Blood Stress Hormonal Indicators and Meat Quality Attributes of Broiler Chickens Processed Using Electrical and Controlled Atmosphere Stunning Systems

by

Montana Rain Riggs

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 10, 2022

Keywords: poultry, stunning, stress,
meat quality, processing

Copyright 2022 by Montana Rain Riggs

Approved by

Dianna V. Bourassa, Chair, Associate Professor of Poultry Science
Rüdiger Hauck, Assistant Professor of Poultry Science
Bethany I. Baker-Cook, Assistant Professor of Poultry Science

ABSTRACT

Increased consumer concern for animal welfare has led some poultry producers to alter their stunning methods from electrical stunning (ES) to controlled atmosphere stunning (CAS). However, there is little peer-reviewed research available comparing the two methods within a commercial setting under U.S. parameters. We conducted two studies to evaluate both CAS and ES methods and their impact on circulating blood-stress hormones and meat quality aspects of broiler chickens within a commercial setting. As a brief explanation, our first experiment was conducted within a commercial facility, where blood samples were collected from broilers stunned by either CAS or ES at lairage, pre-stun, and post-stun. Two separate trials were conducted, Trial 1 having the same flock analyzed for each treatment, and Trial 2 with each treatment sample collected from birds of differing flocks. CORT, ACTH, EPI, and NOREPI concentrations were analyzed by ELISA. We observed that CORT decreased following ES in both Trials 1 and 2. In Trial 2 EPI increased post-stun. Neither ACTH nor NOREPI differed over time in either trial for ES birds. For CAS, CORT concentrations decreased post-stun in Trial 1, but did not differ in Trial 2. ACTH concentrations post-stun increased in Trial 1 but decreased in Trial 2. EPI and NOREPI concentrations did not differ over time for CAS birds. Based on these results, CORT, ACTH, EPI, and NOREPI did not respond in the same manner and trends differed between stunning methods. Results indicate that neither method of stunning was clearly preferable based on measurement of blood hormone indicators of a stress response. For our second study, occurrence of visible wing damage was evaluated post-defeathering and breast fillet meat quality was evaluated through measurement of pH, CIE-LAB values, and drip loss. Values were determined both at deboning and 24 hours after deboning. Blood glucose

concentrations (mg/dL) from CAS and ES birds differed only at post-stun, with glucose from birds stunned by CAS significantly higher than ES (418, 259, $P<0.0001$). Breast fillet quality did not differ between broilers stunned by either electrical or CAS. CAS carcasses had significantly more visible wing damage than ES carcasses (4.3%, 2.4%, $P<0.0001$). Drip loss did not differ between breast fillets of CAS or ES broilers. The implications of increased blood glucose concentration post-CAS are currently unknown and will require further evaluation. Increase in visible wing damage observed post-defeathering from CAS carcasses indicated a need for equipment parameter adjustments and further evaluation. Overall, the results of both studies indicate that there are some differences in blood-stress indictors at differing timepoint of either CAS or ES, as well as significantly more wing damage and glucose after CAS. Further evaluation is required to determine the exact reasoning behind these results.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to Auburn University, for offering an amazing learning space and the resources necessary to complete my research. I would also like to thank the staff and faculty of the Auburn Poultry Department and the Charles J. Miller Jr. Poultry Center for their efforts in my research, and in keeping the department successful. I am especially grateful to my advisor, Dr. Dianna Bourassa, for all the effort and help provided in ensuring the completion of this work. I would also like to recognize the opportunity Dr. Bourassa has given me to continue my education at Auburn for my Master's degree, and for the incredible guidance I received as her advisee. There has been no one else who has made this experience more pivotal for myself during my journey into academia.

I would like to thank Dr. Rüdiger Hauck and Dr. Bethany I. Baker-Cook for their willingness to serve on my Master's committee. They went beyond the expected and provided exceptional guidance, advice, and support throughout this process. I am so grateful for all they have taught me throughout this experience, as it is invaluable.

I am also grateful for the support received from the other graduate students and undergraduates who have helped complete this work; Andrea Urrutia, Marco Reina, Juan Figueroa, Rachel Osborne, Ally Jackson, and Amrit Pal. None of this would be possible without them. Beyond just their efforts in assisting me with my research, they have made the academic work environment a positive, supportive, and cohesive one.

I would also like to thank my friends and family, who have given me a supportive environment and a shoulder to lean on. Specifically, my mother Victoria Riggs, and my father Zebediah Riggs, who have also been supportive in every way possible. They encouraged me to

continue my studies and made sure that I was confident enough to do so. Without them, this would not have been possible, and I am so grateful that they were a part of this experience.

I would like to recognize the support given to me by my friends. A huge thank you goes to Teresa Turner, Emily Spann, Kristen Sink, Davis Butz, Kaicie Chasteen, Sam Sharma, Andrew Barnard, Rachel Smith, Sarah Hellmann, Emma Hellmann, Annie Hellmann, Daniel Paddock, Sevda Sadik, Tarik Sadik, Dean Khulusi, Kaitlin Meeks, Olivia Bachman, Luke Bartner, Geoff Bartner, and Scot O'Muilleoir. These individuals have made my time at Auburn an experience I couldn't have imagined to have. Without them this experience would be vastly different, and with them, it has been some of the greatest years I've come by. Their support, advice, and goodwill were priceless in terms of importance as a student.

Finally, I would like to state my gratitude towards my partner Andrew Bartlett, who has been nothing short of patient and supportive. A great thanks to him for being someone I know I could rely on for help, advice, guidance, and a simple shoulder to lean on when needed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SEPs	Somatosensory impulses
AC	Alternating current
DC	Direct current
CAS	Controlled atmosphere stun
ES	Electrical stun
LAPS^{TM}	Low atmospheric pressure stun
HPA	Hypothalamus-pituitary-adrenal
CRH	Corticotropin-releasing hormone
cAMP	Cyclic-adenomonophosphate
ACTH	Adrenocorticotropic hormone
CORT	Corticosterone
EPI	Epinephrine
NOREPI	Norepinephrine
SAM	Sympathomedullary
ATP	Adenosine triphosphate

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Consumer preference for humanely raised animal products has risen exponentially, with many willing to spend more money on products raised ethically (Spain et al., 2018). The United States poultry industry is one of the largest producers of poultry meat products, with over 9.2 billion broilers processed in 2020 (USDA, 2021). Processing is a main component of production where birds are transported at market age for slaughter, and upon arrival, go through initial processing. During this, there are multiple stages in which the bird must be taken through when alive. Broilers are first loaded into the plant's stunning system, which renders the bird unconscious. This is done prior to the neck cut for exsanguination to ensure painless death. For the stun to be considered successful two main sensory functions are temporarily lost: 1) the reticular activating system, and 2) somatosensory impulses (SEPs) (Raj and O'Callaghan, 2004a). The stunning of birds prior to slaughter is common practice within the poultry industry and is almost always implemented, with the exception of some religious slaughter practice facilities such as Halal or Kosher plants.

Various methods of stunning have become available to the poultry industry and continue to be modified and researched the ensure optimal poultry welfare. Currently most poultry processing plants in the United States utilize electrical water-bath stunning (Berg and Raj, 2015). This involves birds being live shackled by the processing plant employees, which means the birds have be removed from the transportation modules prior to entry to the plant. This step is then followed by the birds' heads being dipped into ionized water with electrical current. Electrical parameters are measured by the interactions of voltage (electrical force), amperes

(current), frequency (Hz), and resistance (Ohm) (Bilgili, 1992). Currents also may be defined as either alternating (AC) or direct (DC) and will have varying effects on the total electrical current applied to each bird. When the stun is successfully administered, the current applied results in immediate unconsciousness, and the duration of unconsciousness will last throughout the following process of exsanguination until death (Bilgili, 1992).

The parameters for an electrical stunning system vary due to legal restrictions placed by country. In the United States, stunning is not required but is commonly used for animal welfare and meat quality benefits, and typically utilizes a low voltage, high frequency (12–38 V, ≥400 Hz) electrical stun (Humane Methods of Slaughter Act, 1978; Ali et al., 2007). Successful electrical stunning induces near-instantaneous unconsciousness in broilers (< 1 second), ensuring no pain is felt during the subsequent neck-cut required for exsanguination (Lines et al., 2011).

There are some proposed alternative methods of stunning to electrical-waterbath stunning. One is controlled atmospheric stunning (CAS). This involves broilers being loaded into the stunning system within their transportation modules. Then, CAS involves an increase in carbon dioxide, argon, or nitrogen to create a hypoxic environment. In doing so, the change in atmospheric gases renders the birds unconscious. Typically, the gas utilized is carbon dioxide due to wide availability and lower cost. Multiple phases are found within the system with a variable increase of concentrations of hypercapnic and/or anoxic gases. Another alternative to electrical-waterbath stunning is hypobaric hypoxia, also known as low atmospheric pressure stunning (LAPS^{TM}). LAPS^{TM} is similar to CAS in that broilers are loaded into the stunning system within their transportation modules. Within the system the atmospheric pressure is gradually reduced by a vacuum, which leads to an overall reduction in oxygen, which induces unconscious by hypoxia. These methods are all utilized in the United States within commercial

poultry processing. While electrical-waterbath stunning is most commonly used, CAS has become the most common alternative method of stun, cited as a potentially more humane method (Gregory, 2005). CAS is also becoming utilized in the U.S. due to the Better Chicken Commitment, where many customers have agreed by 2024 to purchase only poultry meat products from birds stunned by CAS systems.

The electrical current utilized by the U.S. has been questioned in its efficacy. Wilkins et al. (1998) found that frequencies higher than 50 Hz (AC) had a shorter post-stun recovery time, which is critical for animal welfare as broilers should be unconscious during the neck-cut and bleed out for successful stunning practice. Raj and O'Callaghan (2004b) found similar results with frequencies of 400 Hz (AC) and higher, where broilers stunned under these parameters did not show epileptic brain activity or inactivation of somatosensory evoked potentials, indicating the possibility of sensibility to pain during bleed-out.

Further research has shown evidence of distress for birds when processed at an electrical stunning operation (Boyd, 1994; Erasmus et al., 2010). Birds being shackled while conscious through human contact heightens the severity of the stress response (Kannan et al., 1997). A study performed evaluating the different circulatory concentrations of stress indicators (plasma corticosterone, glucose, and heterophil:lymphocyte ratio) and found that shackling had provided the largest increase, indicating that this step is detrimental in terms of animal welfare (Bedanova et al., 2007). Stressors/Stimulus like live-shackling and tend to induce the fight-or-flight (autonomic) response (Whittow, 2000). Birds also have pain receptors within their legs (distal tarsometatarsus affected during poultry shackling) and the use of live shackling has been suggested to cause pain (Gentle and Tilson, 2000). Another concern for animal welfare in relation to electrical stunning is the occurrence of pre-stun shock. Pre-stun shock can occur

during electrical stun when a bird makes improper, premature contact with the ionized water-bath, typically with their wings. Pre-stun shock is often due to flock size variability, where height calibrations of the electrical stunning system are inadequate for smaller birds within the flock (Raj and O'Callahan, 2004b). This causes a shock and usually is followed by an adverse reaction of flapping and lifting of the head to avoid further discomfort. The 'raising head' reaction can lead to the bird missing the stun entirely, which leads to conscious animals receiving the neck-cut (Anastasov and Wotton, 2012). Consequently, recent adaptations in electrical stunning systems have implemented ramps and drop-points into the ionized water-bath, reducing the occurrence of pre-stun shock (Bilgili, 1999).

CAS has been introduced as an alternative to electrical water-bath systems within the United States due to these varying concerns of animal welfare. In the United States, carbon dioxide is the most commonly used gas for stunning. Carbon dioxide has also been found to lead to the least amount of carcass damage in comparison to the inclusion of other gasses, such as nitrogen or argon (McKeegan et al., 2007). For the purposes of this review, the only CAS systems discussed will involve carbon dioxide. The various concentrations of carbon dioxide define the three transitional periods of CAS: induction, transition, and completion. During the induction phase, the birds are exposed to a hypercapnic environment, with carbon dioxide levels between 20% and 40%. This phase lasts approximately two minutes. By the end of the induction period the birds are supposed to have lost posture, which is an indicator of loss of consciousness (Terlouw et al., 2016). The second phase, transition, is the shortest phase being only one minute in length. During transition the carbon dioxide is increased to 40% to 50%. Since a sudden large increase in carbon dioxide can create involuntary convulsions in the unconscious birds, possibly hindering meat quality, the transition phase is included to allow for incremental supplementation.

Finally, the completion phase is included for two minutes with carbon dioxide levels ranging from 65% to 85%. The time of exposure to this concentration of carbon dioxide ensures that: 1) the birds' involuntary, autonomic respiratory response is eliminated, and 2) the stun is irreversible (Grandin and Cockram, 2020). In the post-completion phase, the birds are no longer able to regain consciousness. This is done to guarantee unconsciousness for the neck cutting and exsanguination that immediately follows. This method of stun is considered advantageous towards animal welfare for multiple reasons. One being the reduction in human to bird contact; live shackling is not necessary for CAS birds because they proceed through the system within their transportation coops. The birds remain in their coops until they leave the CAS system and are then shackled while unconscious before exsanguination. Another reason CAS is regarded highly in terms of animal welfare is the lack of pre-stun shock mentioned previously, since there is no electricity involved.

However, there are drawbacks of CAS in terms of animal welfare that need further analysis. The immediate exposure of carbon dioxide during the induction phase typically results in adverse physical reactions from the birds. Gasping and shaking of the head are noted in high repetition during research trials (McKeegan et al., 2006) and are indicative of discomfort. This is potentially due to the reaction following rapid inhalation of elevated levels of carbon dioxide and water within the mucosal lining of the respiratory tract, creating carbonic acid (Anton et al. 1992). Respiration during exposure to high carbon dioxide concentrations causes an increase of both intracellular and extracellular acidosis, which is the primary factor for the loss of consciousness. However, acidosis within the mucosal membranes of poultry directly stimulates trigeminal nociceptor response (Gent et al., 2020). The trigeminal nociceptors of the somatic nervous system, which conduct the initial reception of discomfort to brain (anterior cingulate

cortex, thalamus and insula), are activated prior to unconsciousness (McKeegan et al., 2005), potentially indicating discomfort due to the carbonic acid produced during respiration during CAS – therefore, there is a potential for discomfort. Disorientation, breathlessness, and signs of general anxiety have also been noted as an adverse reaction to inhalation of CO_2 in poultry (Gent et al., 2020), and may be indicative of adverse effects upon animal welfare when utilizing CAS for stunning poultry.

Based on the above information, these findings have made CAS a questioned alternative of stunning, even with the current method of electrical stunning within many processing plants. The objective of this review is to compile the existing knowledge of key aspects of both electrical stunning and CAS, the potential biomarkers found to quantify a stress response of poultry from stunning, and the effect on each method's potential benefit for poultry meat quality:

1) Analyzing Circulatory Indicators of a Stress Response within the Blood – Hypothalamus-Pituitary-Adrenal Axis
2) Analyzing Circulatory Indicators of a Stress Response within the Blood – Sympathomedullary Pathway
3) Muscle Meat Quality Between Methods of Stunning
4) Physiological Responses from Stunning on Meat Quality

1.2 KEY ASPECTS OF BOTH CONTROLLED ATMOSPHERE AND ELECTRICAL STUNNING AND THEIR IMPACT UPON BOTH BROILER STRESS BIOMARKERS AND MEAT QUALITY

1. Analyzing Circulatory Indicators of Stress within the Blood – Hypothalamus-Pituitary-Adrenal Axis

When categorizing the per-acute stressors of both CAS and electrical stunning, they are considered indirect labile stressors of the environment; meaning these changes are unpredictable and only last seconds to minutes. Current research which quantifies physiological indicators of a poultry stress response in blood tends to exclude indirect labile stressors. Research performed comparing the neurological and endocrine responses of avian species during predictable long-term and unpredictable short-term changes are intrinsically different (Wingfield, 2013). Most studies identify the reactive scope of glucocorticoids and induce enough of a stress response to allow for homeostatic overload (i.e. excessive glucocorticoid concentrations over long periods of time, leading to pathological complications). These results are therefore incomparable for stunning-induced stress response research (Wingfield, 2013) as the short-term disturbances of CAS and electrical stunning only briefly, if at all, invoke enough glucocorticoid release to reach the reactive homeostasis range. The reactive homeostasis range is defined as the concentration range of the reactive glucocorticoid required to induce further physiological response to environmental change (Romero et al., 2009). By analyzing various pathways of endocrine function and biochemical reactions in stress responses, a few methods of quantification have been utilized for the analysis of the avian stress response.

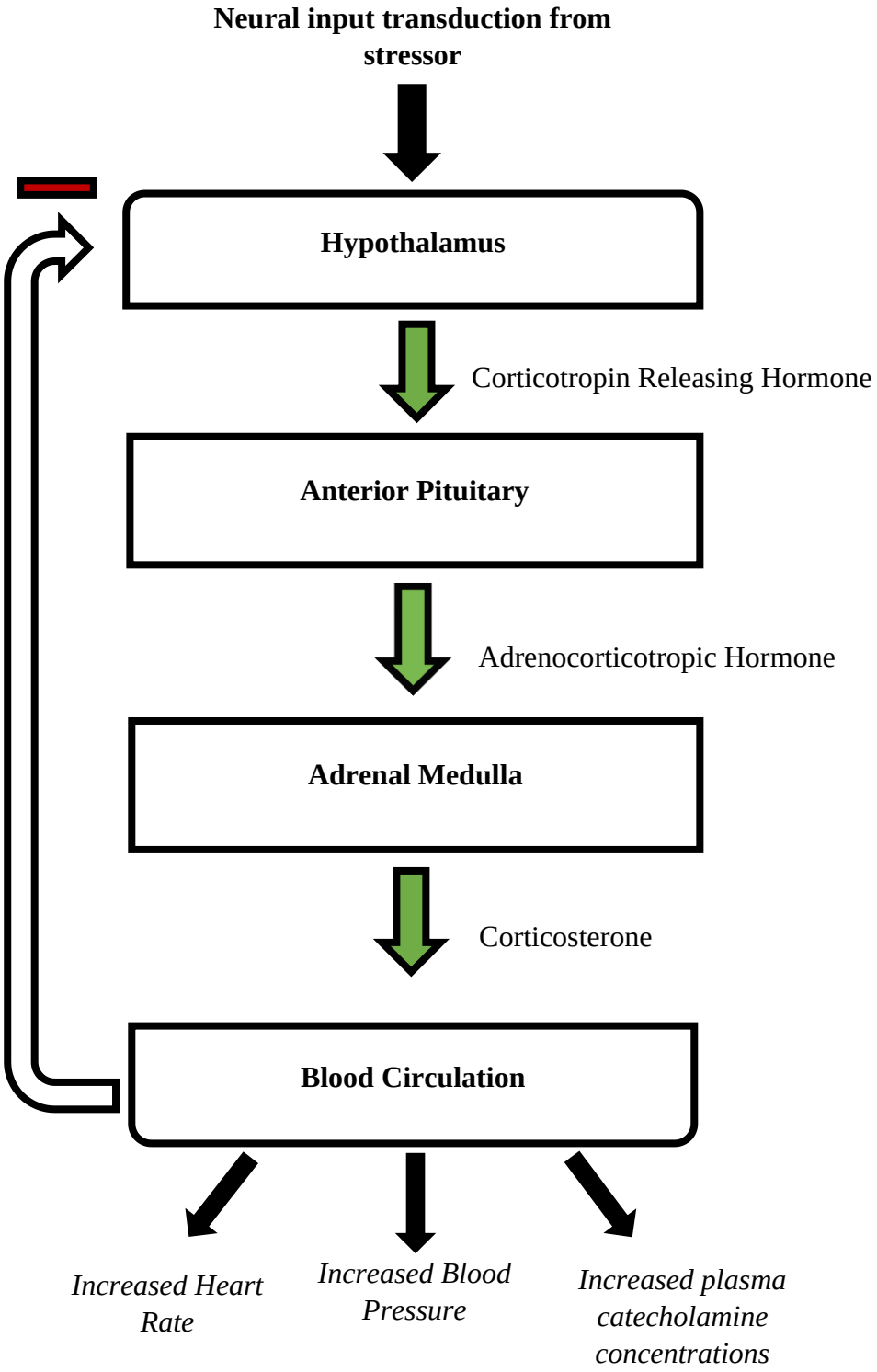
Physiological indicators of a stress response within circulation are mostly released from the hypothalamus-pituitary-adrenal (HPA) axis cascade, as represented in Figure 1.1. The hypothalamus of the brain is located directly below the thalamus and above the pituitary gland. The hypothalamus transduces signals from the environment to respond to stressors. When a stimulus or stressor is deemed dangerous the hypothalamus releases corticotropin-releasing hormone (CRH) to the corticotropes of the pituitary gland. The corticotropes, of the pituitary cephalic lobe, have a type-1 CRH receptor that CRH binds, which leads to an increase of cyclic-

adenomonophosphate (cAMP). The cAMP increase then directly triggers an increase of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the bloodstream (Kuenzel et al., 2013). In a stress response, one of ACTH's main target organs is the adrenal gland.

