of the behavioural responses observed could be pain related, possibly resulting from painful expansion of trapped air in body cavities. Vizzier-Thaxton et al. (2010) noted that the anatomy and function of the avian respiratory tract with interconnecting airsacs and lungs makes it unlikely that significant amounts of gas would be trapped in the abdomen, while hemorrhagic lesions were found in the lungs, brain, and heart of animals undergoing rapid decompression (Van Liere, 1943).

Pain is difficult to assess as it cannot be measured directly, but behaviour is the parameter most often used to assess animal pain (Rutherford 2002) and signs of stress during stunning in poultry include head shaking (Erhardt et al., 1996; Raj, 1996), gasping (Raj and Gregory, 1990), yawning (Erhardt et al., 1996), vocalisation (Zeller et al., 1988), sneezing (Hoenderken et al., 1994) and defecating (Morton et al., 1998). Some of these signs may also indicate pain or varying degrees of discomfort, or may reflect physiological responses. Quantitative differences may be significant from a welfare point of view, as well as the time at which they occur during the stunning process.

Analgesic intervention has been widely used in a range of contexts in animal welfare research, for example to examine pain associated with lameness (e.g. Hocking et al., 1997). It is widely recognised that the abolition of suspected pain related behaviour with analgesic is circumstantial evidence of pain (Rutherford, 2002; Walker et al., 2014). However, analgesic drugs may have behavioural effects unrelated to pain and nociception, and some also have general sedative or side effects. Thus, care must be taken with the choice of agent and the dose applied. The primary objective of this study was to investigate whether subjecting birds to LAPS with and without administration of an opioid analgesic would affect their behavioural responses, especially those suspected to relate to pain and discomfort. Butorphanol was chosen for this trial, as it is a Kappa opioid receptor agonist and a mu opioid receptor antagonist with characterised pharmacokinetics (Guzman et al., 2014) and is the currently recommended opioid for use in birds (Paul-Murphy and Fialkowski, 2001; Paul-Murphy, 2013). We used a low-moderate dose (Paul-Murphy, 2013) to minimise sedation and side effects. Broilers were exposed to LAPS in pairs to maximise visibility of their reactions to the process while eliminating isolation stress. A blocking design was used in which birds receiving analgesic or sham treatments were randomly allocated to three types of pairs (analgesic/analgesic, analgesic/sham, or sham/sham)). This robust design, random allocation and blinding of behavioural observers to pair type allowed us to reliably determine

the effects of analgesic intervention on behaviour during LAPS, and thus contribute to a thorough welfare assessment of the process.

2. Material and methods

2.1 Animals and housing

Ninety Cobb 500 male broiler chickens (Gallus gallus domesticus) from the female breeder line were used in this study. They were sourced from a commercial hatchery and were wing tagged at 4 weeks of age. The birds were 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/m2). Clean pine shavings were used for litter. Single-pass ventilation was maintained at a constant rate of 6 m3/min in all chambers. The photoperiod was 23L:1D for d 1 to 4, and 16L:8D thereafter. Chambers were equipped with 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. Environmental controls for climate were maintained to follow recommended management practices (Cobb, 2012). Birds and environmental controls were monitored twice daily by trained staff. The trials were undertaken in Arkansas, USA, and therefore were not subject to UK legal requirements through DEFRA or Home Office regulations. The experimental design and animal husbandry was performed following the EU Directive on the Protection of Animals used for Scientific Purposes (EU 2010/63) for guidance. The experiments were specifically authorized by the University of Arkansas Institutional Animal Care and Use Committee (Protocol 15031).

2.2 LAPS process

The LAPS chamber was developed by Technocatch LLC in Mississippi, USA the system and the pressure curves applied by the process are patented (Cheek & Cattarazzi, 2010). The 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 (2.1mm lens) 18 infra-red illuminators, Model #RVS-507, RVS Systems) was fitted into the chamber to observe the birds (fixed centrally on the front wall of the chamber allowing full view of the relevant tier). A manually operated door is present that allows the entry of the transport module and seals them into the chamber to begin the process. 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), slightly different pressure reduction curves must be applied to achieve the same hypobaric effect under different ambient conditions. As discussed by Holloway (unpublished results), water in the LAPS chamber may also lead to modification of the rate of decompression based on temperature. Ambient temperature and humidity were recorded for each LAPS cycle and means were 13.5 ± 0.5 °C and 76.3 ± 0.6%, respectively. In this study, all 45 LAPS runs were carried out within a single temperature setting.

2.3 Experimental procedure

The experimental birds were randomly selected from the flock by a random number generator (Microsoft Excel 2010) based on wing tag number. They were systematically and equally allocated via a Latin-Square design across two treatments (analgesic - A, sham - S) and then allocated by individual wing tag number into three types of blocked pairs (analgesic/analgesic (AA), analgesic/sham (AS), and sham/sham (SS)) and pair kill order following a Graeco Latin-Square design (Martin & Bateson 2007). There were 15 replications of each block (AA, AS, and SS), each containing a pair of birds. The birds underwent LAPS in 45 consecutive pairs over two days (day 1 = 23 pairs; day 2 = 22 pairs) at 36-37 days of age (mean bodyweight 2.30 ± 0.12 kg). To mimic commercial transport and lairage conditions, experimental birds for each day were removed from the flock and transported and held in poultry transport crates (97 x 58 x 27 cm, maximum 8 birds per crate) prior to LAPS. Thus birds had food and water withdrawn for between 2-6 hrs before LAPS, dependent on the pair kill order.

