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EFFECTS OF PREMORTEM STRESS ON HEAT SHOCK PROTEIN ABUNDANCE,

OXIDATION, AND COLOR IN THE *LONGISSIMUS LUMBORUM* OF HOLSTEIN

STEERS FOLLOWING HARVEST

by

Reganne K. Briggs

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal Nutrition

Approved:

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2020

ABSTRACT

Effects of premortem stress on heat shock protein abundance, oxidation, and color in the

longissimus lumborum of Holstein steers following harvest

by

Reganne K. Briggs, Master of Science

Utah State University, 2020

Major Professor: Dr. Kara Thornton-Kurth
Department: Animals, Dairy, and Veterinary Sciences

Undesirable variation in beef tenderness and stability of flavor and color may be

associated with the abundance of heat shock proteins (HSP) and oxidation in muscle

following harvest. This study aimed to determine whether premortem stress impacts HSP

abundance and oxidation in the skeletal muscle following harvest. Initial *longissimus*

lumborum (LL) biopsy samples were taken prior to premortem stress. Forty Holstein

steers were administered adrenocorticotropic hormone (ACTH; 0.1 IU/Kg BW) to mimic

acute premortem stress. Serum cortisol was measured every 0.5 h from -2 to 6 h relative

to the ACTH challenge. Skeletal muscle and blood samples were collected at four

different harvest times (2, 12, 24 and 48 h post-challenge; n=10). Samples were collected

from the LL immediately after harvest and after 14 d of aging. Steers from the different

harvest times differed (P < 0.05) in serum cortisol response 0.5 to 1 h after administration

of ACTH. As such, steers were grouped based on cortisol response 0.5 h after initiation

of ACTH challenge (lowest, lower, higher, and highest; n=10). Statistical analyses

evaluated the impacts of time of harvest, initial cortisol response, and their interaction.

Protein abundance of HSPβ1, P-HSPβ1, HSPβ5, DJ1, and troponin I was analyzed in muscle samples taken prior to the ACTH challenge, at harvest, and after 14 d aging. In addition, complete blood count (CBC), steak color, myofibrillar fragmentation index (MFI), and thiobarbituric acid reactive substances (TBARS) were analyzed. Harvest time following the ACTH challenge affected ($P < 0.05$) relative protein abundance of P-HSPβ1. Protein abundance of P-HSPβ1 was increased ($P < 0.01$) in animals harvested 48 h after the ACTH challenge, however after 14 d of aging P-HSPβ1 was increased ($P < 0.01$) in animals harvested 12 h after the ACTH challenge. Additionally time of harvest affected ($P < 0.05$) DJ1 and troponin I at harvest and after 14 d of aging. The abundance of DJ1 was increased ($P < 0.05$) in animals harvested 24 h after the ACTH challenge at harvest and after 14 d of aging. Troponin abundance was also elevated ($P < 0.05$) in animals harvested 24 h after the ACTH challenge at harvest. However, after 14 d of aging animals harvested 48 h after the ACTH challenge had increased ($P < 0.05$) abundance of troponin. Harvest time and cortisol response did not affect ($P > 0.05$) relative abundance of HSPβ1 and HSPβ5. Regarding steak color, time of harvest had an effect ($P < 0.01$) on a* and b*, but no effect ($P > 0.05$) on L*. Animals that were harvested 24 h and 48 h after the ACTH challenge had increased ($P < 0.05$) a* and b* values. Time of harvest and cortisol response affected ($P < 0.05$) TBARS concentration in the serum relative to the ACTH challenge as animals that were to be harvested 24 h after the challenge had increased ($P < 0.05$) concentration of TBARS. Lastly, time of harvest had an effect ($P < 0.05$) on MFI. These data indicate that harvest time and cortisol response following a stressful event may affect the quality of meat.   (92 pages)

PUBLIC ABSTRACT

Effects of premortem stress on heat shock protein abundance, oxidation, and color in the

longissimus lumborum of Holstein steers following harvest

Reganne K. Briggs

Consumers consider tenderness and steak color to be two important attributes associated with meat quality. However, inconsistencies in both tenderness and steak color remain despite similar production practices of beef cattle. Stressful events before harvest may impact meat quality by initiating certain pathways such as abundance of heat shock proteins (HSP) and oxidation within the skeletal muscle. Heat shock proteins have been associated with tenderness while oxidation may affect steak color and flavor. In this study, 40 Holstein steers were administered adrenocorticotrophic hormone (ACTH) to mimic a stressful event before harvest. Animals were harvested at different times following the ACTH challenge (2 h, 12 h, 24 h, and 48 h; n=10). In addition, animals were grouped based on cortisol response 0.5 h after initiation of the ACTH challenge (lowest, lower, higher, and highest; n=10) because initial serum cortisol concentrations were variable following the challenge. Skeletal muscle tissues were collected from the *longissimus lumborum* (LL) before the ACTH challenge, at harvest, and after 14 d of aging. Blood samples were also collected. We investigated how time of harvest and cortisol response affected HSP abundance, tenderness, oxidation, and steak color. Time of harvest affected the relative abundance of one of three HSP analyzed, Phosphorylated-HSPβ1 (P-HSPβ1). The abundance of P-HSPβ1 was increased at harvest in animals harvested 48 h after the ACTH challenge, while after 14 d of aging animals that were

harvested 24 h after the ACTH challenge had increased abundance of P-HSPβ1. Additionally, time of harvest affected tenderness assessed by the protein troponin I, a regulatory protein in the muscle, and myofibrillar fragmentation index (MFI). Animals that were harvested 24 h after the ACTH challenge had increased abundance of troponin at harvest and increased MFI after 14 d of aging. Both time of harvest and cortisol response affected oxidation assessed by DJ1, protein which is a marker of oxidation, and thiobarbituric acid reactive substances (TBARS). Animals that were harvested 24 h after the ACTH challenge had increased abundance of DJ1 and increased concentration of TBARS in the serum. In addition, steak color was affected by both time of harvest and cortisol response. Animals that were harvested 24 h and 48 h after the ACTH challenge had yellower and redder steaks than animals harvested 2 h and 12 h after the challenge. These finding indicate that time of harvest and cortisol response following a stressful event impacts meat quality, however more research is needed to fully understand the impacts of premortem stress on meat quality.

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Reganne K. Briggs

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INTRODUCTION

Despite similar production practices, meat products from beef cattle exhibit undesirable variation in tenderness and stability of flavor and color. Tenderness has been reported as one of the most important characteristics of meat according to consumers. It has been stated that inconsistencies in tenderness is a major problem in the beef industry (Koohmaraie et al., 2005). The conversion of muscle to meat is a complex process that includes predictable metabolic pathways that have been studied at length (Laville et al., 2009). Meat is tenderized through proteolysis of myofibrillar and associated structural proteins such as calpains, cathepsins, caspases, and their counter parts (Koohmaraie & Geesink, 2006). The processes of proteolysis and development of tenderness are largely studied, however inconsistencies in tenderness still exist (Ouali et al., 2006). Inconsistencies may be a result of the effect that external factors, such as premortem stress, have on proteolysis of myofibrillar and structural proteins of meat.

Prior to harvest, many livestock animals experience both physiological and physical stress (Dhabhar, 2018). Physiological stress may include transportation, handling, and novel experiences (Nwe et al., 1996), while physical stress may include water restriction, feed withdrawal, extreme temperatures, and morbidity (Grandin, 1997). Premortem stress has the potential to decrease meat quality through different pathways within the animal such as heat shock proteins (HSP).

Heat shock proteins are abundant and highly conserved proteins that are quickly synthesized by the onset of various stressors (Xing et al., 2018). Heat shock proteins appear as soon as the cell is in danger, possibility as a result of stress, and contribute to

proper conformation of proteins and preservation of their biological function (Herrera-Mendez et al., 2006). Recent research suggests that small heat shock proteins (SHSP) may play a role in the development of meat tenderness, however their exact role is unknown (Lomiwes et al., 2014). Some HSP are both constitutively expressed and expression is upregulated in response to stressful conditions in order to better protect proteins from damage (Kristensen et al., 2003). Heat shock proteins appear as soon as the cell is in danger from various stressors, and contribute in protecting certain myofibrillar proteins such as desmin, titin, and actin (Herrera-Mendez et al., 2006). Oxidative stress, following a stressful event, may also play a role in the development of tenderness, color stability, and flavor of meat from beef cattle. The process of oxidation can be related to oxidative stress and occurs under postmortem conditions. Oxidation of myoglobin causes color deterioration, and lipid oxidation causes undesirable flavors and rancidity development in meat (Ponnampalam et al., 2017). Like tenderness, steak color is also an important characteristic of meat that consumers consider while purchasing meat (Suman & Joseph, 2013). Premortem stress may cause a cascading effect of certain pathways to decrease quality of meat such as tenderness and color stability, however the effects that premortem stress has on SHSP and oxidative stress is currently unknown.

LITERATURE REVIEW

Conversion of Muscle to Meat

The conversion of muscle to meat includes intricate biochemical and metabolic processes that have been studied and described for the last three decades. Biochemical and metabolic processes induced by the animals' tissues that occur at harvest attempt to retain homeostatic control (Warner, 2016). Harvest of an animal initiates metabolic processes in the muscle that change its *in vivo* nature. Animal muscles do not suddenly lose all functionality and become meat, however, for a period of hours or days a series of physical and chemical changes take place to convert muscle to meat (Sayas-Barberá et al., 2010). The process of the conversion of muscle to meat can be divided in to three phases: the pre-rigor phase, the rigor phase, and the resolution or tenderization phase (Bhat et al., 2018).

In the living animal, muscle is used for work through contraction and relaxation mechanisms. The energy needed in the contraction and relaxation process progresses from hydrolysis of adenosine triphosphate (ATP). This energy uses the transport of active sodium and potassium in order to maintain the membrane potential for the transport system of calcium during relaxation and in the formation of activated myosin (Sayas-Barberá et al., 2010). In a resting muscle, ATP keeps the muscle in a relaxed state by preventing the formation of cross-bridges between myosin and actin filaments that forms actomyosin (Paredi et al., 2012).

After harvest, supply of oxygen and energy rich compounds, such as glucose, are not transported to cells, and metabolic products are not removed. It is known that lack of

oxygen and buildup of metabolic products in the muscle are the cause of chemical and physical modifications responsible for the conversion of muscle to meat. Supply of oxygen ceases after harvest, consequently, muscle cannot receive energy from breathing. Initially, some accessory ATP is regenerated through the conversion of creatine phosphate (CP) into creatine and the transfer of its phosphate to ADP to form ATP (Sayas-Barberá et al., 2010). In the liver, CP is synthesized and then stored in the muscle in order to satisfy fluctuating energy demand until sources are depleted (Braden, 2013). As a consequence of decreased oxygen and CP supply, metabolism must be anaerobic, and ATP can only be regenerated through breakdown of glycogen by glycolysis (Sayas-Barberá et al., 2010). Concentration of ATP is maintained by the breakdown of glycogen until lack of substrate or pH conditions inhibit glycolysis (Paredi et al., 2012). As glycogen is broken down, lactate accumulates because the elimination of products produced from anaerobic metabolism does not function (Sayas-Barberá et al., 2010). As a consequence of lactate accumulation, pH deceases from approximately 7.2 to 5.3-5.7 resulting in muscle acidification. Muscle acidification causes loss in water holding capacity, calcium release, and leads to cross-bridges being formed between myosin and actin filaments (Paredi et al., 2012). Once ATP is depleted, myosin remains bound to actin, and rigor mortis is the result, with stiff and inextensible muscles (Warner, 2016).

The onset of rigor mortis marks the most rigid interaction in muscle and its toughest state. The development of rigor mortis is influenced by many factors, such as species of animal, muscle type, muscle temperature, and the glycogen and CP reserves of the muscle at the time of slaughter. Elevated glycogen and CP content increase the time it

takes for rigor mortis to appear. Temperature is another essential factor in the development of stiffness and glycolysis. Consequently, decrease in pH is slower when the meat temperature is decreased. The biochemical processes stop completely when the meat is frozen (Sayas-Barberá et al., 2010). Once muscles are completely inextensible, rigor has reached completion. This process is basically irreversible, however when the channels are old and with time, certain inherent enzymes can cause degradation of the contracted structure. The resolution of rigor is still not completely understood. This is a period in which muscle flexibility begins to return, likely due to damage to muscle proteins (Braden, 2013).

Rigor resolution is partially obtained by the proteolysis of proteins associated with the Z-disks of the sarcomere. This proteolysis lends to the fracture of myofibrils and therefore myofibers. There is increasing evidence that selected myofibrillar proteins are denatured in the early postmortem period and proteolysis ultimately leads to a reduction in muscle tension (Braden, 2013). Proteins that are degraded during myofiber degradation are myofibrillar and cytoskeletal proteins, troponin T and I, desmin, vinsulin, nebulin, dystrophin, and titin. Cytoskeletal structures that are degraded are Z- to Z-line attachments, and Z-and M-line attachments to the sarcolemma (Koohmaraie & Geesink, 2006). Endogenous proteolytic enzymes such as, calpains, cathepsins, and caspases are responsible for degradation of muscle fibers. Several studies have provided convincing evidence that most of the tenderness development during aging is the result of post-mortem proteolysis of key structural proteins, including desmin, titin, nebulin, troponin-T and others (Bhat et al., 2018) Rigor resolution and tenderness are preceded by

proteolytic enzymes that alter the protein structure of muscle. Major changes in muscle protein architecture are associated with the conversion of muscles into meat and ultimately result in tenderness (Paredi et al., 2012).

Tenderness

Tenderness is one the most important organoleptic qualities consumers consider when eating red meats (Ouali et al., 2006). In livestock animals, inconsistency and variability in meat texture are identified as two major problems in the meat industry (Sentandreu et al., 2002). However, traditional carcass grading systems are not effective at identifying meat tenderness variation. In fact, marbling accounts for merely 5% of the variation in meat tenderness (Koohmaraie & Geesink, 2006). The process of tenderization, however, is dependent on the enzymatic activity of endogenous enzymes targeting muscle fibers.

Tenderness has two major components: the background toughness, which results from connective tissue characteristics, and the myofibrillar component. The myofibrillar component is closely related to muscle fiber characteristics that control the tenderization phase characterized by post-mortem proteolysis (Bernard et al., 2007). For a protease system to be involved in post-mortem proteolysis and ultimately tenderization, it must be endogenous to skeletal muscle cells, must be able to mimic post mortem changes *in vitro*, and it must have access to myofibrils in tissue (Kemp et al., 2010). Proteolytic systems present in muscle have been studied largely to investigate their possible role in postmortem proteolysis and tenderization. The calpain system, lysosomal cathepsins, and

caspases are largely responsible for the tenderization process in muscle (Koohmaraie & Geesink, 2006).