The adrenal gland is the final organ of the HPA axis and is located just above the kidneys. The adrenal gland is responsible for the secretion and storage of glucocorticoids. The release of these specific stress-respondent glucocorticoids is stimulated by ACTH from the pituitary gland. When ACTH is found in high concentrations throughout the adrenal gland it activates a key factor in the stress response – steroidogenesis. In reference to a stress response, ACTH activates steroidogenesis within the adrenal gland by establishing the uptake of cholesterol (Bauer et al., 2000). This process of neural transduction to steroidogenesis is the basis of stress response in avian species and is important to understand for the proper evaluation of animal welfare and stress.

Corticosterone (CORT) release is a product of ACTH circulation and steroidogenesis. CORT is widely considered the main stress response hormone in avian species. It is derived from the adrenal gland and is a glucocorticoid of the corticosteroid subdivision. More specifically CORT is a 21-carbon, steroid based hormone. It is considered in place of the hormone cortisol found in mammals as it is the predominantly synthesized glucocorticoid in the adrenal gland, where hormones for stress response are synthesized and released (Huibregtse et al., 1973). CORT is directly synthesized and released into circulation by the adrenocortical tissue of the adrenal gland and is the last responding factor of the hypothalamus-pituitary-adrenal axis cascade for a stress response.

Figure 1.1 Outline of the Hypothalamus-Pituitary-Adrenal axis on corticosterone



The overall effects of elevated CORT during an acute stress response include increasing availability of fatty acids as an energy source, increasing heart rate, regulation of blood pressure, and acts as a concentration-dependent mediator for subsequent physiological responses. CORT, when in maximum circulatory concentrations, allows for the continuation of the stress response when stressors are perceived for prolonged lengths of time.

CORT is also considered the 'gold standard' in measuring physiological indicators of a stress response. This is partially because CORT is non-retrospective and therefore the circulating concentrations found are reflective of current adrenal outputs. However, it has been determined that in many avian species, the increase of CORT in response to a stressor can last for upwards of 10 to 12 minutes and make take anywhere from 5 to 10 minutes to reach a quantifiable difference (Sapolsky et al., 2000).

Pinto et al. (2016) analyzed the concentration of circulatory CORT of broilers stunned by either electrical or CAS methods. CORT concentrations were found to be lower in CAS broilers, however it was noted that physical convulsions were more prevalent in comparison to the electrically stunned broilers. In terms of animal welfare, whether the physical convulsions were during a period of consciousness or unconsciousness is critical, and these physical convulsions or potentially exaggerated righting reflexes were noticed during the initial phases of CAS. Therefore, even though CORT concentrations were found to be lower in CAS broilers post-stun, the reactions recorded during phases where there is potential consciousness during CAS may contradict the indication of a stress response given by the results of CORT. Interestingly, when evaluating turkey CORT concentrations before and after CAS, Hänsch et al. (2009) found concentrations to have been affected by transportation and prior handling by the catching crew. CORT concentrations during CAS were not found to differ between samples taken immediately

prior or after completion of stunning. Therefore, CAS did not elicit a CORT response, or CORT may not be reliable for use of a biomarker of a stress response for the duration of stunning. In order to determine if there is a presence of a stress response, and if so, the quantification of that response, it would be of interest to further analyze other potential blood stress response biomarkers.

ACTH, as previously mentioned, is a potential blood stress response biomarker in poultry. With ACTH being the precursor to CORT, it may be applicable as a predeterminant factor of an elicited stress response. Puvadolpirod and Thaxton (2000) found that broiler chicks infused with ACTH under differing doses elicited different stress responses. Those given a continuously higher dose of ACTH (8 or 16 IU) had greater circulatory CORT and glucose than chicks given less ACTH (2 or 4 IU). Given these results, it is implied that a greater concentration of ACTH in blood circulation results in a greater response to a stressor. Though further investigation is required for ACTH concentrations of broilers before and after stunning, Xu et al. (2021) evaluated ACTH concentrations in the blood of Yangzhou geese after AC electrical stunning of differing currents were applied. Xu et al. (2021) found that currents of 70 mA and higher resulted in increased circulatory ACTH in broilers after stunning in comparison to currents of 20 or 40 mA. It is important to note that the frequency was set to 50 Hz for all treatments, and therefore is not comparable to U.S. electrical standards which utilize >400 Hz and pulse direct current (DC). Nonetheless, ACTH concentrations were shown to differ between geese stunned under differing electrical stunning parameters in Xu et al.'s (2021) research, indicating that ACTH may be a viable biomarker for physiological stress responses during stunning of avian species. Though ACTH concentrations were not recorded prior to stunning, it

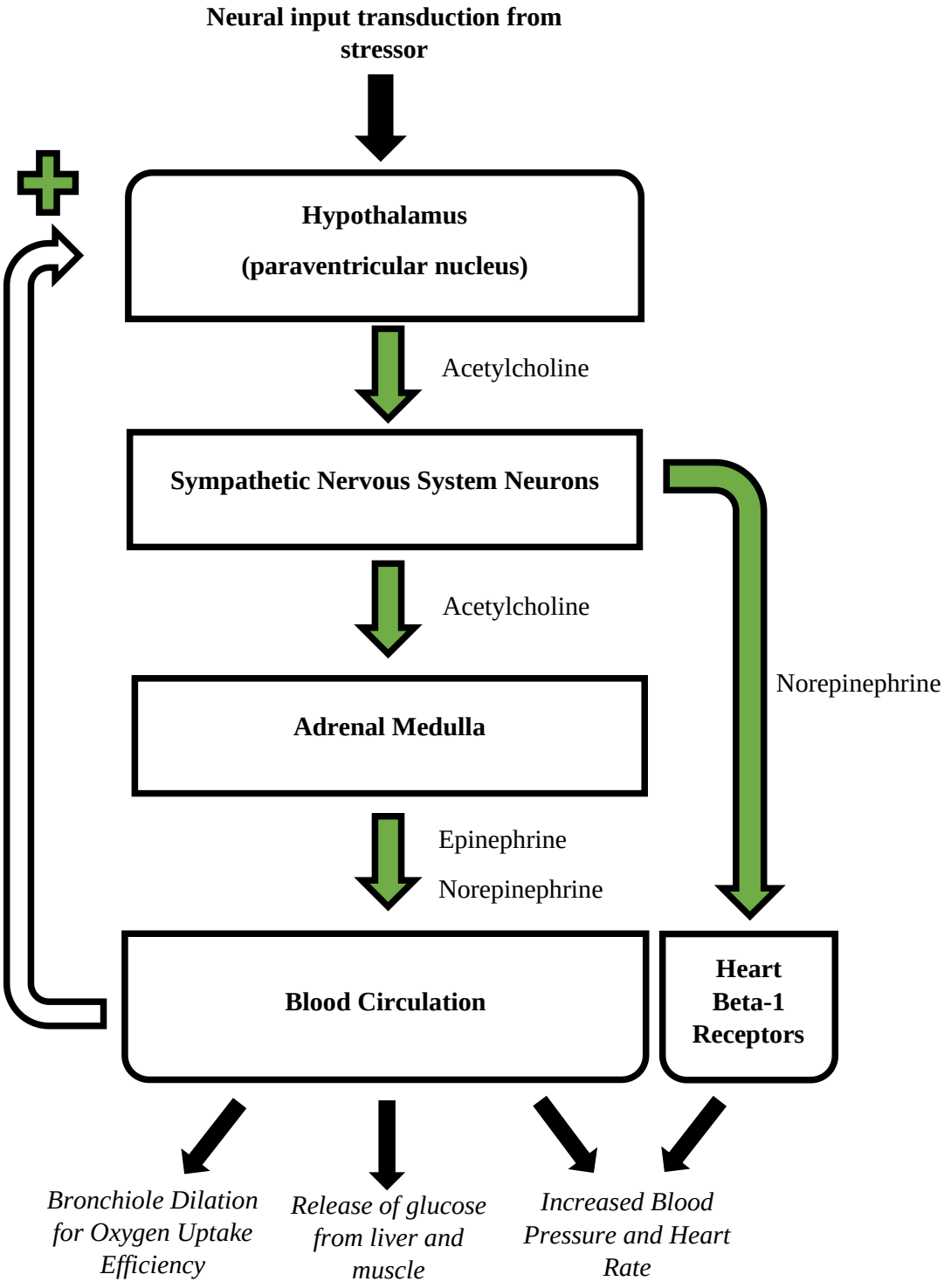
is possible that basal concentrations may have differed, altering the implication of a stress response for these results.

2. Analyzing Circulatory Indicators of Stress within the Blood – Sympathomedullary Pathway

As previously mentioned, the stressors during the CAS and electrical stunning processes are defined as short term and unpredictable from the birds' perspectives. While these disturbances are low/not present in the reactive homeostatic scope, they almost always prompt the "fight or flight" response, also known as the sympathomedullary response (SAM). This is considered the initial physiological response to environmental stressors. The SAM response is categorized separately from other stress responses as: 1) relatively no glucocorticoids are required to cause a SAM response and 2) this response stimulates specific regions of the adrenal glands. The adrenal gland, compromised of adrenocortical cells and chromaffin islets, release various adrenergic hormones for specific circumstances. In the case of a SAM response, the chromaffin cells of the adrenal gland are triggered by the presence of acetylcholine to release epinephrine and norepinephrine (EPI, NOREPI) (What is the Stress Response, 2022). This release of acetylcholine for subsequent EPI and NOREPI circulation is part of the autonomic nervous system in avian species.

EPI and NOREPI are catecholamines secreted by the adrenal glands as a first response to acute stressors (Dennis, 2016). The adrenal gland is almost entirely the sole source of EPI and NOREPI in avian species. The actions of both EPI and NOREPI are directly correlated to the surge in available energy for a response to stressors. EPI and NOREPI are products of the SAM pathway as shown in Figure 1.2. First, NOREPI acts as a neurotransmitter on the cardiac muscles of the heart. Specifically, the β-1 receptor is activated by reception of NOREPI when it is

Figure 1.2 The role of the Sympathomedullary pathway in Epinephrine and Norepinephrine release



released via stimulation of sympathetic neurons; the end results being increased heart rate and strength of contractions (Randall et al., 2002). The α-1 receptors along the heart are also stimulated by EPI when released in the blood. EPI also stimulates α-1 receptors in the smooth muscle of the skin, causing vasoconstriction for increased blood pressure. NOREPI/EPI bind to receptors on both the pancreas and the liver, activate the pancreatic α-2 receptors decreasing insulin release, the β-2 pancreatic receptors increase glucagon release, and the activation of the hepatic β-2 receptors triggers glycogenolysis. These actions produce a surge in free glucose as a short-term energy source for muscles when necessary (Thurston et al., 1993).

Due to the near-immediate release of EPI and NOREPI in response to a stressor, it is possible that these biomarkers are better indicators of a potential stress response during stunning. As previously mentioned, Hänsch et al. (2009) evaluated stress response biomarkers before and after CAS in turkeys. While they found no change in CORT was attributable to the stun itself, EPI and NOREPI significantly increased after completion of stun. The authors note that for analyzing the short time period of CAS itself, EPI and NOREPI were evidently more accurate to determine a stress response. Zulkifli et al. (2019) evaluated concentrations of CORT, EPI, and NOREPI in the blood of broilers after shackling and after electrical stunning. Ten broilers from each timepoint were sampled, and those that were stunned had an estimated current of 240 mA (400 Hz, 50 V AC). Interestingly, their results showed that both EPI and CORT concentrations were significantly higher post-stunning, with no differences between concentrations of NOREPI after shackling or after administered stunning. The authors suggest that 1) EPI is increased in blood circulation due to a psychological stress response while NOREPI is usually released in higher concentrations due to a physical stress response, therefore the lack of NOREPI increase did not indicate a physical stress response (Broom and Johnson, 1993) and 2) the observed

CORT increase after stunning was most likely due to the shackling process, therefore CORT is likely not reliable to evaluate just the stunning component itself. However, the authors do not take into account that NOREPI is released in lower quantities in the blood due to its direct release in the heart β-1 receptors from excitation of the sympathetic nervous system, so NOREPI may still be present in the system's physiological response to electrical stunning.

It is unknown whether CORT is a viable biomarker in determining a broiler stress response during stunning. Further comparison of concentrations of CORT, ACTH, EPI, and NOREPI is necessary to determine the efficacy of these biomarkers in broilers' reaction to an acute stressor. Further evaluating the concentrations of CORT, ACTH, EPI, and NOREPI of broilers stunned by either electrical on CAS may give more insight to the possible stress response associated with either method.

3. Muscle Meat Quality Between Methods of Stunning

The stress response has a critical impact on the overall meat quality of poultry products and can result in various changes of the metabolic muscle reaction (Santonicola et al., 2017). CAS has been proposed as a method of stunning to positively affect animal welfare as an alternative to electrical stunning, and therefore is also correlated with a decreased stress response. Therefore, meat quality of CAS broilers should theoretically be better than of electrically stunned broilers. Fernandez et al. (2010) evaluated fillet and carcass quality of geese and ducks stunned either by electrical or CAS.

Electrically stunned ducks and geese had a faster onset and rate of rigor, and therefore an accelerated drop in pH. Both CAS geese and ducks had the lowest total fractures in humeral bones, however, CAS geese had more engorged wing veins, which may be unfavorable from the

consumers' perception. They found no difference in meat textural analysis or in water loss between any treatment, which was further confirmed with broiler breast meat by Savenije et al. (2002). It is important to note that the electrical stunning parameters used by Fernandez et al. (50 Hz, 130 mA AC) are not comparable to the U.S. standards, as the birds were stunned-to-kill with low-frequency high-voltage parameters.

Raj et al. (1997) found that broken bones, most notably coracoids and scapula, were more prevalent in electrically stunned broilers in comparison to CAS broilers. They also found instances of hemorrhaging of breast muscle, and breast muscle toughness (measured in kg yield force) was higher in electrically stunned broilers. Breast muscle was initially higher in pH for electrically stunned broilers two hours post-debone, however, 24 hours post-debone no difference in pH was found in comparison to CAS broiler breast tissue. However, in this study broilers were electrically stunned at 80 mA and 50 Hz AC, which is not applicable to U.S. industry standards.

Pre-mortem concentrations of hormones and circulating products of a stress response correlate directly with the post-mortem meat biophysiological reaction. While CORT, ACTH, EPI, and NOREPI help induce and upregulate the physiological effects of a stress response, circulatory glucose levels are indicative of excess movement and struggle in response to a stressor. This is critical in terms of overall meat quality, as less available glycogen or glucose due to excess muscle usage peri-mortem will change meat pHu, and impact meat color, drip loss, and toughness (Robergs et al., 2004; Le Bihan-Duval et al., 2008; Ouali et al., 2013).

In one study, meat color, glucose, and CORT concentrations were evaluated between electrically stunned broilers and CAS broilers; glucose and CORT concentrations were not only significantly higher for the electrically stunned broilers, the meat was also darker in color,

indicating higher pH (Pinto et al., 2016). The higher concentration of glucose post-stunning with electrically stunned birds indicates that there was most likely excessive movement prior to stunning, which has been previously noted by Fletcher (1991). Since the birds are live-shackled, it is not uncommon for them to flap in order to attempt to escape and/or readjust posture (Shields and Raj, 2010). The higher concentration of CORT in electrically stunned broilers may indicate a more severe response to a stressor during stunning. However, in other research analyzing the adverse physical reactions induced by carbon dioxide during CAS, birds displayed mild to moderate signs of physical discomfort (McKeegan et al., 2007). Since birds stunned with the controlled atmosphere method are done so within their transportation coops, it is typical to see some excess movement during the induction phase. Initial exposure and consciousness during induction indicates some complications in terms of animal welfare (Hindle et al., 2010). It is also critical to note that unconscious convulsions from hypercapnic conditions in the transition and completion phase may also increase post-stunning glucose concentrations (McKeegan et al., 2007), however these movements would not be applicable for animal welfare. Similar results were found to be beneficial for CAS when analyzing water-holding capacity and tenderness (Fletcher, 2002).

Epinephrine directly stimulates the release of glucose into the blood stream by activating hepatic gluconeogenesis and glycogenolysis (Sardana et al., 1985; Yamada and Noguchi, 1998). Following electrical and controlled atmosphere stunning, epinephrine concentrations were found to significantly increase in broiler blood post-stunning (Zulkifli et al., 2019), with only CAS having an observed increase of pyruvate kinases, indicating an increased use for glycolysis (Uyeda, 2013). Increased rate of glycolysis and use of glucose negatively affects meat quality by

prematurely initiating rigor prior to blood loss, as well as decreasing water holding capacity, which consequentially decreases tenderness (Adeyemi and Sazili, 2014).

High variability among results from available research may be due to the different standards/applications of each stunning method. CAS varies due to the variation in concentrations of gas, gases used, number of phases, and time of exposure in each phase; all of which affect the overall meat quality and stress response biomarker concentrations of broilers, as seen in Tables 1.1, 1.2, and 1.3. Electrical stunning research varies greatly in the parameters set for current type, voltage, and frequency, leading different results in meat quality and stress response biomarker concentrations of broilers (Tables 1.1 and 1.2). Furthermore, when taking these implications of electrical stunning into consideration, U.S. electrical stunning systems utilize low-voltage high-frequency parameters, while most available research comparing either stress response biomarkers or meat quality follow E.U. standards of high-voltage low-frequency. Even so, the variability in CAS system parameters further complicates the accuracy of comparison. More research needs to be conducted in comparing the stress response indicators between methods of poultry stunned with U.S. parameters used in commercial operations. Better animal welfare is beneficial for the animal and the producer. In deciding which method of stunning has the highest achieved welfare, best quality meat, and greatest overall benefits, further analysis is needed to confirm claimed advantages of CAS.