In sequential order, bird pairs were removed from the transport crates and weighed. Dependent on their pre-determined treatment, birds were injected with either butorphanol ('Dolorex', butorphanol tartrate 10mg/ml, Merk) delivered IM in the right thigh at 1mg/kg or saline (veterinary 0.9% Sodium Chloride Injection, Hospira Inc.) delivered IM in the right thigh at equivalent volume to the analgesic treatment based on bird weight. Treatments were staggered to provide a consistent 30 minute interval between injection and LAPS. At the time of injection one bird per pair had its wing tip feathers marked by a black permanent marker (Sharpie® Magnum chisel tip); this marking was to allow better visualisation of individuals during behavioural observations and was randomly allocated by wing tag number, irrespective of treatment. Birds were then housed within their pairs in separate cardboard pet carriers (28 x 35 x 46 cm) until transferred into the LAPS chamber by hand. Each pair of birds was placed in the top right tier (1.53 x 1.21 x 0.23 m) of the US poultry container within the LAPS chamber. Soft polystyrene dividers were used to position the two birds at the front of the tier (available space 0.76 x 1.21 x 0.23 m, resulting in a stocking density of 5.0 Kg/m2

based on average bird weight of 2.3 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, 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) to allow 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 pair of birds. 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.

2.4 Behavioural Observations

An ethogram was developed based on previous behavioural work on LAPS (Mackie & McKeegan, 2016) as well as CAS research (Lambooij et al., 1999; Coenen et al., 2009) (Table 1). Behaviours for both birds in each pair were recorded using The Noldus Observer XT 11.0 programme by a single observer who was blinded to pair number, block type and individual bird treatment. 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. Reasons for birds going out of sight were that they moved behind the other bird or to the far end of the chamber. Data was exported from Observer to Microsoft Excel 2010.

2.5 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 $P<0.05$ threshold level. Summary graphs and statistics were produced at bird level. 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, companion bird ID and pair block type as random effects. All fixed effects were treated as factors and all interactions between factors were included in maximal models. All models included treatment, pair 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. For behaviours which were not exhibited by all birds, the effect of treatment on the proportions of birds showing the behaviour was compared with Chi Square tests using two by two contingency tables.

3. Results

No birds showed any signs of life at the end of the LAPS cycle (absence of rhythmic breathing, absence of corneal or palpebral reflex (EFSA 2013). A total of 17/90 birds went out of sight at some point during observations (by treatment: A = 9; S = 8), with mean total out of sight durations of 29.4 ± 10.9 s for analgesic birds (10.5 ± 3.9% of total observation time) and 90.8 ± 33.1 s for sham birds (33.1 ± 11.6% of total observation time). Based on exclusion criteria (>50% of observation time out of sight), 3 sham birds were removed from analysis to avoid bias. The birds showed a consistent sequence of behaviours during LAPS: ataxia, loss of posture, clonic/tonic convulsions, leg paddling and motionless. Clonic convulsions, sitting, lying, ataxia, loss of posture, loss of jaw tone and motionless were observed in all birds as they underwent LAPS. No birds were observed performing escape behaviour, pecking or panting.

Almost all birds (83/90) exhibited vigilance behaviour at the onset of LAPS, and the total duration, bout duration and number of bouts this behaviour was increased in birds receiving analgesic (total mean duration 37.1 s compared to 30.5 s in controls, Table 2). The mean

latency to show mandibulation was delayed by analgesic treatment (18.8 s vs. 25.5 s, Table 3), while saline treated birds exhibited more counts of mandibulation than analgesic treated birds (mandibulations per bird ranged from 1-12, mean count 2.7 vs. 2.1, Table 4). Mean counts of headshaking were higher in analgesic treated birds compared to saline treated birds (headshakes per bird ranged from 1-7, mean counts 2.4 with analgesic compared to 1.7 in controls, Table 4). Total duration of standing was higher in analgesic treated birds (16.0 s compared to 12.3 s in saline birds, Table 2) there were also longer standing bout durations with analgesic (13.1 s compared to 7.5 s, Table 2).

Analgesic treatment affected the latency to ataxia but the effect in terms of time difference was small (44.5 s for birds receiving analgesia compared to 41.8 s for sham treated birds, overall range 21.4 – 65.2 s, Table 3). Analgesic treatment had no effect on the duration of ataxia (Table 2). Analgesic treatment also had no effect on other latencies related to the onset of unconsciousness (loss of posture or loss of jaw tone; Table 3). Figure 1 shows the patterning of key behaviours relating to loss of consciousness in the first 100 s of LAPS according to treatment, indicating the sequence of behaviour and showing that analgesia treatment was associated with a delay in the latency of some behaviours, but had no effect on latency to loss of consciousness, as indicated by loss of jaw tone and loss of posture. Jumping was not seen until birds started to show ataxia and loss of posture (mean latency 55.4 ± 1.4 s); this was seen in fewer birds receiving analgesic compared to those receiving saline (46.5% vs. 67.5%; Table 5). Saline treated birds also exhibited more jumps than analgesic treated birds (jumps per bird ranged from 1-3, mean count 1.0 compared to 0.5, Table 4).

Slow wing flapping was seen in significantly more birds receiving saline (74%) than analgesic (47%, Table 5), but longer bout durations were observed with analgesic (Table 2). Longer and more bouts of tonic convulsions were also observed in birds received analgesic, but latencies and overall durations were unaffected (Table 3, Table 2). There were no effects of

analgesic on clonic convulsions. Frequency of bouts and bout durations of lying were increased in birds receiving analgesic (75.2 s) compared to controls (72.7 s, Table 2). The total duration of leg paddling was affected by treatment, with analgised birds exhibiting longer durations (9.1 s compared to 6.8 s in saline birds). Latency, bout duration and bout frequency of leg paddling was unaffected by treatment, as was latency to become motionless.