Calpains are intracellular cysteine proteases and are expressed in a tissue in a specific manner (Kemp et al., 2010). Calpains are separated into three different groups, ubiquitous (μ- and m-calpain), tissue specific (3, ncl-2, nc1-4, Lp8s, and Rt88), and atypical calpains (5, 6, and 10). There are three types of calpains that can be found in skeletal muscle: μ-calpain, m-calpain, and calpain 3. Ubiquitous calpains are regulated by calcium ions, phospholipids and calpastatin. They are also involved in in many basic cell functions (Sentandreu et al., 2002). Both, μ- and m-calpains target and cleave the same myofibrillar proteins. However, μ-calpain is activated early on during postmortem, and m-calpain lasts longer following harvest (Kemp et al., 2010). Calpain 3 is only found in skeletal muscle, however, it does not affect tenderness and is not inhibited by calpastatin like μ- and m-calpains (Kemp et al., 2010).

Calpains, especially μ-calpain, play a major role in the development of tenderness (Kemp et al., 2010). Calpains are responsible for 90% of the tenderization that occurs during post-mortem storage and degradation of Z-disks (Taylor et al., 1995). Studies have shown rapid loss of Z-disks in calpain treated skeletal muscle (Sentandreu et al., 2002). Although, calpains can serve as indicators of tenderness, calpastatin is a better indicator because of its inhibitory role against calpain activity (Sentandreu et al., 2002). In fact, tenderness relates inversely to the ratio of calpastatin to calpain among beef, lamb, and pork (Koohmaraie et al., 1991). Calpastatin is a single gene with multiple promoters that generate several different transcripts that are further spliced into multiple mRNAs,

resulting in multiple protein isoforms being derived from a single gene. It has the ability to regulate multiple calpain genes. Calpastatin can be broken down, however the remaining fragments retain inhibitory activity. Calpastatin is unstructured, however when it binds to calpain inhibition activity is activated (Kemp et al., 2010).

Cathepsins are acid proteases located in the lysosome. They may be released into the cytoplasm and intracellular spaces as a consequence of lysosomal disruption following cell death. There are approximately 13 types of cathepsins, however there are only four that are involved in muscle aging, cathepsins B, L, H, and D (Chéret et al., 2007). The interaction between cathepsin and tenderness of meat has been studied in the past, however, new studies suggest that cathepsins do not play a role in tenderization for numerous reasons. There is a lack of activity at postmortem pH and chilled temperatures (Sentandreu et al., 2002). In a recent review, cathepsins were stated to not influence tenderness; however cathepsins B and L activity at 8 h postmortem are positively correlated with tenderness in beef, and cathepsin L hydrolyses the largest number of myofibrillar proteins (Kemp et al., 2010). In addition, cathepsins degrade many other structural and contractile proteins (Sentandreu et al., 2002). Cathepsin B, H, and L are regulated by cystatin, a protease inhibitor (Chéret et al., 2007). Like calpastatin, cystatin is a better indicator for meat tenderness than the activity of cathepsins in the muscle (Sentandreu et al., 2002).

Caspases are neutral cysteine peptidases (Herrera-Mendez et al., 2006), and are likely the cause of apoptosis in muscle cells. They are responsible for the signaling pathway leading to cell death after exsanguination of the animal, however other pathways

may exist (Sentandreu et al., 2002). There are three classes of caspases, caspases that are involved with the inflammatory process, apoptosis initiation, and effector's caspases that disrupt the cell wall when activated. To date, 14 caspases have been identified, some caspases are species specific, for example, caspase 13 is only expressed in bovine (Herrera-Mendez et al., 2006). Caspases can be activated early in pathological events associated with hypoxia, which is similar to the hypoxic conditions in muscle after harvest. In meat animals, the process of exsanguination deprives all cells and tissues of nutrients and oxygen (Kemp et al., 2010). As a result of their role in apoptosis, caspases are considered to impact the process of tenderization (Herrera-Mendez et al., 2006). In a study conducted in 2010, caspases were found in the muscle 8 h after death of the animal, and relationship of caspases and shear force values was negative. These findings indicate that changes in caspase activity and caspase-mediated cleavage take place in muscle during the conditioning period and this could be associated with the development of tender meat (Kemp et al., 2010).

As mentioned before, tenderness is one of the most important qualities that consumers place on meat. It is unanimously recognized that the process of tenderization is enzymatic in nature, and the systems mentioned above are some of the most studied proteolytic systems (Ouali et al., 2006). However, there are still inconsistencies in tenderness of meat. Studies suggest that rate and extent of tenderization is very variable depending on the differences in the enzyme ratios (Kemp et al., 2010). However, there still may be other factors that are responsible for the inconsistencies observed in tenderness.

Premortem Stress

Stress have been defined as a constellation of events that begins with a stimulus, which initiates a reaction in the brain, which results in activation of certain physiologic systems in the body (Dhabhar & Mcewen, 1997). Many livestock animals experience stressful situations before harvest such as transportation, heat stress, handling, and morbidity. It has been reported that transportation is stressful for animals and has implications for their productivity and well-being. Stress factors in transportation include noise, vibration, lack of exercise, prolonged standing, and environmental temperature and humidity (Nwe et al., 1996). However, recent research suggests that handling during loading and unloading may increase stress response in cattle rather than transportation itself (Burdick et al., 2011). Transport itself is not responsible for the impacts on meat quality, but rather the stress that comes from this experience. Other management practices such as restricted water access, amount of resting time, and facilities at the harvest plant can have an impact on meat quality (Pighin et al., 2013). Stressors in livestock have been described in two ways, physiological and physical. Physiological stress refers to stress that may come from restraint, handling, or novel experiences. Physical stress refers to stress that may come from hunger, thirst, fatigue, injury, or thermal extremes (Grandin, 1997). Physiological and physical stress activates the released of stress responses, such as stress hormones, into systemic circulation and peripheral tissues (Dhabhar, 2018). In addition to different types of stressors, premortem stress can be attributed to different factors such as genotype, the animal's temperament, and previous handling experience (Pighin et al., 2013).

Animals that are exposed to stressful situations, such as transportation and handling, react by activating both the sympathetic and the hypothalamic-pituitary-adrenal axis. The activation of the first axis determines the release of epinephrine and norepinephrine in the blood as a preparatory event. The activation of the second axis determines the increase of cortisol-adrenal hormones (Micera et al., 2007). In addition, corticotropin-releasing hormone (CRH) is released from the hypothalamus, causing the release of adrenocorticotropic hormone (ACTH), which signals the release of glucocorticoids from the adrenals (Hulbert et al., 2013). Transportation has been known to induce a rise in plasma cortisol and catecholamines by the hypothalamic-pituitary-adrenal axis (Murata et al., 1987; Nwe et al., 1996). Cortisol is a useful indicator of short-term stress from handling or husbandry procedures. However, cortisol is a time dependent measure that takes 10-20 min to reach peak values (Grandin, 1997).

Depending on the source of stress, animals may experience fear, dehydration, hunger, increased fatigue, and physical injury. These effects of stress contribute to changes in energy homeostasis, intracellular ion dynamic, protease systems, and proteins in skeletal muscle. These factors have the potential to affect the conversion of muscle into meat (Xing et al., 2018). In addition, man-made stressors caused by management, transportation and the harvest process significantly influence the conversion of muscle to meat, its tenderness, and ultimately meat quality (Paredi et al., 2012). It has been reported that conditions established prior to slaughter have an effect on the tenderizing process of meat (Bond et al., 2004). Meat obtained from excitable animals has a tendency to have decreased meat quality such at dark cutting, Increased Warner-Bratzler shear force

values, and altered pH values (King et al., 2006; Pighin et al., 2013). In addition to effects on meat quality, stressors in cattle intensifies the incidence of disease and mortality by disturbing their metabolic and immune system along with alteration of leukocyte profiles (Murata et al., 1987). Catecholamines and cortisol are essential components of adaption to stress, however stress affects the immune system because cortisol and catecholamines reduce immunity (Nwe et al., 1996). In short, poor handling immediately prior to harvest, can ruin efforts made by producers during the long process of growth and fattening (Liste et al., 2009), and controlling stress response is crucial to both animal welfare and meat quality (Xing et al., 2018).

Many studies have been conducted to investigate the effects of lairage and rest time before harvest and at the harvest facility. In general, lairage serves as a rest and recovery time from the previously experienced stressors (Xing et al., 2018). Studies have contradictory results on lairage time and effects on meat quality. In a study involving rabbits and lairage time, results demonstrated that longer lairage time for rabbits at a harvest facility allowed time to recover from the stress of transport (Liste et al., 2009). Recovery rate from stressors during the lairage period may be affected by other factors such as time, environmental conditions, social mixing, and provided water and food (Ekiz et al., 2012). One study demonstrated that lairage periods that were longer than one hour decreased pale, soft, and exudative (PSE) meat, while skin damage increased in pigs (Nanni Costa et al., 2002). In beef cattle, increased lairage time decreased cortisol concentration in the blood (Chulayo et al., 2016), but increased the incidence of dark cutting beef (Tadich et al., 2005). Therefore, proper holding time, agreeable

environmental conditions, and proper handling during lairage are all important for improving animals welfare and meat quality (Xing et al., 2018).

Small Heat Shock Proteins

In 1973, heat shock proteins (HSP) were initially discovered in the tissues of *drosophila melanogaster* during the analysis of proteins following heat stress (Tissiéres et al., 1974). Cell stressors, such as heat shock and nutrient withdrawal, have been shown to induce apoptosis. Hyperthermia, ischemia, and oxidative stress in cells are known to activate heat shock factors. Upon activation, heat shock factors are translocated to the cell nucleus where they form a trimer and are phosphorylated. This allows for binding to heat shock elements of the heat shock protein gene which results in the synthesis of HSP (Lomiwes et al., 2014). Heat shock proteins are abundant and highly conserved proteins that are quickly synthesized by the onset of various stressors. Heat shock proteins are categorized into five different families based on molecular weight measured in kDa: HSP 60, HSP 70, HSP 90, and small heat shock proteins (SHSP) (Xing et al., 2018). Small heat shock proteins are the most variable in size having molecular mass from 12 to 43 kDa (Lomiwes et al., 2014).

In an unstressed cell, HSP are molecular chaperones. As molecular chaperones, HSP assist with protein assembly, protein folding and unfolding, translocation, and interacting with denatured proteins, and preventing protein aggregation which maintains cellular homeostasis (Xing et al., 2018). Small heat shock proteins play a similar role in molecular chaperoning. They bind to and stabilize unstable proteins and facilitate their correct assembly but are not part of the final functional structure. Cells that experience

stress undergo physiological changes that alter the transcription, translation, and folding

of proteins. Ultimately, this leads to the loss of their function. (Lomiwes et al., 2014).

Heat shock proteins appear as soon as the cell is in danger, and contribute to proper

conformation of proteins and preservation of their biological function (Herrera-Mendez et

al., 2006). Together, HSPβ1, HSPβ5, and HSP70 interact and work together to repair

denatured proteins (Mymrikov et al., 2011). Cells and tissues up-regulate expression of

HSP in response to stress, for example, proteome studies of muscle have identified some

SHSP that are up-regulated in post-mortem muscle (Pulford et al., 2008). Small heat

shock proteins are ATP-independent, meaning they are still functional in post-mortem

muscle (Doran et al., 2007; Hartl et al., 2011).

During periods of stress, SHSP can undergo post translational modification which

results in protein phosphorylation. Mitogen activated protein (MAP) kinase activated

protein kinase-2 phosphorylates HSPβ1 and HSPβ5. The phosphorylation of SHSP

promotes the detachment of oligomeric complexes into smaller oligomers, dimers, and

even monomers. In addition, SHSP phosphorylation is associated with the redistribution

of SHSP within a cell. For example, HSPβ1 will translocate from the cytosol to the

nucleus when phosphorylated, and HSPβ5 will translocate from the cytosol to the Z-line

of myofibrils (Lomiwes et al., 2014). Phosphorylation of SHSP results in two different

form of oligomers with differential activity to bind to denatured proteins: low affinity and

high affinity. Monomeric substructure has a high binding affinity to substrate proteins

due to high levels of exposed hydrophobic regions in their structure. As a result of their

high binding affinity, SHSP oligomers consisting of predominantly monomeric

substructures lose their ability to regulate the number of denaturing protein substrates they bind to. On the other hand, dimeric substructures have a decreased binding affinity to denatured proteins. Therefore, optimal chaperoning ability is defined by the optimal balance of monomeric and dimeric substructure of SHSP oligomers (Benesch et al., 2008).

Several proteomic studies have associated the regulation of SHSP with several meat quality attributes including tenderness, color, juiciness, and flavor. In addition, SHSP have proven to play important roles in regulating the development of tenderness during the postmortem period by mediating the apoptosis of muscle cells and binding to myofibrils and regulating protein degradation (Xing et al., 2018). In the postmortem muscle, structural proteins are degraded which leads to tenderization of the meat. This process can be modified by SHSP that protect and stabilize denatured proteins. It is also known that SHSP can form complexes with active caspases to hinder their function, and protect target proteins to prevent or delay degradation by enzymes (Herrera-Mendez et al., 2006). Small heat shock proteins HSPβ1 and HSPβ5 have been extensively studied for their effects on meat tenderness (Lomiwes et al., 2014) These specific SHSP bind and translocate to Z-disk related structures and prevent disruption of the cytoskeleton. In addition, HSPβ1 and HSPβ5 rapidly bind and accumulate on the myofibrils during stress within the muscle to protect the myofibrillar filament organization (Paulsen et al., 2007). Some SHSP, HSPβ1and HSPβ5, can be downregulated and contribute to improved tenderness, juiciness, and flavor of beef (Pulford et al., 2008). It is known that SHSP

contribute to meat quality, especially tenderness, however their exact role is unknown (Lomiwes et al., 2014).

Oxidative Stress

Oxidative stress occurs when there is a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses. During oxidative stress the balance shifts to increased levels of reactive oxygen species than antioxidants (Betteridge, 2000; Mandelker, 2011). Reactive oxygen species, also known as free radicals, are any chemical species that contain unpaired electrons which increase the chemical reactivity of an atom or molecule, and antioxidants are any substance that delays or inhibits oxidation of a substrate. Free radicals can come from the reduction of oxygen during aerobic respiration (Betteridge, 2000). In healthy animals, free radicals are balanced by antioxidant defenses. However, the balance is not perfect which results in tissue damage when antioxidant defenses are inadequate (Mandelker, 2011).

Metabolic and other processes in the muscle result in free radicals and other oxidative compounds such as hydroxyl radicals, peroxyl radicals, superoxide anions, hydrogen peroxide, and nitric oxide. These free radicals trigger oxidative stress resulting in modified proteins, DNA, and lipids which may affect the quality of meat products (Rowe et al., 2004; Xing et al., 2018). Oxidative stress damages cell membrane and mitochondrial integrity through lipid peroxidation, which increases the risk of oxidative reactions during the postmortem aging of meat and processing of meat products (Xing et al., 2018). The process of oxidation can be related to oxidative stress and occurs under postmortem conditions. Oxidation causes color deterioration, undesirable flavors, and

rancidity development in meat (Ponnampalam et al., 2017). Furthermore, oxidation is a major cause affecting muscle protein functionality, and meat quality (Xing et al., 2018).