4. Physiological Responses from Stunning on Meat Quality

Increased consumer demand for animal welfare during the practice of meat production has caused this alteration in stunning methods, consequentially company concern for the alteration in meat quality and profitability has become prevalent. Pre-slaughter stress responses are critical to reduce at the processing plant, not only for animal welfare, but for meat quality

(Ali et al., 2008). Meat quality is usually defined by overall meat characteristics including its physical, chemical, morphological, biochemical, microbial, sensory, technological, hygienic, nutritional, and culinary properties (Ingr, 1989). In poultry production, the following factors are main determinants of concern: visual appearance (color), firmness, juiciness, tenderness, smell, and flavor (Mir et al., 2017). Meat quality is entirely based on consumer preference at the point of purchase; making it critical for poultry producers to uphold high meat quality of their products.

Since CAS has recently been strongly considered within the U.S. poultry industry, it has been questioned whether the alternative peri-mortem conditions for slaughter will alter final meat quality. There are many parameters under the overall definition of 'meat quality'. Water retention/drip loss, pH values, muscle color (L*a*b* values), and carcass condition are considered some of the most valuable aspects in determination of quality. Conditions of the animal at slaughter are major determinants of muscle meat quality (Mir et al., 2017). This is mostly due to the post-mortem biochemical processes that alter the muscle-meat physiology.

Adenosine triphosphate (ATP) is depleted post-mortem, causing muscles to go into rigor. The muscles become rigid due to the inability to relax when ATP reserves are depleted (Hall and Guyton, 2015). After exsanguination, muscles are no longer given a source of oxygen through respiration and circulatory blood-oxygen. When oxygen is no longer available, the muscle will produce ATP through anaerobic glycolysis to avoid muscle-cell death. This will then lead to a decrease in the muscle pH. The rate of this pH decline is impacted by peri-mortem physical activity and the levels of lactic acids present in the muscle. After 24 hours the meat will have gone out of rigor and an ultimate pH will be reached.

The pH value of meat is impacted by the physiological state of the muscle at slaughter. Muscle glycogen levels at time of slaughter are the main determinant of the pHu level of the product. As mentioned previously, glycolysis is the main production method of ATP in anaerobic conditions post-mortem, eventually yielding two ATP molecules and two molecules of pyruvic acid. When oxygen levels are normal, the pyruvic acid is removed by the circulatory system. However, within the post-mortem muscle tissues, the pyruvic acid is converted to lactic acid (Melkonian, 2022). The lactic acid buildup will lower the pHu of the muscle. Less glycogen storage or circulatory glucose at the time of death will yield a higher pHu than the average, which is typically 6.2 after completion of rigor (Dransfield and Sosnicki, 1999). Broilers with higher rates of glycolytic activity and lower muscle pH before slaughter have been previously correlated with a higher incidence of breast muscle hemorrhaging and drip loss (Sandercock et al., 2001).

However, it is important to note the pHu of the muscle correlates to the organoleptic properties of the muscle meat product as well. Froning (1995) has reported that stunning conditions are one of the main factors influencing poultry meat color. Color is vital in consumer preference at point of purchase, and it has been shown that consumers in the U.S. will generally prefer a lighter color (higher L* value) chicken product (Fletcher, 2002). A strong negative correlation has been found between lightness and pH, where lighter colored meat will have a lower overall pH (Fletcher, 1999). It is proposed that the low pH of the muscle will increase the overall reflectance of the fibers themselves (Swatland, 2008). It is possible that this is due to the lower rate of protein denaturation at high pH levels, which would not allow for aggregation of proteins and decrease the refraction of light perceived from the carcass (Hultin, 1984).

Interestingly, comparisons of different stunning parameters for electrical waterbath systems do not seem to have varying ultimate meat quality attributes. Craig and Fletcher (1997) observed no differences between breast muscle pH or color between broilers stunned by either low voltage or high voltage waterbath stunning systems. Papinaho and Fletcher (1995) observed similar results, finding no differences 24 hours post-stunning from broiler breast fillets from broilers stunned by low or high amperage (0 mA, 50 mA, 100 mA, 150 mA, or 200 mA). However, they did notice initial pH values after slaughter were significantly higher in higher mA stunning settings, indicating a delay of rigor.

Carcass quality may also vary between methods of stunning. Due to the increased flapping from adverse reactions to carbon dioxide during CAS, injury is likely to occur resulting in an overall decrease in carcass quality (McKeegan et al., 2007). However, Fletcher (2002) reported that there were less carcass downgrades from CAS broilers due to the infrequency of broken bones and bruising. This may be comparable to Siqueira et al.'s (2017) results, that the increase in frequency of both AC and DC electrical stunning led to an increase in bruises on the breast and wing. Therefore, when looking at carcass damage and bruising between U.S. electrical stunning parameters and CAS, the results were observed to favor CAS broilers. Though, when analyzing the number of broken bones resulting from either method, Gregory et al. (1991) found that high-frequency stunning (1500 Hz AC) led to less broken bones than low-frequency (50, 200, and 350 Hz) stunning within an electrical stunning system.

Low voltage, high frequency electrical stunning has been noted to produce meat with better deboning qualities. Contreras and Beraquet (2001) found that low voltage, high frequency electrical stunning of broilers resulted in the least amount of red wing tips, deep muscle hemorrhaging, broken bones, and engorged wing veins when compared to lower frequencies and

higher voltages. Raj et al. (1997) observed that CAS broilers has significantly reduced prevalence of carcass defects or injury when compared to head-only electrically stunned broilers. Though it should be noted that Raj et al. (1997) made these observations on an electrical system under E.U. stunning parameters.

1.3 CLOSING REMARKS

Overall, there is a lack of peer-reviewed published literature comparing CAS and electrical stunning systems under U.S. parameters. This gap in the literature makes it difficult to make direct comparisons in terms of potential animal welfare and meat quality benefits. First, it is imperative that the benefit of CAS in terms of welfare is further researched so that poultry producers within the U.S. may feel confident in their choice to switch stunning methods. This research may be made more reliable by choosing to investigate other acutely released blood-stress response indicators, such as ACTH, EPI, and NOREPI. Though there is not much research available on the impact stunning has upon these biomarkers, it may be novel in research for per-acute stress responses in broilers and may allow researchers to better quantify stress response in these situations. Second, it is important that both CAS and electrical waterbath stunning systems are researched in their effects upon meat quality and carcass damage under U.S. parameters. Since electrical waterbath stunning under U.S. parameters has been arguably shown to reduce meat quality defects (Table 1.3), producers may be hesitant to switch stunning systems to the more expensive CAS. However, with the rise in consumer demand for ethically raised meat, the push for installation of CAS systems would require further research in the potential unknown cost in meat quality issues that can arise during CAS. Further research must be performed in order to compare both stunning systems under U.S. parameters for both potential animal welfare and meat quality benefits.

1.4 REFERENCES

Adeyemi, K. D., and A. Q. Sazili. 2014. Efficacy of carcass electrical stimulation in meat quality enhancement: A review. Asian-Australas. J. Anim. Sci. 27:447–456.

Ali, M. S., G. H. Kang, and S. T. Joo. 2008. A review: influences of pre-slaughter stress on poultry meat quality. Asian-Australas. J. Anim. Sci. 21:912–916.

Ali, A. S. A., M. A. Lawson, A. H. Tauson, J. F. Jensen, and A. Chwalibog. 2007. Influence of electrical stunning voltages on bleed out and carcass quality in slaughtered broiler chickens. Arch. Geflügelk. 71:35-40.

Anastasov, M., and S. Wotton. 2012. Survey of the incidence of post-stun behavioural reflexes in electrically stunned broilers in commercial conditions and the relationship of their incidence with the applied water-bath electrical parameters. An. Welf. 21:247–256.

Anton, F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO_2 pulses applied to the nasal mucosa. Pain. 49:53-60.

Bauer, M. P., J. T. Bridgham, D. M. Langenau, A. L. Johnson, and F. W. Goetz. 2000. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol. Cell. Endocrinol. 168:119-125.

Bedanova, I., E. Voslarova, P. Chloupek, V. Pistekova, P. Suchy, J. Blahova, R. Dobsikova, and V. Vecerek. 2007. Stress in broilers resulting from shackling. Poult. Sci. 86:1065–1069.

Berg, C., and M. Raj. 2015. A review of different stunning methods for poultry-animal welfare aspects (Stunning Methods for Poultry). Animals. 5:1207-1219.

Bilgili, S. F. 1992. Electrical stunning of broilers - basic concepts and carcass quality implications: a review. J. Appl. Poult. Res. 1:135-146.

Bilgili, S. 1999. Recent advances in electrical stunning. Poult. Sci. 78:282–286.

Boyd, F. 1994. Humane slaughter of poultry: The case against the use of electrical stunning devices. J. Agric. Environ. Ethics. 7:221–236.

Broom, D.M., and K. G. Johnson. 1993. Stress and Animal Welfare. Kluwer Academic, Dordrecht, Netherlands.

Contreras C. C., and N. Beraquet. 2001. Electrical stunning, hot boning and quality of chicken breast meat. Poult. Sci. 80:501–507.

Craig, E.W., and D. L., Fletcher. 1997. A comparison of high current and low voltage electrical stunning effects on broiler breast rigor development and meat quality. Poult. Sci. 71:1178-1181.

Dennis, R. L. 2016. Adrenergic and noradrenergic regulation of poultry behavior and production. Domest. Anim. Endocrinol. 56:94-100.

Dransfield, E., and A. Sosnicki. 1999. Relationship between muscle growth and poultry meat quality. Poult. Sci. 78:743–746.

Erasmus, M. A., P. V. Turner, and T. M. Widowski. 2010. Measures of insensibility used to determine effective stunning and killing of poultry. J. Appl. Poult. Res. 19:288-298.

Fernandez, X., E. Lahirigoyen, A. Auvergne, C. Molette, M. Bouillier-Oudot, 2010. The effects of stunning methods on product qualities in force-fed ducks and geese. 1. Carcass downgrading and meat quality. Animal. 4:128-138.

Fletcher, D. 1991. Ante mortem factors related to meat quality. Pages 11–19 in: Proceedings of the 10th European Symposium of Poultry Meat. T. G. Uijttenboogaart and C. H. Veerkamp, ed. Spelderholt Centre for Poultry Research and Information Services, Beekbergen, The Netherlands.

Fletcher, D. L. 1999. Broiler breast meat color variation, pH, and texture. Poult. Sci. 78:1323–1329.

Fletcher, D. L. 2002. Poultry meat quality. World's Poult. Sci. J. 58:131–145.

Froning, G. W. 1995. Color of poultry meat. Poult. Avian. Biol. Rev. 6:83–93.

Gentle, M. J., and V. L. Tilston. 2000. Nociceptors in the legs of poultry: implications for potential pain in pre-slaughter shackling. Anim. Welf. 9:227-236.

Gent, T.C., S. Gebhardt-Henrich, S. L. A. Schild, A. A. Rahman, and M. J. Toscano. 2020. Evaluation of poultry stunning with low atmospheric pressure, carbon dioxide or nitrogen using a single aversion testing paradigm. Animals. 10:1308.

Grandin, T., and M. Cockram. 2020. The slaughter of farmed animals: practical ways of enhancing animal welfare. CABI.

Gregory, N. G. 2005. Recent concerns about stunning and slaughter. Meat. Sci. 70:481-491.

Gregory, N. G., L. J. Wilkins, and S. B. Wotton. 1991. Effect of electrical stunning frequency on ventricular fibrillation, downgrading and broken bones in broilers, hens and quails. Br. Vet. J. 147:71–77.

Hall, J. E., and A. C. Guyton. 2015. Guyton and Hall Textbook of Medical Physiology. Philadelphia, PA: Saunders/Elsevier, 2011. MD Consult. Web.

Hänsch, F., B. Nowak, and J. Hartung. 2009. Evaluation of a gas stunning equipment used for turkeys under slaughterhouse conditions. Livest. Sci. 124:248-254.

Hindle, V. A., E. Lambooij, H. G. M. Reimert, L. D. Workel, and M. A. Gerritzen. 2010. Animal welfare concerns during the use of the water bath for stunning broilers, hens, and ducks. Poult. Sci. 89:401–412.

Huang, J. C., J. Yang, M. Huang, K. J. Chen, X. L. Xu, and G. H. Zhou. 2017. The effects of

electrical stunning voltage on meat quality, plasma parameters, and protein solubility of broiler breast meat. Poult. Sci. 96:764–769.

Huang, J. C., M. Huang, P. Wang, L. Zhao, X. L. Xu, G. H. Zhou, and J. X. Sun. 2014. Effects of physical restraint and electrical stunning on plasma corticosterone, postmortem metabolism, and quality of broiler breast muscle. J. Anim. Sci. 92:5749–5756.

Huibregtse, W. H., F. Ungar, and D. S. Farner. 1973. Adrenal steroidogenesis in the white-crowned sparrow (Zonotrichia leucophrys). Comp. Biochem. Physiol. B. 44:1051-1056.

Hultin, H.O. 1984. Postmortem biochemistry of meat and fish. J. Chem. Educ. 61:289.

Humane Methods of Livestock Slaughter Act of 1978. Pub. L. No. 95–445. 92 USC 1069–1070.

Ingr, I. 1989. Meat quality: defining the term by modern standards. Fleisch. 69:1268–1277.

Kannan, G., J. L. Heath, C. J. Wabeck, and J. A. Mench. 1997. Shackling of broilers: effects on stress responses and breast meat quality. Br. Poult. Sci. 38:323-332.

Kuenzel, W. J., S. W. Kang, A. Jurkevich. 2013. Neuroendocrine regulation of stress in birds with an emphasis on vasotocin receptors (VTRs). Gen. Comp. Endocrinol. 190:18-23.

Le Bihan-Duval, E., M. Debut, C. M. Berri, N. Sellier, V. Santé-Lhoutellier, Y. Jégo, and C. Beaumont. 2008. Chicken meat quality: genetic variability and relationship with growth and muscle characteristics. BMC Genet. 9:53.

Lines, J. A., A. B. M. Raj, S. B. Wotton, M. O'Callaghan, and T. G. Knowles. 2011. Head-only electrical stunning of poultry using a waterbath: a feasibility study. Br. Poult. Sci. 52:432–438.

McKeegan, D. F., J. M. Demmers, T. G. Wathes, M. Christopher, B. R. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271-286.

McKeegan, D., J. McIntyre, T. Demmers, J. C. Lowe, C. Wathes, P. Broek, A. Coenen, and M. J. Gentle. 2007. Physiological and behavioural responses of broilers to controlled atmosphere stunning: Implications for welfare. Anim. Welf. 16:409-426.

McKeegan, D. E. F., F. S. Smith, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2005. Behavioral correlates of olfactory and trigeminal gaseous stimulation in chickens, Gallus domesticus. Physiol. Behav. 84:761-768.

Melkonian, E. A., and M. P. Schury. 2022. Biochemistry, Anaerobic Glycolysis.in StatPearls. StatPearls Publishing, Treasure Island (FL).

Mir, N. A., A. Rafiq, F. Kumar, V. Singh, and V. Shukla. 2017. Determinants of broiler chicken meat quality and factors affecting them: a review. J. Food Sci. Technol. 54:2997–3009.

Ouali, A., M. Gagaoua, Y. Boudida, S. Becila, A. Boudjellal, C. H. Herrera-Mendez, and M. A. Sentandreu. 2013. Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci. 95:854–870.

Papinaho, P. A., and D. L. Fletcher. 1995. Effect of stunning amperage on broiler breast muscle rigor development and meat quality. Poult. Sci. 74:1527–1532.

Pinto, M., D. Bitencourt, E. Ponsano, M. G. Neto, and I. Bossolani. 2016. Effect of electrical and controlled atmosphere stunning methods on broiler chicken behavior at slaughter, blood stress indicators and meat traits. Braz. J. Vet. Res. Anim. Sci. 53:1678-4456.

Puvadolpirod, S., and J. P. Thaxton. 2000. Model of physiological stress in chickens 2. Dosimetry of adrenocorticotropin12. Poult. Sci. 79:370–376.

Raj, A. B. M., and M. O'Callaghan. 2004a. Effects of electrical water bath stunning current frequencies on the spontaneous electroencephalogram and somatosensory evoked potentials in hens. Br. Poult. Sci. 45:230–236.

Raj, M., and M. O'Callaghan. 2004b. Effect of amount and frequency of head-only stunning currents on the electroencephalogram and somatosensory evoked potentials in broilers. An. Welf. 13:159–170.

Raj, A. B. M., L. J. Wilkins, R. I. Richardson, S. P. Johnson, and S. B. Wotton. 1997. Carcase and meat quality in broilers either killed with a gas mixture or stunned with an electric current under commercial processing conditions. Br. Poult. Sci. 38:169–174.

Randall, D. J., W. W. Burggren, K. French, and R. Eckert. 2002. Eckert Animal physiology: Mechanisms and adaptations. New York, NY: W.H. Freeman and Co.

Robergs, R. A., F. Ghiasvand, and D. Parker. 2004. Biochemistry of exercise-induced metabolic acidosis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287:502–516.

Romero, L. M., M. J. Dickens, N. E. Cyr. 2009. The reactive scope model- a new model integrating homeostasis, allostasis and stress. Horm. Behav. 55:375-389.

Sandercock, D. A., R. R. Hunter, G. R. Nute, M. A. Mitchell, and P. M. Hocking. 2001. Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: implications for meat quality. Poult. Sci. 80:418–425.

Santonicola, S., M. F. Peruzy, M. Girasole, N. Murru, M. L. Cortesi, and R. Mercogliano. 2017. Preliminary study on physicochemical and biochemical stress markers at poultry slaughterhouse. Ital. J. Food Saf. 6:6346.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

Sardana, M. K., S. Sassa, and A. Kappas. 1985. Hormonal regulation of heme oxygenase

induction in avian hepatocyte culture. Biochem. Pharmacol. 34:2937-2944.

Savenije, B., F. J. Schreurs, H. A. Winkelman-Goedhart, M. A. Gerritzen, J. Korf, and E. Lambooij. 2002. Effects of feed deprivation and electrical, gas, and captive needle stunning on early postmortem muscle metabolism and subsequent meat quality. Poult. Sci. 81:561–571.

Siqueira, T. S., T. D. Borges, R. M. M. Rocha, P. T. Figueira, F. B. Luciano, and R. E. F. Macedo. 2017. Effect of electrical stunning frequency and current waveform in poultry welfare and meat quality. Poult. Sci. 96:2956-2964.

Shields, S. J., and A. B. Raj. 2010. A critical review of electrical water-bath stun systems for poultry slaughter and recent developments in alternative technologies. J. Appl. Anim. Welf. Sci. 13:281-299.

