Pathological consequences of Low Atmospheric Pressure Stunning in broiler chickens

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Abstract

Low Atmospheric Pressure Stunning (LAPS) is a novel approach to pre-slaughter stunning of chickens using progressive hypobaric hypoxia by the application of gradual decompression (280s cycle) according to a set of prescribed pressure curves. LAPS produces a non-recovery state. Concerns have been raised relating to the possible

pathological and welfare consequences of expansion of air in the body during LAPS. In a randomized trial we compared the gross pathology of broilers exposed to LAPS with a control group euthanized by intravenous injection of pentobarbital sodium (60 mixed sex broilers per treatment). The birds were exposed to each treatment in triplets and all birds were subject to necropsy examination to detect and score (1 to 5, minimal to severe) haemorrhagic lesions or congestion for all major organs and cavities (e.g. air sacs, joints, ears and heart) as well as external assessment for product quality (e.g. wing tips). Behavioural data (latency to loss of posture and motionless) and chamber cycle data (temperature, humidity, pressure and oxygen availability) confirmed that LAPS had been applied in a manner representative of the commercial process. All of the organs observed were structurally intact for both treatment groups. No lesions were observed in the external ears, oral cavity, tracheal lumen, crop, and air sacs of birds from either treatment group. There was no difference between treatments in the wingtips, nasal turbinates, thymus, biceps femoralis, and colon. Haemorrhagic lesions were observed in the calvaria, brains, hearts and lungs of both treatment groups, but lesions in these areas were more severe in the LAPS treatment group. It was not possible to distinguish between pathological changes induced by decompression or recompression. In the barbiturate group, more severe haemorrhagic lesions were observed in the superficial pectoral muscles as well as greater congestion of the infraorbital sinuses, liver, spleens, duodenum, kidneys and gonads. These findings provide evidence that LAPS did not result in distension of the intestines and air sacs sufficient to cause changes, which were grossly visible on post mortem examination. There was also no evidence of barotrauma in the ears and sinuses. The pathological changes observed in the barbiturate treatment were as expected based on barbiturate toxicity. LAPS appears to produce pathological changes by a variety of well-established mechanisms, and while these pathological data have limited value as welfare indicators, the results confirm that organ integrity was not compromised by the process.

Keywords: Hypobaric hypoxia; poultry; product quality; slaughter; animal welfare.

Implications

Responding to concerns that injuries may be caused as a result of gas expansion in body cavities during low atmospheric pressure stunning (LAPS) in chickens, this study shows that the process does not compromise organ integrity. The findings lend support to behavioural and physiological welfare assessments that LAPS is a humane system of controlled atmosphere stunning (CAS) for birds. LAPS shares with other types of CAS the welfare advantage that live birds are not inverted and shackled. LAPS also has the potential to replace other systems of stunning chickens such as electric waterbaths, and improve welfare at slaughter.

Introduction

Low Atmospheric Pressure Stunning 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 in the atmosphere to achieve a progressive hypobaric hypoxia (Martin *et al.*, 2016c). Similarly to CAS systems which utilise exposure to hypoxic and/or hypercapnic gas mixtures (Coenen *et al.*, 2009; McKeegan *et al.*, 2007), LAPS irreversibly stuns poultry in their transport crates (McKeegan *et al.*, 2013b; Martin *et al.*, 2016c), thus avoiding welfare concerns associated with the shackling of conscious poultry (Sparrey and Kettlewell, 1994; Gentle and Tilston, 2000), and ensuring all birds are stunned prior to exsanguination.

The welfare consequences of LAPS have been thoroughly examined in broilers in a series of studies (Martin *et al.*, 2016a, 2016b, and 2016c) involving assessment of behaviour, electroencephalogram (EEG) and electrocardiogram (ECG) responses. Collectively, these studies demonstrate that responses to LAPS are consistent and indicative of a process that is largely equivalent to CAS with anoxic gases. Recently, LAPS has been added to EU Council Regulation Number 1099/2009 on the Protection of animals at the time of killing, indicating that the process is considered to be a stunning method which can be performed without inducing avoidable fear, anxiety, pain, suffering and distress (European Council, 2018).