Oxidative stress may be a result of diet, breed, and handling methods prior to harvest. However, environmental temperature and oxidized diets have the most impact on oxidative stress in animals (Xing et al., 2018). Although oxidative stress affects the quality of meat, it is impossible to measure oxidative stress directly in livestock animals (Betteridge, 2000). However, studies show that specific oxidative stress biomarkers help predict meat quality. Blood isoprostanes are prostaglandin like compounds that are produced by direct free radical development of lipid oxidation, may serve as an indicator of oxidative stress (Ponnampalam et al., 2017). Determining lipid peroxidation is also an important consideration in evaluating oxidative stress, since membrane phospholipids are one of the targets of free radicals (Aktas et al., 2011). Protein deglycase (DJ1) may also be an indicator of oxidative stress. It is known for its relationship to Parkinson's disease, which is caused by oxidative stress. An anti-oxidative defense, DJ1, protects cells from oxidative stress. DJ1 can then be used as a oxidative stress sensor once it becomes oxidized under oxidative stress (Saito et al., 2016).

Preventing oxidative stress is another area of study that is of value to the livestock industry. Administering antioxidants may prevent free radicals from damaging tissues by scavenging them, promoting their decomposition, or preventing the formation of them. The most common form of antioxidant in livestock are vitamins and minerals. Vitamins A, D, E and the mineral selenium increase antioxidant defense against free radicals and ultimately oxidative stress (Aktas et al., 2011). Vitamin A can act as a chain breaking

antioxidant by combining with peroxyl radicals. Peroxidation of the cell membrane ceases as vitamin A attaches to free radicals (Palace et al., 1999). Vitamin D assists in protecting cells against damage caused by ultra violet light, which is linked to peroxidation of cell membranes (Wiseman, 1993). Vitamin E inhibits free radical induced generation of peroxyl radicals which protects cells from peroxidation and oxidative damage of proteins and DNA. Selenium is also known to have antioxidant abilities. A selenium deficiency increases peroxidative damage and mitochondrial dysfunction (Fang et al., 2002). The vitamins and minerals discussed are only a few of the known dietary antioxidants. Other antioxidants like β-carotene and coenzyme Q are present within cell membrane to disrupt oxidative stress. Oxidative stress is an important contributor to tissue damage which leads to decreased meat quality (Betteridge, 2000).

Steak Color

Steak color is the most important factor that consumers consider when purchasing meat (Suman & Joseph, 2013). In the muscle, myoglobin is responsible for both oxygen storage and oxygen delivery and contains one heme group (Faustman & Cassens, 1990). In meat, myoglobin acts as the major pigment responsible for the bright cherry color consumers expect when purchasing 'wholesome and fresh' meat (Faustman & Cassens, 1990; Suman & Joseph, 2013). In myoglobin, the iron in the heme group may exist in a reduced or oxidized form, ferrous and ferric respectively. Ferrous heme iron lacks a sixth ligand and is called deoxymyoglobin. Meat with deoxymyoglobin as the primary pigment will appear purplish-red in color. When oxygen binds to the sixth binding site of ferrous heme iron, oxymyoglobin is formed which results in the desirable cherry-red color of

fresh meat. These two reduced forms of myoglobin readily oxidize the heme iron to the ferric state and water occupies the sixth coordination position. This results in metmyoglobin and undesirable brownish-red meat.(Faustman & Cassens, 1990). Reflectance is commonly used to measure steak color; this accurately indicates what a consumer may see. Three common measurements are used when using reflectance, L* indicates lightness, where increased values are lighter; a* measures redness, where positive values are more red and negative values are more green; b* measures yellowness, where positive values are more yellow and negative values are more blue (Mancini, 2009).

Several factors contribute to meat color such as pH, lipid oxidation, antioxidants, muscle source, and mitochondrial activity. In addition, animal factors such as management, diet, and genetics can also influence meat color (Suman & Joseph, 2013). In postmortem muscle, low pH values favor the oxidation of myoglobin. Beef with an ultimate pH greater than 5.8 has more color stability than beef with a decreased pH. In addition, low pH of post mortem muscle favors the release of the free radical, superoxide anion, which encourages oxidation (Faustman & Cassens, 1990). It has been reported that muscles with the greatest color stability were more likely to have less lipid oxidation and oxymyoglobin oxidation (Faustman, Sun, Mancini, & Suman, 2010). Antioxidants haven been shown to increase color stability of meat. However, some muscles accumulate antioxidants faster than others. Therefore, consideration should be taken to determine the length of antioxidant supplementation. Vitamin E is a popular antioxidant to feed to animals to improve color stability by decreasing lipid oxidation (Suman et al., 2014).

Management of animals before harvest may impact steak color. Dark cutting meat, also known as dry, firm, and dark (DFD) meat, is an ongoing issue that the livestock industry faces. 'Dark cutters' are animals that have a meat quality defect characterized by increased muscle pH, high water holding capacity, and dark red learn color. It is commonly known that decreased muscle glycogen before slaughter is the cause of dark cutting meat. Additionally, premortem stress has the potential to result in dark cutting meat. In a study conducted in 2005, animals that were subjected to restraint and isolation stress had darker *longissimus lumborum* (LL) steaks than unstressed animals (Apple et al., 2005). In addition, animals that were stressed had greater bound moisture in the meat which signals toward dark cutting meat (Apple et al., 2005).

According to consumers, tenderness is one of the most important organoleptic qualities of beef. The process of tenderization occurs during the conversion of muscle to meat as a result of proteolysis of myofibrillar and associated structural proteins within the muscle (Laville et al., 2009). It has been shown that premortem stress may affect the quality of meat, including dark cutters and oxidation of tissues (Paredi et al., 2012; Xing et al., 2018). Such stress may include physical and physiological stressors (Dhabhar, 2018). Evidence shows that SHSP appear as soon as the animal is stressed and cells are in danger, and contribute to protecting protein structure and integrity (Herrera-Mendez et al., 2006). More research needs to be conducted to determine how SHSP affect the quality of meat following a stressful event. Understanding how HSP and oxidative stress are involved in the development of tenderness will provide a deeper understanding of the

development of meat quality and potentially provide more avenues to improve meat quality in the future.

MATERIALS AND METHODS

All animal experiments will be conducted following procedures approved by the

USDA-ARS Livestock Issues Research Unit (LIRU), protocol #1808S. A total of forty

Holstein steers, weighing approximately 180 kg each, were obtained from an outside

source for use in this study. Although, industry harvest weight steers would have been

ideal for analyzing meat quality parameters, 180 kg Holstein steers were used based on

ease of handling and availability. After arriving to the USDA-ARS LIRU, animals were

given one week to acclimate to individual pens while having *ad libitum* access to a

general grower ration and water. All animals were housed in an indoor, thermoneutral

climate controlled environment during the duration of the study.

Initial Skeletal Muscle Samples

After the one-week acclimation period, skeletal muscle biopsies were collected

from the LL following previously described methods (Thornton et al., 2012); this sample

served as an initial control prior to stress challenge. In brief, animals were led calmly to a

cattle chute and immobilized. The loin area of the animals was clipped, washed, and

disinfected. Lidocaine was given to anesthetize the area before performing a 1 cm

incision using a sterile scalpel blade. Samples were immediately snap frozen in liquid

nitrogen and stored at -80°C for subsequent analyses.

ACTH Challenge

Prior to the ACTH challenge, animals were fit with a rectal temperature

monitoring device as describe by (Reuter et al., 2010) and an indwelling jugular vein

catheter for serial blood collection following previously described methods (Carroll et al.,

2009). The following day, all cattle were challenged with bovine ACTH at a dose of 0.1 IU/kg of body weight to initiate stress. Following the ACTH challenge, animals were serially harvested in groups of 10 animals at the following time points after 6 h of stress from ACTH: 2 h, 12 h, 24 h, and 48 h (Figure 1).

Blood Analyses

A ProCyte DX hemotology analyzer was used to measure complete blood count (CBC) every 2 h from -2 to 6 h relative to induction of the ACTH challenge at 0 h. In addition, serum cortisol was measured every 0.5 h from -2 to 6 h relative to induction of the ACTH challenge at 0 h following methods previously described (Carroll et al., 2009). Between blood samples, all catheters were flushed with 5 ml of saline (0.9% w/v NaCl) followed by 5 ml of heparinized saline (1 ml of heparin 10,000 IU/ml in 500 ml of saline) to replace fluid volume and to maintain catheter patency.

Sample Collection

At the end of the treatment phase, cattle were harvested at the USDA-ARS LIRU near Lubbock, TX. During exsanguination, blood samples were collected and processed to produce serum. Within 30 min of exsanguination, a skeletal muscle sample from the LL was collected. A further portion of the LL was held under vacuum and refrigeration (2-4°C) for 14 d and a final LL muscle sample was collected. All muscle samples collected for protein and oxidative measures were immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Western Blotting

Skeletal muscle samples were ground under liquid nitrogen and total protein was extracted following previously described procedures (Thornton et al., 2017). In brief, approximately 60 mg of ground tissue was transferred to 15 µL/mg tissue total protein extraction buffer; 50 mM Tris-HCl (pH 7.52), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Tergitol, 0.1% SDS, and 0.5% sodium deoxycholate. Immediately before use, phosphatase and protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA) were added to the extraction buffer. Samples were homogenized and placed on a rocking platform for 10 min at 4°C. All samples were centrifuged at 10000 x g for 10 min at 4°C. The supernatant was removed and stored at - 80°C. Total protein was quantified using a Pierce™ BCA Protein Assay Kit, subsequently samples were diluted to a concentration of 10 ug/uL. Proteins were separated using SDS - 10% polyacrylamide gels for 90 min at 140 V at 4°C suing a Bio-Rad Mini PROTEAN tetra cell gel box in running buffer. The proteins were then transferred to an immobilidon- polyvinylidene fluoride (PVDF) membrane using a Bio-Rad trans-blot apparatus with transfer buffer. Samples were transferred at 4°C for 90 min. Western blot analyses were completed on samples collected prior to the ACTH challenge, immediately following harvest, and after 14 d of aging to measure abundance of HSPβ1, Phosphorylated-HSPβ1 (P-HSPβ1), HSPβ5, DJ1, and troponin I. using specific primary and secondary antibodies presented in Table 1.

All membranes for all target proteins were incubated in 5% non-fat milk plus 1X tris-buffered (1X TBS) saline for 1 h at room temperature. The membranes were then

incubated with antibody specific dilution of primary antibody overnight at 4° C. Membranes were briefly washed in 1X TBS and incubated with an antibody specific dilution of anti-rabbit antibody (HSPβ1, P-HSPβ1, DJ1, and troponin I) or anti-mouse antibody (HSPβ5) conjugated to horseradish peroxidase (HRP) for 2 h at room temperature. After a brief wash, bound antibodies were detected using a C-DiGit® Blot Scanner by LI-COR. The chemiluminescence band intensity was quantified with Image Studio™. The protein abundance values were adjusted based on internal standard abundance for each blot.

Myofibrillar Fragmentation Index

Skeletal muscle from samples aged for 14 d post mortem were ground under liquid nitrogen and myofibril fragments were extracted using a protocol adapted from Culler et al. (Culler et al., 1978). In brief, ground tissue was submerged in MFI buffer (100 mM KCl, 20 mM KPO_4^- pH 7, mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3). Tissue and MFI buffer were homogenized for 30 s. Samples were centrifuged twice at 1000 x g for 15 min at 4°C; supernatant was decanted, and fresh MFI buffer was added each time after samples were centrifuged. Myofibrillar fragments were then quantified using the Pierce™ BCA Protein Assay Kit. After quantification, samples were diluted to 500 ug/mL, plated, and read at 540nm on a BioTek Synergy H1 plate reader (BioTek, Winooski, VT) to find Myofibrillar Fragmentation Index (MFI). Samples were then measured using methods from the above-mentioned protocol.

Steak Color

Meat color was measured from all 14 d aged steaks taken from the LL at 0, 1, 2, 3, 4 retail d. Steaks were placed onto foam trays with absorbent pads, over-wrapped with a PVC film (oxygen-permeable polyvinyl chloride fresh meat film; 15,500 to 16,275 cm^3 O$_2$ m–2 24 h–1 at 23°C, E-Z Wrap Crystal Clear Polyvinyl Chloride Wrapping Film, Koch Supplies, Kansas City, MO). After packaging, steaks were placed in a coffin-style open display case maintained at 2°C ± 1 under continuous lighting (1612 to 2152 lx, Philips Deluxe Warm White Fluorescent lamps; Andover, MA; color rendering index = 86; color temperature = 3000 K). All packages were rotated daily to minimize variances in light intensity and/or temperature caused by specific case locations. The surface color was measured using a HunterLab MiniScan XE Plus spectrophotometer (Model 45/0 large area view, 2.5-cm diameter aperture, Illuminant A, 10° Observer; HunterLab, Reston, VA) on respective display days. Both reflectance spectra from 400 to 700 nm (10 nm increments) and CIE L*, a*, and b* values were measured on each steak at three random locations and the subsamples were averaged for statistical analyses.

TBARS

Thiobarbituric acid reactive substances (TBARS) were measured in serum at 2 h increments throughout the study, at exsanguination, and in stored muscle tissue utilizing a modification of the method proposed by others (Buege & Aust, 1978; Luqué et al., 2011). A standard curve was prepared using a trichloroacetic acid (TCA)/ thiobarbituric acid (TBA) reagent (15% TCA (w/v) and 20 mM TBA in DI water) and a diluted 1, 1, 3, 3-tetra- ethoxypropanone (TEP) standard. Six standards and a blank were prepared to have

the following concentrations of TEP: 0 µM, 0.17 µM, 0.34 µM, 0.68 µM, 1.36 µM, 2.72 µM, and 5.45 µM. To prepare muscle tissue samples for evaluation, muscle was frozen and homogenized to a fine powder. Samples (2.5 g) were weighed into 50 mL centrifuge tube with 7.5 mL of cold DI water. Tubes were then capped and vortexed for 90 sec. Mixed samples were centrifuged for 15 min at 4500 x g. Following centrifugation, 2 mL supernatant were aliquoted to labeled 15 mL centrifuge tubes, and 4 mL of TCA/TBA reagent, and 100 µL of butylated hydroxyanisole (BHA) were added. Samples were vortexed approximately 1 min. For the serum, 250 µL of samples were pipetted into screw-cap microcentrifuge tubes with 500 µL of TCA/TBA reagent and 10 µL of BHA and vortexed for approximately 1 min. All prepared samples and standards were heated for 1 h in a 90°C water bath, then submerged in an ice water bath for 20 min to stop the reaction. For standards, tubes were then allowed to come to ambient temperature before 200 µL of each standards were plated for evaluation. Heated and cooled serum and tissue samples were centrifuged for 15 min at 4500 x g, then 200 µL of supernatant was plated onto a 96 well plate for evaluation at 531 nm. Standard curves were used to determine concentrations of malondialdehyde (MDA) in all samples.

Statistical Analysis

Statistical analysis for all measurements were analyzed using the PROC MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). The main effect of time of harvest (n=10) was analyzed with each individual animal serving as a random variable. Initial cortisol response was found to differ ($P < 0.05$) between time of harvest groups and as such, the effect of initial cortisol response was used as a co-variate. A total of four

cortisol response groups (n = 10) were assigned: lowest, cortisol = 13.94 ng/mL; lower,

cortisol = 22.40ng/mL; higher, cortisol = 34.81 ng/mL; and highest, cortisol = 55.41

ng/mL. The final statistical model assessed the main effects of harvest time, initial

cortisol response and their interaction. The fold change of protein abundance was

measured by calculating the fold chance from samples collected before the ACTH

challenge to samples collected at harvest and after 14 d of aging. Steak color, TBARS,

and CBC were analyzed using repeated measures and PROC MIXED where harvest time,

cortisol response, and their interaction were used as fixed effects and individual animal

served as a random effect in the model. In all analyses, when treatment differences were

found to be significant ($P < 0.05$), least square means were separated using Tukey-

Kramer adjustments. A $P < 0.05$ was considered statistically significant, whereas a $P <$

0.10 was considered a trend in the data. All data are presented as the least square mean $\pm$

SEM.

