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Effects of an injectable zinc solution at weaning as an alternative castration method in beef cattle

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Effects of an injectable zinc solution at weaning as an alternative castration method in beef cattle

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ABSTRACT

Two experiments were conducted to investigate a 1 mL intratesticular zinc (100 mg Zn) injection administered into each testicle at weaning (**Z**) and its effects on growth, health, behavior and testosterone. In the first experiment, beef bulls were assigned randomly to treatment at birth: 1) surgically castrated at birth (**S**; n = 37) or 2) Z (n = 37). Testicular thickness differed by day ($P < 0.01$) for Z. Testosterone concentrations were greater in Z by d 77 and remained so through d 280 ($P = 0.02$) compared to S. Zinc injected calves were heavier at the trail's conclusion ($P \leq 0.04$), had greater ADG overall ($P < 0.01$), heavier hot carcass weight ($P = 0.01$), and greater lean muscle area ($P = 0.01$) compared to S; but a lower marbling score ($P < 0.01$). Yield Grade, dressed carcass yield and fat thickness were similar ($P \geq 0.14$). Zinc injected calves had greater haptoglobin (**Hp**) concentrations ($P < 0.01$). Zinc injected calves had greater concentrations of white blood cells on d 1 and 2 and greater concentrations and proportions of neutrophils on d 1, 2 and 3 ($P < 0.01$). Conversely, S had a greater percentage of lymphocytes on d 1, 2 and 3 and a lower ratio of neutrophils to lymphocytes during that same time ($P < 0.01$). Zinc injected calves spent more time on their side or sternum on d 1 and more time on their side on d 2 while S spend more time standing during that time ($P < 0.01$). During d 3, 4, 5, and 6, Z stood more while S laid on their sternum ($P < 0.01$). In trail 2, beef bulls were allocated randomly to treatment one week post weaning: 1) banded (**B**; n = 42) or 2) Z (n = 39). Body weights were similar ($P \geq 0.39$) but ADG improved for Z compared to B ($P = 0.05$). Testosterone concentrations were greater in Z compared to B ($P \leq 0.02$). Testicular width in Z differed by d ($P < 0.01$). Zinc castrated calves spent more time on their side compared to B one day post castration ($P = 0.03$) until d 3 and 4 where B spent more time on their side ($P \leq 0.02$). Banded calves stood more the first two days ($P \leq 0.01$). On days 3, 4, 5, and 6, Z stood more ($P \leq 0.01$) while B were on their sternum ($P \leq 0.03$). Banded calves had lower Hp concentrations compared to Z ($P \leq 0.01$). Total white blood cell concentrations, proportions and

concentrations of neutrophils, and proportion of lymphocytes were greater in Z compared to B on d 1, 2, and 3 ($P \leq 0.01$). There was no interaction between treatments over time for IL1$_\beta$, IL6, and TNFα expression ($P \geq 0.83$). The findings in both studies indicate that intratesticular Zn injections at weaning can improve growth performance and some carcass attributes but produce a heightened inflammatory and immune response and cause discomfort. Injecting zinc, as formulated and administered in these trails, does not result in complete castration of beef calves at this age and therefore cannot be considered a true castration alternative.

Key words: behavior, growth, health, zinc castration

DEDICATION

This dissertation is dedicated to my Grandmommy: Linda Gayle Short. Without her support and encouragement, I would have never imagined that I could have attempted even half of the milestones that I have accomplished thus far. Her contagious smile and words of affirmation are sorely missed. She embodied grace and beauty and practiced kindness with everyone. She was truly an example of hard work, perseverance and strength. It's said that nobody is perfect, but she was awfully close. Without her, the completion of my Ph.D. would not have been possible. Grandmommy, I love you the most!

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CHAPTER I.

INTRODUCTION

Animal castration is a frequently practiced and necessary husbandry technique conducted on all meat producing animals. It is performed each year on roughly 16 million bull calves in the U. S. alone (USDA, 2018). Removing testicles from meat animals is a centuries old procedure utilized to improve genetic selection, reduce aggressive temperament, and adjust for unwanted sexual behavior (Appleby, 1986); and it improves meat quality with enhanced fat deposition and dispersion while reducing instances of dark-cutter beef (Field, 1971). Intact males have a lower live market value because buyers will assume the costs and subsequent issues like illness, stress and weight reduction that come with castrating males. Their value is also discounted at harvest (Faulkner et al., 1992) due to a tougher, less palatable, lower quality and overall, less uniform consumable product (Field, 1971; Klosterman et al., 1954; Seideman et al., 1982).

There are various castration methods that have proven to be effective including surgical, banding, or external clamping (Stilwell et al., 2008). While these are commonly accepted methods, the process of castration, regardless of method, causes pain and stress which can decrease growth performance by reducing ADG (Fisher et al., 1996) due to a decrease in the animal's ability to walk and graze more frequently (Keane, 1999). It has been observed that castrating as early as possible has mitigated the negative effects on growth performance (Jago et al., 1996; Fisher et al., 2001). Additionally, cattle health can become a concern with increased instances of bovine respiratory disease and subsequent treatment and labor costs (Daniels et al., 2000), negative immunomodulatory effects (Roberts et al., 2015; Chase et al., 1995), and increased physiological stress parameters such as cortisol, fibrinogen, haptoglobin, and substance P (Roberts et al., 2015; Fisher et al., 1997; Ballou et al., 2012; Coetzee, 2011). However, bulls fed for the purpose of

slaughter have improved feed efficiency, heavier live weight and carcass weights, improved yield and leaner meat products (Seideman et al., 1982).

Due to the pain that is associated with castration, the industry has received continued scrutiny from consumers and animal activist groups. It is because of this growing concern that the inclusion of pain mediation tactics at the time of castration has become a practice that certain countries have enforced. While the US and Canada currently lack legislation regarding anesthetic use during castration (Webster et al., 2013); the European Commission in 2001 suggested that if painful husbandry practices such as castration, dehorning, or branding could not be avoided, local anesthetics, or local anesthetic in addition to systemic analgesia should be used to reduce pain (European Commission, 2001). Chemical castration has been investigated as a means to pacify welfare concerns without compromising growth and harvest attributes in beef cattle. Recent studies utilizing intratesticular zinc solution injection in feedlot cattle did not improve meat quality characteristics compared to bulls, nor did it reduce testosterone; it did however, keep growth aspects that are found in bulls (Ball et al., 2018b). A preliminary study utilizing the same injectable zinc solution in pre-weaning bull calves (≈ 113 kg) reported similar serum testosterone concentration compared to surgically castrated calves, however, the study did not extend to investigate the effects of growth performance beyond weaning nor did it consider carcass quality (Ball et al., 2018a). Therefore, further research implementing this injectable castration method in weaned calves (≈ 227 kg) is necessary to see if serum testosterone can be reduced while improving meat quality and animal welfare.

CHAPTER II.

LITERATURE REVIEW

Castration Overview

Castration is a widely used animal husbandry practice to alter behavior and improve meat quality. In beef cattle, castration is the action in which the use of the testicles is lost, and is performed on approximately 16 million calves in the United States annually (USDA, 2018). There are various castration methods that include the removal of the testicles entirely, damaging them irreparably, or causing testicular tissue to atrophy, which can be executed by means of physical, chemical, or hormonal castration (Currah et al., 2009). A survey of veterinarians indicated that producers perform 83% of castrations in perinatal calves while 68% of calves weighing 270 kg or more were castrated by a veterinarian (Coetzee et al., 2010).

Physical castration is the most commonly used castration method (Coetzee et al., 2010) and involves surgical removal of the testicles, applying a constricting elastic band around the base of the scrotum, and external clamping or bloodless castration (Stilwell et al., 2008). One of the most common castration techniques (57%) is knife or surgical castration where the spermatic cord and testes are removed by severing the scrotum with a blade followed by emasculator utilization (36%) whose function is to both cut and crush the spermatic cord and detach the testis (Coetzee et al., 2010). The most commonly used, non-surgical method, was reportedly elastrator rubber rings (44%) in calves less than 90 kg, which was followed by the burdizzo clamp (21%).

Chemical castration results in irreparable damage and loss of function and is typically induced by an injection of a toxic agent into the testicular parenchyma. Testicular cell destruction is accomplished through caustic or osmotic processes by injecting lactic acid, calcium dichloride (Martens et al., 2011), sodium chloride (Neto et al., 2014) or zinc (Ball et al., 2018a). When

compared to surgical castration, chemical castration requires almost twice the healing time (Fordyce et al., 1989), which could be attributed to the increased inflammation response (Oliveira et al., 2017), and could be impacted by age (Ball et al., 2018b).

Hormonal castration, or immunocastration, involves an injection of immunocontraceptives in order to induce antibody production against gonadotropin releasing hormone (GnRH) so that endogenous hormone production decreases (Fisher et al., 1996). Immunocastration has reportedly increased live weight and hot carcass weight, and improved average daily gain (ADG), and dressing percentage when compared to calves that were surgically castrated (Amatayakul-Chantler et al., 2013). Although testosterone production is reduced within the first six months after immunocastration, it is considered a less effective technique due to persistent sexual behavior, need for repeated injections, and consumer concerns (Stafford, 2007).

There are numerous factors influencing castration method selection. These factors include animal handling ease and risk to operator, calf weight and scrotal circumference, castration technique experience, time, costs, facility, adverse effects, and painfulness (Coetzee et al., 2010). The meat animal agriculture sector is faced with the daunting task of feeding a growing population under immense scrutiny of animal handling procedures (Bayvel, 2004). It will come as no surprise that future decisions on castration method will be selected with heavy emphasis on consumer perception of appropriate animal welfare procedures.

Issues with Castration

Although castration is a management practice that is widely accepted amongst various countries, the negative impacts associated with castration are well documented. Castration, regardless of method, induces a period of stress, produces neuroendocrine changes, negatively impacts growth performance, and cattle exhibit behaviors associated with pain and discomfort

(Stafford and Mellor, 2005). The duration of stress is subject to castration method. Regardless of type, castration impacts growth performance by increasing weight loss (Bretschneider, 2005), and by temporarily reducing ADG (Fisher et al., 1996) and feed intake (Earley and Crowe, 2002) due to a decrease in walking and grazing time (Keane, 1999). These symptoms are heightened with an increase in age. Growth performance measurements in relation to castration are drastically improved in calves castrated closest to birth or prior to weaning when puberty is prevalent (Bretschneider, 2005). The improvement is well documented when comparing growth performance parameters between calves castrated at or near birth to those castrated at weaning (Champagne et al., 1969; Worrell et al., 1987).

Results for growth performance can also be impacted by stress and inflammation associated with castration or additional animal husbandry practices performed at the time of castration. Immune function is a complex system that can be divided into two responses; innate and adaptive. Innate immunity is the initial line of defense against foreign pathogens and is non-specific. This type of defense includes physical barriers and mucous membranes. The innate immune cells (phagocytic leukocytes, dendritic, and natural killer cells) initiate the inflammatory response by presenting antigens to the adaptive immune system. The adaptive immune system is a more specific response. The inflammatory response is initiated by the production of pro-inflammatory proteins, or cytokines (primarily interleukin-1 [**IL1**], interleukin-6 [**IL6**], and tumor necrosis factor – alpha [**TNF-α**]). These cytokines initiate the production of acute phase proteins (Baumann and Gauldie, 1994). The production of inflammatory proteins require energy and therefore consume the energy that would have otherwise been utilized for body weight gain and use it to support the immune system (Johnson, 1997). Additionally, cytokine production reduces growth and body weight gain by decreasing circulating concentrations of insulin-like growth

factor-1 (Broussard et al., 2001). This is accomplished when cytokines activate receptors on various target cells which activate the hypothalamic-pituitary-adrenal axis (Gruys et al., 1999) and suppress hepatic cell sensitivity to growth hormone (Broussard et al., 2001).

Acute phase proteins are synthesized in response (but are not limited) to IL1, IL6, and TNF-α production and initiate the acute phase response. Interleukin-1 increases the whole-body amino-acid flux and activates the pituitary-adrenal system (Gruys et al., 2005). Tumor necrosis factor-alpha causes glucagon-induced hyperglycemia and amino acid uptake by the liver in addition to muscle catabolism (Gruys et al., 2005). Finally, IL-6 plays a major role in the mediation of hepatocytic secretion of a majority of the acute phase proteins (Heinrich et al., 1998). Commonly measured acute phase proteins include but are not limited to: haptoglobin, ceruloplasmin, and fibrinogen.

Haptoglobin is the most frequently assayed acute phase protein to detect stress in cattle. This protein is almost impossible to detect in the blood of unstressed cattle however, which makes it a reliable protein to diagnose stressed cattle since concentrations elevate rapidly during inflammatory distress (Conner and Eckersall, 1988). Haptoglobin forms complexes with free-hemoglobin in the blood which reduces available iron which hinders bacterial growth (Eaton et al., 1982). This protein serves as an anti-inflammatory by binding to CD_{11b}/CD_{18} integrins which are major receptors on the cell membranes of leukocytes (El Ghmati et al., 1996). Measuring haptoglobin is advantageous in that it has a very rapid response time post stimulation.

Ceruloplasmin is a glycoprotein produced by the liver and is the primary copper-transporter-protein in mammals. It performs as an antioxidant with its primary function as a carrier of copper to tissues during an inflammatory response (McCord and Fridovich, 1968). Unfortunately, ceruloplasmin concentrations are difficult to distinguish between inflammation and

dietary copper intake and is detected in both stressed and unstressed animals. Its role as an acute phase protein is subject to nutritional copper status (Arthington et al., 1996).

Fibrinogen is similar to ceruloplasmin in that it can be measured in both stressed and unstressed animals. It is an important protein for blood clotting and tissue repair and is recognized as a marker of inflammation in cattle (Sutton and Hobman, 1975). Some studies suggest that fibrinogen and ceruloplasmin are linked, although the mechanisms in which they are related are not well understood, and suggests that fibrinogen concentrations might not only be related to inflammation, but that it might fluctuate due to diet (Arthington et al., 1996).

Research has indicated a difference in acute phase protein response in association with castration method (Warnock et al., 2012). When comparing the inflammation response between surgically castrated calves and banded calves, those that were banded had a lower concentration of haptoglobin and ceruloplasmin compared to surgical castration. Furthermore, studies suggest that during surgical castration, the inclusion of pain remedy (oral meloxicam) decreased serum haptoglobin concentrations and the acute phase inflammatory response and cortisol concentrations dropped quicker (Roberts et al., 2015). These findings suggest that pain management practices prior to castration might prove to be advantageous against stress and inflammation.

Pain Management

Pain is a sensation that is the body's defense mechanism towards tissue damaging stimuli. There are three main stages for pain perception which first includes pain sensitivity. The second is the signal transmission from the periphery to the dorsal horn located in the spinal cord by means of the peripheral nervous system. Those signals are then transmitted to the brain by the central nervous system (Yam et al., 2018). There are three classifications of pain (1) nociceptive pain which is the bodies' sensory nervous system response towards potentially harmful stimuli, (2)

neuropathic pain, which is associated with nerve damage or impairment, and (3) inflammatory pain, which is the natural response produced by tissues against harmful stimuli (Woolf et al., 1998). Furthermore, there are three events in which pain signals are transmitted in the presents of stimuli; transduction, transmission, and modulation (Yam et al., 2018).

The basic mechanisms of pain include neurons, axons (group A, B, and C fibers), action potential, synaptic transmissions, and route of pain transmission (Yam et al., 2018). Sensory neurons, interneurons, and motor neurons are the primary components that connect, receive, and process nociceptive information. All neurons consist of soma, axon, and dendrites which connect to transmit chemical and electrical signals via synapses. The synaptic signals are sent from a neuron and are received by dendrites and soma of surrounding neurons and then transmitted within the neurons by axons. This transmission causes a brief pulse known as action potential. This chain reaction acts as a pathway to carry the signal (Yam et al., 2018). Axons are myelinated or unmyelinated nerve fibers that are broken into A, B, and C sub fibers which are myelinated, moderately myelinated, and unmyelinated, respectively. Action potential is the release of neurotransmitters at the axon terminal which is initiated by nociceptive signals and Ca^{2+} (Yam et al., 2018).