Decompression has the potential to cause adverse effects and clinical signs. Potential gross pathology can vary with the rate of decompression and final vacuum level (Bankcroft and Dunn, 1965; Bankcroft *et al.*, 1968; Catron *et al.*, 1984). The pressure curves used in LAPS produce slow decompression which minimises the risk of discomfort, pain and suffering during the period before the animal loses consciousness (Battula *et al.*, 2008; Purswell *et al.*, 2007; Vizzier-Thaxton *et al.*, 2010). By contrast, explosive decompression (less than one second to near vacuum), and rapid decompression (several seconds to very low vacuum) both produce extensive pathology due to severe organ damage and haemorrhages resulting from expansion of trapped gases in the heart, lung and brain in mammals (van Liere, 1943; Burch *et al.*, 1952; Booth, 1978). Rapid decompression to near vacuum (at altitude above 63,000 feet (19,202 m; Armstrong's line)) can also result in the production of bubbles in water vapour in blood and joint fluids known as ebullism (Murray *et al.,* 2013). These bubbles can cause mechanical damage to organs and tissues (Murray *et al.,* 2013). Ebullism is unlikely to occur in LAPS since the equivalent maximum altitude is well below Armstrong's line (Murray *et al.,* 2013; Cheek and Cattaruzzi, 2017). Rapid recompression from a low vacuum has also been shown to produce organ damage and hemorrhage, which may occur both before and after death (Booth, 1978). Loss of consciousness due to slow rates of decompression, such as those experienced by man during ascent in an unpressurised aircraft, is usually uneventful but can be associated with clinical signs such as tooth and middle ear pain, abdominal discomfort and joint pain, although these are more common with descent of aircraft rather than ascent. Decompression to around 21 kPa, which occurs in LAPS, is equivalent to ascent to about 11,950 m (39,200 feet) and produces hypoxic effects akin to ascent to such at altitudes in unpressurized aircraft (Woodrow and Webb, 2011). There is extensive literature on the pathology of effects of acute hypoxia in man and its role in myocardial and cerebral ischemia (Michiels, 2004). Acute hypoxia hasalso been evaluated in a variety of mammalian experimental models to determine, for example, its role in cerebral apoptosis (Nakajima *et al.*, 2000), and subendothelial oedema (Astrup 1972).

There is little published information on pathology of acute hypoxia in birds. Vizzier-Thaxton *et al.* (2010) grossly examined more than 10,000 birds following LAPS conducted in a crate in a commercial processing plant and found that wing tip damage was slightly greater than with electrical waterbath stunning. It was noted, however, that the wings of these birds could impact on the walls of the crate. No gross damage was seen in the lungs, liver, heart and intestines of 40 birds sampled following commercial treatment with LAPS in that study. Extensive studies reported that the meat quality of birds subject to LAPS was similar to the meat quality of birds subjected to electrical stunning methods (Schilling *et al.,* 2012).

There is only limited information on the effect of exposure to hypoxic and hypercapnic gas mixtures on the pathology, behaviour and neurological responses of birds with which to compare LAPS (European Food Safety Authority (EFSA), 2017). Sparks *et al.* (2010) reported on the pathology of 20 birds post exposure to stunning with 45% carbon dioxide. All had mild congestion of the breast muscles, and focal areas of haemorrhage were found on the lateral aspects of the hip, thigh or leg muscles of nine birds. The livers of the birds tended to be dark and congested, and the spleens were moderately enlarged. The lungs of all 20 birds were orange-pink in colour, and a small quantity of dark fluid was noted in the pleural spaces of four birds.

The clinical signs and pathology produced by decompression relate principally to hypoxia, although the effects of trapped gas expansion, decompression sickness (DCS) and ebullism may occur (Murray *et al.,* 2013). DCS can result when pressure is reduced in body fluids saturated with inert gas, which can occur due to exposure to high altitude or more commonly, as a result of return to atmospheric pressure following deep diving (Murray *et al.,* 2013). There is not a close relationship with the observation of gas bubbles in blood and onset of clinical symptoms and signs (Woodrow and Webb, 2011). DCS can occur in man in ascent to 11,000 m, but effects are not seen until about 10 to 15 minutes from start of ascent, and more commonly appear over a course of hours.