RESULTS

Cortisol

The concentration of cortisol in the serum collected every 0.5 h relative to the ACTH challenge was affected by both harvest time ($P < 0.001$) and time ($P < 0.001$) when analyzed using repeated measures, Figure 2. Differences between groups that were to be harvested at different times were present 0.5 h after initiation of ACTH challenge such that animals that were to be harvested 48 h after the ACTH challenge had an increased ($P < 0.05$) serum cortisol concentration compared to animals that were to be harvested 12 h after the ACTH challenge, Figure 2. However, no differences between harvest time groups were observed the remainder of the ACTH challenge, Figure 2.

Protein Abundance

Time of harvest did not ($P > 0.05$) impact relative HSPβ1 abundance in samples collected at harvest nor samples aged for 14 d in the LL, Figure 3. In addition, relative abundance of HSPβ1 was not affected ($P > 0.05$) by initial cortisol response when analyzed in samples collected at harvest or after 14 d of aging, Figure 3.

Time of harvest affected ($P < 0.05$) relative P-HSPβ1 abundance in the LL at harvest such that animals that were harvested 48 h after the ACTH challenge had increased ($P < 0.05$) abundance of P-HSPβ1 compared to animals that were harvested 12 h after the ACTH challenge, Figure 4. Relative P-HSPβ1 abundance of samples aged for 14 d was also affected ($P < 0.05$) by time of harvest, where animals that were harvested 2 h and 48 h after the ACTH challenge had decreased ($P < 0.05$) abundance of P-HSPβ1 compared to animals that were harvested 12 h after the ACTH challenge, Figure

4. Cortisol responses 0.5 h after initiation of the ACTH challenge had an effect ($P < 0.05$) on abundance of P-HSPβ1 at harvest, however no differences ($P > 0.05$) were seen in cortisol quartile groups after 14 d of aging, Figure 4.

Analysis of abundance of HSPβ5 from samples collected at harvest and after 14 d of aging was not different ($P > 0.05$) relative to time of harvest, Figure 5. Furthermore, analysis of relative HSPβ5 abundance was not different ($P > 0.05$) at harvest or after 14 d of aging between animals with different initial cortisol responses, Figure 5.

Relative abundance of DJ1 was different ($P < 0.05$) at both harvest and after 14 d of aging between animals harvested at different times after the ACTH challenge, Figure 6. After 14 d of aging, samples from animals that were harvested 24 h after the ACTH challenge had increased ($P < 0.05$) abundance of DJ1 compared to the other three harvest times, Figure 6. Initial cortisol response did not ($P > 0.05$) impact abundance of DJ1 at harvest or after 14 d of aging, Figure 6.

Time of harvest had an effect ($P < 0.05$) on relative troponin abundance in the LL at harvest, Figure 7. Animals that were harvested 24 h after the ACTH challenge had increased ($P < 0.05$) abundance of troponin compared to animals that were harvested 2 h and 12 h after the ACTH challenge, Figure 7. Cortisol response had no effect ($P > 0.05$) on troponin abundance at harvest, Figure 7.

Time of harvest had an impact ($P < 0.05$) on abundance of the entire band of troponin; samples from animals harvested 48 h after the ACTH challenge had increased ($P < 0.05$) abundance of troponin when compared to animals that were harvested 12 h

after the ACTH challenge, Figure 8. Relative abundance of the entire band of troponin after 14 d of aging was not ($P > 0.05$) impacted by initial cortisol response, Figure 8.

Time of harvest affected ($P < 0.01$) abundance of the lower band of troponin after 14 d of aging such that samples from animals that were harvested 48 h after the ACTH challenge had increased ($P < 0.05$) abundance compared to samples from animals harvested at the other three times, Figure 9. No differences ($P > 0.05$) were observed in the abundance of the lower troponin band relative to initial cortisol response, Figure 9.

Complete Blood Counts

When analyzed with repeated measures, time of harvest affected ($P < 0.01$) red blood cell concentration, Figure 10. Animals that were to be harvested 24 h after the ACTH challenge had a decreased ($P < 0.05$) concentration of red blood cells when compared to animals that were to be harvested at the other three time points, Figure 10. Additionally, time of harvest affected ($P < 0.01$) hemoglobin concentration when analyzed with repeated measures; animals that were to be harvested 2 h after the ACTH challenge had increased hemoglobin concentration compared to animals that were to be harvested 24 and 48 h after the ACTH challenge, Figure 10. Additionally, animals that were to be harvested 12 h after ACTH challenge has increased hemoglobin compared to those that were harvested 24 h after ACTH challenge, Figure 10. Time of harvest also had an effect ($P < 0.05$) on hematocrit such that animals harvested 48 h after the ACTH challenge had a decreased hematocrit compared to those harvested 2 and 12 h after the challenge, Figure 10. Platelet concentration was also affected ($P < 0.05$) by time of harvest, although no differences ($P > 0.05$) between groups that were to be harvested at

different times were observed, Figure 10. The final concentration of hemoglobin, hematocrit, and platelets did not differ ($P > 0.05$) between harvest times, however red blood cell concentration did ($P < 0.05$), Figure 10.

Concentration of white blood cells, neutrophils, monocytes, eosinophils, and lymphocytes were each affected ($P < 0.05$) by time of harvest when analyzed as repeated measures, Figure 11. White blood cells were increased ($P < 0.05$) in animals harvested 2 h after ACTH challenge when compared to animals harvest 12 h after the ACTH challenge, Figure 11. Animals that were harvested at 24 h after the ACTH challenge had increased ($P < 0.01$) neutrophil concentrations compared to animals harvest 12 h after the challenge, Figure 11. Animals that were harvested 2 and 48 h after receiving ACTH had increased ($P < 0.05$) monocytes compared to those animals that were harvested 12 and 24 h after the challenge, Figure 11. Eosinophils were increased ($P < 0.05$) in animals harvested 12 h after the ACTH challenge when compared to animals harvested at the three other time points, Figure 11. Additionally, concentration of white blood cells, neutrophils, monocytes, and eosinophils changed ($P < 0.05$) over time, Figure 11. In each of these, concentration increased ($P < 0.05$) 2 h after the ACTH challenge and then decreased after that time point, Figure 11. There was no effect ($P = 0.31$) of time of harvest on concentration of basophils, Figure 11. Concentration of lymphocytes and basophils were not ($P > 0.05$) when analyzed over time, Figure 11. The final concentration of white blood cells, neutrophils, monocytes, eosinophils, lymphocytes, and basophils did not differ ($P > 0.05$) between harvest times, Figure 11.

Steak Color

The a* value, redness, of steaks was affected by time of harvest ($P <$ 0.01) and retail day ($P < 0.01$) affected a* values, Figure 12. On d 4 of retail display steaks from animals that were harvest 24 h and 48 h after the ACTH challenge had an increased ($P < 0.05$) a* value than steaks from animals harvested 2 h and 12 h after the ACTH challenge, Figure 12. In addition, a harvest time*cortisol response interaction ($P <$ 0.01) was observed for a* values, Figure 12. Cortisol response affected a* values ($P <$ 0.05) over time such that those with an increased initial cortisol response had an increased ($P < 0.05$) a* compared to those with a lower and the highest cortisol response, Figure 12.

Time of harvest affected ($P < 0.01$) and retail day of display ($P < 0.01$) affected b* values, Figure 13. After 4 d of retail display, steaks from animals that were harvested 24 h and 48 h after the ACTH challenge had increased ($P < 0.05$) b* values than steaks from animals harvested 2 h and 12 h after the ACTH challenge, Figure 13. Cortisol response also affected b* values ($P < 0.05$) such that animals that had a higher initial cortisol response had an increased b* compared to animals with the lower and highest initial cortisol response, Figure 13. A harvest time*cortisol response interaction ($P <$ 0.01) was also observed for b* values, Figure 13.

The values for L* were not affected by time of harvest ($P > 0.05$), retail day of display ($P > 0.05$) or initial cortisol response ($P < 0.05$) when analyzed as repeated measures, Figure 14. However a harvest time*cortisol response interaction was detected ($P < 0.05$) for L* values of steak, Figure 14.

Oxidation

Time of harvest had an effect (P < 0.01) on concentration of TBARS, Figure 15. Concentration of TBARS were increased in the serum of animals harvested 24 h and 48 h after the ACTH challenge when compared to those harvest 2 and 12 h after the challenge, Figure 15. Additionally, cortisol response had an effect (P < 0.05) on TBARS concentration in the serum such that animals that had a lower concentration of cortisol had increased concentrations of TBARS compared to animals that had a higher initial cortisol response, Figure 15. A harvest time*cortisol response interaction (P < 0.01) was also observed for TBARS concentration in the serum, Figure 15.

Myofibrillar Fragmentation Index

Time of harvested affected (P < 0.05) MFI such that animals that were harvested 24 h following the ACTH challenge had an increased MFI value compared to animals that were harvested 12 h after the ACTH challenge, Figure 16. However, cortisol response did not affect (P > 0.05) MFI, Figure 16.

DISCUSSION

The rate and extent of post-mortem breakdown of myofibrillar proteins are major determinants of end-product tenderness (Koohmaraie & Geesink, 2006; Laville et al., 2009). Although the processes of proteolysis and development of end-product tenderness are largely studied, inconsistencies in tenderness still exist (Ouali et al., 2006). External factors, such as premortem stress, may be involved in decreased meat quality by modulation of HSP abundance. Heat shock proteins are highly conserved proteins that are both constitutively expressed and expression is upregulated by the onset of stressful conditions (Kristensen et al., 2003; Xing et al., 2018). As molecular chaperones, HSP assist with proper protein assembly, protein folding and unfolding, translocation, interaction with denatured proteins, and preventing protein aggregation, which maintains cellular homeostasis (Xing et al., 2018). Small heat shock proteins, a family of HSP, play a similar role in molecular chaperoning by binding to and stabilizing unstable proteins and facilitating their correct assembly, but are not part of the final functional structure (Lomiwes et al., 2014). It is known that SHSP are ATP-independent, demonstrating they can still be active in post-mortem tissues (Doran et al., 2007; Hartl et al., 2011). In fact, proteomic studies of muscle have identified that some SHSP are up-regulated in post-mortem muscle (Pulford et al., 2008). Following a stressful event, oxidative stress may affect tenderness, color stability, and flavor of meat by causing color deterioration, undesirable flavors, and rancidity development in meat (Ponnampalam et al., 2017). It is known that tenderness and steak color are both important qualities to consumers, however inconsistencies in meat quality remain (Koohmaraie & Geesink, 2006; Suman &

Joseph, 2013). Premortem stress may result in activation of pathways that decrease overall meat quality. The goal of this research was to determine the role of sHSPs and oxidative stress relative to meat quality following a stressful event prior to harvest.

Animals used in the present study were smaller and younger than industry standard finished feedlot animals. In addition, animals used were a dairy breed rather than a beef breed. To the authors' knowledge no research observed meat quality traits on animals this size or age in relation to stress. As such, many comparisons are made with beef breeds or finished feedlot animals.

In the present study, most blood components in the serum differed over time based on time of harvest relative to the ACTH challenge. A study involving Limousine feedlot beef cattle that were transported for 5 h observed increased WBC concentration 48 h following transportation (Giannetto et al., 2011). However, in the present study animals that were allowed 2 h rest following the ACTH challenge had increased concentration of WBC compared to animals that were harvested 12 h after the challenge. Another study observed the effects of lairage duration following transportation in Limousine bulls and found that lairage decreased platelet count, but hematocrit increased from unloading the animals to harvest (Liotta et al., 2007). In the present study, platelet count was not affected by harvest time, however hematocrit was increased in animals that were harvested 2 h and 12 h after the ACTH challenge compared to animals that were harvested 48 h after the ACTH challenge. This could be a result of minor dehydration due to stress during the ACTH challenge. Other researchers found that heifers separated into low or high residual feed intake (RFI) groups showed increased concentrations of RBC

and HCT in the low RFI group relative to an ACTH challenge (Kelly et al., 2017).

Additionally, Kelly et al. (2017) observed that heifers belonging to the low RFI group

had decreased plasma cortisol. The concentration of RBC was highest over time in

animals that were harvested 2 h after the ACTH challenge in the present study.

Additionally, animals that were harvested 2 h and 12 h after the ACTH challenge had

increased hematocrit compared to animals that were harvested 48 h after the challenge.

Interestingly, animals that were harvested 12 h after the ACTH challenge had a decreased

cortisol response compared to animals that were harvested 48 h after the challenge. A

study that observed the effects of a lipopolysaccharide (LPS) challenge on steers fed a

control diet or a *Saccharomyces cerevisiae* fermentation product saw no treatment effects

on RBC, hemoglobin, hematocrit, WBC, neutrophils, lymphocytes, monocytes, or

eosinophils (Burdick Sanchez et al., 2020). However, researchers observed increased

platelet count during the LPS challenge for the steers supplemented with the

Saccharomyces cerevisiae fermentation product; in addition this group of steers had

increased cortisol concentration in the serum 0.5 h after initiation of the LPS challenge

(Burdick Sanchez et al., 2020). In the present study, time of harvest did have an effect on

platelet concentration, however there were no differences between harvest times during

the ACTH challenge. A study that observed the effects of cold stress and ACTH

administration in Japanese Black steers saw that monocytes, eosinophils, and neutrophils

increased, while lymphocytes decreased after ACTH was administered in a cold

environment (Ishizaki & Kariya, 1999). The current study showed a slight increase in

eosinophil concentration at harvest compared to eosinophil concentration throughout the

ACTH challenge, while no apparent changes were observed in monocytes, neutrophils, and lymphocytes. These differences may be due to the additional cold stress the Japanese Black steers were experiencing. Taken together the results of this study demonstrate that some blood components are affected slightly during a stressful period. However, intense stress or a increased cortisol response compared to the current study may affect blood components differently.

Cortisol concentration relative to the ACTH challenge showed that the ACTH challenge did indeed cause a peak in serum cortisol that was evident 0.5 to 1 h after ACTH was administered. The hormone ACTH stimulates the adrenal gland, which synthesizes and releases adrenaline, noradrenaline, and cortisol (Binsiya et al., 2017). Although animals were the same breed, uniform in size, the same age, and sourced from one farm, differences in cortisol response were observed between harvest time groups 0.5 h after initiation of the ACTH challenge. However, cortisol concentration did not differ at harvest. This may be the result of differing anxiety behaviors between the harvest time groups as a difference in cortisol response was observed 0.5 h after the initiation of the ACTH challenge. A behavioral study of Angus x Hereford cows showed that animals that were more anxious (i.e. ruminating less and increased vocalization during restraint) had increased concentration of cortisol in the serum (Bristow & Holmes, 2007). The baseline cortisol concentration of the animals in the present study is similar to other studies (Båge et al., 2000; Schwinn et al., 2018; Verkerk et al., 1994); however the peak cortisol concentration in the present study is slightly decreased than previous studies that performed an ACTH challenge (Båge et al., 2000; Schwinn et al., 2018). This could be

attributed to the environment of the animals during the ACTH challenge and dose of ACTH. The animals in the present study were housed in an indoor thermoneutral climate during the ACTH challenge.