Spain, C. V., D. Freund, H. Mohan-Gibbons, R. G. Meadow, and L. Beacham. 2018. Are they buying it? United states consumers' changing attitudes toward more humanely raised meat, eggs, and dairy. Animals. 8:128.

Swatland, H. J. 2008. How pH causes paleness or darkness in chicken breast meat. Meat Sci. 80:396-400.

Terlouw, C., C. Bourguet, and V. Deiss. 2016. Consciousness, unconsciousness and death in the context of slaughter. Part II. Evaluation methods. Meat. Sci. 118:147–156.

Thurston, R. J., C. C. Bryant, and N. Korn. 1993. The effects of corticosterone and catecholamine infusion on plasma glucose levels in chickens (*Gallus domesticus*) and turkey (*Melagridis gallopavo*). Comp. Biochem. Physiol. C. 106:59-62.

United States Department of Agriculture. 2021. Poultry - Production and Value 2020 Summary. ISSN: 1949-1573. Page 7. https://www.nass.usda.gov/Publications/Todays_Reports/reports/plva0421.pdf

Uyeda, K. 2013. Pyruvate Kinase, Editor(s): William J. Lennarz, M. Daniel Lane, Encyclopedia of Biological Chemistry (Second Edition). Academic Press. Pages: 719-721.

What is the Stress Response - Simply Psychology. Available at https://www.simplypsychology.org/stress-biology.html (accessed 13 November 2022).

Whittow, G. C. 2000. Sturkie's avian physiology. San Diego: Academic Press.

Wibawati, A. P., M. Mufasirin, and S. Estoepangestie. 2019. Stunning of broiler and its effect on meat pH value and corticosterone hormone: study of animal welfare. Indian. Vet. J. 96:17–19.

Wilkins, L. G., N. G. Gregory, S. B. Wotton, and I. D. Parkman. 1998. Effectiveness of electrical stunning applied using a variety of waveform-frequency combinations and consequences for carcase quality in broiler chickens. Br. Poult. Sci. 39:511-518.

Wingfield, J.C. 2013. Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. Funct. Ecol. 27:37-44.

Xu, L., H. Yang, X. Wan, X. Zhang, Z. Yang, Z. Wang. 2021. Effects of high-frequency electrical stunning current intensities on pre-slaughter stunning stress and meat lipid oxidation in geese. Animals. 11:2376.

Xu, L., L. Zhang, H. Y. Yue, S. G. Wu, H. J. Zhang, F. Ji, and G. H. Qi. 2011. Effect of electrical stunning current and frequency on meat quality, plasma parameters, and glycolytic potential in broilers. Poult. Sci. 90:1823–1830.

Yamada, K., and T. Noguchi. 1998. Nutrient and hormonal regulation of pyruvate kinase

gene expression. Biochem. J. 337:1–11.

Zulkifli, I., Z. Wakiman, A. Q. Sazili, Y. M. Goh, and A. Jalila A. 2019. Effect of shackling, electrical stunning and halal slaughtering method on stress linked hormones in broilers. S. Afr. J. Anim. Sci. 49:599-603.

Table 1.1 Corticosterone Evaluated Post-Stun

Methodology	Parameters	Concentration	Findings	Reference
Electrical Water-Bath	15 V, 750 Hz, DC	No Stun: 159 ± 11.2 ng/mL No Stun w/ Restraint: 117 ± 7.7 ng/mL With Stun: 136 ± 9.4 ng/mL Stun w/ Restraint: 127 ± 11.8 ng/mL	No stun with restraint treatment had the lowest circulating CORT concentrations	Huang et al., 2014
Electrical Water-Bath	(5, 15, 25, 35, and 45) V, 750Hz, DC	5V: 185.2 ± 11.8 g/mL 15V: 155.6 ± 10.5 g/mL 25V: 142.8 ± 9.80 g/mL 35V: 155.7 ± 13.6 g/mL 45V: 158.4 ± 11.2 g/mL	Only the 5V (lowest voltage) treatment broilers had significantly higher circulation concentrations of CORT No differences in treatments 15, 25, 35, 45V	Huang et al., 2017
Electrical Water-Bath	(35, 50, 65) V, (160, 400, 1,000) Hz, AC	35V: 46.30 ng/mL 50V: 41.81 ng/mL 65V: 41.26 ng/mL 160Hz: 41.71 ng/mL 400Hz: 44.15 ng/mL 1,000Hz: 43.5 ng/mL	No significant difference between CORT concentrations when frequency was altered, or in the interaction between frequency and voltage CORT significantly decreased when voltage was raised	Xu et al., 2011
Electrical Water-Bath	60 V, 267.9 Hz	No Stun: 45.23 ± 11.60 ng/mL Stun: 46.37 ± 24.47 ng/mL	No difference was found in CORT concentrations if stunning was or was not administered prior to neck-cut	Wibawati et al., 2019
Electrical Water-Bath	50 V, 400 Hz, AC	After Stun: 19.12 ± 1.31 ng/mL After Slaughter, Stun: 15.46 ± 1.20 ng/mL	After administered stun broilers had the highest concentration of CORT among any treatment group	Zulkifli et al., 2019

		After Slaughter, No Stun: 14.14 ± 1.25 ng/mL	CORT was no different from stunned or not stunned broilers once slaughter was complete	
Controlled Atmosphere	CO_2: 30%, 40%, 50%, 60% Control (EU Regulation): 40% CO_2 + 30% O_2 + N2	Control:42.76 ng/mL 30%: 43.41 ng/mL 40%: 44.86 ng/mL 50%: 41.86 ng/mL 60%: 38.54 ng/mL P=0.31	No differences in CORT were observed when CO_2 concentrations were altered.	Xu et al., 2011
Controlled Atmosphere Electrical Water-bath	CO_2, CO_2 + Ar, 220V + 60 Hz AC, Control (No Stun)	CO2: 72.49 ± 35.82 ng/dl CO2 + Ar: 55.71 ± 31.38 ng/dl Electrical: 104.13 ± 64.39 ng/dl Control: 50.65 ± 22.41 ng/dl P<0.05	The only difference in plasma CORT was found in broilers stunned by electrical water-bath, which had the highest circulating concentrations	Pinto et al., 2016

Table 1.2 Adrenocorticotropic Hormone, Epinephrine, and Norepinephrine Evaluation Post-Stun

Methodology	Stun Parameters	Hormone(s) Evaluated	Species	Results	Findings	Reference
Electrical Water-Bath	50 V, 400 Hz, AC	EPI NOREPI	Broiler	(EPI) After Stun: 651 ± 76.14 After Slaughter, Stun: 455 ± 19.97 After Slaughter, No Stun: 511 ± 47.70 (NOREPI) After Stun: 1671 ± 72.58 After Slaughter, Stun: 1511 ± 102.9 After Slaughter, No Stun: 1504 ± 72.58	EPI was highest immediately after stunning was completed, but decreased once slaughter was complete. No difference between post-slaughter EPI concentrations of stunned or not stunned birds. NOREPI did not differ from any time points or treatments.	Zulkifli et al., 2019
Electrical Water-Bath	(30, 60, 90, 120V), 500Hz, AC	ACTH	Geese	No Stun: 27.10 pb/mL 30V: 28.02 pb/mL 60V: 24.20 pb/mL 90V: 32.67 pb/mL 120V: 34.57 pb/mL P<0.01	ACTH concentrations were highest in broilers stunned by 120V, and lowest in those stunned by 60V. No stun was comparable to 30V, 90V	Xu et al., 2021

Table 1.3 Meat Quality Parameters Measured After Stunning

Methodology	Stun Parameters	Meat Quality Parameters	Results	Findings	Reference
Electrical Water-Bath	(35, 50, 65) V, (160, 400, 1,000) Hz, AC	Meat Color, pH, Drip Loss	Color (b*, 24 hours): $P<0.05$ pH: P=0.19 Drip Loss: P=0.87	Only b* color value was significantly different, when evaluated at 24 hours post-stun, and decreased as voltage increased	Xu et al., 2011
Controlled Atmosphere	CO_2: 30%, 40%, 50%, 60% Control (EU Regulation): 40% CO_2 + 30% O_2 + N_2	Meat Color, pH, Drip Loss	Color (L*, 45min & 24 hr): $P<0.05$ pH: $P<0.05$ Drip Loss (Pectoralis major, Musculus iliofibularis): P=0.01, $P<0.01$	Darkest at 30% CO_2, and increased coincided with increase of CO_2 pH was unaffected at 45min post-mortem, but was lowest 24hr post-mortem in birds stunned by CO_2 50% and 60% Drip loss in meat was lowest in 30% CO_2 and highest in 50% CO_2	Xu et al., 2011
Electrical Water-Bath	60 V, 267.9 Hz	pH: 3 mins, 8 hour, 24 hour post-mortem (With and without stun)	No Stun (3min, 8Hr, 24Hr): 6.75 ± 0.19, 6.23 ± 0.12, 5.78 ± 0.07) Stun (3min, 8Hr, 24Hr): 6.58 ± 0.23,	Each treatment, across all three time points, decreased in pH. No difference was observed for pH of	Wibawati et al., 2019

			6.26 ± 0.10, 5.71 ± 0.09	muscle between either treatment at any timepoint.	
Electrical Water-Bath	(5, 15, 25, 35, and 45) V, 750Hz, DC	Wing damage (Score 0-5), Color, pH,	Wing Damage: Lowest, 5V (2.82 ± 0.22) Highest, 15V (4.78 ± 0.45), 25V (4.25 ± 0.36) Color: No difference at 2Hr or 24Hr pH, 2Hr: Lowest, 5V (5.82 ± 0.17) Highest, 15V (6.17 ± 0.13), 25V (6.22 ± 0.12) pH, 24Hr: No Difference	Largest area of wing damage was found in broilers stunned with 15V or 25V, smallest wing damage was observed in broilers stunned with 5V. No difference observed in color by any voltage. Initial 2Hr pH had the highest in broilers stunned by 15V and 25V, and lowest in 5V, but at 24Hr there was no difference.	Huang et al., 2017
Electrical Water-Bath	70V, 300 or 650Hz, AC or DC	Wing Bruising (%) Color	AC (WD%, L*): 300Hz – 0.00, 63.30 ± 0.48 650Hz – 11.55, 57. 77 ± 0.47 DC (WD%, L*): 300Hz – 6.68, 60.76 ± 0.48 650Hz – 9.09, 58.58 ± 0.48	Wing bruising was highest in AC, high frequency (650Hz) stunning methods. L* was the only Lab* value affected by treatments. L* was lowest in DC and AC 650Hz	Siqueira et al., 2017

				electrically stunned broilers. DC was significantly different from AC only when 300Hz was applied during stunning	
Controlled Atmosphere	*CO_2 + O_2* Chamber 1-CO_2 40·0 ± 0·6%, O_2 30·0 ± 0·4%; Chamber 2-CO_2 80·0 ± 0·7% *Ar + O_2* Chamber 1-CO_2 30·1 ± 1·7%, O_2 1·3 ± 0·6%, Ar 58·6%	Carcass Damage (Skin Perforation), Tenderloin Hemorrhage	Carcass Damage: P <0.001 Tenderloin Hemorrhage: P=0.05	The addition of Ar with CO_2 resulted in significantly greater skin perforations and tenderloin hemorrhaging.	McKeegan et al., 2007

CHAPTER 2: EVALUATION OF CIRCULATING BLOOD HORMONES IN BROILER CHICKENS STUNNED BY EITHER ELECTRICAL OR CONTROLLED ATMOSPHERE METHODS UNDER COMMERCIAL CONDITIONS

AUTHORS: Montana R. Riggs*, Rüdiger Hauck[†,*], Bethany I. Baker-Cook*, Rachel C. Osborne*, Amrit Pal*, Maria T. Bethonico Terra*, Gracie Sims*, Andrea Urruita*, Leticia Orellana-Galindo*, Marco Reina*, Juan F. DeVillena‡, Dianna V. Bourassa*

*Department of Poultry Science, Auburn University College of Agriculture, Auburn, Alabama, 001009, United States

[†]Department of Pathobiology, Auburn University College of Veterinary Medicine, Auburn, Alabama, 36849, United States

‡International Center for Food Industry Excellence, Department of Animal and Food Sciences, Texas Tech University, Lubbock, TX 79409, United States

2.1 ABSTRACT

Increased consumer concern for animal welfare has led some poultry producers to alter their stunning methods from electrical stunning (ES) to controlled atmosphere stunning (CAS). CAS is suggested to be more humane than ES, which should lead to a lower level of blood hormones indicative of a stress response. The objective of this study was to assess the impact of ES or CAS on levels of circulating blood hormones. Two separate trials were conducted, Trial 1 having the same flock analyzed for each treatment, and Trial 2 with each treatment sample collected from birds of differing flocks. Blood was collected from 30 broilers (15 ES, 15 CAS) at lairage and 60 broilers (30 ES and 30 CAS) post-stun for both Trial 1 and 2. Trial 2 included 60 additional broilers (30 ES and 30 CAS) immediately pre-stun. Corticosterone (CORT), adrenocorticotropic hormone (ACTH), epinephrine (EPI), and norepinephrine (NOREPI) were measured by ELISA. Data were analyzed using generalized linear models with a significance at $P \leq 0.05$. Means were separated by Tukey's HSD. CORT decreased following ES in both Trials 1 and 2. In Trial 2 EPI increased post-stun for ES broilers. Neither ACTH nor NOREPI differed over time in either trial for ES birds. For CAS, CORT concentrations decreased post-stun in Trial 1, but did not differ in Trial 2. ACTH concentrations post-stun increased in Trial 1 but decreased in Trial 2. EPI and NOREPI concentrations did not differ over time for CAS birds. Based on these results, CORT, ACTH, EPI, and NOREPI did not respond in the same manner and trends differed between stunning methods. Results indicate that neither method of stunning was clearly preferable based on measurement of blood hormone indicators of a stress response and further research is needed to identify the most appropriate indicator of a stress response during stunning for slaughter.

Keywords: Poultry, Controlled Atmosphere Stunning, Electrical Stunning, Hormone

2.2 INTRODUCTION

Various methods of broiler stunning have become available to the poultry industry and continue to be modified and researched to ensure optimal poultry welfare. Currently most broiler processing plants in the United States utilize electrical water-bath stunning (Berg and Raj, 2015). This method induces unconsciousness by exposing birds to an electrical current through ionized water. However, research has shown evidence of distress for birds when processed at an electrical stunning operation (Boyd 1994; Erasmus et al., 2010).

An alternative method of stunning is Controlled Atmosphere Stunning (CAS). CAS uses an increasing concentrations or high concentrations of inert gases, typically carbon dioxide, to render birds unconscious by hypoxia. Multiple phases are applied within the system with a variable increase of concentrations of inert gases. The gradual increase in CO_2 concentrations slowly induces unconsciousness and prevents a return to consciousness prior to shackling and neckcut/slaughter. This method of stunning is considered advantageous for animal welfare for multiple reasons. It reduces human to bird contact and removes the need for live shackling because birds are stunned within their transportation modules. The birds remain in their transport modules until they leave the CAS system and are then shackled while unconscious before exsanguination. For electrical stunning, birds are shackled while conscious, which has been previously shown to lead to a stress response (Kannan et al., 1997; Bedanova et al., 2007). Shackling birds while conscious has also been suggested to cause discomfort due to stimulation of nociceptors in the legs, which are receptors triggered in response to the mechanical agitation (Gentle and Tilston, 2000).

Another concern for animal welfare in relation to electrical stunning is the occurrence of missed stunning (Raj, 2004). Broilers missing the stun due to size variability within the flock can

lead to pain during exsanguination. This issue of broilers missing or receiving an improper stun due to flock variability should not occur when utilizing CAS because each bird will be exposed to the controlled atmosphere to ensure unconsciousness (Raj and Gregory, 1990).

However, since it has been reported by Gerritzen et al. (2000) that a sudden large increase in atmospheric carbon dioxide can cause convulsions, CAS systems have been questioned in their efficacy for animal welfare improvement. The immediate exposure of carbon dioxide during induction of unconsciousness typically results in adverse physical reactions from birds. Gasping and shaking of the head are noted in high frequency during research trials and are indicative of discomfort (McKeegan et al., 2006). This may be due to the water within the mucosa of the respiratory system reacting with high levels of CO_2 creating carbonic acid (Anton et al., 1992). Inspiration/inhalation of high CO_2 concentrations causes an increase of both intracellular and extracellular acidosis, which is the primary factor for the loss of consciousness (Martoft et al., 2002). However, acidosis within the mucosal membranes of poultry directly stimulates the trigeminal nociceptor response. These findings have made controlled atmosphere stunning a questioned alternative of stun, which may, in part, be responsible for slow adoption of this technology in the U.S.

There is little research available comparing the impact of electrical stunning applied under U.S. parameters and CAS used under commercial conditions on circulating blood hormones indicative of a stress response. The current parameters utilized by U.S. processing plants are typically low voltage-high frequency (12–38 V, ≥400 Hz). Low voltage stunning provides meat quality benefits, through the reduction of product damage (Siqueira et al., 2017). However, low voltage stunning must be coupled with a swift neck cut to prevent the birds from regaining consciousness prior to death.

Corticosterone (CORT) has been considered the gold standard blood hormone to indicate stress in poultry (Puvadolpirod and Thaxton, 2000; Siegel, 1985). CORT is released by the adrenal glands in response to a stressor through the hypothalamus-pituitary-adrenal axis. High circulatory concentrations of CORT are indicative of a physiological reaction to a stressful situation. Zulkifli et. al (2019) reported that shackling prior to electrical stunning caused an increase of CORT concentrations in broilers. Shackling is considered stressful for conscious broilers and is a negative aspect of electrical stunning on broiler welfare (Bedanova et al., 2007). When comparing CORT concentrations of broilers undergoing either electrical stunning or CAS, Pinto et al. (2016) found higher concentrations in electrically stunned broilers when compared to CAS after the stun was administered. The same result was found by Vizzier-Thaxton et al. (2010) when comparing CORT concentrations of electrically stunned and low-atmosphere pressure stunned (LAPS) broilers, where CORT was also higher for electrically stunned broilers after stunning. However, these studies were not performed under a commercial setting, used either high voltage or unreported electrical stunning parameters, and utilized different concentrations of atmospheric gases within the CAS treatments.

Circulatory CORT concentrations have typically been found to increase in under ten minutes post-exposure to a stressor (Sapolsky et al., 2000). This makes utilizing CORT as a marker for broiler stress during stunning potentially unreliable, as the CAS process typically takes approximately five minutes. Electrical stunning varies, but broilers are typically stunned less than one minute post-shackling. Because of the short amount of time between exposure to a stressor and blood collection, CORT may not be a reliable indicator of a broiler stress response caused by bird handling and shackling in this context. Evaluation of other stress-linked blood hormones may be beneficial in determining a broiler stress response. The precursor of CORT

within the HPA axis, adrenocorticotropic hormone (ACTH), has potential as an indicator of an acute stress response (Olanrewaju et al., 2006). Circulatory ACTH must accumulate before CORT enters circulation and has been used to invoke a response in prior studies evaluating broiler stress responses (Edens and Siegel, 1975; King and Chen, 1998). ACTH also initiates the 'fight-or-flight' response for birds to react quickly to life-threatening stressors (Martin, 1978). The main hormones involved in this response are epinephrine (EPI) and norepinephrine (NOREPI), both catecholamines used for increased respiration, blood flow, and heart rate, which are released less than one minute post-ACTH circulation (Kadono, et al., 1964; Sapolsky et al., 2000). These blood hormones may be beneficial in determining stress responses of broilers during stunning given their acute release in comparison to CORT.