The aim of this study was to identify and assess the severity of pathological changes induced by LAPS in standard commercial slaughter-weight broilers (approximately 2.5-4.5kg). Our objective was to generate data to directly address concerns about organ integrity and potential barotrauma in the ears and sinuses during LAPS. Since slaughter-age broilers may have underlying pathologies, we employed a control group: broilers from the same flock euthanized by intravenous injection of pentobarbital sodium (BARB). Barbiturate overdose was chosen because it is recognised as a humane approach (American Veterinary Medical Association (AVMA) 2013 and 2016; European Council, 2018) and it does not cause hypoxia or organ distention. Gross pathology evaluations of specified organ systems focused on the detection and scoring of congestion or haemorrhage, which could indicate a lack of blood vessel integrity or other organ damage. We paid particular attention to body regions considered to be at risk of damage during decompression. The organs and body sites specifically examined in each bird included wingtips, ears, oral cavity, infraorbital sinus, nasal turbinates, tracheal lumen, crop, thymus, calvarium and bone trabeculae of the skull, brain, heart, spleen, liver, left and right lung, air sacs (cervical, interclavicular, thoracic and abdominal), kidneys, duodenum, pancreas, colon, bursa of Fabricius, gonad, and superficial pectoral muscle, biceps femoris, and the opened stifle joint.

Material and methods

Animals and husbandry

A total of 120 as hatched commercially bred broiler chickens (Ross 708) were randomly selected from a flock reared at Mississippi State University and moved to the premises of Technocatch (limited liability company (LLC)) in Kosciusko, Mississippi, United States at 45 days of age. The birds were wing-tagged and randomly assigned to 20 groups of 6 birds by a random number generator (Microsoft Excel). Post-mortem examinations of the gonads revealed that the male: female ratio was approximately 3:1, irrespective of treatment allocation (LAPS – 47 males, 13 females; and BARB – 45 males, 15 females). Bird weights were as expected for a U.S. commercial system; at the time of killing their mean weight was 3.65 ± 0.5 kg (range 2.4 – 4.8 kg). The birds were housed in 20 pens (1.22 m x 1.22 m), for 24 h before the trials. The birds were placed on clean wood shavings bedding, and had *ad-lib* access to water and standard commercial diet. The birds were kept under a 6 h darkness per 24 h lighting regime. Before undergoing LAPS, the birds were feed-restricted for 7-9 hours to mimic commercial practice, in which birds would be caught, transported and held in lairage.

Low atmospheric pressure stunning process

Technocatch LLC in Mississippi, United States, developed the LAPS system and the pressure curves applied by the process are patented (Cheek and Cattaruzzi, 2017). The LAPS chamber and its monitoring and control systems have been described previously (Holloway and Pritchard, 2017). The current study used a scaled-down research unit (as previously used in Martin *et al.* 2016a, 2016b and 2016c), which is identical to units used commercially, except for its reduced size and 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. Once started, the process can only be stopped in the event of an emergency. An infrared camera (130° camera with 18 infra-red illuminators, Model #RVS-507, RVS Systems) was fitted into the chamber to allow observation of the birds. The LAPS cycle takes exactly 280 s and consists of two phases. Firstly, the vacuum chamber pressure is reduced from atmospheric pressure to an absolute vacuum pressure of ~250 Torr (~33 kPa) in ~67 s. In the second phase, a sliding gate valve is partially closed, gradually reducing the effective pumping speed by 'choke flow', to a minimum chamber pressure of ~150 Torr (~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. Cold air is denser and therefore contains more oxygen than warm air, and birds respond differently to LAPS at different temperatures (Martin *et al.,* 2016a); therefore slightly different pressure reduction curves must be applied to achieve the same hypoxic effect under different ambient conditions, Based on ambient temperature, the computer control programme selects the appropriate pressure curves to ensure that all birds are irreversibly stunned. According to ambient temperature, one of six possible temperature settings was applied in this study (setting 1, applied between 20.7°C and 31.2°C). Within the LAPS chamber, the temperature, relative humidity, oxygen levels and the pressure profile of each cycle was recorded. LAPS was performed in complete darkness, in the same manner as it would be performed commercially (Martin *et al.*, 2016b).

Experimental procedure

Ethical approval for the study was granted by the University of Glasgow Veterinary Ethics Committee (Reference 06A17). Three birds per group were systematically randomized to receive either LAPS or barbiturate (BARB) interventions. The two different treatments (LAPS and BARB) were applied alternately to subgroups of 3 birds per pen (as allocated by wing tag number). Birds were placed in individual open boxes and subjected to antemortem inspection. Any clinical signs (such as dullness or depression, sneezing, coughing, swollen sinuses, conjunctivitis, injury, plumage quality and abnormal faeces) were recorded. Each group of three birds undergoing LAPS was placed in the chamber and the cycle was activated. The corresponding triplet of birds allocated to BARB were killed sequentially, starting at the beginning of the LAPS cycle. Procedures relating to euthanasia of birds using barbiturate injection were conducted by a licensed veterinarian. Pentobarbital sodium (Vet One® Euthanasia solution 390mg/ml, United States) was injected into the alar (wing) vein at a dose of 300 mg/kg. Speed of injection was sufficient to ensure rapid euthanasia. Death was verified by the absence of nictitating membrane reflex and lack of respiratory movement.