As molecular chaperones, SHSP bind to and stabilize unstable proteins (Lomiwes et al., 2014). Various stressors result in the synthesis of HSP (Xing et al., 2018). The increase of cortisol prompts cellular adaptation, which is accomplished through synthesis of HSP (Das et al., 2016). Cells and tissues upregulate expression of SHSP in post-mortem muscle (Pulford et al., 2008). In the present study, relative HSPβ1 did not differ at harvest nor after 14 d of aging based on time of harvest or cortisol response, however other studies showed increased HSPβ1 abundance caused by various stressors. Heat stress caused by increased ambient temperatures in Brazil was measured using rectal temperature in Nelore and Caracu (Pires et al., 2019). Increased heat stress resulted in increased relative HSPβ1 gene expression measured in the serum (Pires et al., 2019). Expression of HSPβ1 mRNA in peripheral blood lymphocytes increased after 9 h of transportation and gradually declined in Xia Nan cows (Deng et al., 2013). The difference in findings may be a result of breed of animal and/or the tissue analyzed. Additional research would be beneficial to determine how stress specifically impacts abundance of HSPβ1 in skeletal muscle.

During periods of stress, HSPβ1 can undergo post translational modifications which results in phosphorylation (Lomiwes et al., 2014). In response to oxidative stress, HSPβ1 can be phosphorylated and in this form it interacts with actin and protects it form fragmentation (Huot et al., 1996). In the present study, P-HSPβ1 abundance differed

based on time of harvest. At harvest, animals that were harvested 48 h after the ACTH challenge had increased abundance of P-HSPβ1 than animals that were harvested 12 h after the ACTH challenge. However, after 14 d of aging animals that were harvested 12 h after the ACTH challenge had increased abundance of P-HSPβ1 than animals that were harvested 2 h and 48 h after the challenge. To the knowledge of the authors no other research has been analyzed on the effects of various stressors and time of harvest on P-HSPβ1 abundance. However, one study observed that P-HSPβ1 protects actin from fragmentation induced by oxidants (Huot et al., 1996). Additional research needs to be completed to determine how stress and time of harvest impact P-HSPβ1 abundance.

The SHSP, HSPβ5 rapidly binds to and accumulates on myofibrils during stress to protect the myofibrillar filament organization (Paulsen et al., 2007). In the present study the abundance of HSPβ5 did not differ based on time of harvest nor cortisol response. Little research has been done on the abundance of HSPβ5 and time of harvest or stress in cattle, however studies have looked at other species. Pietrain x Erhaulian barrows were transported for variable times and it was found that barrows that were transported 1 h and 4 h had decreased abundance of HSPβ5 in the *longissimus dorsi* than barrows that were not transported, however animals that were transported 2 h did not have different -HSPβ5 abundance than animals that were not transported (Yu et al., 2009). The differences in HSPβ5 abundance from the present study may be a result of species of animal used. More research is needed to fully understand the effects of stress on HSPβ5 abundance in cattle.

In the present study, MFI and troponin abundance were used to assess tenderness. Time of harvest affected MFI, however cortisol response did not affect MFI. The

tenderness measurement, Warner-Bratzler shear force (WBSF), was not used in the study due to inferior size of loins collected from the Holstein steers. A strong negative correlation between MFI and WBSF has been well documented (Culler et al., 1978; Whipple et al., 1990). However, another study showed that WBSF was negatively correlated to MFI, but there was not a strong correlation between WBSF and MFI after 14 d of aging (Shackelford et al., 1991). In the present study, MFI was increased in animals that were harvested 24 h after the ACTH challenge, indicating that steaks from this group of animals have increased tenderness due to increased myofibrillar fragmenting. Other studies have observed that meat tenderness was affected following a stressful event. Following transportation for 4 h, Hereford and Braford steers were allowed to rest in lairage for various lengths and steers that were in the long lairage group showed decreased WBSF values than steers from the short lairage group (del Campo et al., 2010). Another study showed that Nellore steers with increased chute scores or flightier temperaments had increased WBSF values (Coutinho et al., 2017). In a study using Omani rams, animals that were not transported on day of harvest and allowed to rest for 48 h prior to harvest had increased MFI than animals that were transported 3 h on day of harvest (Kadim et al., 2009). Although MFI is shown to be correlated to WBSF and is a common measurement of tenderness, MFI is a result rather than a cause of postmortem aging (Shackelford et al., 1991). As such, future studies need to include additional measurements, such as WBSF, to determine how time of harvest following a stressful event effects end-product tenderness.

As mentioned above, troponin degradation was used in the present study to assess tenderness. The troponin complex is a principle regulatory component of the thin filament in skeletal muscle, and contains three subunits (troponin I, troponin C, and troponin T) (Farah & Reinach, 1995). Increased degradation of troponin is related to increased tenderness of meat (Carlson et al., 2017). The present study used troponin I in troponin degradation analysis. Troponin I is involved in the inhibition of troponin C, which binds Ca^{2+} and removes Troponin I inhibition, and troponin T, which binds to tropomyosin (Farah & Reinach, 1995). In the present study, animals that were harvested 48 h after a stress exposure had increased relative abundance of troponin I in 14 d aged samples. In a study that characterized pre-harvest stress by dark, firm and dry (DFD) meat in Rubi Gallega cattle observed that troponin C was increased in normal meat compared to the DFD meat in the *longissimus thoracis* (LT) (Franco et al., 2015). In addition, researchers observed that DFD meat was more tender than normal meat (Franco et al., 2015). Another study showed that Angus influenced heifers designated for kosher slaughter are calmer than non-kosher animals, however kosher heifers had increased WBSF values and increased troponin T abundance in the LL than non-kosher animals (Hayes et al., 2015). The increased WBSF values and troponin abundance in the calmer kosher heifers could be a result of heavier muscled and larger carcass weights in the kosher heifer group. The effects of a stressful event prior to harvest on troponin abundance and MFI have not been studied at length. More research in this area would be beneficial to understand this interaction more clearly.

The highly conserved protein, DJ1, is present in the cytoplasm as well as in the intracellular organelles and protects against oxidative stress (Gagaoua et al., 2015). In the present study, animals that were allowed to rest for 24 h following a stressful event had the highest abundance of DJ1 after 14 d of aging compared to the other three harvest times. Little to no research has been done on the effects of premortem stress on DJ1 abundance in cattle. However, several studies have been done on the relationship of DJ1 and tenderness of meat. Some researchers have reported that tenderness was associated with abundance of DJ1 (Jia et al., 2009; Picard et al., 2014). Interestingly, the animals that were allowed to rest for 24 h after the ACTH challenge had the highest MFI compared to the other three treatment groups, which agrees with other studies that protein abundance of DJ1 is linked to increased tenderness. In addition to DJ1 abundance, TBARS were used as a measurement of lipid peroxidation in the serum relative to the ACTH challenge. Time of harvest and cortisol response had an effect on TBARS concentration in the present study. Animals that were to be harvested 24 h after the ACTH challenge had an increased concentration of TBARS 8 h after initiation of the challenge and at harvest, and animals that had a decreased cortisol response has an increased concentration of TBARS 2 h before initiation of the challenge. Researchers saw three-fold increase in MDA in crossbred steers after transportation compared to before transportation (Chirase et al., 2004). In addition, another study observed a strong positive correlation between concentration of cortisol and TBARS in calves after 2 h transportation (Wernicki et al., 2006). In cows infected with *Theileria annulata* researchers saw increased lipid peroxidation measured in hemolysate than healthy cows

(Grewal et al., 2005). In other species such as broiler, researchers observed an increase in TBARS in heat stressed broilers compared to non-heat stressed birds (Altan et al., 2003). It is apparent that certain stressors can increase oxidation of tissues which may affect meat quality (Rowe et al., 2004; Xing et al., 2018). However, more research would be beneficial in understanding the effects of stress on the abundance of DJ1 in skeletal muscle and concentration of TBARS in serum.

Steak color is an important quality of meat that consumers consider when purchasing meat (Suman & Joseph, 2013). In the present study, time of harvest and cortisol response had an effect on b* and a*, however L* was not affected. Animals that were harvested 24 h and 48 h after the ACTH challenge had steaks that were more red and yellow in color than steaks from animals that were harvested 2 h and 12 h after the ACTH challenge. This is similar to what other studies have observed. One study observed that Hereford and Braford steers that were transported 4 h had increased a* and b* values in the long lairage group, however L* values were not significant between the short and long lairage times (del Campo et al., 2010). In another study, Bos indicus x Bos taurus cross cattle were allowed to rest in lairage for 3 h or 18 h after transportation and the steaks collected from the LL were aged for either 1 d or 14 d (Ferguson et al., 2007). Researchers observed a lairage*aging interaction for a* and b* and animals that were held in lairage longer had increased a* and b* values in 14 d aged samples (Ferguson et al., 2007). However other researchers observed different results than the present study. In a study involving Hungarian Simmental bulls, researchers observed the effect of lairage time after long distance transportation on steak color measured 24 h and 7 d after harvest

(Teke et al., 2014). This group saw increased L* values 24 h after harvest in bulls that were held in lairage for 48 h and 72 h compared to bulls that were held in lairage 24 h, however no differences were seen in a* values based on lairage time (Teke et al., 2014). Furthermore, researchers did not see differences in steak color 7 d after harvest (Teke et al., 2014). Friesian steers that were transported 3 h or 16 h and kept in lairage for 3, 6, 12, or 24 h had differences in L* values (Gallo et al., 2003). Animals that were transported for 3 h and harvested 12 h after transportation had increased L* values that the other treatment groups (Gallo et al., 2003). Additionally, researchers observed decreasing L* values in cattle the longer they were in lairage after 16 h of transportation (Gallo et al., 2003). These differences may be a result of differing lairage times and aging periods from the current study. Measurement of color closer to harvest would have been beneficial to compare steak color over time. More research studying the effects of rest time after a stressful event on steak color is needed to understand the relationship between stress and steak color.

The results of this study demonstrate that time of harvest and cortisol response following a stressful event have an effect on meat quality. Small heat shock proteins that have been shown to be involved with meat quality were affected by time of harvest and cortisol response. Time of harvest and cortisol response affected the abundance of P-HSPβ1 in the LL. However, abundance of HSPβ1 and HSPβ5 in the LL were not affected by time of harvest nor cortisol response. Tenderness assessed by troponin I abundance and MFI was affected by time of harvest following a stressful event. In addition, oxidation measured by the protein DJ1 and TBARS, was affected by time of harvest and

cortisol response following a stressful event. Steak color was also affected by time of harvest and cortisol response. Waiting 2 h to 12 h, rather than 24 h to 48, after a stressful event may result in better quality beef. The findings of this study are significant because they demonstrate that time of harvest and cortisol response following a stressful event effects meat quality. Additional research, especially on industry finished beef cattle, is needed to determine how premortem stress and cortisol response is involved in meat quality in order to provide consumers beef with consistent quality.

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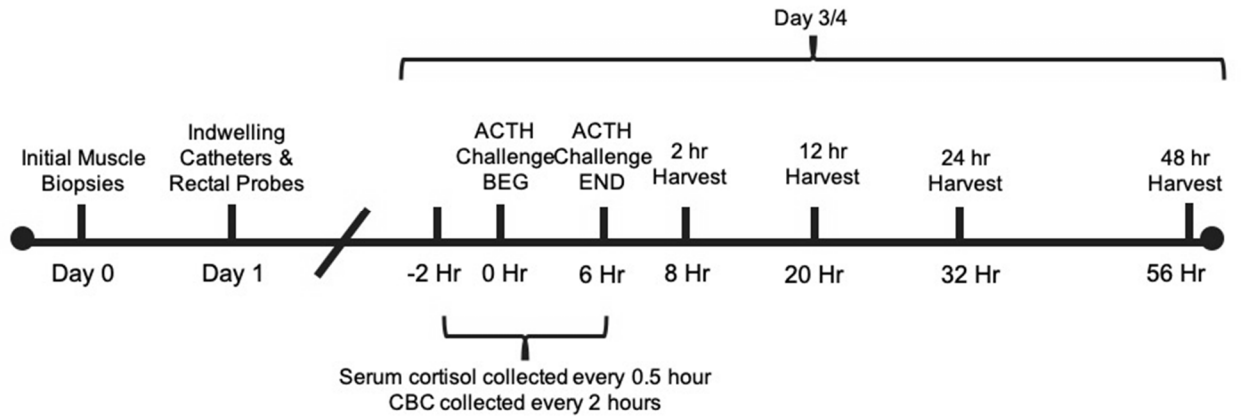
Figure 1. Timeline of sample collection during the adrenocorticotropic hormone challenge.