The latency to the first deep inhalation behaviour was 82.5 s in saline treated birds, greater compared to 101.8 s analgesic treated birds (Table 3), but counts of this behaviour (counts per bird ranged from 1-8) were not affected by treatment (Table 2). The duration of open bill breathing bouts was shorter in analgesic treated birds (8.1 s compared to 6.8 s in saline treated birds, Table 2). Only four birds vocalised; three of the vocalisations occurred during clonic convulsions suggesting that they may have been involuntary. The fourth bird vocalised once at 14 s into LAPS.

Fixed effects had minimal influence on behaviour latencies; however some factors affected certain behaviours. Bird weight affected latency to ataxia ($F_{1,84} = 7.77$, $p = 0.021$) and mandibulation ($F_{1,41} = 17.7$, $p < 0.001$) and was negatively correlated with both, but not significantly ($r = -0.109$, $p = 0.322$ and $r = -0.123$, $p = 0.428$, respectively). The onset of open-bill breathing ($F_{1,67} = 8.63$, $p = 0.005$) and deep inhalation ($F_{1,48} = 9.41$, $p = 0.002$) were positively related to bodyweight, with significant positive correlation with first deep inhalation ($r = 0.354$, $p = 0.014$). Ambient temperature had no effect on the majority of behavioural latencies except for time to become motionless ($F_{1,84} = 5.51$, $p = 0.022$) which was non-significantly negatively correlated ($r = -0.098$, $p = 0.373$). Latency to slow wing flapping was also related to ambient temperature ($F_{1,51} = 2.33$, $p < 0.001$) with a non-significant positive correlation ($r = 0.075$, $p = 0.600$). In terms of behaviour durations, fixed effects did not explain a significant proportion of the data except for ambient temperature ($F_{1,64} = 5.00$, $p = 0.028$) and humidity ($F_{1,64} = 4.26$, $p = 0.042$) which were related to the durations of tonic

convulsions, although neither had significant correlations (r = 0.178, p = 0.159; r = -0.138, p = 0.278 respectively).

The majority of fixed effects and interactions had no significant effect on the total counts of behaviour including jumping, mandibulation, head shaking or deep inhalation behaviours. The only significant effects were between mandibulation and bird weight ($F_{1,44}$ = 3.11, p = 0.008), ambient humidity ($F_{1,44}$ = 7.68, p = 0.007) and ambient temperature ($F_{1,44}$ = 6.42, p = 0.011), as well as between headshaking and ambient humidity ($F_{1,51}$ = 5.22, p = 0.025).

4. Discussion

A consistent series of behavioural responses were seen during LAPS, similar to previous reports (Vizzier-Thaxton et al., 2010; Mackie and McKeegan 2016). The responses also closely resembled those observed during exposure to controlled atmosphere stunning with inert gases such as Argon and Nitrogen (Raj et al., 1991; Gerritzen et al., 2000; McKeegan et al., 2007). Previously, EFSA (2004) opined that “anoxia is not aversive to poultry and does not induce any signs of respiratory distress prior to loss of consciousness”. Mackie and McKeegan (2016) discussed the welfare implications of behavioural responses to LAPS but noted that further work would be required to determine if any of them are specifically pain related. Our expectation was that the most likely pain related behaviours would be headshaking, vocalisation and escape behaviour. In general, administration of butorphanol had no effect on the type and patterning of behavioural responses during LAPS compared to control birds, but there were differences in behaviour latencies, counts and durations. While bout durations and frequencies of some behaviours were affected by analgesic, total durations were generally unaffected except for vigilance, standing and leg paddling.

Pain related behaviour in birds has been previously identified in a variety of contexts, and includes active escape/withdrawal, guarding, sick bird posture, freezing and vocalisation (Gentle, 2011; Paul-Murphy, 2013). Since these responses were not seen during LAPS, this

study presents an opportunity to use analgesic intervention to identify potential pain related behaviour. There is a danger that using the effects of analgesic treatment on behaviour to recognise pain becomes a circular argument (i.e. pain is something removed by an analgesic; an analgesic is something which removes pain; Bateson, 1991). It is also important to note that analgesic drugs may have behavioural effects unrelated to pain and nociception. The analgesic applied in this study was potentially optimal, systemic and centrally acting with proven effectiveness in clinical contexts (Paul-Murphy, 2013). Butorphanol has been shown to have high bioavailability following IM administration in psittacines and raptors (Guzman et al., 2011; Gustaven et al., 2014), though Paul-Murphy (2013) notes that dosage of butorphanol for effective analgesia needs to be balanced with sedation and respiratory depression, which may vary between avian species.

Latencies to ataxia, mandibulation and deep inhalation were slightly delayed by analgesic treatment, however the duration of ataxia and other behaviours related to loss of consciousness were unaffected. These delayed initial responses raise the question of whether butorphanol had a sedative effect. Previous work administering butorphanol IM to Kestrels at 1, 3 or 6 mg/kg did not change mean sedation-agitation scores, except in at 6mg/kg 1.5 hours after injection (Guzman et al., 2014), but responses to this compound are likely to be species specific (Paul-Murphy, 2013). Possible sedation effects of the analgesic are not supported by results showing that analgised birds spent more time vigilant at the start of the LAPS cycle, and the latency to become vigilant was unaffected by treatment. In some species such as dogs (Hofmeister et al*.,* 2006) butorphanol can produce side effects such as dysphoria where the animals appear agitated and disorientated. This could provide an explanation for some of the differences in behaviour seen, but such dysphoric effects have not been reported in birds (Hawkins, 2006).One of the most obvious candidates for pain related behaviour during LAPS is headshaking, which has been previously associated with disorientation, discomfort, respiratory distress (Webster and Fletcher, 2001) or arousal (Hughes, 1983). Nicol et al., (2011) found that head shaking may be a valid indicator of a