After the killing treatments were applied to each group of 6 birds, the birds were immediately inverted and hung on shackles and exsanguinated by severing the carotid artery and jugular veins, taking care not to damage the trachea or the oesophagus. Bleeding times ranged from 2-18 minutes (mean (±SD): LAPS = 9.8 ± 6.2 mins; BARB = 6.5 ± 5.9 mins). Birds remained on the shackle line until they were removed for necropsy.

Behavioural observations

Video footage of each LAPS run was recorded. During the trials, the birds were also watched in real time on a monitor to check for unexpected behaviour (none was seen). Subsequently, the video files were analysed by a trained single observer and the time from the onset of LAPS to loss of posture (inability to regain/maintain a controlled posture) and motionlessness (no discernible body or breathing movements) was recorded for each bird, where visible.

Necropsy assessments

The post-mortem examinations were carried out by two independent pathologists, board-certified by the American College of Poultry Veterinarians. Dead birds from each treatment group were randomly presented to the pathologists, who were blinded to the LAPS or BARB interventions. Necropsy was conducted within 30 minutes of LAPS birds being removed from the vacuum chamber or death following barbiturate overdose.

The organs and body sites specifically examined in each bird included wingtips, ears, oral cavity, infraorbital sinus, nasal turbinates, tracheal lumen, crop, thymus, calvarium and bone trabeculae of the skull, brain, heart, spleen, liver, left and right lung, air sacs (cervical, interclavicular, thoracic and abdominal), kidneys, duodenum, pancreas, colon, bursa of Fabricius, gonad, and superficial pectoral muscle, biceps femoris, and the opened stifle joint.

Lesions were scored on a scale of 0 to 5, with 0 representing normal; 1 minimal; 2 mild; 3 moderate; 4 marked; and 5 severe. More specifically, for haemorrhage in the lung, the lesions were scored as 0 for no haemorrhage; 1 for haemorrhage involving 1 to 20% of the parenchyma; 2 for involvement of 21 to 40%; 3 for involvement of 41 to 60%; 4 for involvement of 61 to 80%; and 5 for involvement of 81 to 100%. For haemorrhage into the calvarium, the lesions were scored based on a similar 20% incrementally increasing scale. For haemorrhage in the heart, lesions were scored as

0 for no haemorrhage; 1 for a small focus of myocardial and possibly epicardial petechial haemorrhage; 2 for multifocal petechiation of the myocardium/epicardium; 3 for moderate multifocal to confluent petechial to ecchymotic haemorrhage of the myocardium/epicardium; 4 for marked coalescing foci of haemorrhage; and 5 for severe coalescing to diffuse haemorrhage. For reddened joint fluid, lesions were scored as 0 for normal, clear, straw-coloured synovial fluid; 1 for mild pink discoloration of the synovial fluid; 2 for mild diffuse pink opacity of the synovial fluid; 3 for moderate diffuse pink to red opacity of the synovial fluid; 4 for marked red opacity of the synovial fluid; and 5 for dark red opacity consistent with haemorrhage into the joint.

Statistical analysis of results

The pathology data was recorded on scoring sheets and the raw data was transferred to Excel spreadsheets. Statistical analysis was carried out using Genstat (19^{th} Edition). Statistical significance was termed by a threshold of 5% probability based on F tests. A *P* value >0.05 and <0.10 was defined as a statistical trend. Statistical comparisons of pathological variables by treatment were conducted via Generalised Linear Mixed Models (GLMMs), using either the logarithm link function and Poisson distribution or the logit link function and binomial distribution. All models included pen number as a random effect, and bird weight, temperature and relative humidity as co-variates. All fixed effects were treated as factors (e.g. treatment and assessor) and classed as categorical classifications and all interactions between factors were included in maximal models.