Specifically, regarding pain perception in castration, the physical act and stimuli of castration is transduced into action potentials on Aδ and C nerve fibers. The action potential created from electrical signals from the nerve are transmitted to the dorsal horn of the spinal cord and are then transmitted to the brain (Anderson and Muir, 2005). Nociceptive pain is the first stage of pain sensation which are associated with Aδ and C nerve fibers (Ma and Zhang, 2010) and can be transmitted to inflammatory pain. Inflammation is a natural response to tissue trauma which stimulates an immune response. Pain associated with inflammation is subcategorized into acute,

which would most likely be observed during surgical castration and chronic, which we might see with banding cattle. Acute inflammatory pain occurs for a short period of time and is initiated by Aδ fibers accompanied by leukocytes and plasma while chronic pain is mediated by C fibers and is a prolonged pain experience (Basbaum et al., 2009).

Pain management and animal husbandry practices are growing concerns against the animal agricultural industry. The meat animal sector of agriculture is under a small microscope with growing awareness of issues with agricultural practices to those outside of the industry. Information is readily shared at hand with activist organizations utilizing social media platforms to spread information. While each sector has its own issues, whether it's the dairy industry, feedlot, or cow/calf, a common major concern with these groups across all is the pain that is associated with castration.

In an attempt to appease this growing concern, the AABP (American Association of Bovine Practitioners) suggests that long-lasting nonsteroidal anti-inflammatory drugs (NSAID) be used to extend the postoperative period analgesia (AABP, 2014). Analgesic and anti-inflammatory medications are effective for mild to moderate somatic pain and are divided into non selective cyclo-oxygenase (COX) inhibitors and selective COX-2 inhibitors which is determined by the chemical nature of NSAIDs (Vane et al., 1998). Regardless of COX classification, the purpose of NSAIDs is to inhibit COX enzymes in order to reduce prostaglandin and thromboxane synthesis (Modi et al., 2012).

Up until recently, there were no analgesic drugs approved for cattle by the U.S. Food and Drug Administration (FDA; Compendium of Veterinary Products, 2010); however, in 2018, the FDA approved the use of Banamine® Transdermal (flunixin transdermal solution) to mitigate pain in meat animals. Flunixin meglumine is a NSAID drug that inhibits COX enzymes which are

responsible for the conversion of arachidonic acid to prostaglandins (Boynton et al., 1988) and has been approved to treat pyrexia associated with respiratory disease, mastitis, and inflammation associated with endotoxemia (Smith et al., 2008).

Even with the previously mentioned resources at hand, a survey indicated that local anesthetics like lidocaine are used only 22% of the time prior to castration. Furthermore, 83% of producers that do utilize an anesthetic, administer the anesthesia prior to surgical castration, with a greater percentage administering the anesthetic to heavier calves (Coetzee et al., 2010.) The lack of anesthetic utilization could be attributed to historical practices, cost, time, and the fact that anesthetic use isn't mandatory.

While there are recommendations made by the AVMA and AABP to reduce pain during castration, there is currently no mandated use of local anesthetics at the time of castration in the United States. European countries have already initiated a mandate that requires anesthetic use at the time of castration, we can assume, that while there is no regulation of it worldwide, that it is not a matter of *if* we will be required to follow anesthetic guidelines, but *when*.

Carcass Quality

Carcass configuration and meat quality are important aspects to consumers and producers alike. It also contributes to one of the reasons as to why we castrate males. Castration status and carcass quality are well documented and heavily researched. Bulls fed out for slaughter are known to be more feed efficient, higher yielding and leaner products; however, they are also known for their darker appearance, tougher texture, lacking fat distribution within the muscle, and in some cases, a less desirable taste (Seideman et al., 1982).

Bulls are known to have a heavier muscled physic. Skeletal muscle consists of 74% water, 18% proteins, 4 to 5% lipids, 1% carbohydrates, and 1% of other substances such as vitamins. The

percentage varies for species, breed, and muscle location and type (Picard and Gagaoua, 2020). Individual muscle fibers are subcategorized into contractile, cytoskeletal, sarcoplasmic and regulatory, with the sarcomere (the smallest contractile unit of muscle) playing an influential role on meat quality (Ertbjerg and Puolanne, 2017). Muscle development occurs during the embryonic, fetal, and adult stages. Postnatal growth is specifically hypertrophic with observed changes in muscle fiber type as age progresses. There are two main phases of metabolic and contractile property changes in muscle. Within the first-year glycolytic metabolism increases and oxidative metabolism decreases, which is associated with intense muscle growth (Picard et al., 2010). More specifically the changes in muscle fiber are mainly attributed to decreased percentage of fast oxidoglycolytic IIA fibers and increase in fast glycolytic IIX fibers. The reverse is true during the second phase, where there is a decrease in IIX fibers and an increase in I and IIA fibers due to a decreased growth rate after the first year (Picard and Gagaoua, 2020). It is well documented that castration status influences fiber type which is largely due to androgens. Androgens induce greater cross-sectional area and muscle hypertrophy in bulls compared to steers (Wegner et al., 2000; Jiang and Ge, 2014). Steers possess a greater amount of fast glycolytic fibers IIX and greater amounts of glycolytic activity of the enzymes compared to bulls (Brandstetter et al., 1998; Picard et al., 1995; Young and Bass, 1984). The differences in fiber type due to castration and androgen production are more clear after puberty as indicated by differences in 12 to 16 mo old steers containing a greater percentage of IIX fibers and a lower percentage of type I fibers compared to young bulls which can be explained by lower oxidative activity by isocitrate dehydrogenase and a greater glycolytic activity from lactic dehydrogenase and a more hypertrophic role of testosterone as evidenced by denser cross sectional areas (Brandstetter et al., 1998).

The sarcomere has many structural components to it, all of which contribute to quality attributes such as water holding capacity, tenderness, color, and cooked quality. Striated filaments in the sarcomere are called myofibrils. These myofibrils contain light and dark bands or I-bands and A-bands respectively. Within the A-band, there is a central region called the H-zone that also contains the M-zone within itself. Additionally, myosin (a motor protein) assembles into thick filaments on the A-band which overlap to thin filaments on the I-band known as actin monomers. Together, these make up the contractile component of sarcomeres. Lastly, a lateral boundary forms where the thin filament (actin) is attached and this is known as the Z-disk (Ertbjerg and Puolanne, 2017).

The biological functions that occur postmortem play an important role in meat quality. Rigor mortis is the muscle stiffness that occurs after all muscle fibers enter rigor. During rigor mortis, myoglobin-bound oxygen is depleted and mitochondrial respiration ends. This causes a shift from aerobic metabolism to anaerobic in which there is the disappearance of creatine phosphate which causes a decline in ATP and an increase in free Ca^{2+} in sarcoplasm. Finally, the muscle becomes stiff with the formation of actomyosin (Ertbjerg and Puolanne, 2017).

Castration status plays a role in how meat quality is affected, such as pH, tenderness, water holding capacity and both raw and cooked color. Muscle pH is one of the most influential factors affecting meat quality because it influences the other aspects such as color and tenderness. Ultimate pH is directly influenced by muscle glycogen depletion and cooling temperature. There are numerous factors that affect glycogen depletion such as transportation stress (Honkavaara et al., 2003), animal handling prior to slaughter (Hambrecht et al., 2005), muscle fiber and muscle type (Hambrecht et al., 2005), and breed and temperament (Gardner and Thompson, 2003) to name a few. Glycogen is the metabolic substrate that produces post mortem lactate acid and enables the

decline of muscle pH. When glycogen stores are insufficient at the time of slaughter, due to reasons previously mentioned, ultimate muscle pH is affected and can result in undesirable meat characteristics such as dark-cutting meat which is dark in color (Lawrie, 1958) and has a reportedly weaker flavor (Dransfield, 1981). Specifically, regarding muscle fiber type, it is well known that pH decline can be greater in meat with a greater percentage of fast-twitch glycolytic fibers, like we observe in castrated beef cattle (Picard and Gagaoua, 2020). Oxidative fibers, like those in bulls, have low glycogen storage which decreases the rate of glycolysis and lactate acid production which results in a slow pH decline (Wicks et al., 2019). This causes the carcasses of intact males to have a greater than desired ultimate pH (>5.5) and can negatively impact color and tenderness.

Meat color in the fresh market is critical to consumer perception and decision making (Carpenter et al., 2001). Myoglobin is the meat protein responsible for color in raw and cooked meat. The redness of meat depends on the concentration of myoglobin that is mainly found in oxidative red fibers (Astruc and Venien, 2017). The ultimate pH of oxidative fibers causes a greater consumption of oxygen on the surface of the exposed muscle and reduces light scattering resulting in a darker purple/red appearance (Hughes et al., 2020; Purslow et al., 2020). This is because Type 1 oxidative fibers compete with myoglobin for oxygen (Picard and Gagaoua, 2020).

Meat tenderness is an important economic and consumer aspect of beef quality which can be affected by nutrition, age, and castration status. While there is contradicting research regarding muscle fiber type and their impact on meat tenderness, proteolytic activity has been extensively discussed regarding meat tenderness. It should be mentioned that there is a relationship with greater calpain/calpastatin action in fast-twitch glycolytic muscles (Ouali and Talmant, 1990). Ultimate pH and proteolytic enzymatic function are related and both contribute to tenderness. Meat tenderization is the direct result of Ca^{2+} activated calpain proteases and both Z-disk and

myofibrillar proteins under high pH conditions (Wicks et al., 2019). Tenderness is also affected by other factors such as collagen content, muscle type and location, and nutrition. Meat tenderness and nutrition have been extensively investigated. Research has shown that high concentrate diets that steers consume result in an increased measurable tenderness compared to grass-based diets like that of cows and bulls (Bruce et al., 1991; Allingham et al., 1998; Mitchell et al., 1991).

Not only is animal welfare a consumer consideration when choosing a product, but so is meat quality. If alternative castration methods are going to become commercially practiced, they must produce a product that is equally palatable. A study investigating the effects of immunocastration with gonadotropin releasing factor vaccine on carcass and meat quality utilized 288 *Bos indicus* bulls. The bulls allocated to immunocastration were roughly 20 mo of age and received two doses of the GnRF vaccine, the first dose on d 0 and the other on d 91. Bulls were slaughtered on d280. The authors reported that immunocastrated bulls had heavier live weights at slaughter, heavier hot carcass weights, improved ADG, and a higher dressing percentage compared to bulls that were surgically castrated on d 91. The investigators also found that there were no differences in meat quality aspects of the *Longissimus dorsi* when analyzed for meat color, fat color, cooking loss, and Warner-Bratzler Shear Force tenderness analysis. This study concluded that there were no adverse effects of immunocastrating with GnRF and that it might improve carcass attributes without compromising meat quality standards (Amatayakul-Chantler et al., 2013). Previous studies contradict this current study and claim that utilizing immunocastration over surgical castration did not improve carcass characteristics (Hernandez et al., 2005; Ribeiro et al., 2004) nor did it effect color, cooking loss, or tenderness (Ribeiro et al., 2004) which would still suggest that immunocastration does not compromise meat quality standards and might be an alternative solution to surgical castration.

Economics

Raising bull calves is more costly at any stage of production than raising steers. Order buyers typically pay less for bull calves than they would steers because they have the added cost of castration, temporary weight loss, and higher risk of morbidity than steer calves. Therefore, castration is a necessary husbandry practice, consequently retaining bulls throughout any sector of the beef cattle industry is costly. Furthermore, due to the superior quality and improved carcass characteristics, steers are consequently sold at increased prices at live markets compared to bulls (AVMA, 2009). Massey and associates (2011) evaluated price differentials based on feeder calf prices in 2009. They compared the cost of ownership retention and castration time and reported that bull calves entering the feeder market should receive an $0.11/kg discount relative to similar body weight feeder steers weighing 209 kg. This discount reportedly climbed in 170-kg calves to $0.12/kg and declined to $0.08/kg for calves in the 250-kg category. When ownership retention was discussed, the discounts increased for average weight calves to $0.14/kg, decreased to $0.04/kg for light and increased again to $0.17/kg for heavy calves.

It is well documented that the age in which castration is performed has a significant effect on growth performance, morbidity, and carcass quality and therefore has an economic impact. Castrating as close to birth as possible reduced the instances of weight loss associated with castration (Bretschneider, 2005). As age increases, so does weight loss associated with castration. When calves are castrated at weaning (between 6 and 9 mo old) growth performance is temporarily decreased due to the stress and reduced feed intake and increases even more so with cattle that are castrated post puberty (Bretschneider, 2005). Although weight loss associated with castration is well documented and a great concern to producers, research shows that calves that are castrated after the onset of puberty finish at similar weights of those calves castrated prior to puberty (Jago

et al., 1996; Fisher et al., 2001). Without taking into account the animal welfare aspect of time of castration, these findings would imply that although there is an obvious loss in weight with cattle castration, compensatory gain is in effect for those castrated any time after birth and finish at the same time and weight as those that are castrated earlier on. However, this doesn't factor for carcass quality.

It is not only advantageous to castrate early for welfare and growth performance purposes, but for carcass quality as well. Due to protein anabolic effects of testicular hormones and their association with positive nitrogen balance (Galbraith et al., 1978) it is well understood that slaughtered bulls produce a greater quantity of much leaner and tougher consumable product compared to steers (Klosterman et al., 1954; Brown et al., 1962; Rhodes, 1969; Field et al., 1971). When evaluating timing of castration and carcass quality, Massey and associates (2011) reported that calves castrated during the backgrounding phase had no adverse impact on carcass quality, however, increased morbidity and decreased ADG which decreased hot carcass weight and increased the number of days on feed. Seidman and others (1982) summarized the advantages and disadvantages of growth, carcass merit, and consumer palatability of bulls and steers. Their table suggests that bulls are highly advantages in growth rate, feed conversion, leaner carcasses (35% leaner) and yield, however, they lack marbling, fat cover, and exhibit a darker lean color which could attribute to their disadvantage in tenderness, color, and texture.

Conventional castration methods alone are relatively inexpensive; however, when you add the cost of veterinary involvement and analgesic use, the price per head increases and is an undesirable added cost to the producer. Conversely, some research suggests that consumer prices may be driven by ethical practices (Stookey, 2005) meaning that consumers are willing to pay a

premium for the reassurance that the product they are consuming has been handled in a manner that enhances the animal's quality of life (Lagerkvist and Hess, 2011).

While not monetary, there have been studies that indicate growth advantages with the use of pain mediation. This improvement may compensate for the cost of anesthetized castration. Earley and Crowe (2002) administered ketoprofen and a local anesthetic prior to castration and reported an increase in ADG compared to those that did not receive an anesthetic. Similar results were found in a study using dairy bull calves where ADG was improved anywhere between 30 to 35% in those that received a local anesthetic block at the spermatic cord during surgical castration (Fisher et al., 1996).

Hormone Response and Behavior in Bull Calves

An additional issue with keeping bulls intact is their behavioral response. Problems with keeping bulls intact increases the management problems associated with aggressive and sexual behavior and can result in unwanted breeding (Appleby, 1986). These issues are heightened with age and the onset of puberty and are attributed to testosterone. Testosterone's major functions include: secondary sex characteristic development, expression of sexual behavior, embryonic differentiation and maintenance of the male duct system and external genetalia, accessory gland functions, function of the tunica dartos muscle in the scrotum, and spermatogenesis (Bearden and Fuquay, 1997).

Testosterone is an androgen which is vital for the development and functionality of the male reproductive organs and is produced primarily by the testicles in Leydig cells. The Leydig cells are located in the testicular interstitium and produce approximately 95% of circulating testosterone with the remaining production deriving from adrenal steroidogenesis (Luetjens and Weinbauer, 2012). The starting point for de novo androgen synthesis in Leydig cells is cholesterol

which is stored in lipid droplets. In order for cholesterol to convert into testosterone, five enzymatic steps occur in which cholesterol is converted to pregnenolone which is then dehydrated to progesterone and then converted to testosterone (Luetjens and Weinbauer, 2012).