417 less preferred environment and high rates of head shaking may indicate poor welfare. Only
418 around half of birds showed this behaviour (as reported previously, Mackie and McKeegan,
419 2016) and the proportion of birds exhibiting the behaviour was unaffected by treatment; in
420 fact its frequency was increased in birds receiving analgesia. This does not fit with it being
421 pain related behaviour abolished by analgesia, and it is possible that the observed increase
422 may be related to dysphoria and/or a sensation of disorientation. Headshaking is also a
423 behaviour that is routinely seen in controlled atmosphere stunning of chickens with both inert
424 and hypercapnic gas mixtures (McKeegan et al 2007; Abeyesinghe et al 2007). Interestingly,
425 birds receiving analgesia spent more time standing at the start of the LAPS cycle. While
426 none of the birds was obviously lame, several sources of leg pain may be present and these
427 may have been relieved by butorphanol in treated birds.
428
429 Administration of butorphanol has been shown to cause lowering of the heart rate, tidal
430 volume, and inspiratory and expiratory times in psittacines (Curro et al., 1994), but such
431 opioid side effects appear to be less pronounced in chickens (Concannon et al., 1995). In
432 contrast to previous work describing behavioural responses to LAPS (Mackie and
433 McKeegan, 2016), in this study we attempted to distinguish between deep inhalation and
434 open bill breathing. Analgesic treatment was associated with a delayed latency to deep
435 inhalation and increased duration of open bill breathing bouts (but not total duration). These
436 differences suggest that there were some physiological side effects of the drug which
437 affected the response to hypobaric hypoxia, possibly due to respiratory depression. While
438 53% of birds performed deep inhalation behaviours, 77 to 83% of birds exhibited open bill
439 breathing and similar responses have been seen in response to controlled atmosphere
440 stunning using hypoxic gas mixtures (e.g. McKeegan et al., 2007) suggesting they probably
441 relate to anoxia.
442
443 A wide range of behavioural responses were seen in all birds, with a few (e.g. standing)
444 exhibited only in a small proportion of birds and were generally unaffected by treatment.

Exceptions to this were slow wing flapping and jumping, both behaviours associated with ataxia and loss of posture. Fewer birds receiving analgesia showed jumping (20 compared to 27) and slow wing flapping behaviour (20 compared to 31) compared to controls, which suggests these may be pain related. The latencies of these behaviours show that they occurred, on average, after the onset of ataxia and they did not appear to be escape behaviours. The results may reflect a smoother induction to unconsciousness in analgised birds, with butorphanol possibly having an effect similar to a premedication.

No panting behaviour was shown and only 4 birds vocalised, although it was apparent that the three of the vocalisations may have been unconscious forced exhalation by the birds due to simultaneous vigorous wing flapping and clonic convulsions as all but one vocalisation was observed after loss of jaw tone, ataxia and loss of posture had occurred, suggesting the birds were no longer conscious (McKeegan *et al*., 2013; Sandercock *et al*., 2014; Martin, 2015).

There were some effects of temperature and humidity but many of the underlying correlations were not significant. The LAPS system operates a series of decompression curves according to ambient temperature, and these have been previously shown to affect some behaviour latencies and durations (Mackie and McKeegan, 2016). In this study, only one curve was applied so determining the effects of ambient temperature and humidly was not our aim. Bird weight effects on ataxia, mandibulation, open bill breathing and deep inhalation were apparent in the current study, but a more powerful factorial study would be needed to investigate these relationships further.

5. Conclusion

There are few studies on the side effects of butorphanol in chickens, which limits our ability to draw firm conclusions from this study. Another obvious limitation is the lack of a positive control and thus any conclusions depend on acceptance of the fact that butorphanol is an

effective analgesic in chickens. Apart from the ethical concerns raised by deliberate induction of pain, it is not clear what sort of pain model would be relevant to this study. With these limitations in mind, it may still be argued that the results do not provide convincing evidence that birds undergoing LAPS are experiencing pain. While there were effects of analgesia on some aspects of behaviour, and jumping and slow wing flapping was reduced, these effects may be explained by the potential sedative, dysphoric and physiological side effects of butorphanol. In particular, obvious pain related behaviours such as escape/withdrawal and freezing were not seen at all, while others such as head-shaking and vocalisation were not reduced with analgesic intervention during LAPS. EEG data (McKeegan *et al*., 2013; Martin *et al*., submitted) demonstrates the maintenance of slow wave EEG patterns induced by darkness in the early part of LAPS (while birds are still conscious); desynchronisation of the EEG resembling 'waking' from sleep would be expected during aversive or painful stimulation (Gentle, 1975). These findings support the notion that during the period of the gradual reduction of pressure in LAPS the behavioural responses seen are primarily related to exposure to hypoxia rather than hypobaric conditions. The patterns of behaviour are also similar to those seen in normobaric hypoxia using inert gases, and thus in terms of welfare, this stunning method could be considered to be equivalent to controlled atmosphere stunning with inert gases.

Acknowledgements

This study was funded by TechnoCatch LLC. The funders had no role in the study design, data interpretation or publication. We thank David Pritchard for discussions and comments on the draft paper.

References

Abeyesinghe, S.M., McKeegan, D.E.F., McLeman, M.A., Lowe, J.C., Demmers, T.G.M., White, R.P., Kranen, R.W., van Bemmel, H., Lankhaar, J.A.C., and Wathes, C.M., 2007. Controlled atmosphere stunning of broiler chickens. I. Effects on behaviour,

physiology and meat quality in a scale system at a processing plant. Br. Poult. Sci., 48, 406 – 423.

AVMA, 2013. Guidelines for the Euthanasia of Animals: 2013 Edition American Veterinary Medical Association ISBN 978-1-882691-21-0 https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

Bateson, P., 1991. Assessment of pain in animals. Anim. Behav., 42, 827–839.