Results

Cycle parameters

The mean vacuum pressure and rate of pressure change versus pumping times for the 20 completed LAPS runs showed a typical deviation of ± 1 Torr and ± 1 s (Figure 1). Ambient pressure was 101.9 to 104.3 kPa (764.0 to 766.70 Torr). Ambient temperature ranged from 20.7 ºC to 31.2 ºC with an average of 27.6 ºC. Relative humidity varied from 32.7% to 59.8% with an average of 39.9%. Figure 2 shows the mean temperature and humidity profile in the chamber during the LAPS runs, while Figure 3 illustrates the mean measured percentage oxygen and equivalent percentage oxygen corrected for chamber pressure and water vapour. The data show that the LAPS process was equivalent to those described in Holloway and Pritchard (2017) and the studies reported in Martin *et al.* (2016a, 2016b and 2016c).

Behaviour

Behavioural observations were obtained for 33/60 birds for loss of posture and 29/60 birds for time to motionless, as birds regularly went out of view or were obscured by other birds. Mean (±SE) values for loss of posture were 78 ± 11.7 s (range 56-106 s) and 206 ± 29.8 s (range 131-260 s) for motionless. These are as expected for this LAPS curve.

Pathology

No major ante mortem signs (e.g. wounds, injuries etc.) were reported in any birds prior to exposure to either treatment (BARB or LAPS); however minor bruising was noted in thirty birds from the BARB treatment group and twenty-seven in the LAPS treatment group. Ante mortem examination outcomes and assessor had no effect on the post mortem pathology results, for all measures and irrespective of treatment. The eardrums and all of the organs and tissues observed were structurally intact in both treatment groups. No lesions (i.e. all score 0) were observed in the external ears, oral cavity, tracheal lumen, crop, and air sacs of the birds from both treatment groups. Table 1 summarises the scores for the other organs. Evaluations of the wingtips, nasal turbinates, thymus, stifle joint, colon and duodenum yielded very similar scores for the two treatment groups and were not significantly different (Table 1).

While haemorrhagic lesions were observed in the calvaria, brains, hearts and lungs (left and right) of both treatment groups, lesions in these areas were more severe in the LAPS treatment group. Ninety-three percent, three percent and twenty-three percent of chickens in the LAPS treatment group had marked to severe haemorrhage of the calvarium (median (and interquartile range (IQR)) lesion scores: LAPS = 4 (4,4); BARB = 0 (0,0), $P < 0.001$), heart (median (and IQR) lesion scores: LAPS = 1 (1,2); BARB = 0 (0,0), $P < 0.001$) and lungs (left (L) and right (R) (median (and IQR) lesion scores: LAPS = (L) 1 (0,2) and (R) 2 (1,4.75) (; BARB = (L and R) 0 (0,0), $P<0.001$) respectively. Severe haemorrhage was not observed in these areas in the barbiturate treatment group. By contrast, more severe haemorrhagic lesions were observed in the superficial pectoral muscles of the barbiturate group compared with the LAPS treatment group (median (and IQR) lesion scores: LAPS = 0 (0,0); BARB = 0 (0,1), $P = 0.003$).

Congestion was more severe in the BARB treatment group compared with LAPS for the infraorbital sinuses (median (and IQR) lesion scores: LAPS = 0 (0,0); BARB = 0 (0,1), $P = 0.004$), liver (median (and IQR) lesion scores: LAPS = 0 (0,1); BARB = 2 (1,3), $P < 0.001$), spleen (median (and IQR) lesion scores: LAPS = 0 (0,1); BARB = 1 (0,2), $P < 0.001$), kidneys (median (and IQR) lesion scores: LAPS = 0 (0,0); BARB = 0

(0,1), *P* = 0.004), pancreas (median (and IQR) lesion scores: LAPS = 0 (0,0); BARB = 0 (0,1), *P* = 0.012) and gonads (median (and IQR) lesion scores: LAPS = 0 (0,1); BARB = 1 (0,1.75), *P* = 0.002).