Luteinizing hormone (**LH**) is a dimeric glycoprotein involved in the regulation of testosterone synthesis. It binds to specific receptors located on Leydig cells in order to synthesis and release testosterone for transportation (Luetjens and Weinbauer, 2012). Transportation is carried out mostly by testosterone bound to albumin or sex hormone binding globulins (**SHBG**) produced by hepatocytes. Testosterone is delivered to capillaries where disassociation occurs. The hormone binding site is altered at this stage and allows testosterone to either diffuse freely into target cells or bind together with SHBG and megalin which is an importer protein (Hammes et al., 2005).

There are many hormonal influences and endocrine reactions that impact bull calf development and subsequent changes in puberty; most of which involves testosterone or androgen production. These changes alter sex drive and aggression in bulls. The hypothalamus releases gonadotropin-releasing hormone (**GnRH**) that travels to the anterior pituitary via the hypophyseal portal blood system. Here, follicle stimulating hormone (**FSH**) and LH are released. As pre-pubertal bulls age, the release of hypothalamic GnRH increases which subsequently increases the pulsatile secretion of LH (Rodriguez and Wise, 1989) which in turn initiates the onset of puberty with testicular maturation in bulls and increasing serum testosterone concentrations as previously described (McCarthy et al., 1979; Amann et al., 1986).

Puberty in male beef calves occurs between 4 and 8 months of age (Rodriguez and Wise, 1989) due to the combination of increased circulating testosterone concentrations, which begins around 6 wk of age and increases rapidly by wk 42 (Evans et al., 1996), increased LH serum

concentrations between wk 10 and 20 (McCarthy et al., 1979; Evans et al., 1993; Evans et al., 1996), and decreased concentrations of circulating FSH observed between wk 14 to 30 (Evans et al., 1996). Testicular maturation occurs when the testes secrete steroids due to increased concentrations of FSH and LH (Evans et al., 1996). Testicular maturation results in spermatogenesis.

Spermatogenesis in bulls is a process that occurs over a 61-d period with 3 different stages: 1) spermatocytogenesis, or mitotic proliferation, 2) meiosis, and 3) spermiogenesis or cytodifferentiation with 21, 23, and 17 d respective durations. It is a process of germ cell multiplication and differentiations that results in the production of male gametes known as spermatozoa which are released at the apical pole of Sertoli cells in the seminiferous tubules (Staub and Johnson, 2018). During the first stage of spermatogenesis, known as spermatocytogenesis, stem germ cells initiate the process by executing the first mitotic division that produces spermatogonia. Spermatogonia then undergo additional mitotic divisions that generate preleptotene spermatocytes. The preleptotene spermatocytes cross the blood-testis barrier and engage in the second phase, the meiotic prophase. During this phase, the first division of meiosis includes successful differentiation of germinal calls into leptotene, zygotene, pachytene, or diplotene before undergoing two additional meiotic divisions: one to reduce chromosome number and separate homologous chromosomes, and the second to separate daughter chromatids. Both steps result in the production of round haploid spermatids. Lastly, spermiogenesis is the differentiation of round spermatids into various degrees of elongation and then to spermatozoa to be released into the lumen of seminiferous tubules during spermiation (Staub and Johnson, 2018).

Growth and hormonal maturation impact bull behavior and handling. Many of the gender-related behaviors that are associated with bulls such as aggression, are due to testosterone

(Dykeman et al., 1982; Katz and McDonald, 1992). Therefore, castration is a necessary action if such behaviors want to be avoided. Bull behavior may also affect the time in which a producer chooses to castrate. It has been noted that aggressive behavior increases during the peripubertal period (Baker and Gonyou, 1986; Price and Wallack, 1991) and peeks prior to harvest (roughly 22 mo of age) with increased sexual behavior between 8 to 13 mo of age (Finnerty et al., 1996). Therefore, castration upon arrival for either stocker or feedlot operations is imperative to ensure the safety of the handlers and other animals. Additionally, as previously addressed, meat quality declines with increased age and time in which bulls are left intact.

We know that typical castration methods such as surgical or banding methods successfully reduce unwanted sexual and aggressive behavior due to the removal of the testicles and subsequent reduction in testosterone, however, in order for chemical castration to become commercially acceptable and justified to producers like traditional methods, aggressive behavior and sexual viability must be altered. Jago and associates (1997) immunocastrated calves with GnRH at 2, 4 and 7 mo of age and observed sexual and aggressive behavior (among other parameters) and indicated that immunocastrating, regardless of age, improved sexual and aggressive behavior as indicated by fewer mount intentions, attempts, and successful mounts. Reduced instances of bunting (lowering of head and using to strike), head pushing, fighting, pawing, head rubbing, and fence pacing were also observed. Additionally, they observed less pasture damage with immunocastrates compared to bulls. This would be an economic benefit to producers as well. While these findings were not permanent, immunocastrating did alter bull behavior during pre and post puberty and suggest that the findings could be attributed to delayed onset of puberty due to differences in castration time. A similar study where calves were immunocastrated with GnRH at 4 mo of age and boosted at 12 mo reported similar findings where aggression such as butting and

sparring bouts were reduced in calves that were chemically castrated when compared to bulls (Price et al., 2003). These findings are further supported by Finnerty et al. (1996) and Huxsoll et al. (1998). The results from the previously mentioned studies may imply that chemical castration (with GnRH) might be a viable solution to mitigate sexual and aggressive behavior associated with intact bulls.

Zinc

Zinc is a trace mineral that is required for numerous transcription factors and is involved in most metabolic pathways which depend on Zn-requiring proteins (Beattie and Kwun, 2004; Cousins et al., 2006). This trace mineral aids in gene expression, appetite control, fat absorption, antioxidant defense, immune function, and reproduction. The Zn dietary requirements for cattle are 20 to 30 mg/kg of diet dry matter. Pertaining to livestock, the most important functions of Zn are those that limit immune function and hinder an aspect of production.

The immune system requires specific Zn enzymes to undergo rapid cell proliferation and protein synthesis. In the innate immune response, Zn maintains cellular integrity and hastens epithelial tissue repair. More specifically, Zn deficiency decreases natural killer cell function and impairs cell cytotoxicity and immune signaling, impacts the neuroendocrine immune pathway, and alters cytokine production in mast cells (Muzzoili et al., 2009; Mocchegiani et al., 2003; Mariani et al., 1999). Not only is innate immune response impacted by Zn deficiency, but it also impacts hormones and the adaptive immune response.

The hypothalamic-pituitary-gonadal axis produces hormones that directly interact with hormone receptors on immune cells like natural killer cells. When natural killer cells are hormonally activated, they produce cytokines that mediate adaptive immune responses (John et al., 2010). The adaptive immune response is subdivided into groups based on lymphocytes. These

include humoral immunity in which B cells differentiate into immunoglobulin secreting plasma cells and cell mediated immunity in which helper cell function and cytotoxic effects are mediated by T cells (Haase and Rink, 2009).

Zinc deficiency negatively impacts lymphocyte proliferation which can be attributed to thymic atrophy. When Zn levels are inadequate, glucocorticoid concentrations are elevated accelerating apoptosis in thymocytes and causing thymic atrophy (DePasquale-Jardieu and Fraker, 1979). Thymic epithelial cells secrete thymulin which is a hormone responsible for the differentiation and function of T cells. Thymulin requires Zn as a cofactor which exists in plasma as a zinc-bound active form and a zinc-free inactive form (Hasse and Rink, 2009). When plasma Zn concentrations are low, thymulin is unable to aid in T cell differentiation which causes a decline in T cell function.

In addition to immune function, Zn plays a vital role in reproduction. When Zn deficiency occurs, spermatogenesis is impaired and the testes, epididymis and prostate do not develop normally. Additionally, serum testosterone is reduced. Zinc is essential for maintaining the lining of reproductive tissue (Colagar et al., 2009) and amasses in the testis during early spermatogenesis. More specifically, Zn accumulates in the germ cells causing an increase in concentration in the testes prior to spermatogenesis; hence the association between hindered spermatogenesis and Zn deficiency (Yamaguchi et al., 2009). It is incorporated in the flagellum and late spermatids and is located in the outer dense fibers of the spermatozoa tail (Fahim et al., 1993a) and is important for sperm motility (Hidiroglou and Knipfel, 1984).

Furthermore, Zn plays a role in thyroid function and its production of thyroid releasing hormone. In times of deficiency, the production of thyroid releasing hormone is reduced and can also result in low testosterone production (Yan et al., 2010). Testosterone production and secretion

takes place in Leydig cells where Zn is primarily found, in addition to late type B spermatogonia and spermatids (Fallah et al., 2018).

Although required for reproductive development in low doses, high levels of Zn injected into the testicles have been reported to inhibit the division and replication of germ cells and causes the fragmentation of the nucleus and cellular membranes (Fahim et al., 1993a; Bloomberg, 1996). Specifically, regarding testicular development, high concentrations of Zn have reportedly disfigured sertoli cell shape (Kheradmande et al., 2010) and have disrupted intracellular junctions (Ren et al., 2003). High concentrations of zinc also hinder androgen development by inhibiting the binding of testosterone to 5 alpha-reductase enzyme and thereby reducing serum concentrations of dihydrotestosterone (Fahim et al., 1993b). Dihydrotestosterone (DHT) development can also be inhibited by the reduced activity of the 6 alpha-reductase enzymes completely by elevated levels of zinc (Leathem, 1970).

Overview of Zinc and Chemical Castration

Zinc gluconate as a form of chemical castrations has been investigated in canines and felines. In cats, Oliveira and associates (2013) made a single injection into each testis with either isotonic saline or zinc gluconate. They recorded reproductive measurements prior to injections, at 60 days, and at 120 days. Their study resulted in decreased testicular size, reduced male behavior, azoospermia in 73% of subjects, and reduced penile spines in 55% with an additional 36% completely absent. However, testosterone concentrations did not differ between treatments at any time point. An additional follow up study with a similar experimental design measured histopathological and ultrastructural changes. They found that injecting zinc gluconate into each testicle resulted in atrophic and dilated seminiferous tubules, a decreased number of germ cells, a varying degree of cytoplasmic vacuolization in Sertoli cells, along with a loss of nuclear

chromatin, smooth endoplasmic reticulum and mitochondrial degeneration in Leydig cells. They concluded overall that zinc gluconate impaired spermatogenesis in cats and could be a permanent solution for castrations (Fagundes et al., 2014).

Even earlier than cats, intratesticular injections of a zinc-based solution were shown to impair spermatogenesis in canines (Oliveira et al., 2007). The histopathological findings of Oliveira and others (2007) were very similar to the findings in the report from Fagundes and colleagues (2014). They too concluded that utilizing a zinc-based solution as a means to sterilize dogs was a viable option and possibly irreversible. Prior research utilizing zinc gluconate neutralized by arginine into the tail of the epididimydes resulted in azoospermia (Fahim et al., 1993a). A histological examination of the testes and epididimydes indicated that cellular structure of the testis was preserved; however, the rete testis was atrophied along with the fibrous tissue in the epididimydes. These previous studies advocate for the efficacy of a zinc-based solution for sterilization and castration. These findings offer an economically feasible alternative to painful castration methods while addressing animal welfare issues.

Currently, injectable sterilization is not available commercially for use in beef cattle. A novel injectable product consisting of zinc acetate neutralized by L-histidine (Calviex™) has been approved by the FDA for proof-of-concept investigation in beef and dairy bull calves and has recently been studied in cattle upon feedlot arrival. This trail reported similar testosterone concentrations throughout the finishing period in feeder bulls (≈ 317 kg) injected intratesticularly with this zinc solution and in bulls that remained intact. Additionally, zinc injected and intact bulls had similar average daily gains, growth, and carcass characteristics. Histopathological results indicated testicular atrophy as indicated by lighter and smaller testis compared to bulls. Furthermore, testis from injected calves revealed degenerative changes in testicular tissue with the

loss of sperm producing spermatogonia and absence of definable sperm formation and maturation. They discussed that atrophy of testicular tissue might be specific to injection site. Warner-Bratzler shear force analysis reported similar shear values of steaks from both intact bulls and from injected bulls. Finally, a consumer taste panel reported an "off flavor" for steaks from injected bulls (Ball et al., 2018b). However, use of this injectable zinc solution in pre-weaning bulls (≈ 113 kg) resulted in lower serum testosterone concentrations compared to males left intact, and testosterone concentrations were similar to calves that had been surgically castrated (Ball et al., 2018a). Therefore, additional research is needed to investigate the effects that injectable sterilization would have when used in bulls at weaning (≈ 227 kg body weight). We hypothesize that injecting bulls at weaning will mitigate the negative effects of testosterone and inflammation that was observed in older feedlot bulls.

Justification

Consumers pose a threat to current sustainable agriculture. Scrutiny from consumers and animal rights groups has made attention to animal pain associated with conventional castration techniques an important issue. Therefore, novel research is needed to determine if injectable sterilization can minimize the negative physiological and immunological alteration in castrated bulls at weaning. In order for the US beef cattle industry to make effective decisions regarding best management practices, additional scientific data are required to determine if injectable chemical sterilization is effective for castration in cattle at the time of weaning.

Our objective is to determine the effects that a novel castration method (Calviex™ vs. conventional banding) at weaning has on health, growth performance, average daily gain, feed intake, behavior, serum testosterone and haptoglobin concentrations, cytokine expression, and carcass traits of male beef cattle. The data from these studies could lead to a more humane method

of castration ensuring enhanced animal welfare and could result in a more economical production system without compromising growth performance and carcass quality.

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CHAPTER III

[1]Injectable zinc as an alternative castration method at weaning in beef bulls: effects on testosterone concentrations, growth performance and carcass characteristics

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ABSTRACT: This study aimed to investigate an injectable zinc alternative administered at weaning for castration and its impact on testosterone concentrations, growth performance and carcass quality. Crossbred male beef calves (n = 74) were assigned randomly at birth to treatments: 1) surgically castrated at birth (**S**) or 2) a 1 mL intratesticular Zn (100 mg Zn) injection administered at weaning (**Z**). Calves were backgrounded in a dry lot for the initial 43 d and grazed cool season forages for the remainder of the 168-d period; followed by a 140-d finishing phase. At weaning (d -1) BW were measured to assign calves to pens within similar treatments. Four pens were selected (S, n = 18; Z, n = 18) for intensive body weight (**BW**) and testicular thickness (**TT**) sampling one week post injection. Serum testosterone, TT and BW were recorded every 28 d until slaughter. Once harvested, hot carcass weight (**HCW**), dressed carcass yield (**DCY**), lean muscle area, marbling score (**MS**), fat thickness (**FT**) and yield grade (**YG**) were recorded. Data were analyzed using the Mixed procedures of SAS ($\alpha = 0.05$); pen (6 pens with 9 calves and 2 with 10) served as the experimental unit for all analysis. Within the first week of intensive sampling, BW and change in BW were similar ($P \geq 0.19$, $P \geq 0.06$ respectively). Testicular thickness increased ($P < 0.01$) on d 2, peaked by d 7, then fell below d 0 measurements by d 56 and maintained this trend through d 168. Testosterone was similar until d 77 when concentrations in Z calves were greater compared to S calves and remained so through d 280 ($P = 0.02$). Zinc injected calves were heavier on days -1 (weaning), 140, 168 (feedlot), 224, 280, 308 and 318 ($P \leq 0.04$) and had greater ADG the last ten days (308 to 318; $P < 0.01$) and overall ($P < 0.01$). Furthermore, Z calves had heavier HCW ($P = 0.01$) and greater lean muscle area ($P = 0.01$) but a lower MS ($P < 0.01$; small vs modest). The remaining variables such as DCY, FT and YG were similar between treatments ($P \geq 0.14$). Injecting zinc temporarily impeded but did not eradicate testicular function as indicated by increased testosterone production midway through the backgrounding phase; possibly due to

inflammation. Zinc injected calves were heavier at the conclusion of the study and produced heavier carcasses with greater lean muscle area. The findings reported in this study indicate that intratesticular Zn injections at weaning improve growth performance and some carcass attributes. Still, it does not result in complete sterilization and therefore cannot be considered a true castration alternative.