Battula, V., Schilling, M.W., Vizzier-Thaxton, Y., Behrends, J.B., Williams, J.B., and Schmidt, T.B., 2008. The effects of low atmosphere stunning and deboning time on broiler breast meat quality. Poult. Sci., 87, 1202–1210.

Concannon, K.T., Dodam, J.R., and Hellyer, P.W., 1995. Influence of a mu- and kappa-opioid agonists on isoflurane minimal anaesthetic concentration in chickens. Am. J. Vet. Res., 56, 806-811.

Curro, T.G., Brunson, D.B., and Paul-Murphy, J., 1994. Determination of the ED50 of isoflurane and evaluation of the isoflurane-sparing effect of butorphanol in cockatoos (Cacatua spp.). Vet Surg., 23, 429-433.

Cheek, H., and Cattarazzi, B., (inventors and assignees), 2010. United States Process Patent 7662030. Method for humanely stunning and slaughtering poultry using controlled low atmospheric pressure. Feb. 16, 2010.

Close, B., Banister, K., Baumans, V., Bernoth, E., Bromage, N., Bunyan, B., Erhardt, W., Flecknell, P., Gregory, N., Hackbarth, H., Morton, D., and Warwick, C., 1996. Recommendations for euthanasia of experimental animals: Part 1.

Lab. Anim., 30, 293-316

Cobb-Vantress, 2012. Cobb 500TM Performance and nutrition supplement manual. 2nd edition.

Coenen, A.M.L., Lankhaar, J., Lowe, J.C., and McKeegan, D.E.F., 2009. Remote monitoring of electroencephalogram, electrocardiogram, and behavior during controlled atmosphere stunning in broilers: implications for welfare. Poult. Sci., 88, 10-19.

528 European Food Safety Authority, 2004. Welfare aspects of the main systems of stunning and
529 killing the main commercial species of animals. EFSA J., 45, 1–29.
530 European Food Safety Authority, 2013. AHAW Panel (EFSA Panel on Animal Health and
531 Welfare), Guidance on the assessment criteria for studies evaluating the effectiveness
532 of stunning interventions regarding animal protection at the time of killing. EFSA J.,
533 2013, 11, 3486, 41 pp. doi: 10.2903/j.efsa.2013.3486.
534 Erhardt, W.H., Gehra, M., Schaefer, M., Brill, T., and Henke, J., 1996. CO2-Betaeubung zur
535 Schlachtung von Puten. Dtsch Tierarztl Wochenschr., 103, 62-64.
536 Gentle, M., 1975. Using arousal changes in the electroencephalogram to measure taste
537 sensitivity in the chicken. J Physiol., 244, 9-10.
538 Gentle, M.J., and Tilston, V.L., 2000. Nociceptors in the legs of poultry: implications for
539 potential pain in preslaughter shackling. Anim. Welf., 9, 227-236.
540 Gentle, M.J., 2011. Pain issues in poultry. Appl. Anim. Behav. Sci., 135, 232-258.
541 Gerritzen, M.A., Lambooij, E., Hillebrand, S.J.W., Lankhaar, J.A., and Pieterse, C., 2000.
542 Behavioral responses of broilers to different gaseous atmospheres. Poult. Sci., 79,
543 928–933
544 Gustavsen, K.A., Guzman, D.S., Knych, H.K., Petritz, O.A., Olsen, G.H., and Paul-Murphy,
545 J.R., 2014. Pharmacokinetics of buprenorphine hydrochloride following intramuscular
546 and intravenous administration to American kestrels (Falco sparverius). Am. J. Vet.
547 Res., 75, 711-5. doi: 10.2460/ajvr.75.8.711.
548 Guzman, D.S., Flammer, K., Paul-Murphy, J.R., Barker, S.A., and Tully, T.N., 2011.
549 Pharmacokinetics of butorphanol after intravenous, intramuscular, and oral
550 administration in Hispaniolan Amazon parrots (Amazona ventralis). J. Avian Med.
551 Surg., 25,185-91.
552 Guzman, D.S., Drazenovich, T.L., KuKanich, B., Olsen, G.H., Willits, N.H., and Paul-Murphy,
553 J.R., 2014. Evaluation of thermal antinociceptive effects and pharmacokinetics after
554 intramuscular administration of butorphanol tartrate to American kestrels (Falco
555 sparverius). Am. J. Vet. Res., 75,11-8. doi: 10.2460/ajvr.75.1.11.

Hawkins, M.G., 2006. The Use of Analgesics in Birds, Reptiles, and Small Exotic Mammals. J. Exot. Pet Med., 15, 177-192.

Hawkins, M.G., Paul-Murphy, J.R., 2011. Avian analgesia. Vet. Clin. North. Am. Exot. Anim. Pract., 14, 61-80. doi: 10.1016/j.cvex.2010.09.011.

Hoenderken, R.B., Lambooij, B., van den Bogaard, A.E.J.M., and Hillenbrand, S.J.W., 1994. Tierschutzgerechte Gasbetaeubung von Gefluegel. Fleischwirtschaft.,74, 497-500.

Hofmeister, E.H., Herrington, J.L., and Mazzaferro, E.M., 2006. Opioid dysphoria in three dogs. J. Vet. Emerg. Crit. Care (San Antonio), 16, 44-49. doi:10.1111/j.1476-4431.2005.04022.x

Hocking, P., Gentle, M., Bernard, R., and Dunn, L., 1997. Evaluation of a protocol for determining the effectiveness of pretreatment with local analgesics for reducing experimentally induced articular pain in domestic fowl. Res. Vet. Sci., 63, 263-267.

Hughes, B.O., 1983. Head shaking in fowls: the effect of environmental stimuli. Appl. Anim. Ethol., 11, 45-53.