Table 1. Description of Primary Bovine Specific Antibodies and Concentration used for Western Blots

Protein Name[a]	Antibody Company	Host	Product Number	Primary Concentration	Secondary Concentration
HSPβ-1	Invitrogen[b]	rabbit	PA1-25494	1:500	1:1000
PHSPβ-1	Invitrogen[b]	rabbit	PA5-23340	1:2000	1:1000
HSPβ-5	Invitrogen[b]	mouse	MAS-27708	1:4000	1:7500
DJ1	Abcam[c]	rabbit	ab18257	1:1000	1:1000
Troponin	Invitrogen[b]	rabbit	PA5-42108	1:500	1:1000

[a]Heat Shock Protein β-1 (HSPβ-1), Phosphorylated HSPβ-1 (PHSPβ1), Heat Shock Protein β-5 (HSPβ-5), Protein deglycase (DJ1), Troponin I (Troponin)

[b]Invitrogen, Rockford, IL, USA

[c]Abcam, Cambridge, MA, USA
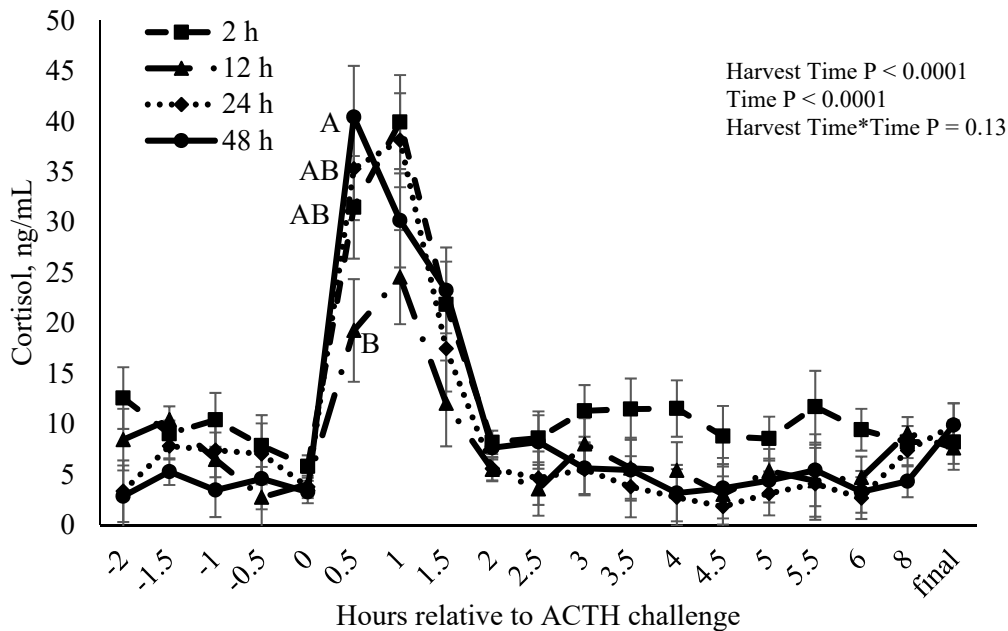
Figure 2. Cortisol concentration in serum relative to beginning of the adrenocorticotropic hormone (ACTH) challenge. Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. Values represent the least squares means ± SEM of cortisol concentration in serum collected relative to the ACTH challenge for harvest times. Points with different letters differ (P < 0.05) between harvest time within that specific time point.
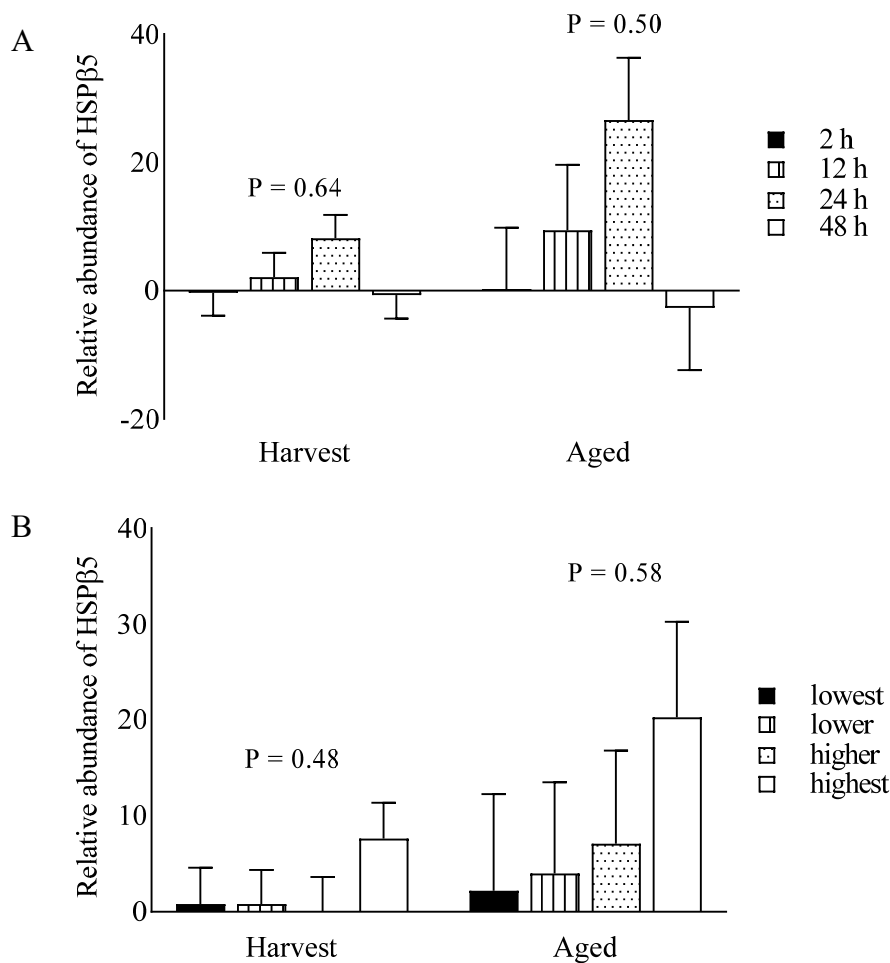
Figure 3: Abundance of heat shock protein β1 (HSPβ1) in the *longissimus lumborum* at either harvest or after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Values represent the least squares mean ± SEM. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B).

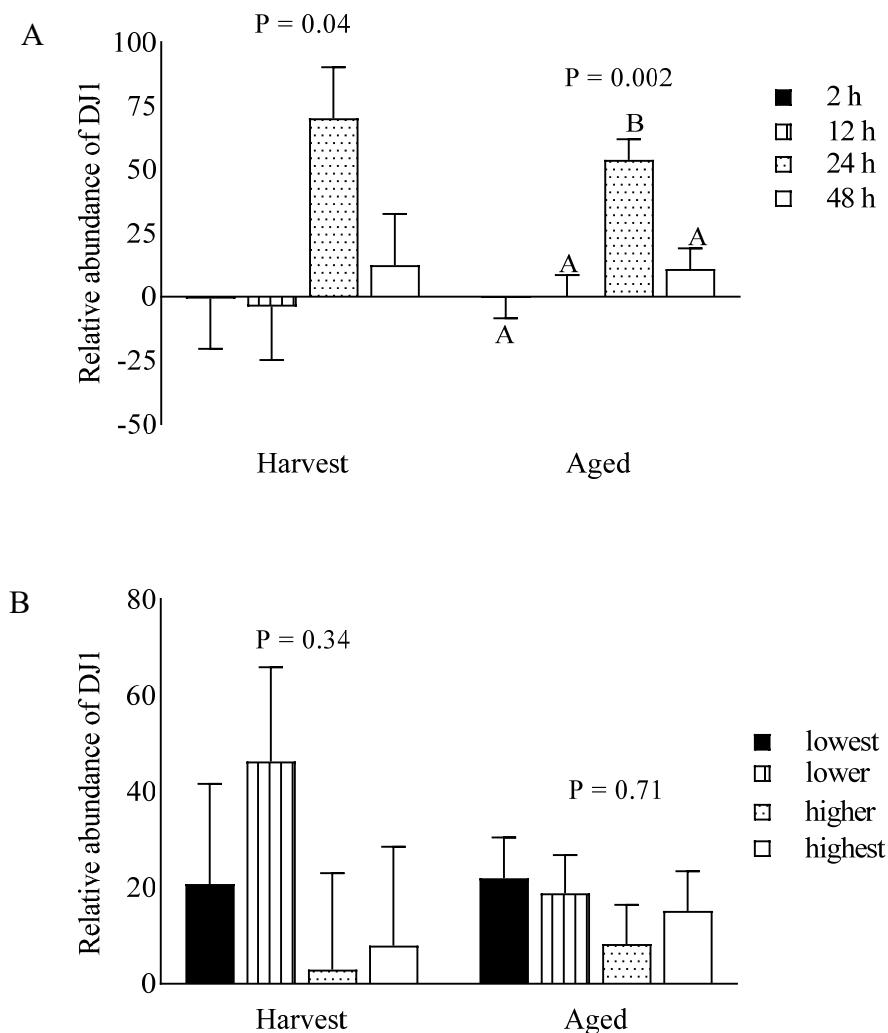**Figure 4.** Abundance of phosphorylated-HSPβ1 (PHSPβ1) in the *longissimus lumborum* at either harvest or after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. Harvest values are graphed on left axis and Aged values are graphed on right axis. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four quartiles (lowest, lower, higher, and highest; n=10). Values represent the least squares mean ± SEM of fold change from initial muscle biopsies. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters differ (P < 0.05) between harvest time or cortisol response within that time point.
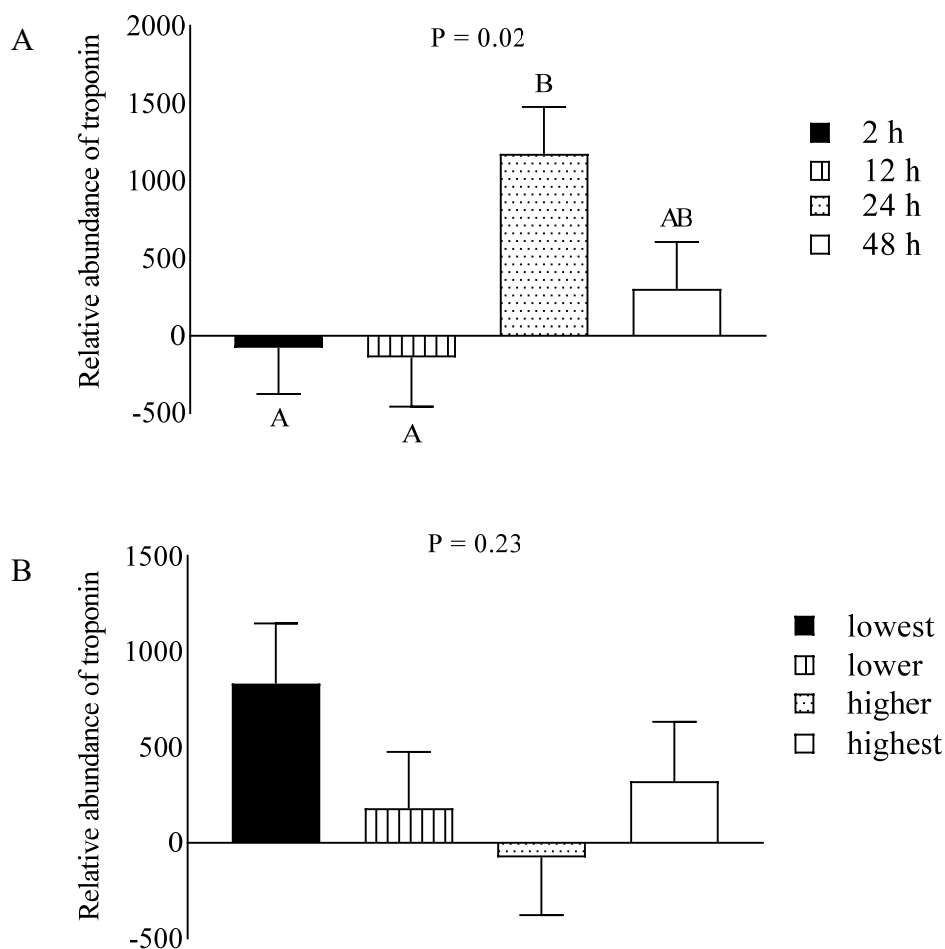
Figure 5. Abundance of heat shock protein β5 (HSPβ5) in the *longissimus lumborum* at either harvest or after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Values represent the least squares mean ± SEM.