Key words: castration, carcass, growth, testosterone, zinc injection

INTRODUCTION

The variances between bulls and steers raised for slaughter regarding growth performance and meat quality are well documented (Bretschneider, 2005; Field, 1971; Seideman et al., 1982). Although bulls excel in feed efficiency, growth performance, meat yield and produce a leaner product, the carcass is often discounted due to instances of DFD (dark firm and dry) and less intramuscular fat distribution (Seidemen et al., 1982). Live market values for bulls are lower due to costs associated with weight loss and illness following castration. Thus, castration methods including physical, chemical, or hormonal are used to mitigate the aforementioned subjects.

Castration is a painful management practice that temporarily restricts an animal's mobility and causes a lapse in feed consumption and reduces ADG (Fisher et al., 1996; Keane, 1999). While issues concerning growth performance and morbidity are somewhat mitigated by castrating calves at a younger age (Bretschneider, 2005; Fisher et al., 1996), it does not address welfare apprehensions pertaining to pain. Select countries enforce pain management tactics at the time of castration to address these concerns (European Commission, 2001).

Chemical castration has been investigated as a means to pacify welfare concerns without compromising growth and harvest attributes in beef cattle. Recent studies utilizing intratesticular zinc solution injection (Calviex, Cowboy Animal Health, Plano, TX) in feedlot cattle did not improve meat quality characteristics compared to bulls, nor did it reduce testosterone (Ball et al., 2018b). Injecting the same solution in pre-weaning bull calves produced similar testosterone concentrations compared to surgically castrated calves, yet, the study did not investigate growth performance beyond weaning or consider carcass quality (Ball et al., 2018a). Further research employing this injectable castration method in weaned calves and its impact on testicular width, serum testosterone, growth performance and carcass merit is warranted.

MATERIALS AND METHODS

This study was conducted in part at the University of Arkansas System Division of Agriculture Southwest Research and Extension Center (Hope, AR) and at the Oklahoma State University Willard Sparks Beef Research Center (Stillwater, OK). All methods and procedures were approved by the University of Arkansas' Institutional Animal Care and Use Committee (approval #19009) prior to the initiation of the study.

Backgrounding Phase

At birth, 74 crossbred beef bulls were allocated randomly to treatments. Experimental treatments included 1) surgically castrated at birth (**S**) where a scalpel was used to remove the testicles or 2) a 1 mL intratesticular zinc (100 mg Zn) injection administered into each testicle at weaning (**Z**). One animal from the S group remained intact through the duration of the study, and two from the Z group died; one from a testicular infection, and the other was euthanized during the finishing phase due to a broken leg. Consequently, these three calves were removed from the trial resulting in a total of 71 animals (S, n = 36; Z, n = 35).

At weaning (d -1), calves were weighed and allocated based on treatments and BW into pens. Intratesticular zinc injections were administered the following day (0). Calves were vaccinated against respiratory (Bovashield Gold 5, Zoetis, Florham Park, NJ) and clostridial-tetanus toxoid (Covexin 8, Merck Animal Health, Madison, NJ) on d 56. Calves were housed in eight 121 m^2 dry-lot pens (10 calves in 2 pens and 9 calves in 6 pens) for 77 d and bunk fed a total mixed ration (Table 1) containing soybean hulls, dried distiller grains, and Bermuda grass hay. A mineral supplement (Sunbelt Custom Minerals, Inc., Sulphur Springs, TX) was included into the total mixed ration. Calves grazed a combination of rye and wheat pastures for the remainder of the 168-d backgrounding period. While consuming cool season forages, calves were supplemented

hay and corn gluten at a rate of 1.8 kg/d with ad libitum access to water and a free choice mineral (Sunbelt Custom Minerals).

Finishing Phase

Calves were shipped 632 km to Stillwater, OK (d 168) where they were received at the Willard Sparks Beef Research Center for the entirety of the finishing phase. Upon arrival, calves were treated for parasites with both an oral drench (Safe Guard, Merck Animal Health) and pour-on anthelmintic combination (treated again on d 252; Standguard, Elanco, Greenfield, IN), vaccinated against respiratory (Titanium 5, Elanco) and clostridial (Vision 7 with SPUR, Merck Animal Health) pathogens, and implanted in the left ear (Revalor-IS, Merck Animal Health). Pens were reallocated based on treatment and arrival weight within backgrounding pen, resulting in two lots with 3 calves, 5 lots with 4 calves, and 9 lots with 5 calves.

Calves were stepped up every 7 d for a 28-d period to a high concentrate diet (Table 2). All diets contained rolled corn, sweet bran and prairie hay mixed with mineral supplement. Diets contained tylosin (60 to 90 mg/d; Tylan-40,), monensin (50 to 480mg/d; Rumensin-90, Elanco), and ractopamine hydrochloride during the last 37 d of the trail (70 to 430 mg/d; Optaflexx, Elanco).

Blood Collection

A subset of 35 (S = 17; Z = 18) calves were selected for serum testosterone analysis. Samples were collected on d 0, 7, 28, and every 28 d after until d 280. Approximately 7 mL of blood was collected via jugular venipuncture into vacuum tubes. Samples were allowed to clot and were then centrifuged at 3,000 x g for 25 min at 23 °C. Serum was decanted into 1.5 mL microcentrifuge tubes and stored at -20 °C until analysis. Testosterone concentrations were determined by a commercial ^{125}I radioimmunoassay kit (ImmuChem Double Antibody Testosterone, MO Biomedicals, LLC, Solon, OH).

Procedures

The same subset was used for intensive sampling during the initial week of the trial for BW and testicular thickness; from the subset, testis thickness measurements were recorded on the Z calves (n = 18) only. Testicular thickness was determined by measuring half way from the base of the scrotum from the front to the back of the right testicle using a digital caliper (Model W80152, Performance Tool, Tukwila, WA). Intensive measurements were collected from the subset only on days 2 and 3. Weights were collected on all animals on days 0, 7, 28 and every 28 days thereafter until the conclusion of the investigation. Body weights were used to determine change in BW within the first week and ADG. Testicular thickness measurements were collected on all Z calves (n = 35) during the same time BW were recorded on all calves.

Calves were harvested (USDA establishment # M86K, Dodge City, KS) upon completion of the finishing phase (d 308) in one load. Harvest data were collected after a 24-h period by trained personnel from the West Texas A&M University (WTAMU) Beef Carcass Research Center (Canyon, TX). The USDA quality, yield grade, LM area and 12th rib fat thickness were determined by video image analysis (VBG 2000; E+V Technology GmbH, Oranienberg, Germany).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) as a completely randomized design. Testosterone concentrations were tested for normality using PROC UNIVARIATE. Pen served as the experimental unit for all variables except testicular thickness. Treatment served as the fixed effect for growth performance and carcass attributes. Testicular thickness was analyzed with day as the lone fixed effect. Serum testosterone concentrations included day as a repeated measure where treatment, day and treatment by day were included in

the model. Least square means were separated using the PDIFF option in SAS. Significance was declared at $P \leq 0.05$ and tendencies were observed between $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Growth Performance

Weaning BW were heavier ($P = 0.04$) in Z calves compared to S calves by 21 kg. When observing BW within the first week of the investigation (Table 3), BW were similar when the zinc injection treatment (d 0) was applied and did not differ between treatments ($P \geq 0.19$) through the remainder of the week. Due to BW differences found at weaning, change in BW was determined during intensive BW sampling (Table 3). While there were no statistical differences between treatments, S calves tended ($P = 0.06$) to have a greater change in BW (4 kg vs -0.1 kg) from d 1 to 2. Likewise, Ball and associates (2018b) reported similar BW observations within the first two weeks of their trial where BW were similar between zinc injected, banded, and intact calves upon arrival to the feed yard and d 14. However, zinc injected calves had a greater ADG during the same time point compared to the two opposing castration methods. The findings for BW within the first week are contradicting to generally accepted knowledge based on numerous studies. Specifically, it has been recognized that weaning weights typically do not differ between calves castrated close to birth and intact males (Bretschneider, 2005; Champagne et al., 1969; Worrell et al., 1978) since the onset of puberty is not reached until roughly 10 mo of age and calves are generally weaned between 6 and 9 mo. Moreover, castration in the current investigation did not induce a period of weight loss that differed from calves castrated near birth. It is well documented that castration in older calves induces a period in which grazing and movement are reduced (Keane, 1999) and temporarily reduce ADG (Bretschneider, 2005; Earley and Crowe, 2002; Fisher et al., 2001).

Body weights reported for all calves (Table 4) for the entirety of the study differed on days -1 (weaning), 140, 168 (feed yard arrival), 224, 280, 308 and 318 where Z calves were heavier compared to S calves ($P \leq 0.05$). Additionally, Z calves tended to have heavier BW on days 0, 77, 116, 196, and 252 ($P \geq 0.06$). Castration treatment had no effect on BW on days 28 and 56 ($P = 0.18$; $P = 0.14$, respectively). Still, when ADG was considered, there were no differences between treatments from weaning through day 252 and from 280 to 308 ($P \geq 0.15$). Average daily gain tended ($P = 0.07$) to be greater in Z calves compared to S calves from d 252 to 280 by 0.3 kg. Z calves had greater ADG compared to S calves when exiting the feedlot ($P < 0.01$, d 318) and overall ($P < 0.01$). Previous research utilizing injectable zinc in feedlot cattle reported no differences in BW compared to banded or intact males until d 112 in the finishing phase. This study reported heavier BW in intact males compared to banded calves for the remainder of the finishing phase. With the exception of d 112 where injected calves were intermediate, injected calves had similar BW ($P < 0.01$) to intact males (Ball et al., 2018b). Feedlot ADG was reportedly greater ($P \leq 0.03$) for injected calves and intact bulls compared to banded calves for all BW intervals except from days 14 to 38 and days 28 to 56. This is somewhat contradicting to the findings of the current investigation. Although BW were reportedly heavier for Z calves compared to S calves throughout various time points during the 318-d investigation, it could be argued that these findings could have been the result of differing initial BW at weaning. When ADG was factored, our findings contradicted those of Ball and associates (2018a) since ADG did not differ between treatments until the final 10 days of the investigation. Therefore, ADG might be a more accurate representation of growth performance in this study. A trial comparing surgical castration and immunocastration (GnRH) in 20 mo old calves reported similar growth performance findings where BW and ADG differed ($P < 0.01$) at the conclusion of the trail (Amatayakul-Chantler et.

al., 2013). However, several studies comparing surgical castration to immunocastration on finishing calves reported no growth advantages with immunocastrating (Adams et al., 1993; Cook et al., 2000).

Testis Thickness

Testicular thickness measurements were also obtained during the first week of the trial and every 28 d afterward and were only collected on Z treated calves. Therefore, differences in testicular thickness in Z calves by day was studied and reported (Figure 1). Testicular thickness differed over time ($P < 0.01$) where baseline measurements were immediately surpassed by d 1 and proceeded to increase until d 7 where measurements peaked, suggesting a potential inflammatory response. Testis thickness began to decline by d 28 but were still thicker than baseline thickness on d 0. Baseline measurements were met by d 56 and continued to decline until d 77 and remained below baseline until feedlot arrival (d 168). Measurements were greater on d 196 and were similar to measurements recorded on d 28 and declined again from d 196 to d 252. Another spike in testis thickness was observed on d 280, also similar to those recorded on d 28 and declined again by d 380. Overall, testis thickness on d 380 was similar ($P = 0.16$) to those recorded on d 0, prior to zinc injection.

The feedlot zinc castration trial previously (Ball et al., 2018b) mentioned also measured testis thickness and reported an effect for treatment, day and a treatment x day interaction. A spike in testis thickness following injection was also observed and did not decrease until d 28, however, they did not differ from intact males. These findings were supported by scrotal circumference measurements where circumference was greater in zinc injected calves compared to banded calves through d 14. By d 14, scrotal circumference was smaller in zinc injected calves when compared

to bulls. Testis thickness remained smaller compared to bulls until the conclusion of the trial (d 168).

Zinc gluconate has been studied as a means to chemically sterilize canines and felines. These studies reported similar findings in that the use of zinc gluconate atrophied the testis and decreased testicular thickness (Fagundes et al., 2014; Fahim et al., 1993; Oliveira et al., 2013; Oliveira et al., 2007). Our findings regarding testis thickness are also comparable to immunocastration research where vaccination against GnRH has reportedly decreased testicular thickness and scrotal circumference in beef bulls (Janett et al., 2012; Monleón et al., 2019). Our results indicate a temporary reduction in testicular thickness for a 91-d period at the conclusion of the backgrounding phase and no difference between initial testicular thickness measurements compared to final measurements. We can conclude that and injectable zinc solution hinders testicular size; yet, because we were unable to obtain testis samples during the harvesting process and a subsequent analysis of the testis did not occur, it is difficult to draw a definite conclusion on the impacts of an intratesticular injection of a zinc solution at the time of weaning and its impacts on testicular atrophy and size.

Serum Testosterone

Serum testosterone concentrations differed between treatments (P = 0.05), by day (P = 0.02) and an interaction between treatment and day (P = 0.02) were detected (Figure 2). Testosterone was undetectable in S calves at weaning and throughout the trial but was similar to Z calves d 0 through d 56. Z castrated calves expressed greater concentrations compared to S calves by d 77 and remained greater through d 280. Serum concentrations in Z calves gradually increased from d 56 and peaked by d 244 and declined thereafter. Ultimately, serum testosterone found in Z calves was similar to weaning concentrations by d 252 (P = 0.20) and d 280 (P = 0.88).

These discoveries would suggest that an intratesticular injection of a zinc solution at weaning might temporarily mitigate testosterone production. The temporary delay of testosterone in Z calves during the backgrounding period might, in part, be clarified by testicular thickness results. There is an observable correlation explicitly in Z calves regarding testicular thickness and testosterone. Specifically, testosterone concentrations were lowest on d 7 which, coincidentally, was the same day in which testis thickness peaked. Testosterone began to incline by d 28 which is also when testicular thickness began to decrease. Furthermore, the anabolic aspects of testosterone might have influenced the differences observed in BW. As previously stated, BW did not differ on d 28 ($P = 0.18$) or on d 56 ($P = 0.14$), however, Z calves tended to have heavier BW than S calves ($P = 0.08$) on d 77 which is also when statistical differences in testosterone concentrations were observed. Z calves tended to have heavier BW on d 116 ($P = 0.06$) and differed statistically by d 140 ($P = 0.04$). The tendency for Z calves to have heavier BW compared to S calves on days 77, 116, 196 and 252 ($P \leq 0.08$) or to statistically differ on days 140, 168, 224, 308 and 318 ($P \leq 0.05$) might be explained by the increase in testosterone concentrations.

Castrating calves with an intratesticular zinc solution upon feed yard arrival temporarily reduced serum testosterone compared to bulls, but was similar by d 112 of the 168-d finishing period (Ball et al., 2018b) which was similar to the current findings in this study where testosterone was temporarily reduced below baseline serum concentrations. The concluding concentrations found in Z calves were numerically lower at the conclusion of the finishing phase in the current study compared to the conclusion of the feedlot study. When calves were administered the same intratesticular zinc solution at 3 mo of age (Ball et al., 2018a) serum testosterone concentrations were similar to surgically castrated bulls. The findings of the two previous studies utilizing an intratesticular injection of a zinc solution coupled with the results of the current study might infer

that this particular castration method is more effective as age decreases which is the general conclusion of most castration research (Jago et al., 1996; Fisher et al., 2001). This theory is further supported by an alternative chemical castration method injecting a solution containing 20% NaCl in calves from birth to 1 mo of age (Neto et al., 2014). The authors concluded that the NaCl solution completely suppressed testosterone comparable to surgically castrated calves and induced sterility indicating that the efficacy of chemical castrations is more pronounces in prepubertal calves.