Discussion

This study provides the first detailed pathological assessment of the effects of LAPS on broiler chickens. Based on behavioural observations (such as time to loss of posture) and LAPS cycle parameters (atmospheric pressure, temperature, relative humidity and oxygen availability) we can be confident that the results can be extrapolated to the commercial LAPS process (Vizzier-Thaxton *et al.,* 2010, Holloway and Prichard, 2017). We observed a range of ante mortem lesions that are often seen in healthy market weight broilers, including minor bruising. Bruising is commonly seen in broilers at slaughter and is a welfare and commercial issue (Northcutt *et al*., 2000). It may be a consequence of injury during transport but is also caused by handling and restraint. In gas stunning, bruises and injury resulting in haemorrhage in muscles and extremities such as wing tips occur due to impacts with the holding crates during clonic convulsions which occur following loss of consciousness (Abeysinghe *et al.,* 2007; McKeegan *et al.,* 2007). The post-mortem incidence of bruises and other injuries is very variable but appears more commonly in anoxic gas systems than those using carbon dioxide (Joseph *et al*., 2016). Gas stunning methods are associated with improved product quality (reduced muscle haemorrhages) but often higher wing tip injury compared to electrical stunning (Food Chain Evaluation Consortium (FCEC), 2008). LAPS also has been shown to improve product quality in relation to muscle haemorrhages but increased rates of wing tip damage have been reported (Schilling *et al.,* 2013). There are no detailed pathological studies of anoxic and hypercapnic gas stunning methods with which to compare our findings using LAPS. Sparks *et al*. (2010) found haemorrhages in several muscles and congestion of the lungs following exposure to lethal quantities of carbon dioxide during whole gassing for depopulation.

During LAPS, there is the possibility of intestinal expansion at lower pressure resulting in distention. Intestinal over-distention, could result in to discomfort and/or pain. Greenwald *et al*. (1969) reported under stimulated flight conditions that 50% of 18 human subjects who agreed to avoid passing gas, experienced abdominal fullness at a pressure of 230 Torr and some reported pain at pressures from 226 to 175 Torr. Although similar pressures are experienced by birds during LAPS direct comparison is difficult to infer as birds have a single coelomic cavity (unlike the separate thoracic and abdominal cavities of mammals) and have the ability to expel air. Speculating on the areas most likely to be affected in birds, the caeca (a blind-ended tube with a narrow outlet) is a candidate. The coprodeum and colon (rectum) may equalise more readily due its proximity with the environment. Whilst there is literature on generalised signs of pain in birds, there is no literature to inform us about the discomfort birds may experience during intestinal distention. Colic in other animals results in guarding behaviour including back-arching and increased attention directed to the affected area. None of these specific behaviours were seen during LAPS (Martin *et al.,* 2016a and 2016b), but we don't know what the equivalent behaviour would be in chickens. In this study there was no evidence of intestinal rupture or damage following LAPS, but there could be expansion with welfare implications without pathological consequences. It could be speculated that the extent of gut fill is a factor in the risk of gut distention causing pain, although this was not examined in this study. The feed withdrawal period used in this study would have produced flattened intestines with minimal premortal distension (Northcutt *et al*., 1997). In practice broilers are always slaughtered with minimal gut fill after a period of food withdrawal prior to catching and transportation (Zuidhof *et al.*, 2004), as emulated in the current study.

The use of scoring systems based on proportion of the organ affected is a widely accepted approach in field and experimental studies of pathology. However, it must be recognised that such measures are judgment based and thus subject to observer bias. In this study, bias was reduced by careful definitions, the use of experienced poultry pathologists and training on the day to ensure agreement on descriptions. Observer effects were included in all statistical models, and had no effect, suggesting minimal observer bias. The numerical scores used allowed for rapid assessment and recording but this was also accompanied by free text for additional observations such as woody breast, skin wounds and bruises. The results indicate that the definitions used were appropriate as a wide range of scores were recorded. Blinding of observers to LAPS or barbiturate treatment allowed valid conclusions to be drawn about the treatments applied. The use of the barbiturate control group not exposed to hypobaria was effective in controlling for pressure effects on intestines and air sacs allowing conclusions to be drawn about the possible effects of hypobaria causing distension of organs due to gas expansion. However, this control treatment did not allow us to determine the effects of normobaric hypoxia. During LAPS, the effects of hypoxia are confounded with effects of hypobaria as the treatment includes simultaneous application of both conditions.