Controlled atmosphere stunning has been suggested to be more humane than electrical stunning, which should in turn lead to a lower level of blood hormone indicators of a stress response. The objective of this study was to assess the impact of either electrical stunning or CAS on the circulating blood hormones CORT, ACTH, EPI, and NOREPI from broiler chickens prior to and following stunning for slaughter in a commercial facility.

2.3 MATERIALS AND METHODS

Experimental Design

The experiment was performed at a commercial processing plant in the United States and consisted of two trials, performed on separate days. For Trial 1, broiler chickens for both electrical stunning and CAS treatments were from the same flock. For Trial 2 broiler chickens were sourced from different flocks due to availability and constraint with the processing facility's production. All flocks had a target weight of 2.7 kg per bird. Two stunning methods

were utilized as treatments: a) electrical stun, or b) controlled atmosphere stun. The electrical and controlled atmosphere (CAS) stunning systems were on separate operational lines. For electrical stunning, birds were removed from their transport crates by tipping, shackled, electrical waterbath stunned at 20 mA/bird for 12 s, and mechanically neck cut. For CAS, birds were stunned in their transport crates by exposure to increasing concentrations of CO_2 from 20% to 85% over the course of 5 min. Following CAS, carcasses were shackled and mechanically neck cut. Blood was collected from broilers at three separate timepoints for each treatment group. The three timepoints were lairage, immediately pre-stun, and post-stun. Samples were taken from a differing broiler at each collection timepoint, and one sample was collected per broiler. At lairage, 30 and 15 blood samples per stunning method were collected for Trial 1 and Trial 2, respectively. The module at lairage was randomly selected amongst the transportation truck. Five broilers were randomly chosen per level within the module. The decision to decrease the number of samples in Trial 2 from 30 to 15 was made upon the statistically calculated requirement for basal concentrations for analysis based on Trial 1 results. Immediately pre-stun in Trial 2, 30 blood samples per stunning method were collected. Broilers were randomly chosen amongst the shackling line (ES) or within the tunnels leading to the CAS system and removed from their transportation tray. Post-stun, 30 blood samples per stunning method were collected for both Trials 1 and 2. Broilers were randomly selected in Trial 1 after the completion of the electrical stun, and in Trial 2 after the neck-cut was applied (ES). For CAS, broilers were randomly selected from various transportation coops, and removed after completion of stun. Blood samples were evaluated for CORT and ACTH in Trial 1 and CORT, ACTH, EPI, and NOREPI in Trial 2. EPI and NOREPI were included within Trial 2 as they were determined to be potential indicators of an acute stress with consideration after Trial 1.

Blood Collection

Blood collection was performed for both trials between 7-8:00 am, EST. For both treatments at lairage, each broiler was individually removed from the transportation coop and cervically dislocated by a trained/qualified on-site professional. Handling of the bird was kept at the minimum possible to minimize the impact of bird handling for blood collection. Immediately after cervical dislocation, the head was removed at point of dislocation and blood was collected.

For the immediately pre-stun timepoint of collection, broilers were removed from the shackles individually just before contact with the electrical waterbath. CAS broilers were individually removed from their transportation modules on the conveyor just prior to gas exposure. Blood was collected by the same procedure as at lairage (cervical dislocation, followed by removal of head at point of dislocation).

At the post-stun location, all birds had been rendered unconscious for subsequent neck-cut. Broilers were individually removed from shackles after the neck-cut for exsanguination for electrically stunned broilers in Trial 1 and Trial 2, and blood was collected. In Trial 1, CAS broilers were removed from the transportation modules after the stun was completed, and heads were removed for blood collection. In Trial 2, CAS broilers were removed from shackles after the neck-cut for exsanguination, and blood was collected, as this was considered beneficial for the flow of operation within the processing facility.

Blood samples (4 to 8 mL) were collected in heparinized tubes (BD Vacutainer Na Heparin N, Becton Dickinson, New Jersey, U.S.A.) gently inverted for 10 seconds to ensure dispersal of heparin, and immediately put on ice. Samples were transported on the same day to the laboratory and centrifuged for 10 minutes at 1370 x g for separation. Blood serum from each

sample was then removed by pipet and placed in clean sterile tubes. The serum samples (n=270) were then stored at -80°C prior to analysis.

Blood Hormone Measurement

CORT, ACTH, EPI, and NOREPI concentrations from blood serum were analyzed using enzyme-linked immunosorbent assays (ELISA). CORT (Wuhan Fine Biotech Co., Wuhan, China), ACTH (MyBioSource, San Diego, California), EPI and NOREPI (Abnova™, Taoyuan City, Taiwan) were quantified by a competitive ELIS, per manufacturer instruction. Serum samples were thawed and centrifuged for 60 seconds at 3500 x g to assure no remnants were included in the assay. For each ELISA plate, optical density was measured using a multiscan plate reader (Spectramax iD3, Molecular Devices). The best fit curve was determined using Curve Expert 1.4 software. The data were transformed based on the most accurate model applied to the standards of each ELISA plate. Table 1 outlines the models that were determined to be of the highest accuracy for each dataset. EPI and NOREPI were only analyzed during Trial 2 because these hormones were added as potential hormones of interest following Trial 1.

Statistical Analysis

A completely randomized design with two treatments (electrical stun or CAS) and either two timepoints (lairage and post-stun, Trial 1) or three timepoints (lairage, pre-stun, and post-stun, Trial 2) was used. Blood hormone concentration data were analyzed by treatment and timepoint using the General Linear Model procedure. Means were separated by Tukey's HSD with significance determined at $P \leq 0.05$. Trends were considered relevant at $P \leq 0.10$. All analyses were conducted using the SAS OnDemand for Academics software (SAS Institute Inc, Cary, NC).

As different birds were utilized for each timepoint and each stunning line, basal concentrations, considered at lairage, were analyzed for statistical difference ($P \leq 0.05$). If no statistical difference was found, statistical comparisons between stunning methods at each timepoint were considered valid. If a significant different was found, comparisons between each stunning method were not considered for further analysis, and instead only considered between the timepoints of the stunning methods themselves.

2.4 RESULTS

Results for CORT, ACTH, EPI, and NOREPI concentrations are presented in Table 2. Results from Trial 1 and Trial 2 are reported separately because one flock of birds was used for Trial 1 for both treatments while two different flocks of birds were used for each treatment in Trial 2 due to logistical needs within a commercial processing facility. Additionally, significant interactions were observed between the main effects of trial, treatment, and timepoint for the blood hormones measured.

Corticosterone

In Trial 1, CORT concentrations decreased for both electrical stunning and CAS treatments between lairage (92.75 ng/mL electrical, 87.55 ng/mL CAS) and post-stun (53.69 ng/mL electrical, 59.48 ng/mL CAS, $P<0.0001$). When comparing CORT concentrations between electrical stunning and CAS treatments at each timepoint, no significant differences were observed at lairage ($P=0.2862$) or post-stun ($P=0.0850$).

In Trial 2, electrically stunned broilers at lairage had a CORT concentration of 98.00 ng/mL which did not differ from pre-stun CORT concentration (112.87 ng/mL). Post-stun, CORT concentrations were found to decrease to 66.42 ng/mL when compared to pre-stun values

(P=0.0159) but did not differ from lairage CORT concentration. For CAS broilers, no differences were observed in CORT concentrations between lairage (65.52 ng/mL), pre-stun (51.04 ng/mL), or post-stun (50.51 ng/mL, *P*=0.2113). When comparing CORT concentrations between electrical stunning and CAS treatments at lairage, there was no difference between electrical stunning and CAS (*P*=0.0850). However, because differences in CORT concentrations between electrical stunning and CAS at lairage were nearing significance and different flocks were used for each treatment it was determined that valid comparisons between stunning types for Trial 2 could not be made.

Adrenocorticotropic Hormone

In Trial 1, ACTH concentrations for the electrical stun treatment did not differ from lairage to post-stun (0.5710 pg/mL, 0.5649 pg/mL; *P*=0.9315). ACTH concentrations increased in the CAS treatment from lairage to post-stun (0.5095 pg/mL, 0.9898 pg/mL; *P*=0.0025). When comparing ACTH concentrations between electrical stunning and CAS treatments at each timepoint, no difference was observed at lairage (*P*=0.2217), however birds in the CAS treatment had higher ACTH concentrations post-stun (*P*=0.0005).

In Trial 2, electrically stunned broilers at lairage had an ACTH concentration of 0.7189 pg/mL which did not differ from concentrations at pre-stun at 0.5376 pg/mL or post-stun at 0.6256 pg/mL (*P*=0.1240). CAS broilers at lairage had a concentration of ACTH of 0.1757 pg/mL, which then decreased at pre-stun (0.1184 pg/mL) and post-stun (0.0979 pg/mL, *P*=0.0052). When comparing ACTH concentrations between electrical stunning and CAS treatments at lairage, broilers to be used for the electrical stunning treatment had a higher mean concentration than broilers to be used for CAS (*P*<0.0001). Because ACTH concentrations

between broilers intended for electrical stunning and CAS treatments were different at lairage, it was determined that valid comparisons between stunning types for Trial 2 could not be made.

Epinephrine

Electrically stunned broilers at lairage had an EPI concentration of 1.285 pg/mL which did not differ from pre-stun EPI concentration (1.021 pg/mL). Post-stun, EPI concentration was found to increase to 1.555 pg/mL when compared to pre-stun values (P=0.0068). No differences were observed for EPI concentrations between timepoints for CAS broilers. When comparing EPI concentrations between electrical stunning and CAS treatments at lairage, broilers to be used for the electrical stunning treatment had a lower concentration than broilers to be used for CAS (P=0.0036). Because EPI concentrations between broilers intended for electrical stunning and CAS treatments were different at lairage, it was determined that valid comparisons between stunning types for Trial 2 could not be made.

Norepinephrine

At lairage, electrically stunned broilers had a NOREPI concentration of 30.01 pg/mL which did not differ from pre-stun (28.16 pg/mL) or post-stun (37.19 pg/mL) NOREPI concentration (P=0.5781). CAS broilers at lairage had a NOREPI concentration of 24.10 pg/mL, with a trend for a difference from the pre-stun or post-stun NOREPI concentrations of 21.92 pg/mL or 15.77 pg/mL, respectively (P=0.0555). NOREPI concentrations at lairage did not differ between the electrical stun and CAS treatments (P=0.5934), and therefore comparisons were made between stunning types. NOREPI concentrations between electrically stunned broilers and CAS broilers did not differ at pre-stun (P=0.2432). However, NOREPI concentrations were

found to be lower at post-stun for CAS (15.77 pg/mL) compared to electrically stunned broilers (37.19 pg/mL, *P*=0.0052).

2.5 DISCUSSION

In Trial 1, CORT concentrations decreased from lairage to post-stun for both electrically stunned broilers and CAS broilers. A trend was observed for higher CORT concentrations of CAS broilers in comparison to electrically stunned broilers post-stun. These results differed from a previous study by Pinto et al. (2016) evaluating CORT concentrations between CAS broilers and electrically stunned broilers, where CORT was higher from blood of electrically stunned broilers. However, CORT concentrations were not evaluated prior to stunning, and it is possible basal concentrations could have differed. Pinto et al. (2016) also used 25% argon within their CAS system, whereas the current study only utilized CO_2. The inclusion of argon in CAS systems has been previously argued to decrease stress in hens and may be why Pinto et al.'s results differed from this current study (Webster and Fletcher, 2001).

The trend of higher CORT concentrations for CAS broilers post-stun in comparison to electrically stunned broilers may be attributed to the difference in time taken to completion of stun. As previously mentioned, CORT concentrations typically take more than 5 minutes to observe an increase in broilers (Sapolsky et al., 2000). In electrical stunning, the time between shackling and post-stun was less than 1 min, whilst the duration of CAS itself was 5 minutes. Since the duration of time between shackling and post-stun for electrical stunning is shorter than the duration of CAS, CORT concentration differences may have only been possible to observe for CAS broilers, regardless of stress response, due to required accumulation time.

CORT concentrations decreased between lairage and post-stun, in Trial 1, and pre-stun and post-stun, in Trial 2. A previous study evaluating circulatory concentrations of corticosterone of broilers with or without restraints during processing followed by receiving either an electrical stun or no stun, found that unrestrained broilers that were electrically stunned had lower CORT concentrations than un-stunned birds that were also unrestrained (Huang et al., 2014). This notable decrease in CORT concentrations following electrical stunning is hypothesized to be due to the inhibition of Ca^{2+} channels during electrical stimulation previously described in rats (Samidurai, et al., 2018), which has previously been shown to impair the pituitary-adrenal stimulated release of CORT (Borycz et al., 1993).

CORT concentrations of CAS broilers either decreased or did not differ between any timepoint suggesting a lack of stress response as measured by circulating CORT concentrations. The lack of live bird handling, decreased bird exposure to light in the tunnel prior to stun, and the use of increasing concentrations of CO_2 for stunning have been reported to benefit poultry welfare (Martin et al., 2016; McKeegan et al., 2007). However, this lack of change observed in CORT concentrations does not suggest that CAS negatively or positively impacts the poultry stress response.

In Trial 2, differences in CORT concentrations between electrically stunned broilers and CAS broilers at lairage were only compared for birds at lairage. Since Trial 2 evaluated broilers from different flocks for each treatment and the baseline levels of CORT of birds from each treatment were nearing significance, the differences between each stunning method treatment could not be evaluated. Since information about these commercial flocks prior to lairage was not available, differences in basal concentrations can only be speculative. Since different flocks were evaluated, this may alter the behavior, sensitivity, or timing of the hypothalamus-pituitary-

adrenal (HPA) axis response to short term stress, therefore any comparisons made may not be valid (Dos Santos et al., 2020; Zheng et al., 2020).

ACTH concentrations did not differ between timepoints for electrically stunned broilers. This could be because ACTH can influence circulatory CORT concentrations up to 30-60 min past the initial cascade response and the half-life of ACTH in small vertebrates is less than 10 minutes (Walker et al., 2015; Lopez and Negro-Vilar, 1988), although cascade response time and half-life for ACTH in poultry is not known. Since CORT was significantly higher at lairage for electrically stunned broilers, the initial ACTH released may have been degraded during waiting time at lairage and during shackling, while CORT can continuously circulate for up to an hour after initial dose. Since electrical stunning following shackling is less than 1 min, an increase in ACTH would not be physiologically necessary due to the already heightened CORT concentrations' ceiling effect upon hormonal release. This could explain the lack of difference in ACTH concentrations during electrical stunning in broilers.

For the CAS broilers, an increase in circulatory ACTH from lairage to post-stun was seen for one trial while a decrease was seen in the other. Conversely CAS broilers circulatory ACTH concentrations in Trial 1 increased from lairage to post-stun. This difference in ACTH response could possibly be explained as a response to stressors during CAS (Canoine et al., 2002). Since ACTH is a direct precursor to trigger CORT release, when CORT levels are low, an increase in ACTH prior to an increase in CORT would be expected (Kunzel et al., 2020). CORT concentrations typically increase about five minutes after exposure to an ACTH increase, which may be why no parallel increase in CORT was observed, as CAS can take upwards of five minutes to completion of stunning (Hermans et al. 2014). However, since broilers are only conscious for 1 to 2 minutes of the CAS process, it is possible this ACTH concentration increase

was a physiological response during the phases of CAS that broilers are unconscious, therefore not impeding on animal welfare. Another explanation could be that the ACTH increase was a result of a biochemical reaction from the increased atmospheric CO_2 concentration. In humans, a single breath of 35% atmospheric CO_2 significantly increases circulatory ACTH in under two minutes and was found to activate the HPA axis response (Kaye et al., 2004). During CAS broilers are exposed to CO_2 concentrations ranging from 20-85%. When inhaled, high concentrations of CO_2 result in respiratory acidosis (Kisaka et al., 2015). A decrease in blood pH was previously found to stimulate ACTH release into circulation in anesthetized sheep (Wood and Isa, 1991). Therefore, the ACTH increase observed during Trial 1 may be a physiological response to blood acidification, caused by the increased CO_2 exposure, rather than a direct response to stressors during CAS.

However, circulatory ACTH from CAS broilers in Trial 2 decreased between lairage and pre-stun/post-stun. In this case, since ACTH is a direct precursor to trigger CORT release, it was expected to see no differences in CORT concentrations at the same timepoints for CAS broilers, as CORT circulates longer than ACTH in the blood (Walker et al., 2015; Lopez and Negro-Vilar, 1988). Interestingly, opposite trends of ACTH concentrations over time were observed for CAS broilers in Trial 1 and Trial 2. One reason for this observation may be the difference in blood collection methods between trials of CAS broilers. Trial 1, broilers were removed from the line after completion of stun and cervically dislocated prior to blood collection. Trial 2, blood was collected after the neck-cut for exsanguination was administered. It is possible that the added time required for broilers in Trial 2 to be shackled after completion of stun and continue to the neck-cut had allowed for ACTH concentrations to deplete due to its relatively short half-life (Lopez and Negro-Vilar, 1988).

When analyzing EPI, it is important to note that only differing flocks at each timepoint were used for analysis. An increase of circulatory EPI was observed in electrically stunned broilers from pre-stun to post-stun, which was unexpected due to the decrease of CORT at the same time periods. However, EPI has a different relative function within the body in comparison to CORT. Specifically, EPI is released within seconds after neural induction of a stressor and only circulates in the system for about 1 to 3 min (Romero and Butler, 2007; Kvetnansky and McCarthy, 2007). The timeframe between the collection points of pre-stun and post-stun within the electrical stun treatment were 10 to 15 seconds, where birds were immediately removed for blood collection. EPI is one of the most acutely released stress biomarkers (Sapolsky et al., 2000), it is possible that the timeframe between pre-stun and post-stun was long enough to invoke an observable EPI response as it is one of the most acutely released stress biomarkers, but the time frame was too short to determine a noticeable difference in CORT. Another possible reason for this increase could be related to an electrical interference upon EPI-relevant receptors. As previously demonstrated by Wakade (1981), direct electrical stimulation of the adrenal medulla resulted in a mass-release of EPI. This was found to be a result of the electrotonic-stimulated depolarization of the medullary cell membrane, where Ca^{2+} channels open for an influx of Ca^{2+}, and subsequent firing of the action potential, which is the stimulatory effect required for EPI release. Consequentially, the relevance of this increased circulatory EPI to bird welfare is unclear/unknown. If the EPI release was propagated by physiological interference from the applied electrical current, then EPI may not be a reliable indicator of a stress response during electrical stunning.

Conversely, there was no difference in EPI concentrations between lairage and pre-stunning, even though the shackling process has been previously shown to elicit a stress response

in broilers (Boyd, 1994; Erasmus et al., 2010). However, shackling is considered the most stress-inducing live handling process during processing (Kannan et al., 1997). It is possible that any circulatory EPI released during live handling may have left circulation before the pre-stun timepoint was sampled, since pre-stun blood samples were collected at the last possible location prior to stun.