Final serum concentrations in Z calves (0.65 ng/mL) were less than reported plasma concentrations (3.96 ng/mL) in post pubertal *Bos Indicus* breeds (Fields et al., 1982). Furthermore, concluding serum concentrations were numerically lower than plasma concentrations reported for bull calves at weaning (6 to 7 mo; 4.2 ng/mL) and older (Lund-Larsen et al., 1977). Our findings are similar to those found in immunocastrated calves where testosterone production is reduced within a 6-mo time period but not eradicated (Stafford, 2007). Although serum testosterone was similar to baseline values, it is clear that testicular function was reduced but not impaired, therefore, the Z calves in this particular study could not be considered castrates.

Carcass Quality

Carcass characteristic (Table 5) in Z castrated calves had heavier HCW (P = 0.01) by 24 kg, and increased LM area by 6.6 cm^2 compared to S calves. Dressed carcass yield, fat thickness and yield grade were similar ($P \geq 0.14$) between castration methods. Surgically castrating male calves at birth improved marbling score ($P < 0.01$) where S calves graded modest compared to Z calves which graded small. It is important to reiterate that final BW and HCW were heavier in Z calves compared to S calves which again contradicts previously reported data. Due to the digression of ADG and feed consumption following castration in weaned bulls, calves castrated near birth generally have improved ADG thereby cancelling any BW advantage observed in bull

calves at weaning. This generally produces similar BW between the two upon arrival to the feed yard and results in similar finishing weights and HCW (Fisher et al., 2001; Jago et al., 1996).

Similar to our investigation, injecting zinc intratesticularly in finishing calves also improved HWC and LM area compared to banded calves and was similar to intact bulls. Moreover, marbling score was improved in banded cattle compared to both bulls and injected calves (Ball et al., 2019). When comparing marbling score in Z castrated calves to those in the finishing study, there is an improvement in carcass quality (slight vs small) in the current study which might suggest that injecting the zinc solution sooner improves carcass quality regarding this specific castration method.

Studies investigating carcass attributes from immunocastrated calves have conflicting conclusions. One study reported heavier HCW and greater dressed carcass yield compared to surgically castrated calves with no adverse effects on meat quality, meat color, fat color, cooking loss, or tenderness (Amatayakul-Chantler et al., 2013). Conversely, alternative studies determined that immunocastrating calves did not improve carcass qualities (Hernandez et al., 2005; Ribeiro et al., 2004) nor did it impact color, cooking loss or tenderness (Ribeiro et al., 2004). This still implies that immunocastration does not compromise meat quality which cannot be said for injecting zinc into the testis at weaning.

IMPLICATIONS

Injecting zinc temporarily impeded but did not irradicate testicular function as indicated by increased testosterone production midway through the backgrounding phase and might be explained by testicular inflammation. Zinc injected calves were heavier at the conclusion of the finishing phase and produced heavier carcasses with greater LM area which cannot be decisively compared to carcass quality reported by bulls in this study since intact bulls were not included, but

could be implied. Our results indicate that intratesticular zinc injections at weaning tend to improve growth performance and improve few carcass attributes compared to surgically castrating calves at birth. Furthermore, injecting testis at weaning resulted in decreased serum testosterone levels and improved carcass quality compared to calves injected upon feedlot arrival. Still, it does not result in complete sterilization and therefore cannot be considered a true castration alternative and warrants further research. Future investigations should include further investigation on the effects of the current intratesticular zinc solution in prepubertal calves and its impacts through the finishing phase and on meat quality. Additional research might include the investigation of multiple injections like that practiced in immunocastration tactics.

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Table 1. Analyzed nutrient composition of common diets (DM basis) in the backgrounding phase.[1]

Item	Drylot[2]	Forage	Hay
DM, %	98.63	95.56	92.14
CP, %	21.21	19.71	12.06
ADF, %	34.67	26.84	35.89
TDN, %	57.81	72.44	62.37
NE_m, Mcal/lb	0.80	--	--
NE_g, Mcal/lb	0.52	--	--
ME, Mcal/lb	1.12	--	--
Ca, %	0.68	--	--
P, %	0.67	--	--
Mg, %	0.42	--	--
K, %	1.65	--	--

[1]Analysis performed by the University of Arkansas Southwest Research and Extension Center, Hope, AR.
[2]Weaning ration fed for first 43 d post weaning; finished on forage diet the remaining 125 d prior to feedlot entry.

Table 2. Analyzed nutrient composition of common diets (DM basis) in the finishing phase.[1]

Item	Step Up Diet 1[2]	Step Up Diet 2	Step Up Diet 3	Step Up Diet 4	Intermediate Diet	Finisher Diet
DM, %	72.2	76.0	75.5	77.1	75.8	75.0
CP, %	14.8	16.3	14.9	13.6	13.5	13.5
ADF, %	23.8	18.4	19.1	13.9	10.4	10.0
TDN, %	69.0	74.6	73.9	83.9	78.6	88.0
NE_m, Mcal/lb	0.73	0.81	0.80	0.94	0.99	99.00
NE_g, Mcal/lb	0.45	0.52	0.52	0.63	0.68	68.00
NEI, Mcal/lb	0.71	0.78	0.77	0.88	0.92	92.00
DE, Mcal/lb	1.38	1.49	1.48	1.68	1.76	1.76
ME, Mcal/lb	1.13	1.22	1.21	1.38	1.44	1.44
Ca, %	0.56	0.89	0.56	0.52	0.53	0.59
P, %	0.50	0.56	0.49	0.50	0.47	0.45
Mg, %	0.20	0.26	0.23	0.21	0.20	0.21
K, %	0.80	0.81	0.74	0.66	0.76	0.74

[1]Analysis performed by Oklahoma State University Willard Sparks Beef Research Center, Stillwater, OK.
[2]Step up 1 fed first 7 d; Step up 2 fed for next 7 d; step up 3 fed for next 7 d; step up 4 fed for next 7 d; intermediate fed for 82 d; finisher fed for remainder 37 d.

Table 3. Effects of castration method at weaning on growth performance in beef calves within the first week post weaning.

	Treatment[1]			
Item	S	Z	SEM[2]	*P* - Value
BW, kg				
Day -1, Weaning	184[b]	205[a]	6.9	0.04
Day 0	183	198	6.7	0.06
Day 1	183	196	9.0	0.30
Day 2	187	196	8.2	0.43
Day 3	189	201	8.6	0.32
Day 7	187	201	8.9	0.28
Chang in BW, kg				
Day -1 to 0	-4.1	-6.8	2.2	0.21
Day 0 to 1	-0.1	-1.5	1.5	0.53
Day 1 to 2	4.0	-0.1	1.5	0.06
Day 2 to 3	1.7	4.8	1.5	0.17
Day 3 to 7	-1.6	0.1	1.7	0.48

[1]S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning.
[2]Pooled standard error of the mean.

Table 4. Effects of castration method at weaning on growth performance through the finishing phase in beef calves.

	Treatment[1]			
Item	S	Z	SEM[2]	*P* - Value
BW, kg				
Day -1, Weaning	184[b]	205[a]	6.9	0.04
Day 0	180	198	6.7	0.06
Day 28	217	231	7.3	0.18
Day 56	250	266	7.7	0.14
Day 77	261	279	7.0	0.08
Day 116	295	315	7.1	0.06
Day 140	321[b]	344[a]	7.3	0.04
Day 168, Feed yard	341[b]	361[a]	7.0	0.05
Day 196	406	428	8.0	0.06
Day 224	471[b]	496[a]	8.3	0.04
Day 252	513	535	8.9	0.08
Day 280	555b	584[a]	9.0	0.02
Day 308	608[b]	641[a]	10.3	0.03
Day 318	614[b]	656[a]	10.1	<0.01
ADG, kg				
Day -1 to 0	-4.0	-6.6	1.3	0.19
Day 0 to 28	1.33	1.18	0.1	0.15
Day 28 to 56	1.2	1.2	0.1	0.44
Day 56 to 77	0.5	0.6	0.1	0.53
Day 77 to 116	0.9	0.9	0.1	0.54
Day 116 to 140	1.1	1.2	0.1	0.34
Day 140 to 168	0.7	0.6	0.1	0.52
Day 168 to 196	2.8	2.8	0.1	0.94
Day 196 to 224	2.3	2.4	0.1	0.37
Day 224 to 252	1.5	1.4	0.1	0.53
Day 252 to 280	1.5	1.8	0.1	0.07
Day 280 to 308	1.9	2.0	0.1	0.40
Day 308 to 318	0.2[b]	0.5[a]	0.1	<0.01
Overall	1.4[b]	1.5[a]	0.1	<0.01

[1]S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning.
[2]Pooled standard error of the mean.
[a-b] Means within a row without common superscript differ ($P \leq 0.05$).

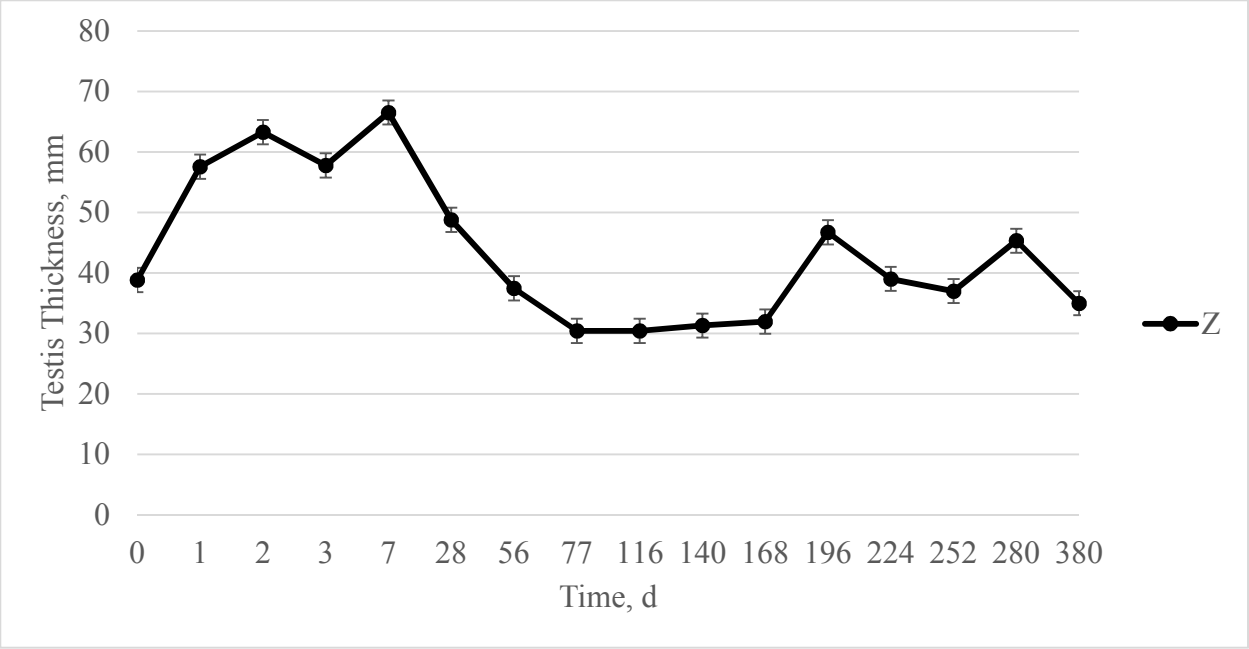

Figure 1. Effects of an intratesticular zinc injection as a method of castration at weaning on testicular width. Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of day ($P < 0.01$) was detected.

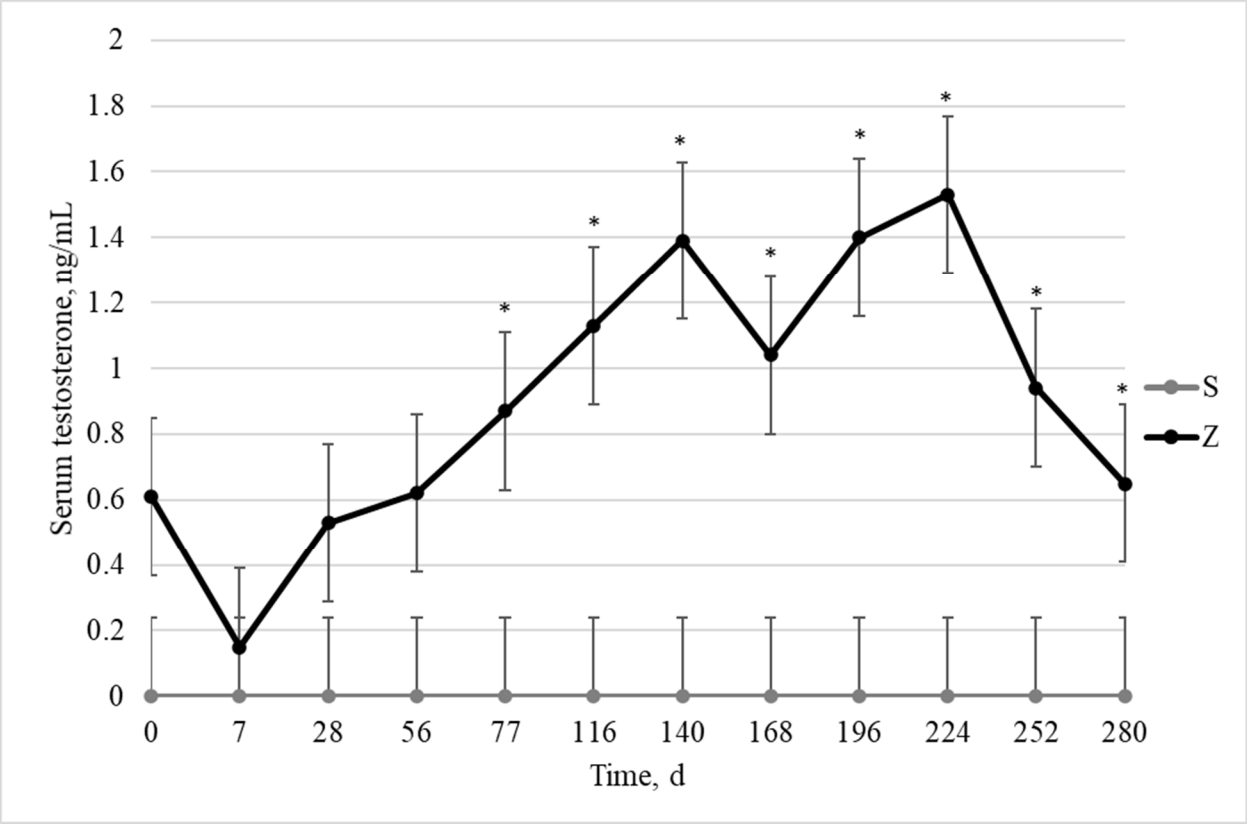

Figure 2. Effects of an intratesticular zinc injection as a method of castration at weaning on serum testosterone concentrations. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment (P = 0.05), day (P = 0.02), and treatment x day (P = 0.02) were detected. *Indicates Z differ from S calves.

Table 5. Effects of castration method at weaning on carcass characteristics.

	Treatment[1]			
Item	S	Z	SEM[2]	*P* - Value
HCW, kg	369[b]	393[a]	6.5	0.01
Dressed Carcass Yield, %	60.1	59.8	0.3	0.46
Marbling Score[3]	537[a]	465[b]	17.0	<0.01
Fat Thickness, cm^2	1.8	1.7	0.1	0.30
Lean Muscle Area, cm^2	81.5[b]	88.1[a]	1.8	0.01
Yield Grade	3.8	3.6	0.1	0.14

[1]S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning.
[2]Pooled standard error of the mean.
[3]300 = Slight[00,] 400 = Small[00], 500 = Modest[00], 600 = Moderate, 700 = Slightly Abundant[00].
[a-b] Means within a row without common superscript differ ($P \leq 0.05$).