After exposure to LAPS, the frequency of congestion and/or haemorrhagic changes was higher in the lungs, heart, brain and calvarium of birds compared with barbiturate treated birds. The pathogenesis of these changes is not clear and could include hypoxia, hypobaria, or both. The haemorrhages may be due to decompression. However, previous studies have reported severe haemorrhages only during rapid or explosive decompression (Booth, 1978; Edelman *et al.*, 1946). Recent reviews of acute hypobaric hypoxia in man relating to decompression to similar altitudes such as occur in LAPS (e.g. air flights or hypobaric chamber pilot training and mountain climbing) do not include information on risks for lung, cardiac or brain haemorrhage. West *et al.* (2007) in his review of conditions relating to longer-term exposure to attitude, such as mountain sickness, does note that this occasionally causes extensive haemorrhages at post mortem. During the period before death, depending on its rapidity and whether breathing is laboured, there is a reduction in the availability of oxygen, which leads to hypoxic changes in many tissues, which can result in hypoxia/agonal haemorrhage. The endothelium of blood vessels is affected and may leak blood which produces the petechial and ecchymotic haemorrhages which are commonly seen in recently killed animals. There is a large volume of literature on these haemorrhagic processes ante mortem, peri mortem and post mortem. There is a view common to human forensic pathologists (Seidl, 2005) and veterinary/avian pathologists (Lowenstein, 1986; McGlavin and Zachary, 2006; Munro and Munro, 2013) that such lesions relate to the process of dying and are not related to cause of death, so their value in relation to assessment of LAPS is limited. Agonal haemorrhage in birds is recognised by Graham (1984) and Lowenstein (1986) in their studies on necropsy of birds and it is noted that agonal haemorrhage of the calvarium may be observed in birds depending on the rapidity of death or method of euthanasia and may be difficult to distinguish from ante mortem head trauma.

The high prevalence of extensive haemorrhagic changes seen in the calvarium of LAPS treated birds appears to be the result of haemorrhage in the underlying trabeculae. Fewer birds had haemorrhages in the brain and they were all score 1 (<20% of organ affected). Possibly, this is trauma sustained during terminal motor activity (i.e. convulsions) but given its consistency this seems unlikely. The lesions were bilateral, sometimes small and sometimes more extensive and situated at junctions of the occipital, parietal and fontal bone, and the fontanel area. They were sited on the top of cranium, which is arguably the part the skull that would be the most exposed to trauma during convulsions. Since slaughter weight broilers are still young and growing quickly, they are at a developmental stage when the process of closure of the junction may be incomplete which may lead to increased sensitivity to trauma. The pathogenesis of the haemorrhagic lesions in the calvarium is likely to be multifactorial and may be due to direct effects of hypoxia on the blood vessels, or due to contusion from head injury during clonic convulsions. It should be noted that static pooling of blood post death might also contribute to the lesions seen at autopsy, but this should apply to both killing treatments. The physiological responses to hypoxia include the relaxation of smooth muscle in peripheral blood vessels including those of the brain and its surrounding structures resulting in their dilation (Hanser and Sander, 2003). Hypoxia can also directly affect metabolism of the endothelial cells and the integrity of their tight junctions, which may lead directly to extravasation of blood (Kaur and Ling, 2008).

Pathological changes were observed post barbiturate anaesthesia, but these are common in a wide range of mammals and birds (e.g. Grieves *et al.*, 2007). Kummerfeld *et al.* (2012) found that barbiturate artefacts such as pooling of blood in organs occurred less in birds than mammals and was reduced if birds were exsanguinated. Despite rapid exsanguination, the barbiturate group still exhibited congestion of the parenchymatous organs due to pooling of blood in organs such as the liver, spleen, pancreas, gonads and bursa of Fabricius. There was also some congestion of the intestines but there were no barbiturate effects on the air sacs. This allowed for a valid comparison with LAPS birds showing that no changes related to expansion of gases causing distension were seen these organs. The findings provide evidence that LAPS did not result in distension of the intestines and air sacs sufficient to cause changes, which were grossly visible on post mortem, examination. It is possible that birds with underlying pathological processes, especially respiratory disease, may respond differently to LAPS and other controlled atmospheric gas stunning systems. Evaluation of controlled atmospheric gas stunning in birds with underlying pathology was not the objective of this research, but could be evaluated in future studies.

The pathogenesis of the haemorrhagic and congestive changes seen in organs post LAPS is probably the outcome of a combination of processes. LAPS produces hypoxia resulting in loss of consciousness. At this stage of LAPS, tissue will be severely hypoxic and readily damaged by physical trauma. Exposure to low pressure may impact on the respiratory tract. In mammals, with closed lung systems, this could be via expansion of organs and cavities but this appears unlikely in the avian lung due to absence of a diaphragm, increased dead space and the complex two-way movements of gases. It should be noted that the effect of pressure to cause damage by the production of gas or water vapour bubbles (so called ebullism) requires significantly stronger and more rapid decompression and occurs over a longer time frame than the 280 seconds used for LAPS (Dunn *et al*., 1965).