Figure 6. Abundance of DJ1 in the *longissimus lumborum* at either harvest or after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Values represent the least squares mean ± SEM. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters differ (P < 0.05) between harvest time or cortisol response within that time point.
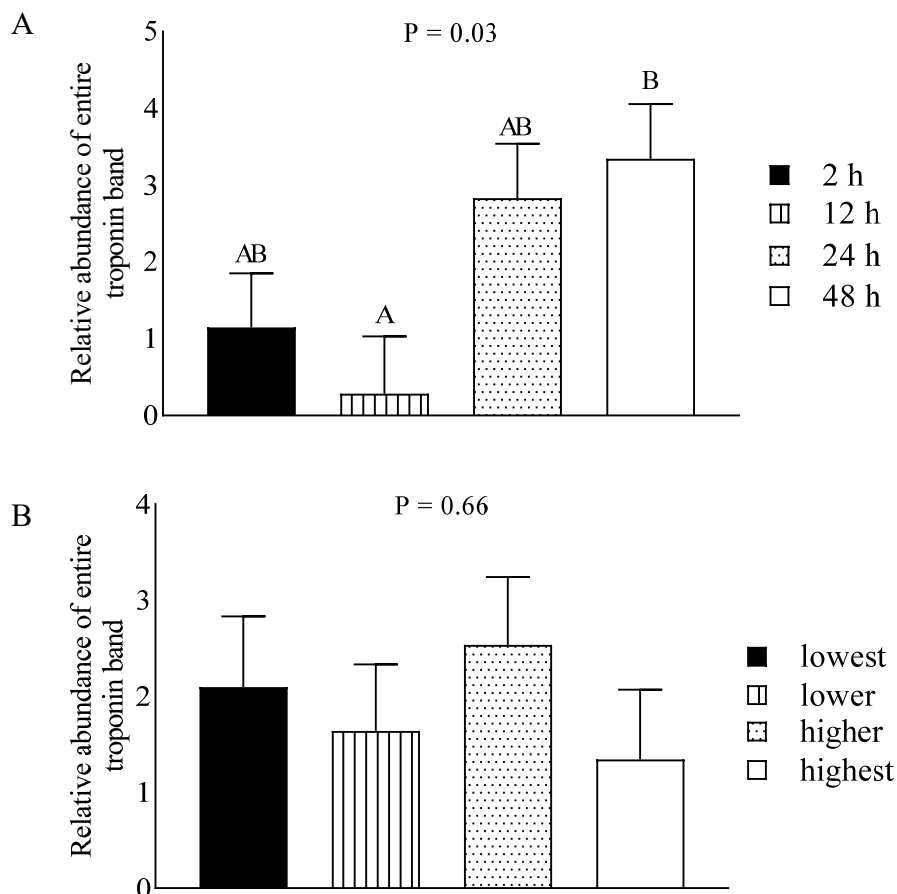
Figure 7. Abundance of troponin in the *longissimus lumborum* at harvest relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Values represent the least squares mean ± SEM. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters differ (P < 0.05) between harvest time or cortisol response within that time point.
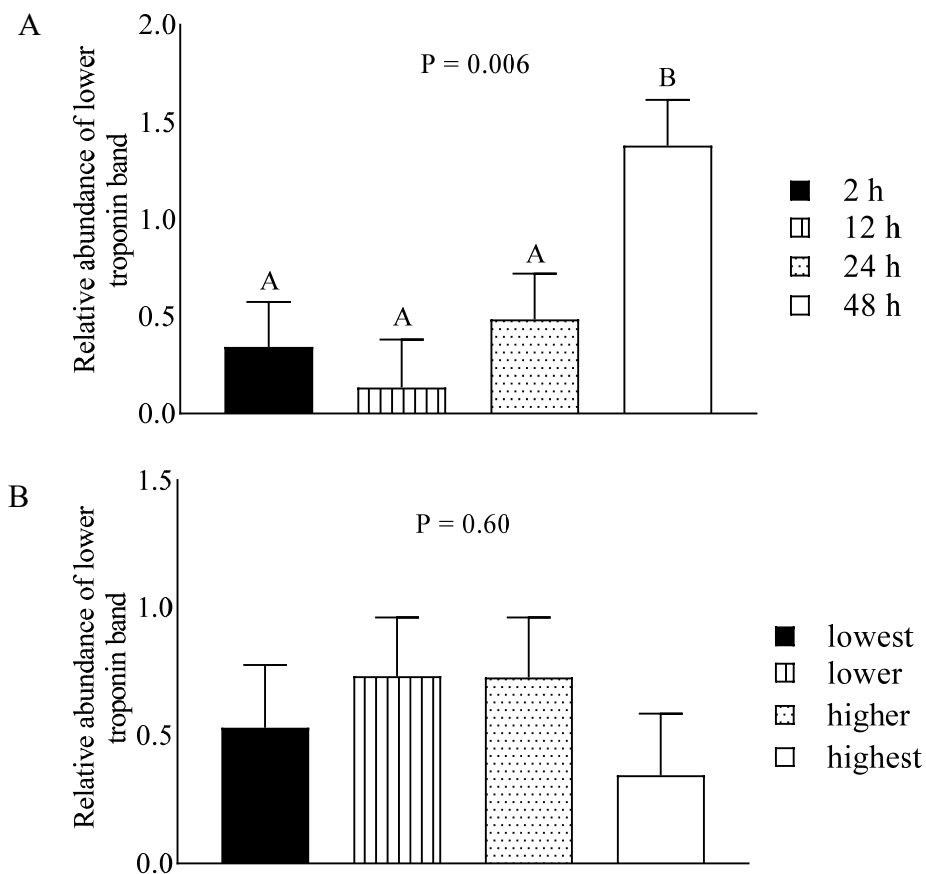
Figure 8. Abundance of entire troponin band in the *longissimus lumborum* after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Values represent the least squares mean ± SEM. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters differ (P < 0.05) between harvest time or cortisol response within that time point.
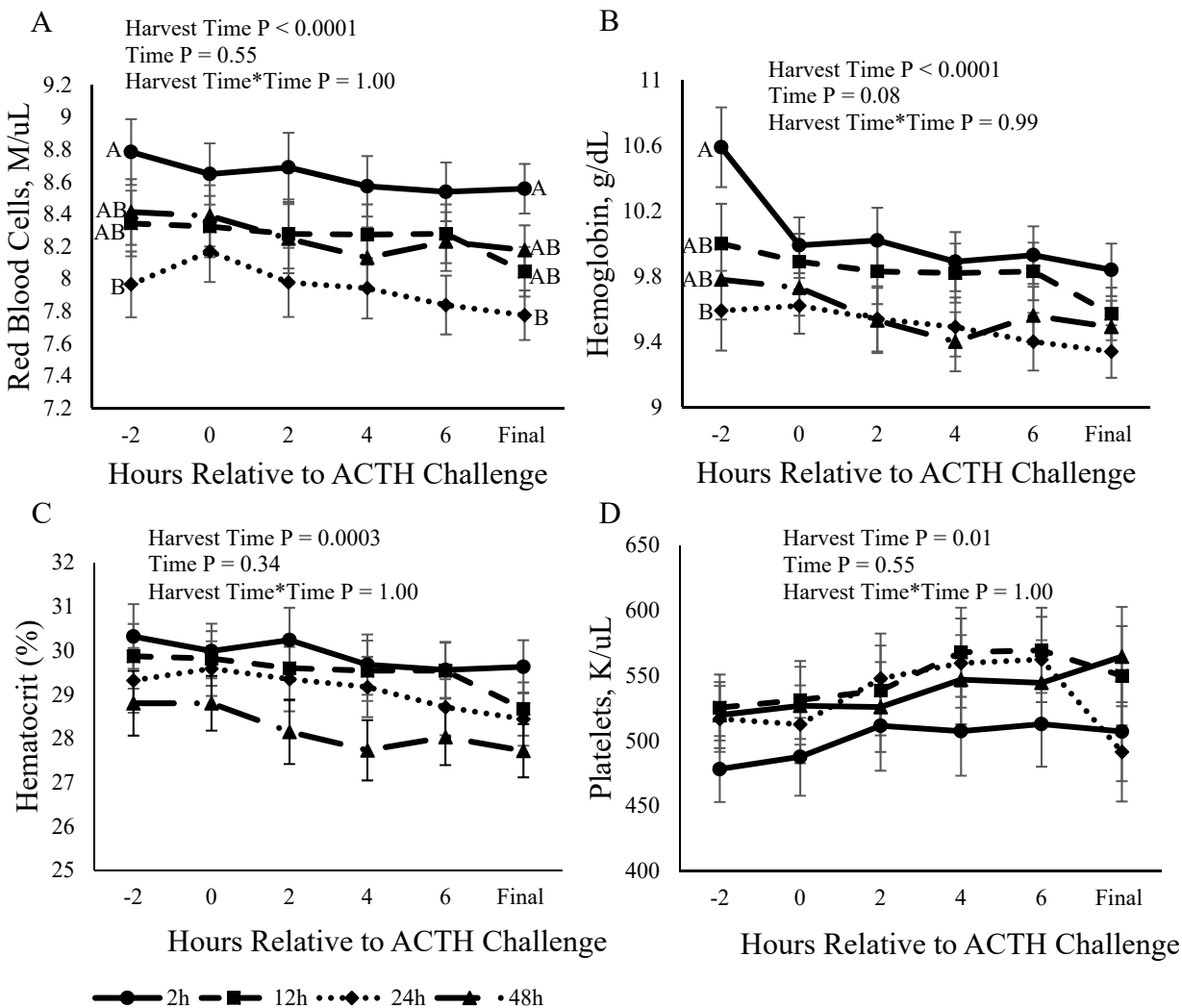
Figure 9. Abundance of lower troponin band in the *longissimus lumborum* after 14 d of aging relative to the initial muscle biopsy collected prior to the adrenocorticotropic hormone (ACTH) challenge. A: Treatment groups consisted of four groups of animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following the ACTH challenge. B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Values represent the least squares mean ± SEM. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters differ (P < 0.05) between harvest time or cortisol response within that time point.