Johnson, C.L., 2013. A review of bird welfare during controlled atmosphere and electrical water-bath stunning. J. Am. Vet. Med. Assoc., 245, 60-68.

Joseph, P., Schilling, M.W., Williams, J.B., Radhkishnan, V., Battula, V., Christensen, K., Vizzier-Thaxton, Y., and Schmidt, T.B., 2013. Broiler stunning methods and their effects on welfare, rigor mortis, and meat quality. World's Poult. Sci. J., 69, 99-112, http://dx.doi.org/10.1017/S0043933913000093.

Lambooij, B., Gerritzen, M.A., Engel, B., Hillebrand, S.J.W., Lankhaar, J., and Pieterse, C., 1999. Behavioural responses during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci., 62, 255-265. http://dx.doi.org/10.1016/S0168-1591(98)00214-7.

Mackie, N., and McKeegan, D.E.F., 2016. Behavioural responses of broiler chickens during Low Atmospheric Pressure, Appl. Anim. Behav. Sci., 174, 90-98.

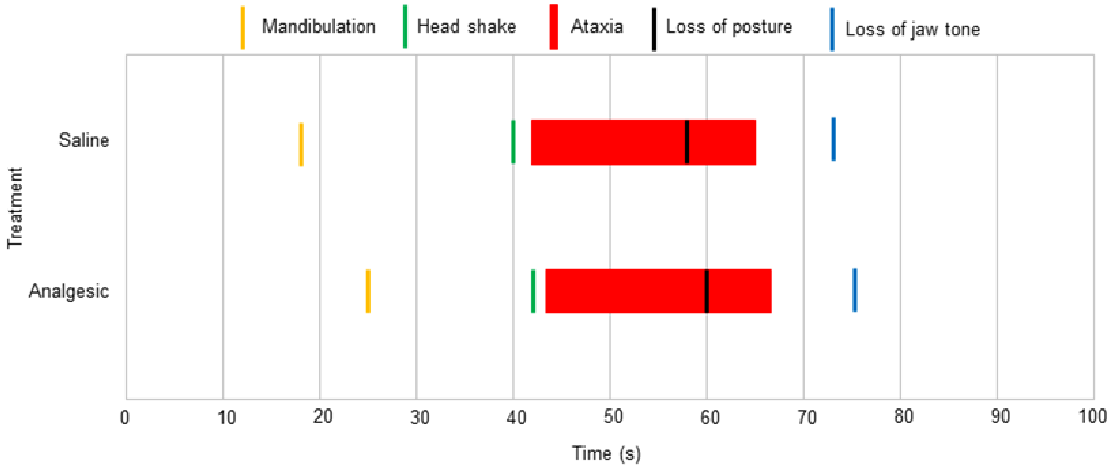
Martin, J.E., 2015. Humane mechanical methods to kill poultry on-farm. PhD Thesis, University of Glasgow, United Kingdom.

584 Martin, J.E., Christensen, K., Vizzier-Thaxton, Y., Mitchell, M.A., and McKeegan, D.E.F.,
585 2016. Behavioural, brain and cardiac responses to hypobaric hypoxia in chickens.
586 Physiol. Behav., (in press).
587 Martin, P., and Bateson, P., 2007. Measuring Behaviour: An Introductory Guide, third ed.
588 Cambridge University Press, Cambridge, United Kingdom.
589 McKeegan, D.E.F., McIntyre, J.A., Demmers, T.G.M., Lowe, J.C., Wathes, C.M., Broek,
590 P.L.C., Coenen, A.M.L., and Gentle, M.J., 2007. Physiological and behavioural
591 responses of broilers to controlled atmosphere stunning: implications for welfare. Anim.
592 Welf.,16, 409-426.
593 McKeegan, D.E.F., Sandercock, D.A., and Gerritzen, M.A., 2013. Physiological responses to
594 low atmospheric pressure stunning and the implications for welfare. Poult. Sci., 92,
595 858-868.
596 Nicol, C.J., Caplen, G., Statham, P., and Browne, W.J., 2011. Decisions about foraging and
597 risk trade-offs in chickens are associated with individual somatic response profiles.
598 Anim. Behav., 82, 255–262.
599 Paul-Murphy, J., and Fialkowski, J., 2001. Injectable Anaesthesia and Analgesia of Birds. In:
600 Recent Advances in Veterinary Anaesthesia and Analgesia: Companion Animals,
601 R.D. Gleed and J.W. Ludders (Eds.) Publisher: International Veterinary Information
602 Service (www.ivis.org), Ithaca, New York, USA.
603 Paul-Murphy, J., 2013. Pain Management. Chapter 8 In: Clinical Avian Medicine Volume 1
604 http://avianmedicine.net/content/uploads/2013/03/08_pain_management.pdf
605 Purswell, J.L., Thaxton, J.P., and Branton, S.L., 2007. Identifying process variables for a low
606 atmospheric pressure stunning-killing system. J. Appl. Poult. Res., 16, 509-513. doi:
607 10.3382/japr.2007-00026
608 Raj, A.B.M., Gregory, N.G., and Wotton, S.B., 1991. Changes in the somatosensory evoked
609 potentials and spontaneous electroencephalogram of hens during stunning in argon-
610 induced hypoxia. Br. Vet. J., 147, 322-330