NOREPI concentrations did not differ over time for electrically stunned broilers. NOREPI and EPI are released simultaneously (Romero, 2010). NOREPI releases at a lower rate in comparison to EPI but has higher overall circulatory concentrations due to its larger overall abundance within the body (Carsia, 2015). The amount of time between sample collection points may not have been adequate for significant differences in concentrations to accumulate since it is released in smaller quantities. This may explain why EPI concentrations between timepoints were different when NOREPI concentrations were not.

CAS broilers had no change in EPI concentrations over time, but there was a trend for decreasing NOREPI concentrations over time. Both EPI and NOREPI are released in response to acute stressors (fight-or-flight). ACTH release also stimulates the release of EPI and NOREPI (Zachariasen and Newcomer, 1974). The decreasing trend observed in NOREPI concentrations combined with the significant decrease observed for ACTH concentrations of CAS broilers in Trial 2 may indicate that CAS did not elicit a stress response. It has been questioned if the inhalation of increased atmospheric CO_2 concentrations during CAS causes irritation through carbonic acid synthesis within the birds' mucosal lining of the upper respiratory tract (Raj, 1998). The decreases observed for these hormones does not suggest a response to discomfort during the CAS process (Jorum et al., 2007). As previously mentioned, EPI concentrations were greater at the post-stun timepoint in comparison to the pre-stun timepoint for electrically stunned

birds. Given the results from this study, it is possible that CAS may be advantageous based on measurements of ACTH and EPI prior to and following stunning. Further analysis, such as analyzing broilers of the same flock between both treatments and differentiating changes in blood hormone concentrations due to a stress response versus a physiological consequence of the method (e.g. electrical shock or blood pH) is necessary to further understand these differing responses.

It is possible that EPI may be a better indicator of an acute stress response in broilers than CORT. In this experiment, an increase in EPI occurred concurrently to a decrease in CORT in the electrically stunned broilers during Trial 2. Of the two biomarkers of stress, EPI is released more rapidly than CORT. So, the increase in EPI observed with the lack of CORT increase, may suggest that EPI is more reliable marker for a short-term stress response, particularly those under the ten minutes. Similarly, ACTH is a precursor for CORT, and thus might be a better biomarker of a short-term stress response. In Trial 1, the CAS broilers, had a higher ACTH concentration post-stun than at lairage, while the opposite was observed for CORT. As the release of ACTH stimulates the release of CORT, an observation of ACTH increase without a CORT increase could indicate the presence of a stressor, but that the time between the stressor and sampling was not sufficient to see an increase in CORT. However, further analysis is required to make definitive assumptions about the quality of EPI and ACTH as an indicator of an acute stress response in placement of CORT.

Conclusion

The differences in concentration changes of each hormone for electrically stunned and CAS broilers indicate that both stunning methods may induce a stress response at different time points. Various responses were observed between stunning methods for broilers of the same

flock. Broilers of differing flocks in Trial 2 for each stun method varied in comparison to Trial 1 results.

For electrical stunning, the increase of EPI observed post-stun may be a negative response to stressors potentially due to bird shackling. For CAS, the time between entering the stunner and exiting the stunner produced increases in ACTH, but only when the *same* flock was evaluated over time. However, at no other time point was an increase for CORT, EPI, or NOREPI observed. Whether the ACTH increase was during the conscious or unconscious period of broilers during CAS is unknown, with each having different implications.

The results observed may be due to the variation in flocks, as stressors experienced by broilers reared separately could vary. Glucocorticoids and, in general, endocrine functionality vary with broilers of differing flocks due to both genetic and epigenetic factors (Aire, 1980; Mormède et al., 2007; Jenkins et al., 2014). By studying a consistent flock, many exogenous factors may be mitigated to help make conclusive analyses. Until then, definitive statements regarding the methodologies utilized in this study cannot be made.

We can conclude that neither method of stunning was clearly preferable based on measurement of blood hormone indicators of a stress response. Both methods of stunning should be further evaluated to conclude what aspect of the process at each timepoint is causing these biomarker changes.

2.6 REFERENCES

Aire, T. A. 1980. Morphometric study of the avian adrenal gland. J. Anat. 131:19–23.

Anton, F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO_2 pulses applied to the nasal mucosa. Pain. 49:53-60.

Bedanova, I., E. Voslarova, P. Chloupek,V. Pistekova, P. Suchy, J. Blahová, R. Dobsikova, and V. Vecerek. 2007. Stress in broilers resulting from shackling. Poult. Sci. 86:1065-1069.

Berg, C. and A. B. M. Raj. 2015. A review of different stunning methods for poultry-animal welfare aspects (Stunning Methods for Poultry). Animals. 4:1207-1219.

Borycz, J., A. J. Bugajski, A. Gadek-Michalska, and J. Bugajski. 1993. Calcium channel blockers impair the pituitary-adrenocortical responses to central adrenergic receptors stimulation. J. Physiol. Pharmacol. 44:161-170.

Boyd, F. 1994. Humane slaughter of poultry: The case against the use of electrical stunning devices. J. Agric. Environ. Ethics. 7:221–236.

Canoine, V., K. Rowe, W. Goymann, and T. Hayden. 2002. The stress response of european stonechats depends on the type of stressor. Behaviour. 139:1303-1311.

Carsia, R. V. 2015. Circulating catecholamines and stress response. In: Sturkie's Avian Physiology. Editor(s): Colin G. Scanes. Academic Press. 6:600-601.

Dos Santos, V. M., B. Dallago, A. Racanicci, Â. P. Santana, R. I. Cue, and F. Bernal. 2020. Effect of transportation distances, seasons and crate microclimate on broiler chicken production losses. PloS One. 15:4.

Edens, W. F., and H. S. Siegel. 1975. Adrenal responses in high and low ACTH response lines of chickens during acute heat stress. Gen. Comp. Endocrinol. 25:64-73.

Erasmus, M. A., P. V. Turner, and T. M. Widowski. 2010. Measures of insensibility used to determine effective stunning and killing of poultry. J. Appl. Poult. Res. 19:288-298.

Gent, T. C., S. Gebhardt-Henrich, S. L. A. Schild, A. A. Rahman, and M. J. Toscano. 2020. Evaluation of poultry stunning with low atmospheric pressure, carbon dioxide or nitrogen using a single aversion testing paradigm. Animals. 10:1308.

Gentle, M. J., V. L. Tilston, and D. E. F. McKeegan. 2001. Mechanothermal nociceptors in the scaly skin of the chicken leg. Neurosci. 106:643-652.

Gentle, M. J. and V. L. Tilston. 2000. Nociceptors in the legs of poultry: implications for potential pain in pre-slaughter shackling. Anim. Welf. 9:227-236.

Gerritzen, M. A., E. Lambooij, S. J. Hillebrand, J. A. Lankhaar, and C. Pieterse. 2000. Behavioral responses of broilers to different gaseous atmospheres. Poult. Sci. 79:928-933.

Hermans, E. J., M. Henckens, G. Ja., M. Joëls, and G. Fernández. 2014. Dynamic adaptation of large-scale brain networks in response to acute stressors. Trends. Neurosci. 37:304–14.

Huang, J. C., M. Huan, P. Wang, L. Zhao, X. L. Xu, G. H. Zhou, and J. X. Sun. 2014. Effects of physical restraint and electrical stunning on plasma corticosterone, postmortem metabolism, and quality of broiler breast muscle. J. Animal Sci. 92:5749–5756.

Jenkins, B. R., M. N. Vitousek, J. K. Hubbard, and R. J. Safran. 2014. An experimental analysis of the heritability of variation in glucocorticoid concentrations in a wild avian population. Proc. R. Soc. B. 281:20141302.

Jorum, E., K. Orstavik, R. Schmidt, B. Namer, R. W. Carr, G. Kvarstein, M. Hilliges, H. Handwerker, E. Torebjork, and M. Schmelz. 2007. Catecholamine-induced excitation of nociceptors in sympathetically maintained pain. Pain. 127: 296-301.

Kadono, H., T. Okada, and K. Ohno. 1964. Effects of certain autonomic drugs on the electrocardiogram of the chicken. Poult. Sci. 43:1265-1272.

Kannan, G., J. L. Heath, C. J. Wabeck, and J. A. Mench. 1997. Shackling of broilers: Effects on stress responses and breast meat quality. Br. Poult. Sci. 38:323-332.

Kaye, J., F. Buchanan, A. Kendrick, P. Johnson, C. Lowry, J. Bailey, D. Nutt, and S. Lightman. 2004. Acute carbon dioxide exposure in healthy adults: evaluation of a novel means of investigating the stress response. J. Neuroendocrinol. 16:256-264.

King, Y. T. and T. C. Chen. 1998. Chemical and physical characteristics of chicken livers following adrenocorticotropic hormone-induced stress. J. Food Sci. 63:589-591.

Kisaka, T., T. A. Cox, D. Dumitrescu, and K. Wasserman. 2015. CO_2 pulse and acid-base status during increasing work rate exercise in health and disease. Respir. Physiol. Neurobiol. 218:46-56.

Kuenzel, W. J., S. W. Kang, and A. Jurkevich. 2020. Chapter Seven - the vasotocinergic system and its role in the regulation of stress in birds. In: Vitamins and Hormones. Editor(s): Litwack G. Academic Press. 113:183–216.

Kvetnansky, R., and R. McCarty. 2007. Adrenal Medulla. In: Encyclopedia of Stress. Editor(s): George Fink. Academic Press. 2:52-59.

López, F. J. and A. Negro-Vilar. 1988. Estimation of endogenous adrenocorticotropin half-life using pulsatility patterns: a physiological approach to the evaluation of secretory episodes. Endocrinol. 123:740-746.

Martin, J., K. Christensen, Y. Thaxton, and D. McKeegan. 2016. Effects of light on responses to Low Atmospheric Pressure Stunning in broilers. Br. Poult. Sci. 57:585-600.

Martin, J. T. 1978. Embryonic pituitary adrenal axis, behavior development and domestication in birds. Am. Zool. 18:489–499.

Martoft, L., L. Lomholt, C. Kolthoff, B. E. Rodriguez, E. W. Jensen, P. F. Jorgensen, H. D. Pedersen, and A. Forslid. 2002. Effects of CO_2 anaesthesia on central nervous system activity in swine. Lab. Anim. 2002. 36:115–126.

McKeegan, D. E. F., J. A. McIntyre, T. G. M. Demmers, J. C. Lowe, C. M. Wathes, P. L. C. van den Broek, A. M. L. Coenen, and M. Gentle. 2007. Physiological and behavioural responses of broilers to controlled atmosphere stunning: Implications for welfare. Anim. Welf. 16:409–426.

McKeegan, D. F., J. M. Demmers, T. G. Wathes, M. Christopher, and B. R. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271-286.

McKeegan, D. E. F, F. S. Smith, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2005. Behavioural correlates of olfactory and trigeminal gaseous stimulation, Gallus Domesticus. Physiol. Behav. 84:761-768.

Mormède, P., S. Andanson, B. Aupérin, B. Beerda, D. Guémené, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. van Reenen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317-339.

Olanrewaju, H. A., S. Wongpichet, J. P. Thaxton, W. A. Dozier, and S. L. Branton. 2006. Stress and acid-base balance in chickens. Poult. Sci. 85:1266-1274.

Pinto, M. F., D. A. Bitencourt, E. Ponsano, M. G. Neto, and I. L. C. Bossolani. 2016. Effect of electrical and controlled atmosphere stunning methods on broiler chicken behavior at slaughter, blood stress indicators and meat traits. Braz. J. Vet. Res. Anim. Sci. 53:1.

Puvadolpirod, S. and J. P. Thaxton. 2000. Model of physiological stress in chickens. 5. Quantitative evaluation. Poult. Sci. 79:363-369.

Raj, A. B. M. and N. G. Gregory. 1990. Investigation into the batch stunning/killing of chickens using carbon dioxide or argon-induced hypoxia. Res. Vet. Sci. 49:364-366.

Raj, A. B. M. 1998. Welfare during stunning and slaughter of poultry. Poult. Sci. 77:1815-1819.

Raj, A. B. M. 2004. “Stunning and slaughter of poultry.”. In *Poultry meat processing and quality.* Edited by: Mead, G. C. Cambridge, UK: Woodhead.

Romero, L. M. 2010. Fight or flight responses. In: Encyclopedia of Animal Behavior. Editor(s): Michael D. Breed, Janice Moore. Academic Press. 1:710-714.

Romero, M. L, and L. K. Butler. 2007. Endocrinology of Stress. Int. J. Comp. Psych. 20:89-95.

Samidurai, M., H. Kang, V. S. Ramasamy, and J. Jo. 2018. Impact of electrical stimulation on cortisol secretion in rat adrenal gland. BioChip J. 12:216–221.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

Siegel, H. S. 1985. Immunological responses as indicators of stress. Worlds Poult. Sci. J. 41:36-44.

Siqueira, T. S., T. D. Borges, R. M. M. Rocha, P. T. Figueira, F. B. Luciano, and R. E. F. Macedo. 2017. Effect of electrical stunning frequency and current waveform in poultry welfare and meat quality. Poult. Sci. 96:2956-2964.

Vizzier-Thaxton, Y., K. D. Christensen, M. W. Schilling, R. J. Buhr, and J. P. Thaxton. 2010. A new humane method of stunning broilers using low atmospheric pressure. J. Appl. Poult. Res. 19:341-348.

Wakade, A. R. 1981. Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. J. Physiol. 313:463-480.

Walker, B. G., S. L. Meddle, L. M. Romero, M. M. Landys, J. Reneerkens, and J. C. Wingfield. 2015. Breeding on the extreme edge: Modulation of the adrenocortical response to acute stress in two High Arctic passerines. J. Exp. Zool. A. Ecol. Genet. Physiol. 323:266–275.

Webster, A. B., and D. L. Fletcher. 2001. Reactions of laying hens and broilers to different gases used for stunning poultry. Poult. Sci. 80:1371-1377.

Wood, C. E., and A. Isa. 1991. Intravenous acid infusion stimulates ACTH secretion in sheep. Am. J. Physiol. Endocrinol. Metab. 260: 154-161.

Zachariasen, R. D., and W. S. Newcomer. 1974. Phenylethanolamine-N-methyl transferase activity in the avian adrenal following immobilization or adrenocorticotropin. Gen. Comp. Endocrinol. 23:193-198.

Zheng, A., S. Lin, S. A. Pirzado, Z. Chen, W. Chang, H. Cai, and G. Liu. 2020. Stress associated with stimulated transport, changes serum biochemistry, postmortem muscle metabolism, and meat quality of broilers. Animals. 10:1442.

Zulkifli, I., Z. Wakiman, A. Q. Sazili, Y. M. Goh, A. Jalila, Z. Zunita, and E. A. Awad. 2019. Effect of shackling, electrical stunning and halal slaughtering method on stress-linked hormones in broilers. S. Afr. J. Anim. Sci. 49:3.

Table 2.1 Transformation models and calculations utilized for each set of circulatory hormone concentrations by treatment and timepoint.

		Method of Stun			
		Controlled Atmosphere		**Electrical**	
Hormone Measured	**Trial**	**Model**	**Equation**	**Model**	**Equation**
Corticosterone	*1*	Harris	$Y = 1 / (a + bx^c)$	Harris	$Y = 1 / (a + bx^c)$
	2	Harris	$Y = 1/(a + bx^c)$	Heat Capacity	$Y= a + bx + (c/x^2)$
Adrenocorticotropic Hormone	*1*	Harris	$Y = 1/(a + bx^c)$	Harris	$Y = 1/(a + bx^c)$
	2	Morgan-Mercer-Flodin	$Y = (ab+cx^d)/(b+x^d)$	Harris	$Y = 1/(a + bx^c)$
Epinephrine	*1*	N/A	N/A	N/A	N/A
	2	Heat Capacity	$Y = a+bx+(c/x^2)$	Heat Capacity	$Y = a+bx+(c/x^2)$
Norepinephrine	*1*	N/A	N/A	N/A	N/A
	2	Harris	$Y = 1/(a + bx^c)$	Morgan-Mercer-Flodin	$Y = (ab+cx^d)/(b+x^d)$

Table 2.2 Concentrations of circulatory blood hormones from broilers at differing timepoints of either electrical stunning or controlled atmosphere stunning.

			Stunning Method		
Hormone	**Stunning Timepoint**		**ES**	**CAS**	**P-Value**
Trial 1					
CORT (ng/mL)	Lairage		92.75 ± 2.44[1a]	87.55 ± 4.11[a]	0.2862
	Post-Stun		53.69 ± 2.94[b]	59.48 ± 3.97[b]	0.0850
		P-value	<0.0001	<0.0001	
ACTH (pg/mL)	Lairage		0.5710 ± 0.032	0.5095 ± 0.038[b]	0.2217
	Post-Stun		0.5649 ± 0.049[y]	0.9898 ± 0.103[az]	0.0005
		P-value	0.9315	0.0025	
Trial 2					
CORT (ng/mL)	Lairage		98.00 ± 17.31[ab]	65.52 ± 8.48	0.0850
	Pre-Stun		112.87 ± 14.59[a]	51.04 ± 4.65	-
	Post-Stun		66.42 ± 7.04[b]	50.51 ± 5.50	-
		P-value	0.0159	0.2113	
ACTH (pg/mL)	Lairage		0.7189 ± 0.091	0.1757 ± 0.026[a]	<0.0001
	Pre-Stun		0.5376 ± 0.040	0.1184 ± 0.012[b]	-
	Post-Stun		0.6129 ± 0.053	0.0979 ± 0.012[b]	-
		P-value	0.1240	0.0052	
EPI (pg/mL)	Lairage		1.285 ± 0.20[ab]	2.043 ± 0.138	0.0036
	Pre-Stun		1.021 ± 0.11[b]	1.984 ± 0.065	-
	Post-Stun		1.555 ± 0.12[a]	1.885 ± 0.10	-
		P-value	0.0068	0.5262	
NOREPI (pg/mL)	Lairage		30.01 ± 11.16	24.10 ± 3.33	0.5934
	Pre-Stun		28.16 ± 4.91	21.92 ± 2.28	0.2432
	Post-Stun		37.19 ± 6.95[y]	15.77 ± 2.01[z]	0.0052
		P-value	0.5781	0.0555	

[1] Values ± SEM

[a-b] Values within a column within a hormone type with different superscripts are significantly different ($P \leq 0.05$).