CHAPTER IV

[1]Injectable zinc as an alternative castration method at weaning in beef bulls: effects on haptoglobin concentrations, immune function and behavioral response during the backgrounding period

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ABSTRACT: This study aimed to investigate an injectable zinc alternative at weaning and its impact on serum haptoglobin concentrations, immune function and behavioral response during a 168-d backgrounding period. Crossbred male beef calves (n = 74) were assigned randomly at birth to treatments: 1) surgically castrated at birth (**S**) or 2) a 1 mL intratesticular Zn (100 mg Zn) injection at weaning (**Z**). Calves were backgrounded in a dry lot for the initial 43 d and grazed cool season forages for the remainder of the 168-d period. At weaning (d -1), BW were measured to assign calves to pens within similar treatments. Four pens were selected (S, n = 17; Z, n = 18) for intensive blood sampling and an alternative four pens (S, n = 13; Z, n = 13) for behavior measurements one week post injection. Complete blood analysis and serum haptoglobin (**Hp**) concentrations were recorded during the first week and again on d 28 and 56. Blood data were tested for normality and were analyzed using the MIXED procedures of SAS. Behavioral data were analyzed using the GLIMMIX procedures. Repeated measures were included for all analysis. Pen served as the experimental unit. Significance was set at $P \leq 0.05$. There was an effect of treatment, time and treatment by time interaction for serum Hp ($P < 0.01$). Zinc injected calves had greater Hp concentrations from d 1 through d 56 with concentrations peaking on d 4 and 7. Serum Hp concentrations for Z calves did not reach baseline levels until d 56. An interaction for total white blood cells and neutrophil concentrations as well as neutrophil proportions ($P < 0.01$) was detected where Z calves had greater concentrations of white blood cells on d 1 and 2 and greater concentrations and proportions of neutrophils on d 1, 2 and 3. Conversely, S had a greater percentage of lymphocytes on d 1, 2 and 3 and a lower ratio of neutrophils to lymphocytes during that same time ($P < 0.01$). Zinc injected calves spent more time on their side or sternum on d 1 and more time on their side on d 2 while S calves spend more time standing during that time ($P < 0.01$). During d 3, 4, 5 and 6, Z calves spent more time standing while S calves spent more time laying

on their sternum ($P < 0.01$). Castrating calves with an injectable zinc solution at weaning displayed a heightened inflammatory and immune response and exhibited behaviors indicative of pain and discomfort. Injectable zinc could not be considered a castration alternative to mitigate pain at this time.

Key words: behavior, castration, inflammatory response, zinc injection

INTRODUCTION

Castration, regardless of method, is a painful but essential management practice that results in a period of stress, produces neuroendocrine changes and exhibits behaviors indicative of pain (Stafford and Mellor, 2005). Alongside castration, painful husbandry practices such as branding and dehorning are under scrutiny. In order to mitigate some of these concerns, many countries have enforced the inclusion of pain mediation tactics during castration (European Commission, 2001).

Acute phase proteins are an acceptable method of detecting stress in cattle. Variations have been detected with castration type (Warnock et al., 2012). Specifically, haptoglobin and ceruloplasmin concentrations were reportedly lower in banded calves compared to surgically castrated ones. Moreover, the inclusion of a pain remedy (oral meloxicam) decreased serum haptoglobin levels and the acute phase inflammatory response and cortisol levels decreased quicker (Roberts et al., 2015). Although pain mediating techniques are not currently required in the U.S., the use of long-lasting nonsteroidal anti-inflammatory drugs (NSAID) have been suggested (AABP, 2014). Additionally, the FDA recently approved analgesic drugs for cattle in 2018.

Even with resources available, it has been reported that local anesthetics are used only 22% of the time prior to castration. Of those that use and anesthetic, only 83% administer prior to surgical castration with an even greater percentage administering an anesthetic to only heavier calves (Coetzee et al., 2010). Thus, a simple and pain mediating castration alternative is necessary. Past research injecting zinc into the testicles of feedlot bulls induced a decidedly greater inflammatory response and behavior suggestive of discontent (Ball et al., 2018). Consequently, the objective of the current investigation was to determine the impacts of an injectable zinc castration solution at the time of weaning on serum haptoglobin concentrations, immune response and the behavioral response.

MATERIALS AND METHODS

This study was conducted in at the University of Arkansas Southwest Research and Extension Center (Hope, AR). All methods and procedures were approved by the University of Arkansas' Institutional Animal Care and Use Committee (approval #19009) prior to the initiation of the study.

Treatments and Diets

At birth, 74 crossbred beef bulls were randomly allocated to treatments. Experimental treatments included 1) surgically castrated at birth (**S**) where a scalpel was used to remove the testicles or 2) a 1 mL intratesticular zinc (100 mg Zn) injection administered into each testicle at weaning (**Z**). One animal from the S group remained intact through the duration of the study, and one from the Z group died from a testicular infection. Both were removed from the trial resulting in a total of 72 animals (S, n = 36; Z, n = 36).

At weaning (d -1), calves were weighed and allocated based on treatments and BW into pens. Intratesticular zinc injections were administered the following day (0). Calves were vaccinated against respiratory (Bovashield Gold 5, Zoetis, Florham Park, NJ) and clostridial-tetanus toxoid (Covexin 8, Merck Animal Health, Madison, NJ) on d 56. Calves were housed in eight 121 m^2 dry-lot pens (10 hd in two pens and 9 hd in six pens) for 77 d and bunk fed a TMR (Table 1) containing soybean hulls, dried distiller grains, and Bermuda grass hay. Mineral (Sunbelt Custom Minerals, Inc., Sulphur Springs, TX) was included into the TMR and contained Ca (14%) and P (7%) from $CaCO_3$ and Ca_2PO_4, Mg (5%) from MgO, NaCl (14%), trace minerals Mn (1,000 ppm) from $MnSO_4$, Fe (2,355 ppm) from $FeSO_4$, Cu (1,250 ppm) from $CuSO_4$, Zn (3,000 ppm) from $ZnSO_4$, Co (20 ppm) from $CoCO_3$, and I (25 ppm) from ethylenediamine dihydroiodide, vitamins (661,500 IU/kg vit. A, 221 IU/kg vit. E, and 66,150 IU/kg vit. D), and an ionophore (1600 mg/ton; Bovatec 91, Zoetis). Calves grazed a combination of rye and wheat pastures for the

remainder of the 168-d backgrounding period. While consuming cool season forages, calves were supplemented hay and corn gluten at a rate of 1.8 kg/hd/d.

Blood Collection

A subset of 35 (S, n = 17; Z, n = 18) calves were selected for serum haptoglobin (**Hp**) and complete blood count analysis. Samples were collected on days 0, 1, 2, 3, 7, 28 and 56. Blood was collected via jugular venipuncture into vacuum tubes. Approximately 7 mL of blood was collected for Hp analysis. Samples were allowed to clot and were then centrifuged at 3,000 x g for 25 min at 23 °C. Serum was decanted into 1.5 mL microcentrifuge tubes and stored at -20 °C until analysis. Haptoglobin concentrations were determined using a commercial, bovine specific sandwich ELISA kit (Immunology Consultants Laboratory, Portland, OR). Whole blood was collected into 6 mL vacuum tubes containing EDTA and processed on an automated hemocytometer (HemaVet HV950; Drew Scientific, Miami Lakes, FL).

Behavior

An additional subset was selected from the remaining pens (S, n = 13; Z, n = 13) for behavior activity 1 wk post weaning. Due to insufficient logging data detected in 5 accelerometers, behavior was measure using 12 S and 9 Z calves. Measurements were obtained using similar methods described by Brown and associates (2015). Accelerometers (HOBOware Pendant G; Onset Computer Corp., Bourne, MA) were attached to the right rear metatarsus with a zip tie through the top loop of the accelerometer and further secured with Vetrap (3M, St. Paul, MN) around the leg and accelerometer with the oval end of the datalogger facing downward. The 3-axis accelerometers were programmed to record the date, time, and both x and y axis positions for 20-s intervals.

Data were downloaded using the HOBOware Lite software (Onset Computer Corp.). Data points were graphed according to metatarsus position do indicate calf positions (lying or standing) and were categorized into 3 cluster positions using the clara function of the cluster package for R (R Project for Statistical Computing, www.r-project.org). Due to the opposing accelerometer placement compared to Brown and associates (2015), graphed cluster data points within cluster 1 (C1) represented the animal lying on their sternum, cluster 2 (C2) represented an animal standing while cluster 3 (C3) was indicative of an animal lying flat on their side. Data were averaged to represent the average X and Y axis points for each minute for statistical analysis.

Statistical Analysis

Blood data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) as a completely randomized design. Blood parameters were tested for normality using PROC UNIVARIATE. Pen served as the experimental unit for all variables. All analysis included day as a repeated measure where treatment, day and treatment by day were included in the model. Least square means were separated using the PDIFF option in SAS for blood results. Significance was declared at $P \leq 0.05$ and tendencies were observed between $0.05 < P \leq 0.10$.

Movement data was categorized into cluster proportions representing the total daily counts for each animal within each category. The aggregate data was analyzed using the GLIMMIX procedure of SAS for binomial distribution as a proportion of total activity. The model included treatment, day and treatment by day interaction. A random statement was used to model day of study as a repeated measure with animal as the subject and default variance component's structure. Least square means were estimated and logit values were converted to proportions using the ilink option. If behavior interaction response differed, differences within day were examined using the

slicediff option. Significance was declared at $P \leq 0.05$ and tendencies were observed between $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Serum Haptoglobin

Haptoglobin is the most frequently assayed acute phase protein to detect stress in cattle. It serves as an anti-inflammatory protein by binding to major receptors on the cell membranes of leukocytes (El Ghmati et al., 1996). The current study measured serum haptoglobin concentrations as a means to determine the inflammatory response to a novel zinc injection serum utilized as a castration alternative at weaning. Due to high concentrations, the Hp values reported are the log transformed data (Figure 1).

There were no differences in Hp concentrations detected ($P = 0.77$) between Z calves and S calves at weaning (d 0). However, concentrations post injection increased intensely in Z calves and continued to climb by d 2 ($P < 0.01$). Concentrations differed between treatments for the remainder of the 56-d collection period ($P < 0.01$) where Z calves expressed elevated concentrations of serum Hp compared to S calves. Haptoglobin concentrations differed by each d ($P < 0.01$) for Z calves until d 56 in which Hp levels declined to baseline measurements.

It is well established that (although there might be variations in duration pertaining to method) castration results in elevated levels of acute phase proteins (Pang et al., 2008; Roberts et al., 2015; Warnock et al., 2012). Haptoglobin concentrations measured in a previous study injecting zinc into the testes of feedlot bulls reported similarly escalated levels of serum haptoglobin (Ball et al., 2018). Concentrations were measured over a 2 wk period where values peaked by day 3 and began to subside until there were no differences between intact males, injected or banded calves on d 14. Although our findings are similar, it is important to point out that the Z

calves in the current study did not reach baseline levels until d 56 and even then, were still greater than S calves. The back transformed values for Hp in Z calves on days 1, 2 and 3 were 1,493,035 mg/dL, 7,877,500 mg/dL and 6,370,963 mg/dL, respectively; all of which were numerically larger than the peak concentrations described on d 3 (1,404,047 mg/dL) by Ball and associates (2018). The extreme variation in values might possibly be explained by the time in which the injections were administered. Weaning is undoubtedly a stressful time in a calf's life. Previous research has shown increased levels of Hp in calves at the time of weaning (Arthington et al., 2005; Arthington et al., 2003). The calves surgically castrated at birth also revealed heightened levels of Hp on d 1 and peaked on d 2 compared to d 7 ($P \leq 0.02$) which can only be explained by stress associated with weaning. Thus, the sharp incline of Hp levels in Z calves in the current study compared to the values in the feedlot investigation might be explained by the compounding stress of both castration and weaning.

Again, elevated serum Hp levels are a normal observation after any form of castration. However, the duration of inflammatory stress reported in the current investigation resulted in a chronic inflammatory state. The length of elevated acute phase protein associated with castration has reportedly improved with the use of analgesics and anesthetics (Fisher et al., 2001; Ballou et al., 2012; Brown et al., 2015). Therefore, the use of an injectable zinc castration method alone is not sufficient in subsiding castration related inflammatory, and is markedly longer in weaning calves than feedlot calves.

Immune Response

Whole blood was analyzed to measure the impact of chemically castrating with zinc would have on the immune response (Table 2). There was an interaction ($P < 0.01$) between d and treatment for white blood cell (WBC; Figure 2) concentrations and neutrophil concentrations (NC;

Figure 3), proportions of neutrophils (NP; Figure 4) and lymphocytes (LP; Figure 5), and the ratio of neutrophils to lymphocytes (NLR; Figure 6). Total WBC concentrations did not differ between Z and S calves on d 0. The total concentrations peaked on d 1 post injection and were greater in Z calves compared to S calves through d 2 ($P < 0.01$). White blood cell concentrations began to decline on d 2 and did not differ by d 3 and through the remainder of the collection period. Similarly, NC and NP did not differ at weaning (d 0; $P \leq 0.73$). Neutrophil concentrations spiked on d 1 post injection ($P < 0.01$) and declined but still remained greater for Z calves compared to S calves on d 3 ($P = 0.05$) until d 7 in which concentrations did not differ ($P = 0.95$). The results for NP were identical to NC. Zinc injected calves had a greater proportion of neutrophils on d 1 and declined but differed through d 3 ($P < 0.01$) until d 7 ($P = 0.76$). Conversely to NP, lymphocyte proportions were greater in S calves compared to Z calves within the same 3 d window ($P < 0.01$). A NLR is used as a measure of stress in animals. Due to the increase in neutrophils and their initial response in the inflammatory process, a higher NLR value is indicative of a stressed animal (Kulberg et al., 2002). The values detected for NP and LP resulted in a difference for NLR within the first 3 d post castration. Due to greater concentrations and proportions of neutrophils, Z calves had a higher NLR through the first wk post castration ($P < 0.01$).

Elevated total white blood cell concentrations in Z calves could be related directly to the elevated concentrations of neutrophils. Neutrophils are the innate immune response's initial action to tissue damage inflammation (Selders et al., 2017). Once the site of inflammation has been reached, neutrophils have been found to remain in the tissue for up to 3 days and are responsible for phagocytosis (Anderson, 2001; Dancey et al., 1976; Mantovani et al., 2011), which is what was observed in the current investigation. Due to the expenditure of neutrophils, it is not surprising

that greater proportions of neutrophils were found in Z calves or that they possessed a greater NLR during the same time frame compared to S calves.

Conflicting data regarding castration type and its influence, or lack thereof, on white blood cell concentrations have been described. Two studies reported no differences in white blood cell concentrations between surgically castrated calves or banded calves (Pang et al., 2009; Wistabu et al., 2004). Similar to our findings, Ratcliff and associates (2014) reported differences in white blood cell concentrations; however, it compared surgically castrated calves to banded calves and compared them over two castrations time points and is therefore difficult to conclusively compare to the current study. Regardless of castrations type, the process itself causes an initial increase in neutrophils and a subsequent increase in the ratio of neutrophils to lymphocytes (Ballou et al., 2012; Ratcliff et al., 2014).

Again, the use of pain mediating techniques such as NSAID (Pang et al., 2006), analgesia used in the testes (Stafford et al., 2002), and infusions of ketoprofen (Ting et al., 2003) have proven to subside the spike in immune response. The results in the current study based on the immune response measured would suggest that some means of inflammatory reducing tactics would still be required in conjunction with chemically castrating with zinc.

Motion Index

Certain aspects of cattle behavior are indications of pain and differ by castration method. Typical observable behaviors include foot stamping, tail flicking, and restlessness (Capucille et al., 2002). Behavior measurements in the current study were detected using 3-axis accelerometers to determine the proportions of time that an animal spent lying down on their side (Figure 7) or their sternum (Figure 8) and standing (Figure 9). The data points were clustered and the total within

clusters was calculated to measure the proportion of time within the day the animal spent exhibiting 1 of the 3 behaviors.