611 Raj, A.B.M., and Gregory, N.G., 1990. Investigation into the batch stunning/killing of chickens
612 using carbon dioxide or argon-induced hypoxia. Res. Vet. Sci., 49, 364-366
613 Raj, A.B.M., 2006. Recent developments in stunning and slaughter of poultry. World's Poult.
614 Sci. J., 62, 467-48. DOI: http://dx.doi.org/10.1017/S0043933906001097.
615 Rutherford, K.M.D., 2002. Assessing Pain in Animals. Anim. Welf., 11(1): 31-53.
616 Morton. D,, Barton-Gade, D., Fernandez, X., Lambooij, B., Raj, M., and Troeger, D., 1998.
617 The use of Mixtures of the Gases CO2, O2, and N2 for Stunning or Killing Poultry -
618 Report of the Scientific Committee on Animal Health and Animal Welfare adopted 23rd
619 June 1998 available at http://ec.europa.eu/food/fs/sc/scah/out08_en.html
620 Sandercock, D.A., Auckburally, A., Flaherty, D., Sandilands, V., and McKeegan, D.E.F.,
621 2014. Avian reflex and electroencephalogram responses in different states of
622 consciousness. Physiol. Behav., 153, 252-289.
623 Sparrey, J.M., and Kettlewell, P.J., 1994. Shackling of poultry: is it a welfare problem?
624 World's Poult. Sci. J., 50, 167-176.
625 Vizzier-Thaxton, Y., Christensen, K.D., Schilling, M.W., Buhr, R.J., and Thaxton, J.P., 2010.
626 A new humane method of stunning broilers using low atmospheric pressure. J Appl
627 Poult Res., 19, 341-348. doi: 10.3382/japr.2010-00184
628 Walker, K.A., Duffield, K.A., Weary, D.M., 2013. Identifying and preventing pain during and
629 after surgery in farm animals. Appl. Anim. Behav. Sci., 135, 259– 265.
630 Webster, A.B., and Fletcher, D.L., 2001. Reactions of laying hens and broilers to different
631 gases used for stunning poultry. Poult. Sci., 80, 1371-1377.
632 Woolley, S.C., and Gentle, M.J., 1988. Physiological and behavioural responses of the
633 domestic hen to hypoxia. Res. Vet. Sci., 45, 377-382.
634 Van Liere, E.J., 1942. Anoxia. Its Effect on the Body. Chicago, University of Chicago Press,
635 United States, 1-269
636 *Zeller, W., Mettler, D., and Schatzmann, U., 1988. Untersuchungen zur Betaeubung des*
637 *Schlachtgefluegels mit Kohlendioxid. Fleischwirtschaft., 68, 1308-1312.*
638
639 **Figure captions**

640

641 **Figure 1** Mean latencies and durations (ataxia only) and the relationship in time of key
642 behaviours related to loss of consciousness during LAPS in saline and analgesic
643 treated birds.



644

645

646

647 Tables

648 **Table 1** Ethogram of bird behaviours during LAPS cycle.

Behaviour	Description	Measures
Vigilance	Alert movements of the head, including 'Notice' as defined by Mackie and McKeegan (2016).	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	Duration Latency

	second.	
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 Summary statistics (mean, SE, minimum and maximum) of behavioural total durations of bouts and individual bouts during LAPS and statistical differences (F statistic and P value) dependent of A/S treatment. Values within a row with different superscripts differ significantly at *p<0.05.*

Measure	Behaviour	N	Analgesic (A)				Saline (S)				*F*	*P*
			Mean	SE	Min	Max	Mean	SE	Min	Max		
Total duration (combined bouts) (s)	Ataxia*	84	23.7	1.7	4.4	65.2	23.5	1.6	3.7	53.0	0.00	0.872
	Leg paddling	65	9.1[a]	1.2	0.3	33.1	6.8[b]	0.8	1.1	17.5	4.04	0.048
	Clonic convulsions	84	23.9	1.7	5.3	65.2	24.2	1.6	3.6	55.7	0.03	0.866
	Tonic convulsions	64	12.6	4.7	2.9	152.9	7.8	0.9	0.1	19.8	1.87	0.175
	Slow wing-flapping	51	2.7	0.3	0.5	6.8	2.8	0.5	0.2	10.8	0.29	0.590
	Sitting	84	60.4	3.7	15.6	168.9	59.0	2.3	37.4	135.9	0.25	0.618
	Standing	22	16.0[a]	4.2	0.9	51.9	12.3[b]	2.9	0.6	20.6	19.39	<0.001

Individual bout duration (s)	Lying	82	76.4	3.0	23.4	120.2	79.1	3.4	23.2	129.6	0.19	0.661
	Open-bill breathing	63	11.0	1.3	3.0	36.4	10.8	1.2	1.8	40.5	0.10	0.716
	Vigilance	83	37.1[a]	1.6	9.6	57.9	30.5[b]	2.0	6.5	50.1	14.50	<0.001
	Leg paddling	65	6.5	0.7	0.3	17.8	4.8	0.6	1.1	17.5	0.15	0.700
	Clonic convulsions	84	7.8	0.4	3.4	15.5	8.8	0.7	1.6	23.5	0.07	0.789
	Tonic convulsions	64	10.9[a]	4.8	3.0	152.9	6.3[b]	0.7	0.1	16.1	7.59	0.007
	Slow wing-flapping	51	3.6[a]	0.4	0.5	6.7	2.1[b]	0.2	0.2	6.3	6.09	0.016
	Sitting	84	51.0	4.1	7.8	168.9	53.2	3.2	18.6	135.9	2.23	0.139
	Standing	22	13.1[a]	3.8	0.9	51.9	7.5[b]	3.0	0.6	20.6	15.65	<0.001
Frequency of bouts	Lying	82	72.7[a]	3.9	11.7	120.2	75.2[b]	4.2	11.6	129.6	18.53	<0.001
	Open-bill breathing	63	6.8[a]	0.6	3.0	18.2	8.2[b]	0.7	1.0	18.1	7.85	0.006
	Vigilance	83	32.7[a]	2.2	3.2	57.9	26.2[b]	2.5	1.7	50.1	5.62	0.020
	Leg paddling	65	1.4	0.1	1.0	4.0	1.2	0.1	1.0	2.0	2.55	0.114
	Clonic convulsions	84	1.1	0.1	1.0	2.0	1.1	0.1	1.0	2.0	0.02	0.890
	Tonic convulsions	64	1.1[a]	0.1	1.0	2.0	1.0[b]	0.0	1.0	1.0	6.10	0.016
	Slow wing-flapping	51	1.3	0.1	1.0	2.0	1.5	0.1	1.0	4.0	1.50	0.225
	Sitting	84	3.1	0.2	1.0	7.0	3.1	0.2	1.0	7.0	0.02	0.881
	Standing	22	1.4	0.1	1.0	4.0	1.3	0.1	1.0	3.0	0.81	0.370
	Lying	82	1.2[a]	0.1	1.0	3.0	1.3[b]	0.1	1.0	3.0	4.41	0.039
	Open-bill breathing	63	1.4	0.1	1.0	3.0	1.6	0.2	1.0	5.0	2.95	0.090
	Vigilance	83	1.6[a]	0.1	1.0	3.0	1.3[b]	0.1	1.0	3.0	5.60	0.020