LAPS appears to produce pathological changes by a variety of well-established mechanisms. Interpretation of the specific effects of LAPS (as opposed to normobaric hypoxia) is made difficult by the lack of any pathological data from birds undergoing controlled atmosphere stunning using gas. Further, because of the nature of the LAPS process, we also are not able to distinguish between pathology changes induced by decompression and recompression, which can have pathological consequences. While these pathological data provide useful information, their value as welfare indicators is limited - behavioural and physiological parameters are far more sensitive measures. Pain, for example, can occur without corresponding pathological consequences and in our previous work on LAPS we have focused on behavioural and physiological responses to assess welfare in period where consciousness is a possibility. These pathological findings demonstrate, however, that organs and cavities are intact following LAPS, providing reassurance that the process does not compromise organ integrity.

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Declaration of interest

The authors declare no conflict of interest.

Ethics statement

All animals in this study were treated according to the ethical standards of the participating countries' regulations. Ethical approval for the study was granted by the University of Glasgow Veterinary Ethics Committee (Reference code 06A17).

Software and data repository resources

The data sets and programs used in the current study are available from the corresponding author on reasonable request.

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Table 1 *Lesion score frequencies for all organs (broiler chickens (Ross 708)) where non-zero scores were obtained for both killing treatments (low atmospheric pressure stunning (LAPS) and intravenous injection of pentobarbital sodium (BARB). F tests and P-values refer to the outcome of Generalised Linear Mixed Models (GLMMs). NS indicates that there was no significant difference between treatments. N=60 in each treatment group.*

Organ/area evaluated	LAPS lesion score						BARB lesion score						F test	*P*-value
	0	1	2	3	4	5	0	1	2	3	4	5		
Wing tips	58	1	1	0	0	0	60	0	0	0	0	0	2.03	NS
Infraorbital sinus	50	9	1	0	0	0	39	15	2	4	0	0	8.50	0.004
Nasal turbinates	36	17	5	2	0	0	33	11	8	7	1	0	3.49	NS
Calvarium	0	2	1	1	42	14	58	2	0	0	0	0	45.69	<0.001
Brain	36	24	0	0	0	0	58	2	0	0	0	0	12.18	<0.001
Thymus	42	14	2	1	1	0	36	18	5	1	0	0	0.28	NS
Superficial Pectoral	59	1	0	0	0	0	44	13	1	2	0	0	8.75	0.003
Biceps femoralis	56	4	0	0	0	0	49	11	0	0	0	0	4.25	0.039
Stifle joint	53	2	2	0	0	0	58	1	0	1	0	0	0.56	NS
Liver	32	15	9	4	0	0	7	9	20	20	4	0	32.53	<0.001
Spleen	39	16	5	0	0	0	18	21	15	6	0	0	17.19	<0.001
Heart	12	29	13	4	2	0	50	8	2	0	0	0	32.74	<0.001
Left lung	23	15	10	2	3	7	49	11	0	0	0	0	38.79	<0.001

Right lung	10	19	10	4	2	15	47	13	0	0	0	0	45.75	<0.001
Duodenum	7	22	23	8	0	0	6	12	26	15	1	0	2.68	NS
Pancreas	50	10	0	0	0	0	41	15	3	1	0	0	6.46	0.012
Colon	39	20	1	0	0	0	31	22	7	0	0	0	2.99	NS
Bursa of Fabricius	35	22	3	0	0	0	28	16	6	10	0	0	4.49	0.036
Kidney	48	8	4	0	0	0	37	13	9	1	0	0	8.42	0.004
Gonad	33	21	5	1	0	0	0	29	6	9	0	0	9.80	0.002

Figure captions

Figure 1 Graphical illustration of low atmospheric pressure stunning (LAPS) chamber mean (± SE) total pressure (Torr) and rate of change of pressure (dP/dt) per second for the 20 LAPS cycles, each with three birds (broiler chickens (Ross 708)) at temperature setting 1.

Figure 2 Graph illustrating of low atmospheric pressure stunning (LAPS) chamber mean (± SE) temperature (ºC) and humidity (%) over time (seconds) for the 20 LAPS cycles each with three birds (broiler chickens (Ross 708)) at temperature setting 1.

Figure 3 Graph illustrating comparison of mean (± SE) measured % oxygen (O2) and equivalent % O2 corrected for low atmospheric pressure stunning (LAPS) chamber pressure and water vapour pressure in the chamber over time (seconds) from the 20 LAPS cycles, each with three birds (broiler chickens (Ross 708)) at temperature setting 1.