Figure 10. Concentration of blood components (A: red blood cells; B: hemoglobin; C: hematocrit; D: platelets) measured every 2 h relative to adrenocorticotropic hormone (ACTH) challenge between animals grouped into harvest time (2 h, 12 h, 24 h, and 48 h; n=10). Repeated measures analyses were completed to determine the effects of harvest time (treatment), time, and treatment*time. Values represent the least squares mean ± SEM for the respected blood component. Points with different letters differ (P < 0.05) between harvest time at each time point.
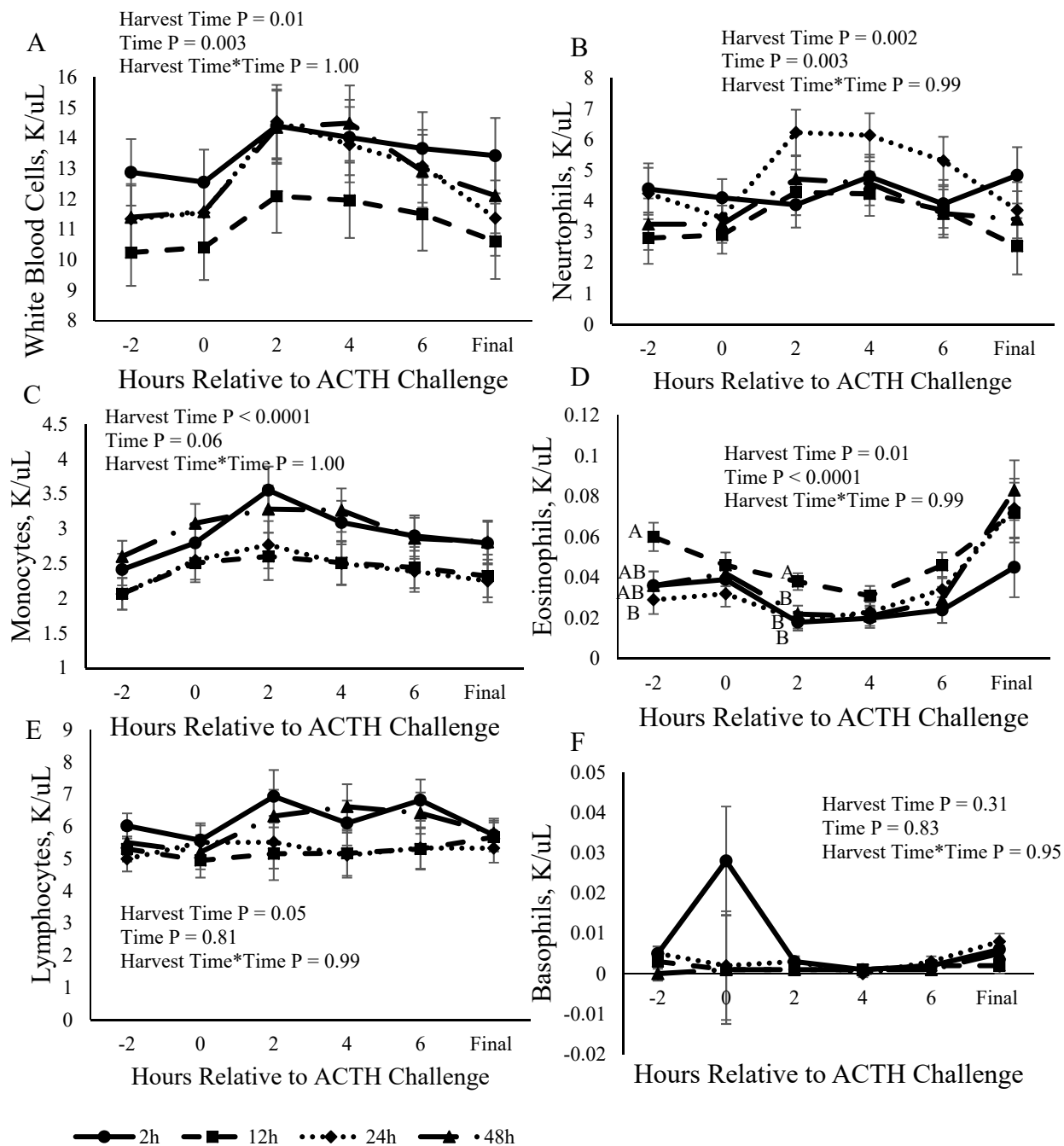
Figure 11. Concentration of blood components (A: white blood cells, B: neutrophils, C: monocytes, D: eosinophils, E: lymphocytes, F: basophils) measured every 2 h relative to adrenocorticotropic hormone (ACTH) challenge between animals grouped into harvest time (2h, 12h, 24 h, and 48h; n=10). Repeated measures analyses were completed to determine the effects of harvest time (treatment), time, and treatment*time. Values represent the least squares mean ± SEM for the respected blood component. Points with different letters differ ($P < 0.05$) between harvest time at each time point.
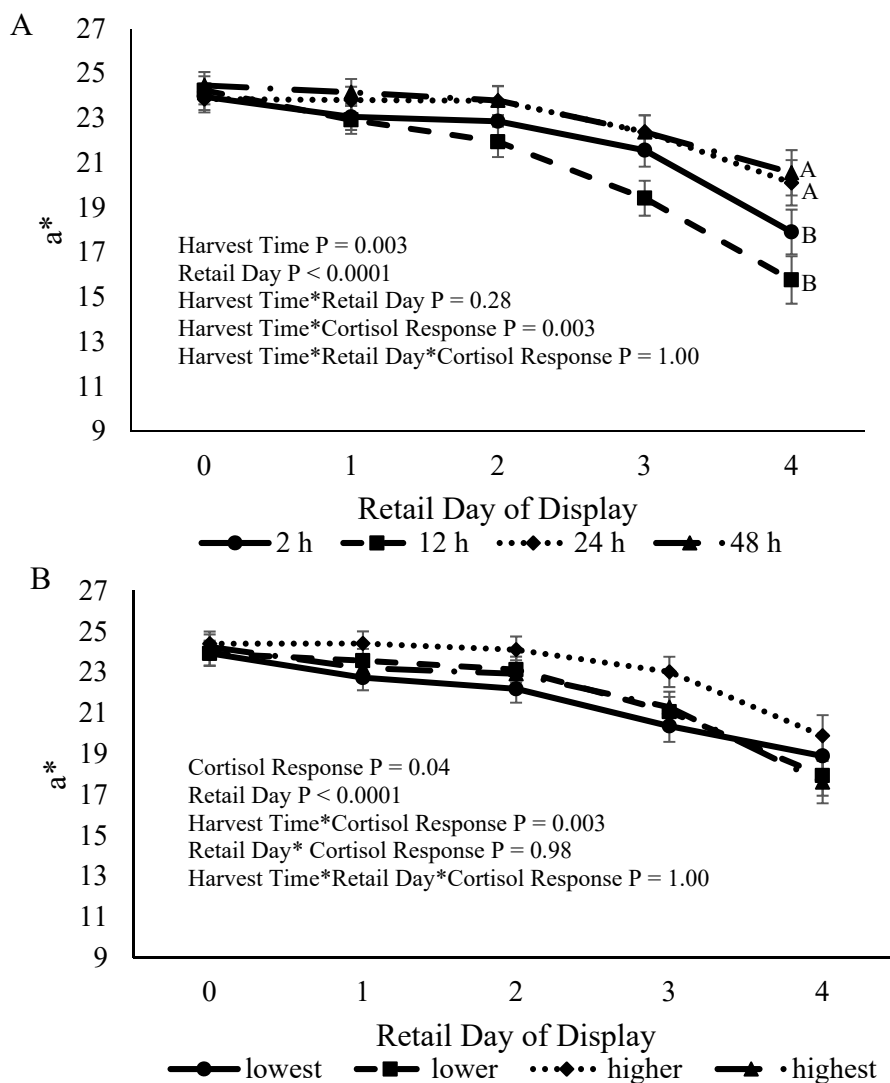
Figure 12. a* (redness) color measurement from the *longissimus lumborum* after 14 d of aging. A: Animals were grouped based on time of harvest following adrenocorticotropic hormone (ACTH) challenge (2h, 12h, 24 h, and 48h; n=10). B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Repeated measures analyses were completed to determine the effects of harvest time (treatment), cortisol, retail day, treatment*retail day, treatment*cortisol, and treatment*retail day*cortisol. Values represent the least squares mean ± SEM of a* measurement. Points with different letters differ (P < 0.05) between harvest time or cortisol response at each time point.
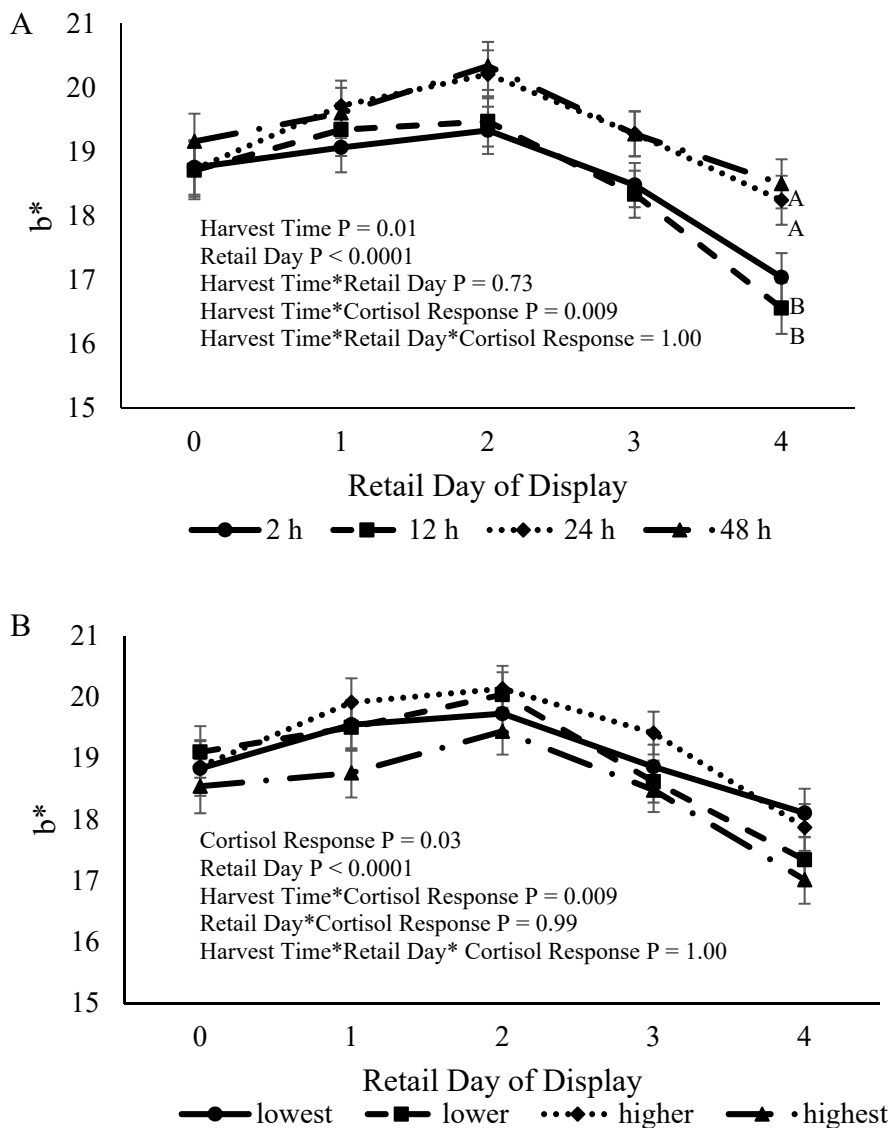
Figure 13. b* (yellowness) color measurement from the *longissimus lumborum* after 14 d of aging. A: Animals were grouped based on time of harvest following adrenocorticotropic hormone (ACTH) challenge (2h, 12h, 24 h, and 48h; n=10). B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Repeated measures analyses were completed to determine the effects of harvest time (treatment), cortisol, retail day, treatment*retail day, treatment*cortisol, and treatment*retail day*cortisol. Values represent the least squares mean ± SEM of b* measurement. Points with different letters differ (P < 0.05) between harvest time or cortisol response at each time point.
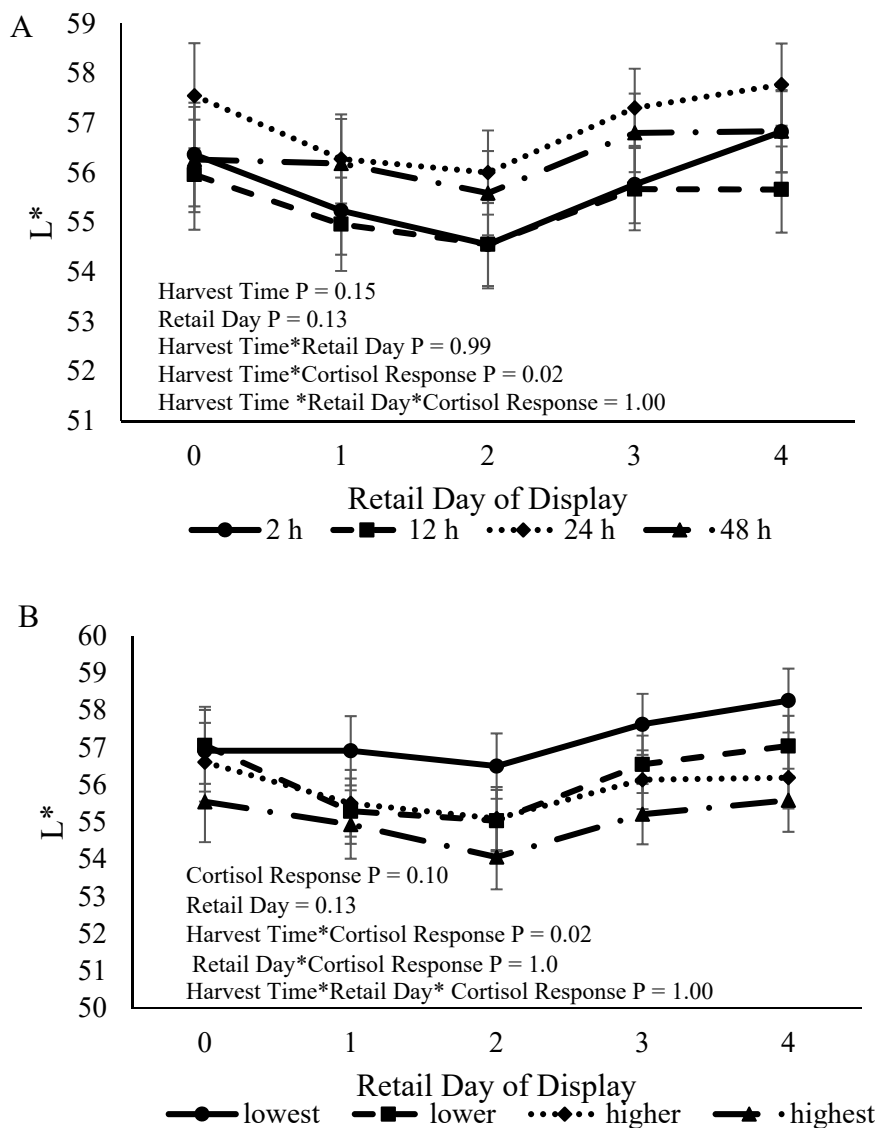
Figure 14. L* (lightness) color measurement from the *longissimus lumborum* after 14 d of aging. A: Animals were grouped based on time of harvest following adrenocorticotropic hormone (ACTH) challenge (2h, 12h, 24 h, and 48h; n=10). B: Animals were grouped based on cortisol response 0.5 h after initiation of ACTH challenge and split into four different quartiles (lowest, lower, higher, or highest; n=10). Repeated measures analyses were completed to determine the effects of harvest time (treatment), cortisol, retail day, treatment*retail day, treatment*cortisol, and treatment*retail day*cortisol. Values represent the least squares mean ± SEM of L* measurement. Points with different letters differ (P < 0.05) between harvest time or cortisol response at each time point.
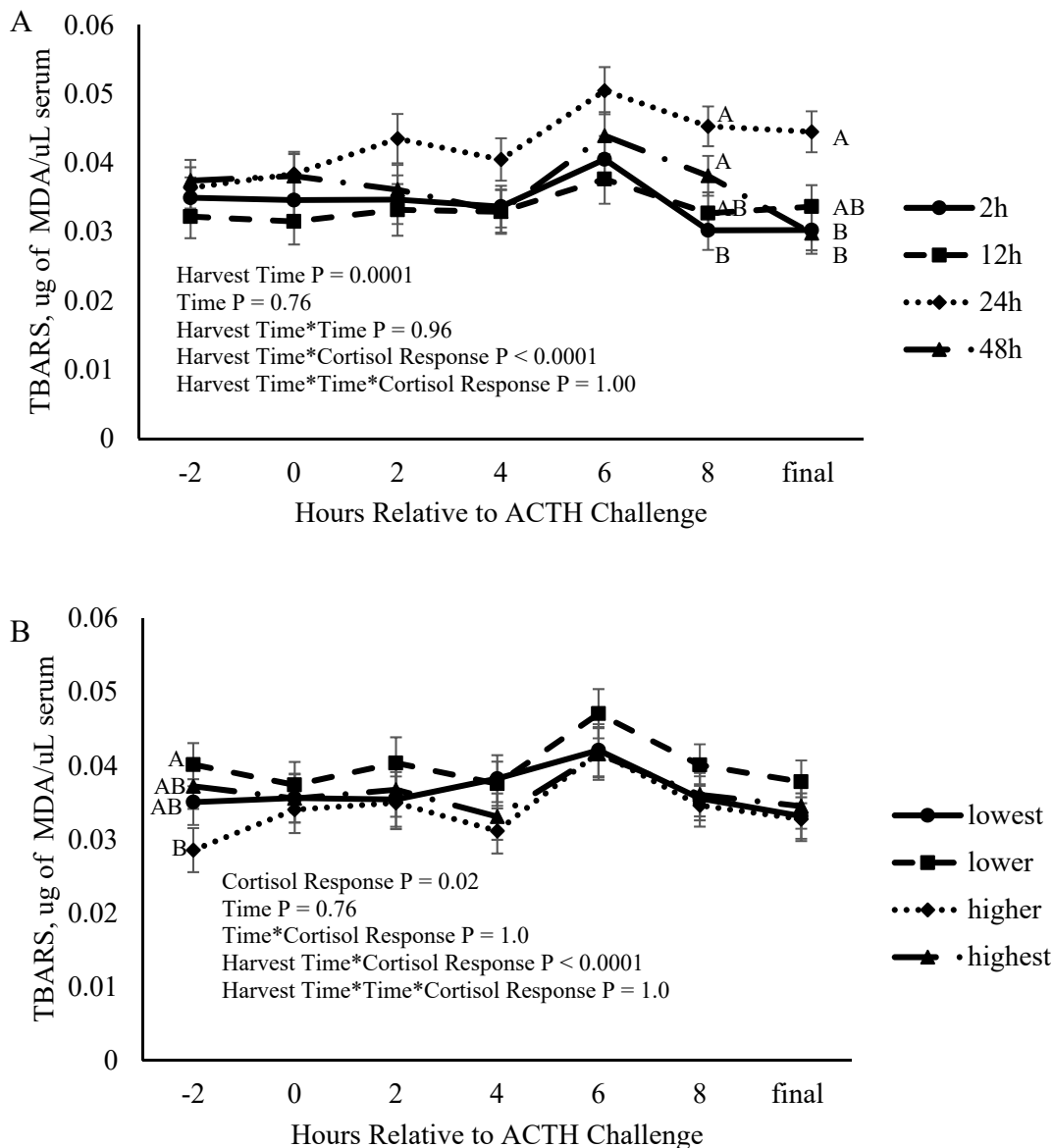
Figure 15. Concentration of TBARS in serum collected every 2 h relative to adrenocorticotropic hormone (ACTH) challenge. Panel A describes TBARS concentration in serum relative to ACTH challenge in animals that were harvested at different time points (2 h,12 h, 24 h, and 48 h; n=10) following the ACTH challenge. Panel B describes TBARS concentration in serum relative to ACTH challenge in animals that had different cortisol responses (lowest, lower, higher, and highest; n=10) 0.5 h after initiation of ACTH challenge. Repeated measures analyses were completed to determine the effects of harvest time (treatment), cortisol, time, treatment*time, time*cortisol, treatment*cortisol, and treatment*time*cortisol. Values represent the least squares mean ± SEM of TBARS concentration in the serum. Points with different letters differ (P < 0.05) within harvest time or cortisol response at each time point.
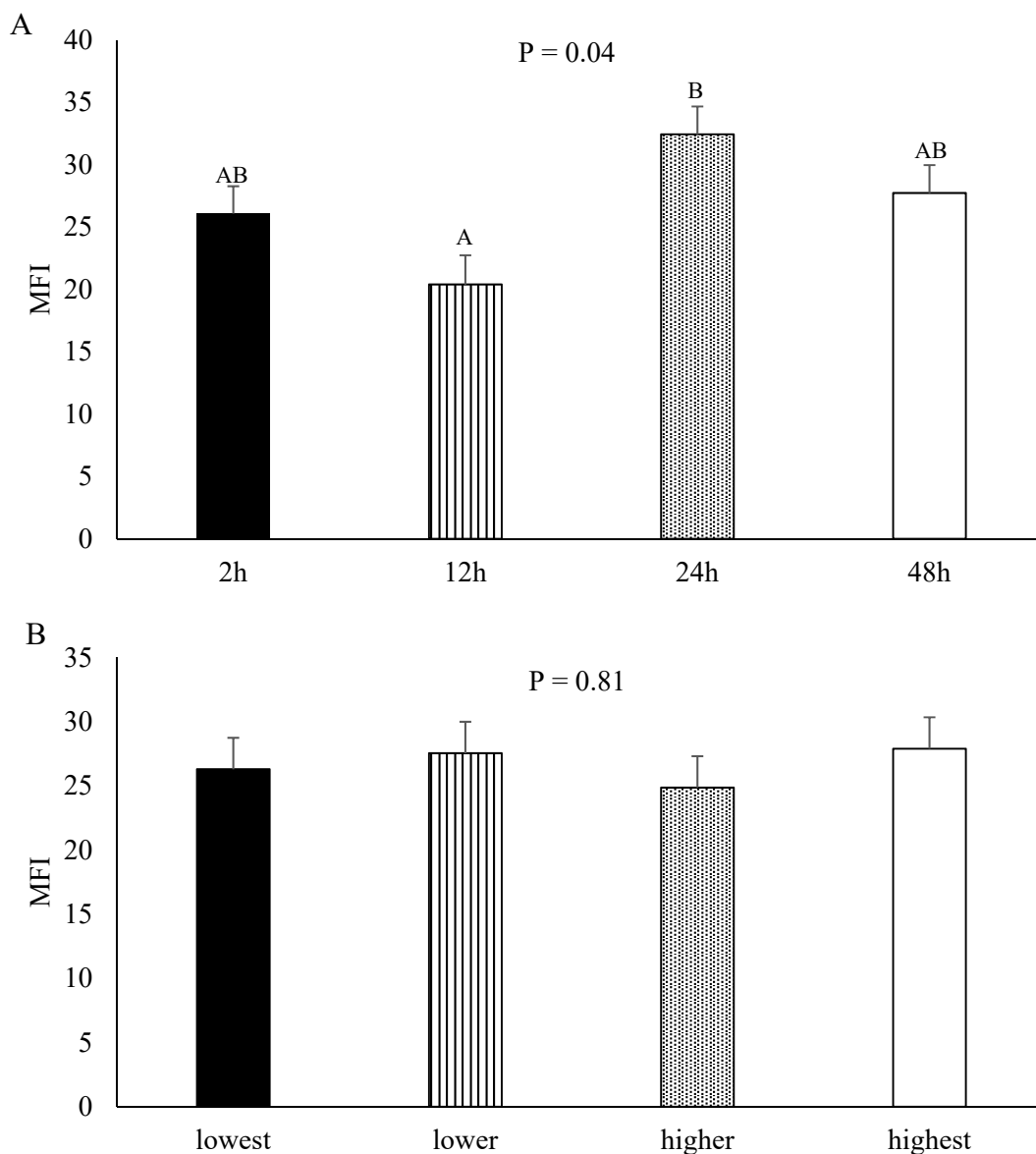
Figure 16. Myofibrillar fragmentation index (MFI) of 14 d aged muscle tissue collected from the LL. Panel A describes MFI of samples collected from animals that were harvested at different time points (2 h, 12 h, 24 h, and 48 h; n=10) following ACTH challenge. Panel B describe MFI of samples collected from animals that had different cortisol responses (lowest, lower, higher, and highest; n=10) 0.5 h after initiation of ACTH challenge Values represent the least squares mean ± SEM of MFI for 14 d aged muscle tissue. The P-value above each time point represents the effect of harvest time (A) or initial cortisol response (B). Bars with different letters are different (P < 0.05) from one another.