Within the first 2 d post castrations, Z calves spent a greater proportion of time lying flat on their side with their legs extended ($P \leq 0.05$) compared to S calves. By d 3 the proportion of time did not differ ($P = 0.13$) between treatments and follow suite for the remainder of the week, although a tendency ($P = 0.07$) was observed on d 4 where Z calves spent less time flat on their side compared to S calves. Since Z calves spent a greater amount of time lying down the first 2 d, S calves spent significantly more time standing during that time period ($P \leq 0.03$), but by d 3, Z calves spent more time standing ($P < 0.01$) compared to S calves. This trend continued through d 6 and did not differ by d 7 ($P = 0.38$). In regard to the proportions of time the animals spent lying on their sternum, Z calves, on d 1, spent more time lying on their sternum compared to S calves ($P = 0.02$) but was similar on d 2 ($P = 0.99$). The inverse was true from d 3 to 6 ($P \leq 0.01$) where S calves spent a greater proportion of time on their sternum compared to Z calves, until d 7 in which standing time did not differ ($P = 0.41$).

Calves lying flat on their side with their legs extended could be an indication of pain and coincides with previously reported behavioral data (Robertson et al., 1994). In this study, the behavioral data followed the trend displayed by the immune and inflammatory results where calves that were chemically castrated with zinc exhibited behaviors indicative of pain within the first 2 d post castration. As previously mentioned, the weaning process in itself is a stressful circumstance. During weaning calves are known to display behaviors of distress such as vocalizing and pacing (Smith et al., 2003). Although vocalization was not measured, it can be surmised that the greater proportions of time spent standing that were determined on d 1 and 2 by S calves was caused by pacing due to weaning stress which was also found in the castration study conducted by Brown

and others (2015). The standing differences subsided by d 3 which was also observed by Smith and others (2003) when investigating weaning stress behavior duration.

After the initial 2 days, a 4-d period where Z calves spent more time standing was observed; this was in part due to the amount of time S calves spent lying on their sternum. However, the behavior during this time could be compared to behaviors exhibited by banded calves (González et al., 2010) but unfortunately over a longer period of time. In agreeance with our findings, using an injectable zinc solution on feeder bulls indicated that zinc castrated bulls induced a short period of lying bouts followed by more time standing compared to banded or intact males (Ball et al., 2018). It is thoroughly documented that castrated calves spend more time standing or pacing post castration due to discomfort (Brown et al., 2015; González et al., 2010; White et al., 2008) and would explain the duration of time spent standing by Z calves.

Zinc injected calves spent a greater proportion of time lying on their sternum ($P = 0.02$) on d 1 followed by a 4-d period (days 3, 4, 5 and 6) where S calves spent greater proportions of time lying on their sternum ($P \leq 0.01$). It is possible that the difference indicated on d 1 was attributed to discomfort similarly to the results found in the feedlot study (Ball et al., 2018), and the decreased proportion of time lying down observed by S calves could be stress induced by weaning (Brown et al., 2015). As Brown and associates pointed out (2015), it is difficult to surmise whether or not and animal lying on their sternum is due to contentment or pain. Similar to our findings, calves castrated near birth spent more time on their sternum at weaning compared to newly castrated calves. It might be implied that calves castrated near birth are more content a few days post weaning due to the lack of castrated associated pain.

IMPLICATIONS

The main purpose of investigating a zinc injection castration alternative is to provide a method of castration that either reduces or resolves stress, pain or adverse growth performance. The objectives of the current study were to explore the impacts that this novel solution has on behavior, serum haptoglobin and the immune function. The hope is that the results from this study could allude to whether this alternative method hinders the acute pain and inflammatory response and the accompanying behaviors in castrated weaned calves.

Using an injectable zinc solution in calves at weaning inflicted a chronic inflammatory response as illustrated by a 56-d period of elevated Hp levels. These outcomes are congruent and supported by the leukocyte levels measured where elevated concentrations and proportions of neutrophils were detected in zinc injected calves. Additionally, the neutrophil to lymphocyte ratio observed in the same calves was elevated which is indicative of a stress response. Subclinical indications of chronic pain and inflammation linked to zinc castration was supported by clinical behavioral observations. Calves castrated at weaning exhibited signs of distress by lying flat on their sides with their legs extended within the first 2 d of the trial, followed by a greater proportion of time standing and is typical behavior found in newly castrated calves. Calves castrated near birth spent more time standing initially followed by, what could be assumed as satisfied behavior, laying on their sternum. The time spent standing within the first days post weaning could be explained by weaning.

These findings would suggest that using a zinc solution as an alternative castration method in weaned calves would not be a suitable alternative. The process inflicted pain, a prolonged period of inflammation and animals exhibited behaviors linked to stress. It is well documented that castrating at an earlier age mitigates the issues observed in the current study (Bretschneider, 2005). Research including analgesic or anesthetics at the time of castration has reportedly improved the

acute phase protein response (Brown et al., 2015), prevents the suppression of leukocyte responses (Ballou et al., 2012) and improves behavioral characteristics (Currah et al., 2009). Our findings indicated that a zinc injectable solution could not be used singularly at weaning to improve the negative aspects of castration, and would need to be applied in conjunction with some form of pain mediating tactic.

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Table 1. Analyzed nutrient composition of common diets (DM basis) in the backgrounding phase.[1]

Item	Drylot[2]	Forage	Hay
DM, %	98.63	95.56	92.14
CP, %	21.21	19.71	12.06
ADF, %	34.67	26.84	35.89
TDN, %	57.81	72.44	62.37
NE_m, Mcal/lb	0.80	--	--
NE_g, Mcal/lb	0.52	--	--
ME, Mcal/lb	1.12	--	--
Ca, %	0.68	--	--
P, %	0.67	--	--
Mg, %	0.42	--	--
K, %	1.65	--	--

[1]Analysis performed by the University of Arkansas Southwest Research and Extension Center, Hope, AR.
[2]Weaning ration fed for first 43 d post weaning; finished on forage diet the remaining 125 d prior to feedlot entry.

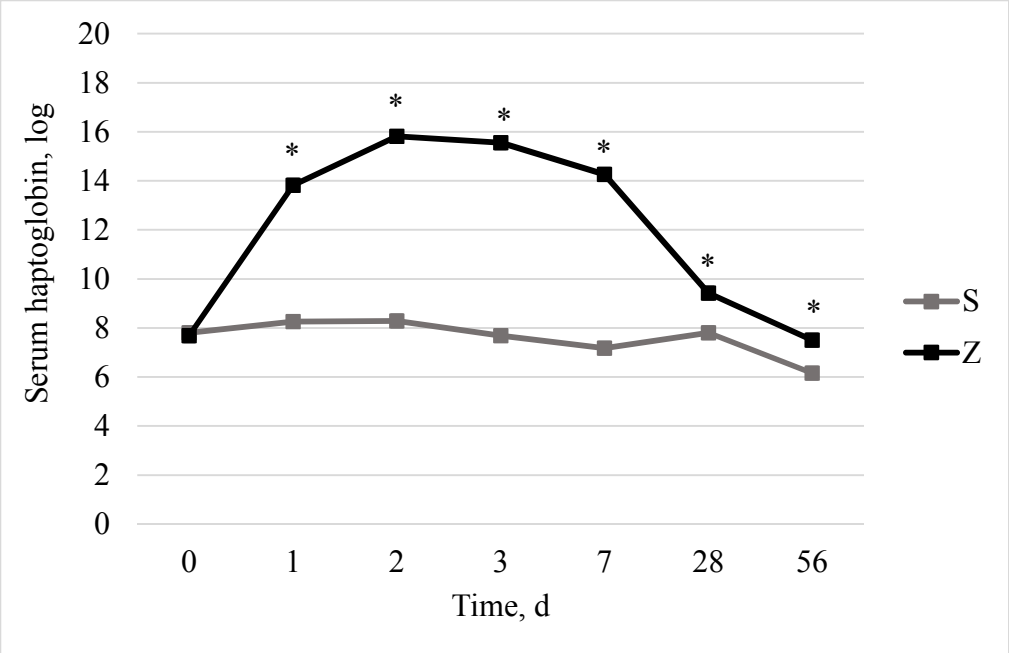

Figure 1. Effects of an intratesticular zinc injection as a method of castration at weaning on serum haptoglobin concentrations. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment ($P < 0.01$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

Table 2. Effects of in injectable zinc solution at weaning on the innate immune response.

	Treatment[1]														*P* - Value		
	Surgical							Zinc									
Item	D0	D1	D2	D3	D7	D28	D56	D0	D1	D2	D3	D7	D28	D56	SEM	TRT	Time	TRT x Time
Concentration, K/µl																		
White Blood Cells	9.19	8.93	8.97	9.80	8.89	9.37	10.70	8.74	14.29	13.01	10.75	8.72	8.94	10.82	0.95	0.35	<0.01	<0.01
Neutrophil	3.54	2.66	2.94	3.31	2.44	3.64	3.84	3.20	9.03	7.02	5.43	2.51	3.62	4.17	0.68	0.17	<0.01	<0.01
Lymphocyte	4.89	5.61	4.36	5.30	5.13	4.74	5.73	4.70	4.32	4.70	4.08	4.97	4.45	5.51	0.34	0.26	0.05	0.15
Monocyte	0.44	0.41	0.69	0.68	0.62	0.56	0.56	0.40	0.29	0.68	0.64	0.50	0.49	0.50	0.07	0.42	<0.01	0.94
Eosinophil	0.29	0.24	0.34	0.40	0.58	0.38	0.52	0.41	0.60	0.51	0.46	0.61	0.33	0.59	0.08	0.27	0.01	0.11
Basophil	0.03	0.01	0.37	0.10	0.13	0.05	0.05	0.02	0.04	0.10	0.14	0.13	0.04	0.05	0.08	0.50	0.15	0.52
Proportions, %																		
Neutrophil	38.22	29.40	32.68	34.64	27.31	38.86	36.08	36.42	62.07	53.64	48.32	28.39	40.11	37.85	2.44	0.07	<0.01	<0.01
Lymphocyte	53.64	62.27	49.95	52.01	57.83	50.72	53.44	53.76	30.97	35.81	36.91	57.53	50.10	51.71	2.25	0.05	<0.01	<0.01
Monocyte	4.81	4.62	8.55	7.14	6.97	5.95	5.17	4.68	2.23	5.95	7.34	5.78	5.89	4.65	0.93	0.42	<0.01	0.29
Eosinophil	3.03	2.60	4.21	4.82	6.45	3.93	4.89	4.87	4.45	3.70	4.49	6.83	3.74	5.37	0.65	0.33	<0.01	0.37
Basophil	0.30	0.10	3.42	1.39	1.43	0.54	0.42	0.27	0.29	0.89	1.94	1.47	0.46	0.42	0.54	0.28	0.02	0.23
N:L Ratio[3]	0.76	0.48	0.71	0.77	0.48	0.78	0.70	0.71	2.14	1.70	1.37	0.5	0.73	0.76	0.11	0.05	<0.01	<0.01

[1]Surgical (S) = calves that were surgically castrated at birth, Zinc (Z) = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning.
[2]SEM = pooled standard error of the mean for treatment x time interaction.
[3]N:L = neutrophil to lymphocyte ratio.

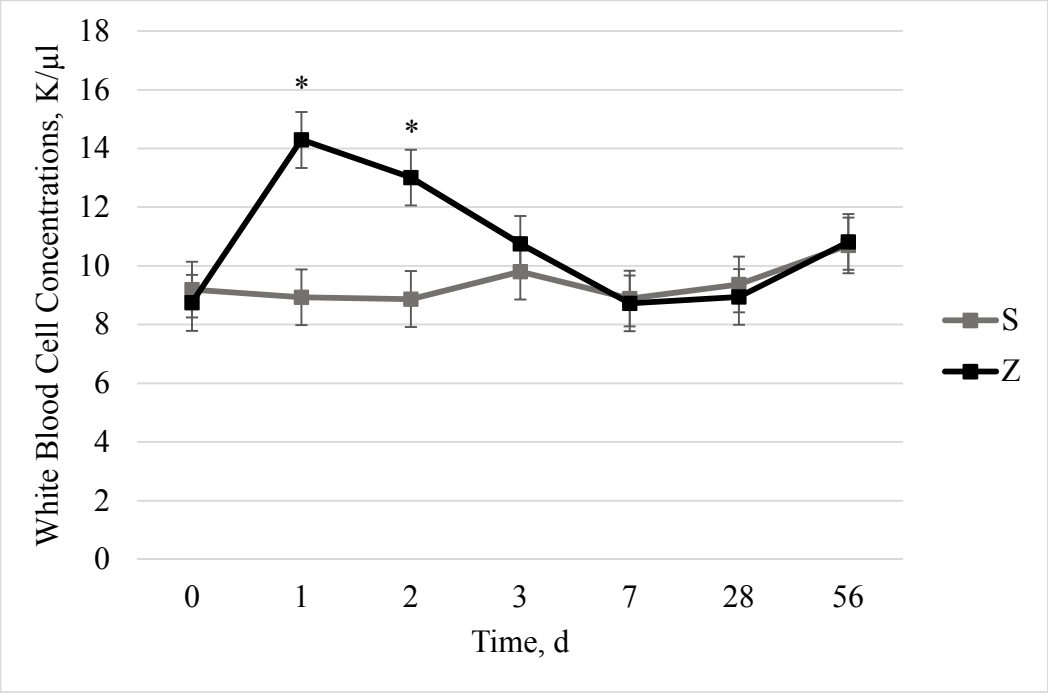

Figure 2. Effects of an intratesticular zinc injection as a method of castration at weaning on white blood cell concentrations. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

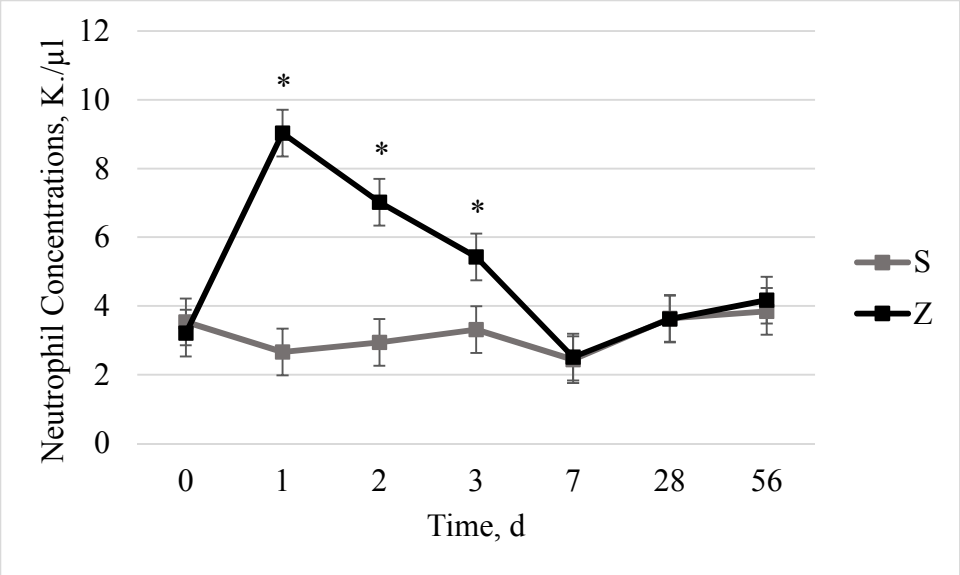

Figure 3. Effects of an intratesticular zinc injection as a method of castration at weaning on neutrophil concentrations. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