[y-z] Values within a row with different superscripts are significantly different ($P \leq 0.05$)

CHAPTER 3: MEAT QUALITY OF BROILER CHICKENS PROCESSED USING ELECTRICAL AND CONTROLLED ATMOSPHERE STUNNING SYSTEMS

AUTHORS: Montana R. Riggs*, Rüdiger Hauck[†,*], Bethany I. Baker-Cook*, Rachel C. Osborne*, Amrit J. Pal*, Maria T. Bethonico Terra*, Gracie Sims*, Andrea Urruita*, Leticia Orellana-Galindo*, Marco Reina*, Juan F. De Villena[2], Dianna V. Bourassa*

**Department of Poultry Science, Auburn University College of Agriculture, Auburn, Alabama, 001009, United States*

[†]*Department of Pathobiology, Auburn University College of Veterinary Medicine, Auburn, Alabama, 36849, United States*

[2]*International Center for Food Industry Excellence, Department of Animal and Food Sciences, Texas Tech University, Lubbock, TX 79409, United States*

3.1 ABSTRACT

Increased consumer concern for animal welfare has led some poultry producers to alter their stunning methods from electrical to controlled atmosphere stunning. The potentially different impacts on meat quality between controlled atmosphere stunning (CAS) and electrical stunning (ES) using current U.S. parameters needs further evaluation, as there is little research available. To assess the impact of each stunning method on meat quality, three trials were conducted in a commercial broiler processing facility. Blood glucose concentrations were measured from broilers stunned by either CAS or ES at the following stages: 1) Lairage 2) Pre-stunning and 3) Post-stunning, using a glucose monitor. Occurrence of visible wing damage was evaluated post-defeathering and breast fillet meat quality was evaluated through measurement of pH, CIE-LAB values, and drip loss. Values were determined both at deboning and 24 hours after deboning. Data were analyzed by GLM or Chi-Square with a significance at $P \leq 0.05$ and means were separated by Tukey's HSD. Blood glucose concentrations (mg/dL) from CAS and ES birds were not different at lairage (284, 272, $P=0.2646$) or immediately prior to stunning (274, 283, $P=0.6425$). Following stunning and neck cut, circulating blood glucose from birds stunned by CAS was significantly higher than ES (418, 259, $P<0.0001$). CAS carcasses had significantly more visible wing damage than ES carcasses (4.3%, 2.4%, $P<0.0001$). Breast fillet pH was lower, L* was higher, and a* was lower at debone for CAS fillets (5.81, 54.65, 1.96) compared to ES fillets (5.92, 53.15, 2.31, $P<0.0001$, $P=0.0005$, $P=0.0303$). Drip loss did not differ between breast fillets of CAS or ES broilers (CAS=4.83, ES=4.84; P=0.0859). Although differences were observed in breast fillet attributes at deboning, differences would have minimal practical application and were no longer present at 24 hours. The implications of increased blood glucose concentration post-CAS are currently unknown and will require further evaluation. However, the

increase in visible wing damage observed post-defeathering from CAS carcasses indicated a need for equipment parameter adjustments during the process from stunning through defeathering when using CAS for broiler stunning. Breast fillet quality did not differ between broilers stunned by either electrical or CAS.

Key words: broiler, controlled atmosphere stunning, electrical stunning, carbon dioxide, meat quality

3.2 INTRODUCTION

From 2011 to 2020 the per capita consumption of broiler meat in the United States has grown 13.9% (USDA, 2021). The increase of broiler production within the United States has similarly increased, in total by 20% over the same time period (USDA, 2021). However, alongside the growth in consumption there has been an increase of concern within the consumers' perspective of animal welfare. Consumer preference for humanely raised animal products has risen exponentially, with some willing to increase spending on products certified under humane credentials (Alonso et al., 2020).

It is common practice within the poultry industry to stun broilers prior to neck-cut and exsanguination. Stunning renders the animal unconscious to avoid unnecessary pain when the neck-cut is administered and aids in automation for neck cut efficiency (Berg and Raj, 2015). Currently, the most common method of stunning in the United States is electrical water-bath stunning. According to industry experts, 95% of commercial broiler production utilizes this method (personal communication). However, research has shown evidence of distress for birds when electrical stunning was used (Boyd, 1994; Erasmus et al., 2010). Birds being shackled

while conscious, and live handling, have been shown to increase circulatory corticosterone and physical stress response indicators (continuous flapping and struggling) (Bedanova et al., 2007; Kannan et al., 1997). Another concern for animal welfare in relation to electrical stunning is the potential for pre-stun shock. Pre-stun shock occurs during electrical stunning when a bird makes improper, premature contact with the ionized water-bath, typically with a wing. This causes an electrical shock and usually is followed by an adverse reaction of flapping and lifting of the head. The lifting of the head may also result in the bird missing the stun entirely. When this occurs, the neck-cut is administered while the bird is conscious, inducing unnecessary pain. There is also potential for smaller-sized broilers within a flock to miss the electrical water bath, and the stun, because the height of the stunning system is unable to accommodate differences in size (Heath et al., 1981). Electrical water-bath stunning has the potential for recovery of consciousness when operated under U.S. parameters if a neck cut is not successfully completed or missed entirely in the appropriate timeframe following stunning (Gibson et al., 2016). U.S. electrical stunning parameters are typically low voltage-high frequency (12-38 V, >400 Hz). This electrical stunning method renders the bird unconscious and has less impact on meat quality than broilers stunned by Controlled atmosphere stunning (CAS) (Kang and Sams, 1999). These animal welfare concerns with electrical stunning have led producers to consider alternative stunning methods.

CAS has increased in popularity due to claims of animal welfare benefits. This method utilizes a gradual, multiphasic change in atmosphere, oftentimes using an increase in carbon dioxide concentrations to induce unconsciousness. One major disadvantage of this method to producers is cost. According to the European Commission Food Chain Evaluation Consortium, CAS systems can cost upwards of $1.5 million U.S.D. for initial capital cost, without factoring in

the long-term requirement of carbon dioxide (FCEC, 2012). While consumer demand for poultry welfare has increased, costs for implementing CAS and concerns regarding product quality have impeded its adoption within the United States.

Alternatively, it has been shown that the physical response to stressors has a critical impact on the overall meat quality of poultry products and can result in various metabolic changes of the muscle (Santonicola et. al, 2017). With the claim of CAS having advantages in animal welfare, this method also has the potential for improved product quality. Studies have found significant improvement in meat product quality, with less carcass damage and rapid initial pH decline for improved deboning when utilizing CAS in comparison to electrical stunning (Raj et al., 1990; Raj et al., 1997). However, Kang and Sams (1999) found that carcass damage, such as bruising, tearing, and broken bones, was lessened with electrical stunning under U.S. parameters (1999) and that the rapid initial pH decline found in CAS stunned broilers has been associated with pale, soft, exudative (PSE) meat (Solomon et al., 1998). However, Kang and Sams utilized a CAS system that required birds be shackled and were only exposed to high CO_2 concentrations for 25 seconds – in comparison to industry practice where birds are sent through CAS systems in transportation modules and exposed for upwards of 5 minutes. These conflicting results may be attributed to variations in gas concentrations (Xu et al., 2011), a difference in flocks of broilers, or differing equipment parameters at each research location. There is limited research comparing meat quality of broiler breast fillets when using either U.S. electrical stunning or CAS. Due to this, there is uncertainty in the benefit of CAS on product quality when compared to U.S. electrical stunning.

To try and get insight into this matter, this study aims to investigate the effect of either electrical stunning or CAS on meat quality by evaluating changes in circulating glucose concentrations, visible wing damage, and breast fillet pH, color, and drip loss.

3.3 MATERIALS AND METHODS

Sample Preparation

The following experiment was performed at a small bird (~2.04 kg live bird weight) commercial processing plant, located within the Southeast region of the United States. Three separate trials were performed within the same facility on separate days in July, August, and November. Female broiler chickens (N = 240 mean weight = 2.7 kg) -were sourced from different flocks were slaughtered by standard industry practice. For Trials 1 and 3, different flocks were used for electrical and CAS treatments. However, for Trial 2 the broilers used for both stunning types were from the same flock.

Birds were assigned one of the two stunning treatments; electrical stun, or controlled atmosphere stun. Post-stunning birds were slaughtered by standard industry practice. Both stunning systems were on separate operational lines. The electrical and CAS stunning systems were on separate operational lines. For electrical stunning, birds were removed from their transport crates by tipping, shackled, electrical water-bath stunned at 20 mA/bird for 12 s, mechanically neck cut, bled for ~90 s, hard scalded at 54ºC for 180 s, then defeathered for 210 s. For CAS, birds were stunned in their transport crates by exposure to increasing concentrations of CO_2 from 20% to 85% over the course of 5 min. Following CAS, carcasses were shackled, mechanically neck cut, bled for ~90 s, hard scalded at 54ºC for 180 s, then defeathered for 210 s.

Following defeathering, carcasses continued through evisceration, immersion chilling, and deboning for both treatments.

Glucose Concentrations

Circulating blood glucose concentrations were evaluated at the following locations for both stunning lines: Lairage (Trial 1, 2, and 3), Immediately Pre-Stunning (Trial 3), and Post-Stunning (Trial 1, 2, and 3). Immediately pre-stunning was only evaluated in Trial 3. This is because it was later determined to be of interest to separate the time period between broilers entering the processing facility until the stunning treatment. At lairage 30, 30, and 15 blood samples per stunning method were collected for Trials 1, 2, and 3, respectively. Immediately pre-stunning in Trial 3, 30 blood samples per stunning method were collected. Post-stunning 30, 30, and 30 blood samples per stunning method were collected for Trials 1, 2, and 3, respectively.

At lairage, each broiler was individually removed from the transport module and cervically dislocated by a trained on-site personnel. The head was then immediately decapitated at the point of dislocation and blood samples were collected from the site of decapitation. For the electrical stunning treatment, at the pre-stunning location broilers were removed from the shackle line immediately before contact with the electrical water-bath. For the CAS treatment, at the pre-stunning location broilers were removed from their transportation tray that was on the conveyor immediately prior to gas exposure. For the electrical stunning treatment, at the post-stunning location broilers were individually removed from shackles after the mechanical neck-cutting and blood was collected from the subsequent processing step. For CAS, carcasses were cervically dislocated, then decapitated for post-stunning blood collection while unconscious. Glucose concentrations (mg/dL) were evaluated at sample collection from blood flow with a handheld EvencarePro glucose reader (Medline Industries, Northfield, IL).

Visible Wing Damage

Carcasses were evaluated for visible wing damage on the shackle line following the last defeatherer. Visible wing damage was visually assessed by a single investigator counting the number of damaged wings using a handheld tally counter over the course of 5 min of operation. Wing damage for this study was defined as any visible damage including dislocation, broken bones, or skin tearing (protruding). A second investigator counted the number of empty shackles within the same 5 min of operation. The total number of wings evaluated during the 5 minutes (birds per minute = 150) of operation was calculated by subtracting the number of empty shackles from the total number of shackles that were observed. The total number of shackles with carcasses was then multiplied by 2 to calculate the total number of wings observed.

Each stunning line was evaluated for a total of 13 repetitions, for five minutes each, for a total of 65 minutes. For Trials One, Two, and Three, there were 2, 5, and 6 repetitions of 5 min observations per stunning type. A total of 18,407 wings are evaluated for the electrical stun line and 22,592 for the CAS line. The difference in total number of wings evaluated between both electrical stun and CAS lines can be attributed to differences in line speeds and empty shackles.

Meat quality attributes

For all three trials, 30 breast butterflies were removed from each processing line at debone. The right fillet was evaluated for pH and color. Fillet pH was determined using a pH piercing probe inserted from the caudal end of the fillet into the center of the breast fillet (Seven2Go S2 pH/mV, Greifensee, Switzerland). The left fillet was weighed (g) and color was measured in triplicate on the dorsal side of the fillet for L*a*b* values at debone (Konica Minolta Chroma Meter CR-400, Tokyo, Japan) and subsequently evaluated at 24 h post-

deboning. The left fillet was then sealed in a ziptop bag and placed on ice within a cooler for subsequent evaluation. Fillet pH, color, and drip loss were subsequently evaluated at 24 h post-deboning in the university laboratory. At 24 h, the stored fillet was weighed and the same procedures for pH and color evaluation were followed. Drip loss was determined by subtracting the weight of the fillet 24 h post-debone from the initial weight of the fillet at debone, then multiplying by 100.

Statistical Analysis

A completely randomized design with 2 treatments (Electrical Stunning or CAS) was used. Glucose data were analyzed by treatment and sample time using the General Linear Model procedure. Initial debone and 24 h post-debone values for pH, CIE L*a*b*, and drip loss data were analyzed by one-way ANOVA. Means were separated by Tukey's HSD with significance determined as $P \leq 0.05$. Visible wing damage data were analyzed using Chi-Square analysis. All analyses were conducted using the SAS OnDemand for Academics software (SAS Institute Inc, Cary, NC).

3.4 RESULTS AND DISCUSSION

Glucose Concentrations

Glucose concentrations differed by trial and treatment. In Trial 1, glucose concentrations (343 mg/dL) were significantly higher than Trials 2 and 3 (298 and 284 mg/dL, respectively). However, because there were no trial by treatment interactions, data for trials were combined. A difference in blood glucose concentrations between trials was not unexpected because different flocks were evaluated at different times of the year between trials.

Broiler blood glucose at lairage, immediately pre-stunning, and post-stunning are shown in Table 1. For electrical stunning and CAS treatments, there were no significant differences in circulating glucose concentrations at lairage (272 and 284 mg/dL, respectively; P=0.2646) or immediately-pre stunning (283 and 274 mg/dL, respectively; P=0.6425). However, blood glucose concentrations post-stunning were significantly higher (P<0.0001) in broilers following CAS (418 mg/dL) compared to electrically stunned broilers (259 mg/dL). When comparing blood glucose between sample location within a treatment, there was no significant differences for electrical stunning. However, blood glucose concentration significantly increased from 274 mg/dL to 418 mg/dL following stunning for CAS broilers (P<0.0001). Because blood glucose concentrations were not different between stunning methods within a timepoint prior to stunning, this indicates that the blood glucose increased during the CAS process.

Previously reported data contradict these findings (Pinto et al., 2016; Xu et al., 2018). Pinto et al. (2016) found that glucose was significantly higher in broilers stunned by electrical stunning compared to those stunned by CAS. This difference in findings may be due to the current study being performed under U.S. electrical stunning parameters (12–38 V, ≥400 Hz), whereas Pinto et al. (2016) used high voltage / low frequency parameters (220 V AC, 60 Hz). The gas type and concentration used for CAS also differed between the studies, as 15% argon gas was included in the CAS system of the cited study. This is an example of the aforementioned issue of how analysis of stunning methods with differing parameters for stun can result in conflicting data. Xu et al. analyzed common mixtures of concentrations and gases, CO_2, O_2, and N (composition or concentration) in comparison to both U.S. and European electrical stunning parameters but found that blood glucose was not significantly different when comparing these methods (2018). This difference from our results, may be due to the method of CAS used. While

the current study was performed with a commercial, multiphasic atmosphere stunner under production conditions, the previous work used a non-commercial chamber. That chamber was filled with CO_2 gas and the birds were immediately exposed to high concentrations (<80%, 90 s exposure). In the current study, gas concentrations were gradually increased throughout the stunning process (20% to 85%, 5 min exposure).

Notably, during this study only Trial 2 had data from the same flock on both stunning lines. While using the same flock for both treatments would eliminate some potential confounding variables and provide more accurate results, data from all three trials followed the same trends as indicated by the lack of significant interactions between trial and treatment. All three trials, whether the same flock was utilized or not, had significantly higher blood glucose concentrations in broilers stunned by CAS at the post-stunning location in comparison to electrically stunned broilers and, more importantly a significant increase when comparing lairage and pre-stun with post-stun at the CAS line.

There is limited data available regarding blood glucose concentrations from broilers stunned by either electrical stun using U.S. parameters or CAS. Results from the current study showed glucose increasing only between pre-stunning and post-stunning in CAS broilers. However, there are a few reasons why CAS could lead to an increase in circulating blood glucose concentrations. One possibility is the lack of restraint during CAS. Since birds are put through the system within their transportation crates, as opposed to live shackling for electrical stunning, there is more freedom for movement during the stunning process (Webster & Fletcher, 2004). Physical movement during an acute stress response, like stunning, rapidly releases glucose from muscle tissue storage at a higher rate than normal activity (Verberne et al., 2016). McKeegan et al. confirmed that various concentrations of CO_2 used with CAS induced strong

respiratory responses, such as gasping, panting, and neck stretching, whereas later phases of increased carbon dioxide induced convulsions and vigorous wing flapping (2007). A visual respiratory response is typically observed during the induction phase of CAS; this phase is where CO_2 is first introduced to the birds and is the only phase where bird should be conscious (to the CO_2) . A physical response to stressors increases the circulation of glucose within the blood. Possible sources of stressors during the conscious induction phase of CAS include the sudden exposure to CO_2, mucosal membrane irritation from carbonic acid production during respiration, and dyspnea (McKeegan et al. 2006; Anton et al., 1992). Physical movements observed prior to loss of consciousness or loss of posture includes stretching of the neck, gasping, and occasionally flapping of the wings (McKeegan et al., 2007; Abeyesinghe et al., 2007). However unconscious movement has also been observed during later phases of CAS, such as clonic or tonic convulsions and/or flapping (Gerritzen et al., 2013; Lambooij et al., 1999). Therefore, if the increase in circulatory glucose was primarily during the unconscious phase, then the increase would not be a response to a stressor, but rather a physical reaction to the lack of oxygen supplied to the brain. It has also been suggested that there is a biochemical reaction occurring due to the sudden change in atmospheric gases inhaled by the bird. However, in a study performed by Hackbarth et al. (2000) this was not observed when rats were euthanized by CO_2 and blood glucose concentrations were analyzed. One group of rats received pentobarbital, a sedative used to control convulsions, whereas the other group did not (control). Both groups were exposed to the same CO_2 concentrations, and there were no differences in blood glucose concentrations or physical reactions when comparing both treatments. Because no differences in physical reactions occurred in mice that were either sensible or insensible to pain, any differences in glucose could not have been from movement itself. Concurrently, there was no

difference in glucose, so attributing a spike in glucose to a biochemical reflex from increased CO_2 in the bloodstream is also invalid. This brings to question whether this study's observation of increased glucose concentrations during CAS occurred in the initial 1-2 minutes or the remaining time where birds were unconscious. Though, it must be noted that rats and broilers have vastly different respiratory anatomy and physiological response and may not behave in the same manner (West et al., 2007).

The precise timepoint when the glucose increase occurred was not determined during the CAS process, so why this increase occurred remains unknown without further research and analysis. Further research could be beneficial to determine whether this increase in blood glucose occurs before or after loss of consciousness therefore indicating whether increased blood glucose during CAS is relevant to animal welfare.

Visible Wing Damage

Percentages of visible wing damage for broiler carcasses after either electrical stunning or CAS are reported in Table 2. Visible wing damage was significantly higher ($P<0.0001$) for broilers stunned by CAS (4.3%) in comparison to broilers electrically stunned (2.4%). There are a few important points to consider due to the data collection methods used in this study. Carcasses from each treatment group were evaluated on separate lines following defeathering, hence, were processed using different equipment. It is possible that the increase in CAS visible wing damage could have occurred due to variations in equipment any time prior to and including the defeatherer. Due to the line speeds in the commercial facility, determining visible wing damage on feathered broilers earlier in the line was not possible.

Distinguishing which type of damage occurred to wings for either stun method will help determine at what point on each stunning line this damage occurred. Because this study did not categorize the wing damage by type of damage, it is difficult to establish what factors influenced the higher occurrence of wing damage for broilers stunned with CAS. Although not measured, it was generally observed that CAS broilers had a high occurrence of broken wing tips. Some previous research has confirmed that excess wing flapping that occurs during CAS did cause/result in wing damage (Gerritzen et al. 2013; McKeegan et al., 2007; Lambooji et al., 1999). Further research, closely studying, categorizing and evaluating wing damage before and after stun in an experimental setting would be beneficial. From the perspective of the poultry integrator, the increase in visible wing damage that occurred on the CAS line would lead to a reduction in yield and final weight of product available for sale. However, if the root cause of the increased wing damage can be determined, these issues could be addressed through targeted adjustments to the offending system.