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

Table 3 Summary statistics (mean, SE, minimum and maximum) of behavioural latencies during LAPS and statistical differences (F statistic and P value) dependent of treatment. Values within a row with different superscripts differ significantly at $P<0.05$.

Latency to behaviours (s)	**N**	**Analgesic (A)**				**Saline (S)**				***F***	***P***
		Mean	**SE**	**Min**	**Max**	**Mean**	**SE**	**Min**	**Max**		

Ataxia	84	44.5[a]	1.3	21.4	65.2	41.8[b]	1.0	28.4	53.0	4.76	0.032
Loss of jaw tone	65	75.1	1.7	61.6	105.1	72.5	1.9	42.1	98.0	0.80	0.375
Motionless	84	144.7	2.5	105.9	180.3	144	2.9	86.1	185.3	0.03	0.870
Leg paddling	65	98.9	3.4	63.4	143.0	92.6	4.3	43.8	155.0	3.93	0.051
Clonic convulsions	85	76.1	2.6	57.2	137.4	78.2	3.3	44.8	147.6	0.49	0.486
Tonic convulsions	64	121.7	3.3	88.5	160.5	119.1	3.5	71.3	163.8	0.23	0.630
Slow wing-flapping	51	64.7	2.5	42.4	91.6	60.8	2.5	6.1	87.5	0.49	0.145
Loss of posture	83	59.6	1.1	46.8	73.8	57.2	0.9	44.0	70.0	3.09	0.083
Mandibulation	41	25.0[a]	3.2	3.6	72.4	18.8[b]	3.7	2.8	62.3	7.40	0.008
Head shake	51	42.1	5.4	7.3	107.1	40.5	3.4	6.5	72.4	0.03	0.869
Open-bill breathing	67	64.2	1.7	43.3	84.0	65.3	1.6	51.9	92.1	0.58	0.448
Deep inhalation	48	101.8[a]	5.1	55.2	141.4	82.5[b]	7.1	4.7	126.1	15.62	0.001
Vigilance	83	2.3	0.5	0.3	17.8	2.6	0.7	0.1	20.1	0.70	0.406
Vocalisations*	4	62.7	7.1	14.6	94.5	103.9	0.0	103.9	103.9	-	-

* No modelling possible for latencies for vocalisations (too few observations (N=4)).

Table 4 Summary statistics (mean, SE, minimum and maximum) of behavioural total counts during LAPS and statistical differences (F statistic and P value) dependent of A/S treatment. Values within a row with different superscripts differ significantly at $p<0.05$.

		Analgesic (A)[3]				Saline (S)[3]				*F* statistic	*P* value
Behaviour[1]	N[2]	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.		
Jump	83	0.5[a]	0.1	0.0	2.0	1.0[b]	0.1	0.0	3.0	10.93	0.001
Mandibulation	44	2.1[a]	0.4	1.0	12.0	2.7[b]	0.5	1.0	8.0	32.33	<0.001
Vocalisation	4	2.0	1.0	1.0	4.0	1.0	0.0	1.0	1.0	-	-
Head shake	51	2.4[a]	0.3	1.0	7.0	1.7[b]	0.2	1.0	5.0	8.69	0.004
Deep inhalation	48	2.0	0.3	1.0	8.0	1.9	0.2	1.0	5.0	1.39	0.241

Table 5 Frequency table demonstrating the proportions of birds which were observed performing (yes), or were not recorded (missing data) due to being out of sight, total number of birds (total) and the percentage of birds which performed the behaviour %).

Behaviour	Analgesic				Saline			
	Yes	Missing data	Total	%	Yes	Missing data	Total	%
Standing	14	2	43	33	8	3	40	20
Leg paddling	32	2	43	74	33	3	42	79
Clonic convulsions	43	2	43	100	42	3	42	100
Tonic convulsions	31	2	43	72	33	3	42	79
Slow-wing flapping	20	2	43	47	31	3	42	74
Notice	42	2	43	98	41	4	41	100
Mandibulation	23	2	43	53	18	4	41	44
Head shaking	23	2	43	53	28	4	41	68
Open-bill breathing	33	2	43	77	34	4	41	83

Deep inhalation	22	3	42	52	26	4	41	63
Jump	20	2	43	47	27	5	40	68
Vocals	3	2	43	7	1	4	41	2
Sitting	43	2	43	100	41	4	41	100
Lying	42	3	42	100	41	4	41	100
Motionless	43	2	43	100	41	4	41	100
loss of jaw tone	30	15	30	100	35	10	35	100
ataxia	43	2	43	100	41	4	31	100
LOP	43	2	43	100	40	5	40	100
Escape	42	3	42	100	42	3	42	100
Peck	42	3	42	100	42	3	42	100
Panting	42	3	42	100	42	3	42	100