Breast Fillet Quality

Color. Breast fillet quality attributes of pH, color, and drip loss from broilers stunned with either electrical or CAS are shown in Table 3. At debone, L* and a* were found to be significantly different between stun methods (P=0.0005, P=0.0303). Breast fillets from electrically stunned birds were darker and more red (53.15, 2.31) than CAS breast fillets (54.65, 1.96). There was no difference in yellowness (b*) at debone. At 24 h post-debone, no differences were found for L*, a*, or b* values between treatments (P=0.0859, P=0.2102, P=0.1415).

While differences were detected for L* and a* values at debone, these differences were small and would likely be undetectable by the human eye. Color/visual aspect is a main factor in guiding consumer product preference (Wideman et al., 2016; Kennedy et al., 2005). However,

these differences in L* and a* are minimal and most likely not applicable to impact quality from a consumer standpoint. When re-evaluated 24 h post-debone, neither L* nor a* were significantly different indicating that fillet color was not influenced by stunning methods.

Raj et al. similarly found no significant differences when analyzing breast fillet color 24 hours post-debone (1997). Pinto et al. observed similar results for initial L* and a* but did not evaluate fillets at 24 hours post-debone (2012). Pinto et al.'s study did have significant differences in both L* and a* of CAS-simulated broiler fillet values in comparison to electrically stunned broilers and found that fillets were lighter and less red. CAS-simulated birds were exposed to 10% initial CO_2 with a gradual increase to 30%, while time of exposure was defined as either observed cessation of breathing (gas killing) or loss of consciousness (gas stunning). The birds that were in the 'gas killing' treatment group that had been exposed to CO_2 the longest had significantly lighter and darker red meat in comparison to electrically stunned birds, whereas the 'gas stunned' group did not show significant differences. This may indicate the exposure time of CO_2 could cause differences in initial meat color attributes. Since they did not evaluate 24 hours post-debone, further investigation would need to be done to determine the effects of exposure time on broiler breast fillet color. Lightness is inversely correlated to pH in poultry meat (Allen et al., 1998; Fletcher et al., 2000) as the myofibrillar proteins in poultry meat tightly bind to water when the pH is above the isoelectric point. This causes more light to be readily absorbed by the muscle, hence, a darker appearance (Cornforth, 1994). This higher L* value in breast meat from CAS broilers observed in our study may be due to the higher levels of circulating glucose observed. High circulating glucose is correlated to rapid-onset post-mortem glycolytic activity, which increases initial lactic acid post-mortem, and therefore may be responsible for the decreased pH values (Fletcher et al., 2000). The change in pH values

observed may be due to higher levels of circulating glucose in broilers stunned through CAS leading to higher initial L* values of broiler breast meat.

Although it has been previously reported by Raj et al. (1998) that broilers stunned through CAS will have a faster initial pH decline, Van Laack et al. (2000) have found pale meat is determined by a L* value higher than 60. Neither L* values for electrical or CAS stunned broiler breast fillets were found to be higher than 60 initially or 24 hours post debone (ES=53.15, 55.68; CAS=54.65; 56.46).

pH. Initial breast fillet pH was significantly higher ($P<0.0001$) for electrically stunned broilers (5.92) when compared to CAS broilers (5.81). As previously seen for L* and a*, the pH value no longer differed between stunning lines when evaluated 24 hours post-debone ($P=0.2615$).

Initial values of lower pH in breast fillets from the CAS treatment align with the trends of lighter breast fillets and higher glucose concentrations post-stunning. Low pH and high L* values have been previously demonstrated to have an inverse relationship (Fletcher et al., 2000). Decreased glucose availability within muscle tissue, due to the physiological demand in response to a stressor (hence higher circulating concentrations), will result in early onset rigor mortis from glycolysis (Sandercock et al., 2001). Salwani et al. (2016) found broilers stunned by CAS had increased activity of pyruvate kinases, indicating an increased use of glycolysis (Uyeda, 2013). Therefore, early onset rigor induced by the increased glycolytic activity during CAS could explain initial pH differences at debone. The ultimate pH would likely not be affected by this, since this increased glycolytic activity was only observed during stunning, which could also explain the lack of significant differences at 24 hours post-debone.

Drip Loss. Drip loss did not differ between breast fillets from broilers stunned by CAS or electrical stun CAS=4.83, ES=4.84; P=0.0859). Typically, higher drip loss is associated with lighter colored meat and lower pH (Woelfel et al., 2002). This trend was previously observed in breast meat from broilers stunned by CAS compared to electrically stunned broilers (Salwani et al., 2015). While our initial pH and L* values were observed to be different, those differences were minor and did not differ 24 hours post-mortem. Therefore, there was no downstream impact observed for drip loss.

3.5 CONCLUSION

There was a clear increase in circulating blood glucose as a consequence of CAS, however, it is unknown whether this is an important factor for animal welfare or product quality. Determining when glucose increases during CAS will allow for a better understanding of the effect of CO_2 exposure on broilers and could possibly lead to improved stunning parameters. The occurrence of wing damage for CAS carcasses was demonstrated to be a critical issue in this commercial processing facility and should be evaluated in depth by categorizing damage by type, as well as evaluating the occurrence of damage before defeathering to isolate the timeframe in which the damage is occurring. Breast fillet meat quality had minimal differences at debone between broilers stunned with either electrical stunning or CAS. Color, pH, and drip loss were not different at 24 hours post-deboning indicating acceptability of breast fillet quality with use of either stunning system for consumers.

3.6 REFERENCES

Abeyesinghe, S. M., D. E. F. McKeegan, M. A. McLeman, J. C. Lowe, T. G. M. Demmers, R. P. White, R. W. Kranen, H. van Bemmel, J. A. C. Lankhaar, and C. M. Wathes. 2007. Controlled atmosphere stunning of broiler chickens. I. Effects on behaviour, physiology and meat quality in a pilot scale system at a processing plant. Br. Poult. Sci. 48:406-423.

Allen, C. D., D. L. Fletcher, J. K. Northcutt, and S. M. Russell. 1998. The relationship of broiler breast color to meat quality and shelf-life. Poult. Sci. 77:361-366.

Alonso, M. E., J. R. González-Montaña, and J. M. Lomillos. 2020. Consumers' concerns and perceptions of farm animal welfare. Animals. 10:385.

Anton F., I. Euchner, and H. O. Handwerker. 1992. Psychophysical examination of pain induced by defined CO_2 pulses applied to the nasal mucosa. Pain. 49:53–60.

Bedanova, I., E. Voslarova, P. Chloupek, V. Pistekova, P. Suchy, J. Blahová, R. Dobsikova, and V. Vecerek. 2007. Stress in broilers resulting from shackling. Poult. Sci. 86:1065-1069.

Berg, C., and A. B. M. Raj. 2015. A review of different stunning methods for poultry-animal welfare aspects (Stunning Methods for Poultry). Animals. 30:1207-1219.

Boyd, F. 1994. Humane slaughter of poultry: The case against the use of electrical stunning devices. J Agric Environ Ethics. 7:221–236.

Cornforth, D. P., A. M. Pearson, and T. R. Dutson. 1994. Color – its basis and importance. Pages 34-78 in Quality Attributes and Their Measurement in Meat, Poultry, and Fish Products. A. M. Pearson and T.R. Dutson ed. Chapman and Hall. London, UK.

Erasmus, M. A., P. V. Turner, and T. M. Widowski. 2010. Measures of insensibility used to determine effective stunning and killing of poultry. J. Appl. Poult. Res. 19:288-298.

FCEC Food Chain Evaluation Consortium. 2012. Study on various methods of stunning for poultry. Accessed Apr. 2022. https://ec.europa.eu/food/sites/food/files/animals/docs/aw_practice_slaughter_study_stunning_poultry_en.pdf

Fletcher D. L., M. Qiao, and D. P. Smith. 2000. The relationship of raw broiler breast meat color and pH to cooked meat color and pH. Poult. Sci. 79:784-788.

Gerritzen M. A., H. G. M. Reimert, V. A. Hindle, M. T. W. Verhoeven, and W. B. Veerkamp. 2013. Multistage carbon dioxide gas stunning of broilers. Poult. Sci. 92:41-50.

Gibson, T. J., A. H. Taylor, and N. G. Gregory. 2016. Assessment of the effectiveness of head only and back-of-the-head electrical stunning of chickens. Br. Poult. Sci. 57:295-305.

Hackbarth, H., N. Küppers, and W. Bohnet. 2000. Euthanasia of rats with carbon dioxide--animal welfare aspects. Lab Anim. 34:91-96.

Heath, G. B., D. J. Watt, P. R. Waite, and J. M. Ormond. 1981. Observations on poultry slaughter. Vet. Rec. 108:97–99.

Kang, I. S., and A. R. Sams. 1999. A comparison of texture and quality of breast fillets from broilers stunned by electricity and carbon dioxide on a shackle line or killed with carbon dioxide. Poult. Sci. 78:1334-1337.

Kannan G., J. L. Heath, C. J. Wabeck, and J. A. Mench. 1997. Shackling of broilers: Effects on stress responses and breast meat quality. Br. Poult. Sci. 38:4.

Kennedy O. B., B. J. Stewart-Knox, P. C. Mitchell, and D. I. Thurnham. 2005. Flesh colour dominates consumer preference for chicken. Appetite. 2005. 44:181-186.

Lambooij E., M. A. Gerritzen, B. Engel, S. J. W. Hillebrand, J. Lankhaar, and C. Pieterse. 1999. Behavioural responses during exposure of broiler chickens to different gas mixtures. Appl. Anim. Behav. Sci. 62:255-265.

McKeegan, D. E. F., J. A. Mclntyre, T. G. M. Demmers, J. C. Lowe, C. Wathes, P. L. C. Van Den Broek, A. M. L. Coenen, and M. J. Gentle. 2007. Physiological and behavioural responses of broilers to controlled atmosphere stunning: implications for welfare. Anim. Welf. 16:409.

McKeegan D. E. F., J. McIntyre, T. G. M. Demmers, C. M. Wathes, and R. B. Jones. 2006. Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. Appl. Anim. Behav. Sci. 99:271–286.

Pinto, M. F., D. A. Bitencourt, E. H. G. Ponsano, M. Garcia Neto, and I. L. C. Bossolani. 2016. Effect of electrical and controlled atmosphere stunning methods on broiler chicken behavior at slaughter, blood stress indicators and meat traits. Braz. J. Vet. Res. Anim. Sci. 53:1-8.

Pinto, M. F., J. P. Nicolau, E. H. Ponsano, and M. Garcia-Neto. 2012. Electrical and CO_2 exposure methods to stun or kill broilers at slaughter: What's the influence on meat characteristics? 58th International Congress of Meat Science and Technology. Montreal, Canada.

Raj ,A. B. M., T. C. Grey, A. R. Audsely, and N. G. Gregory. 1990. Effect of electrical and gaseous stunning on the carcase and meat quality of broilers. Br. Poult. Sci. 31:725-733.

Raj, A. B. M., R. I. Richardson, L. J. Wilkins, and S. B. Wotton. 1998. Carcase and meat quality in ducks killed with either gas mixtures or an electric current under commercial processing conditions. Br Poult Sci. 39:404-7.

Raj, A. B. M., L. J. Wilkins, R. I. Richardson, S. P. Johnson, and S. B. Wotton. 1997. Carcase and meat quality in broilers either killed with a gas mixture or stunned with an electrical current under commercial processing conditions. Br.Poult. Sci. 38: 169–174.

Salwani S., A. Kazeem, S. A. Sarah, J. Vejayan, and I. Zulkifli. 2015. Skeletal muscle proteome and meat quality of broiler chickens subjected to gas stunning prior slaughter or slaughtered without stunning. CYTA. J. Food. 14:1-7.

Sandercock, D. A., R. R. Hunter, G. R. Nute, M. A. Mitchell, and P. M. Hocking. 2001. Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: Implications for meat quality. Poult. Sci. 80:418-425.

Santonicola, S., M. F. Peruzy, M. Girasole, N. Murru, M. L. Cortesi, and R. Mercogliano. 2017. Preliminary study on physicochemical and biochemical stress markers at poultry slaughterhouse. Ital. J. Food Saf. 6:6346.

Solomon M. B., R. L. J. M. Van Laack, and J. S. Eastridge. 1998. Bio-physical basis of pale, soft, exudative (PSE) pork and poultry muscle: A review. J. Muscle Foods. 9:1-11.

USDA, Economic Research Service calculations from USDA, National Agriculture Statistics Service, September 2021.

Uyeda K. 2013. Pyruvate Kinase; Encyclopedia of Biological Chemistry. 2nd ed. Acad. Press. 719-721.

Van Laack R. L. J. M., C. H. Liu, M. O. Smith, and H. D. Loveday. 2000. Characteristics of pale, soft, exudative broiler breast meat. Poult. Sci. 79:1057-1061.

Verberne A. J., W. S.Korim, A. Sabetghadam, and I. J. Llewellyn-Smith. 2016. Adrenaline: insights into its metabolic roles in hypoglycaemia and diabetes. Br. J. Pharmacol. 173:1425-1437.

West, J. B., R. R. Watson, and Z. Fu. 2007. Major differences in the pulmonary circulation between birds and mammals. Respir. Physiol. Neurobiol . 157: 382–390.

Webster, A. B. and D. L. Fletcher. 2004. Assessment of the aversion of hens to different gas atmospheres using an approach-avoidance test. Appl. Anim. Behav. Sci. 88:275–287.

Wideman N., C. O'Bryan, and P. Crandall. 2016. Factors affecting poultry meat colour and consumer preferences - A review. Worlds Poult. Sci. J. 72:353-366.

Wilkins, L.J., S.N. Brown, A.J. Phillips, and P.D. Warriss. 2000. Variation in the colour of broiler breast fillets in the UK. Br. Poult. Sci. 41:308-312.

Woelfel, R., C. Owens, E. Hirschler, R. Martinez-Dawson, and A. Sams. 2002. The characterization and incidence of pale, soft, and exudative broiler meat in a commercial processing plant. Poult. Sci. 81:579-84.

Xu, L., H. Zhang, H. Yue, S. Wu, H. Yang, Z. Wang, and G. H. Qi. 2018. Gas stunning with CO2 affected meat color, lipid peroxidation, oxidative stress, and gene expression of mitogen-activated protein kinases, glutathione S-transferases, and Cu/Zn-superoxide dismutase in the skeletal muscles of broilers. J. Animal Sci. Biotechnol. 9:37.

Xu L., H. Y. Yue, S. G. Wu, H. J. Zhang, F. Ji, L. Zhang, and G. H. Qi. 2011. Comparison of blood variables, fiber intensity, and muscle metabolites in hot-boned muscles from electrical- and gas-stunned broilers. Poult. Sci. 90:1837-1843.

Table 3.1 Blood glucose concentrations from broilers at lairage, immediately pre-stun, and post-stun for electrical stun and controlled atmosphere stun lines.

	Glucose Concentration (mg/dL)		
Location of Sample	**Electrical Stunning**	**Controlled Atmosphere Stunning**	**P-value**
Lairage (n=150)	272 ± 8.17[1]	284[b] 6.43	0.2646
Immediately Pre-Stunning (n=60)	283 ± 8.50	274[b] ± 19.24	0.6425
Post-Stunning (n=180)	259[z] ± 8.22	418[ay] ± 11.75	<0.0001
P-Value	0.2175	<0.0001*	

[a-b]Values within a column with different superscripts are significantly different ($P \leq 0.05$).

[y-z]Values within a row with different superscripts are significantly different ($P \leq 0.05$).

[1] ± Values are standard error.

Table 3.2 Visible wing damage counts and calculated percentages following defeathering from broilers stunned by either electrical stunning or controlled atmosphere stunning.

	Visible Wing Damage		
	Electrical Stunning	**Controlled Atmosphere Stunning**	**P-value**
Damaged Wings	409	796	
Undamaged Wings	17,998	21,796	
Total Wings	18,407	22,592	
Percentage of Damaged Wings	2.4[b]	4.3[a]	<0.0001

[a-b]Values within a row with different superscripts are significantly different ($P \leq 0.05$).

Table 3.3 Color, pH, and drip loss of broiler breast fillets from electrically stunned or controlled atmosphere stunned broilers at debone and 24 hours post-debone.

		Method of Stun		
Meat Quality Attribute	**Time of Sample Collection**	**Controlled Atmosphere**	**Electrical Stun**	**P-Value**
L*	Initial	54.65[a] ± 0.30[1]	53.15[b] ± 0.30	0.0005
	24 Hours Post Debone	56.46 ± 0.31	55.68 ± 0.33	0.0859
a*	Initial	1.96[b] ± 0.12	2.31[a] ± 0.10	0.0303
	24 Hours Post Debone	2.26 ± 0.14	2.08 ± 0.10	0.2102
b*	Initial	7.52 ± 0.18	7.43 ± 0.17	0.7162
	24 Hours Post Debone	9.02 ± 0.23	8.59 ± 0.18	0.1415
pH	Initial	5.81[b] ± 0.02	5.92[a] ± 0.02	<0.0001
	24 Hours Post Debone	5.45 ± 0.03	5.45 ± 0.04	0.2615
Drip Loss %	24 Hours Post Debone	4.83 ± 0.73	4.84 ± 0.80	0.0859

[a-b]Values within a row with different superscripts are significantly different ($P \leq 0.05$).

[1] ± Values are standard error.

CHAPTER 4: CONCLUSION AND FUTURE IMPLICATIONS

Our findings in Chapter 2 indicate that neither method of stunning was clearly preferable based on measurement of blood hormone indicators of a stress response. Our results for EPI did show a greater concentration post-stun in ES broilers, which may indicate that ES elicited a stress-response, or possibly that the electrical current interferes with the release of SAM-products. Further research is necessary to determine the reasoning for the response that we have observed As shown in Chapter 3, there was a clear increase in circulating blood glucose as a consequence of CAS, however, it is unknown whether this is an important factor for animal welfare or product quality. If the broilers were conscious during this increase in glucose, it could indicate conscious animal struggle, possibly as an adverse reaction to pain caused by the CO_2, or a potential biochemical reaction to the increased CO_2. Further research is necessary to determine both the cause of the noted glucose increase, and the time during CAS when it is elicited. In CAS broilers, we observed that wing damage was significantly higher in occurrence. Therefore, these results should be evaluated in depth by categorizing damage by type, as well as evaluating the occurrence of damage before defeathering to isolate the timeframe in which the damage is occurring. We observed that breast fillet quality concerning color, pH, or drip loss did not differ at 24 hours post-debone, and that any differences immediately at debone were minimal and not applicable from a consumer standpoint. Future studies regarding both blood-stress indicator differences between CAS and ES methods. In regard to meat quality differences between the methods, wing damage and glucose increases should be further evaluated during CAS. If the U.S. poultry industry is to follow the growing trend in use of CAS systems, determining more efficient methodologies to decrease wing damage and glucose increases is imperative.