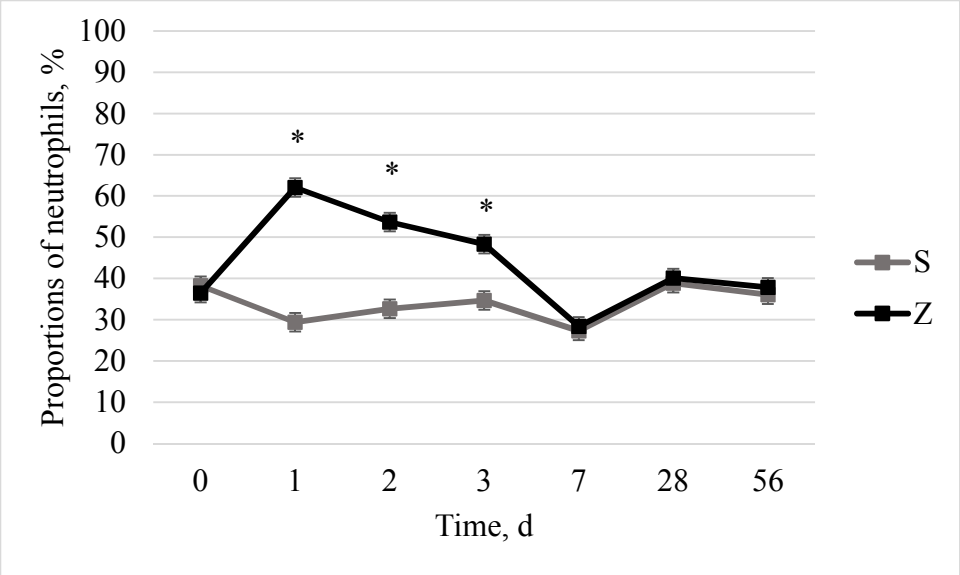

Figure 4. Effects of an intratesticular zinc injection as a method of castration at weaning on neutrophil proportions. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

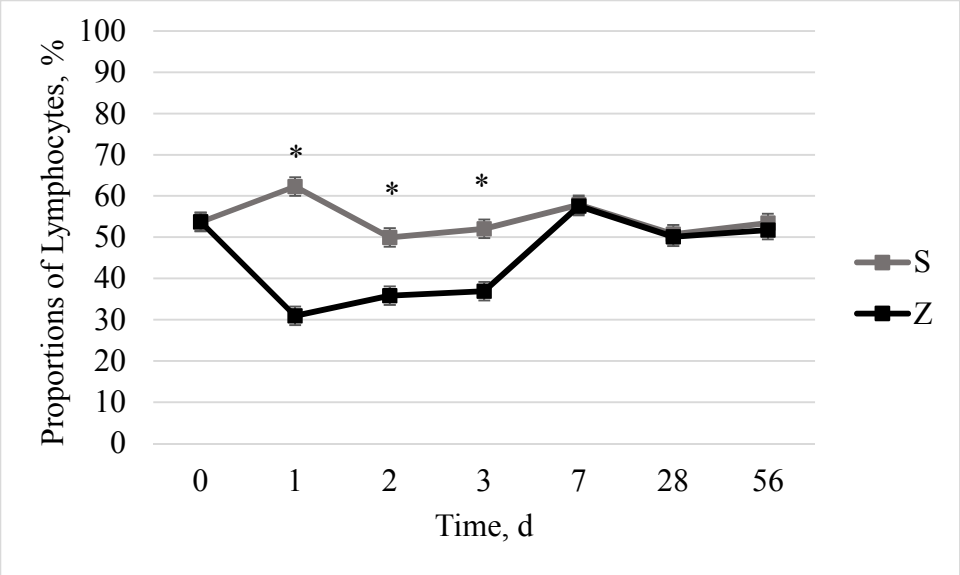

Figure 5. Effects of an intratesticular zinc injection as a method of castration at weaning on lymphocyte proportions. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

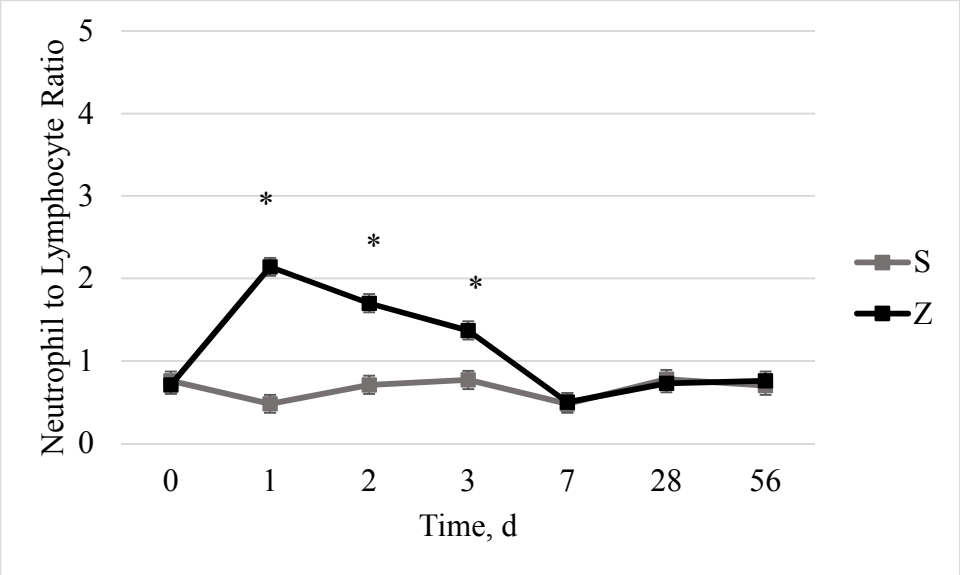

Figure 6. Effects of an intratesticular zinc injection as a method of castration at weaning on neutrophil to lymphocyte ratio. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

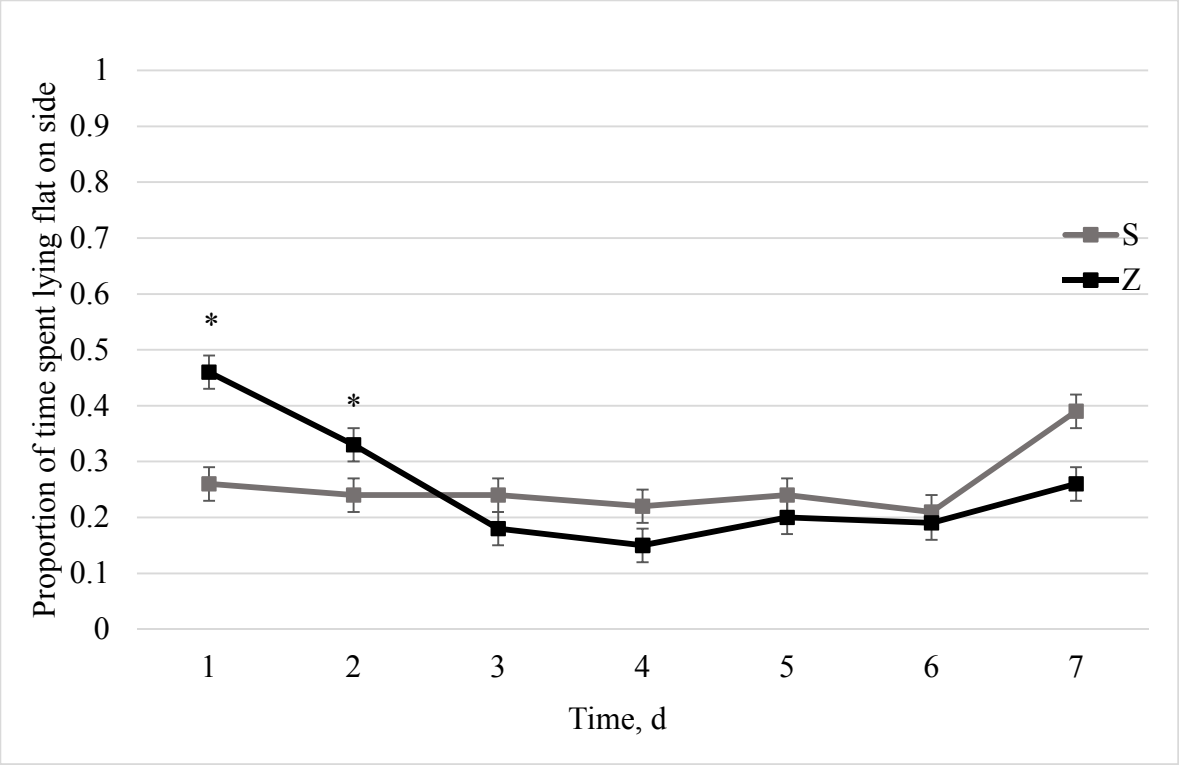

Figure 7. Effects of an intratesticular zinc injection as a method of castration at weaning on the proportion of time the animal spent lying on their side. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

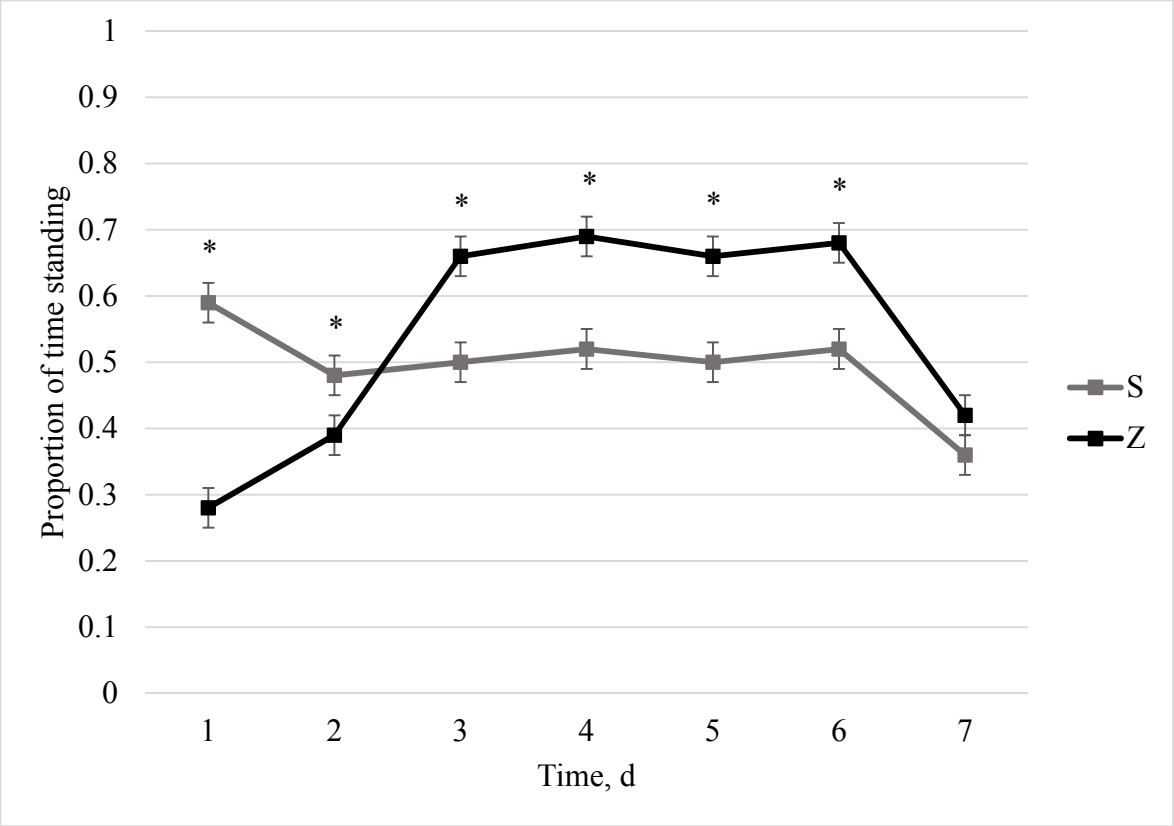

Figure 8. Effects of an intratesticular zinc injection as a method of castration at weaning on the proportion of time the animal spent standing. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

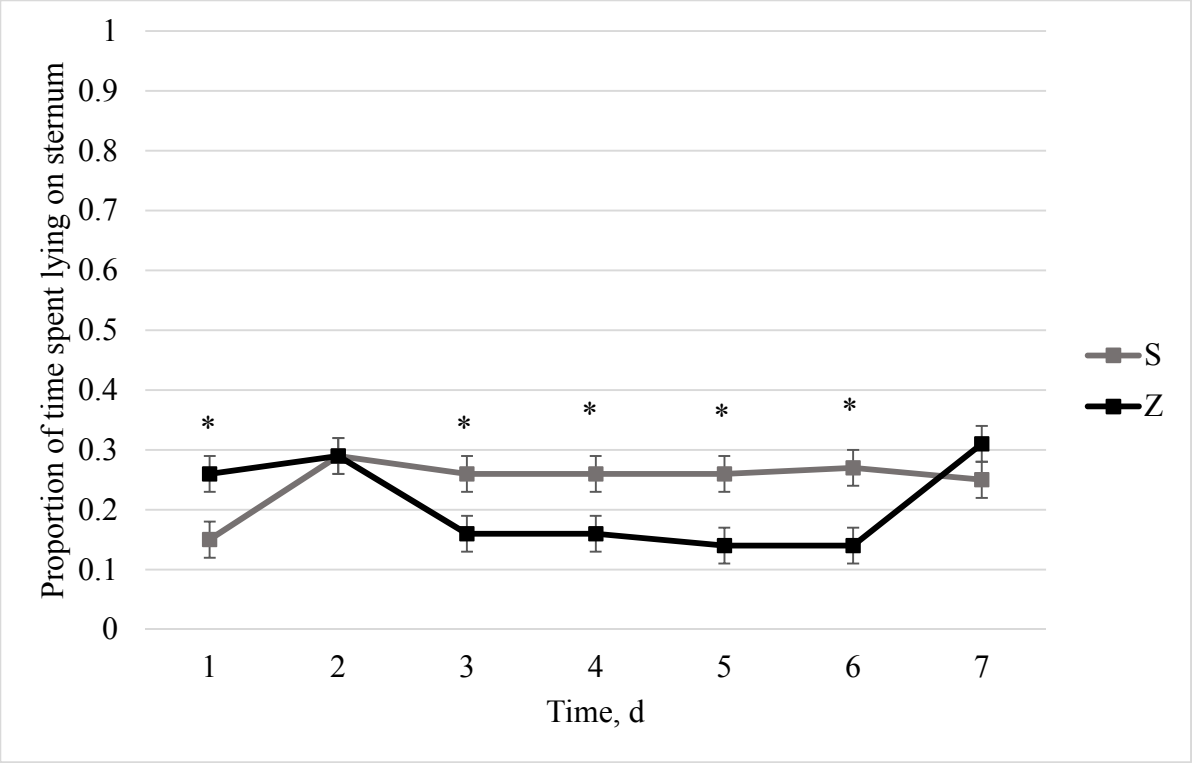

Figure 9. Effects of an intratesticular zinc injection as a method of castration at weaning on the proportion of time the animal spent lying on sternum. S = calves that were surgically castrated at birth, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis at weaning. Effect of treatment x day ($P < 0.01$) were detected. *Indicates Z differ from S calves.

CHAPTER V

Effects of injectable zinc as an alternative castration method compared to banding one week post weaning in beef bulls: effects on health, inflammation, behavior and growth during the backgrounding period

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ABSTRACT: Male beef calves were allocated to 1 of 8 pens based on weaning weight. Pens were assigned randomly to treatment: 1) banded (**B**; n = 42) or 2) a 1 mL intratesticular Zn (100 mg Zn) injection (**Z**; n = 39). Two pens from each treatment were selected for blood collection (B, n = 22; Z, n = 19); the alternative pens were assigned to behavior measurements (B, n = 10; Z, n = 10). Behavior data was collected for one week post castration. Blood was collected on d 0 through the first week post castration and every 28 d for the remainder of the backgrounding phase for complete blood analysis, gene expression, serum haptoglobin (**Hp**), and serum testosterone. Testicular width and BW was also measured at those time points. All blood and growth parameters were analyzed using the MIXED procedures of SAS while GLIMMIX was used for behavioral analysis. Significance was declared at $P \leq 0.05$. Body weights between the two treatments did not differ throughout the 140-d backgrounding phase ($P \geq 0.39$). Overall ADG was greater for Z calves compared to B calves ($P = 0.05$). Testosterone concentrations differed between treatments throughout the 140-d trial ($P \leq 0.02$). Testicular width in Z calves peaked numerically on d 3 and declined to baseline measurements by d 113 ($P = 0.67$). Zinc castrated calves spent more time lying flat on their side compared to B calves one day post castration ($P = 0.03$) until d 3 and 4 where B calves spent more time lying on their side compared to Z calves ($P \leq 0.02$). Banded calves spent more time standing the first two days post castration compared to Z calves ($P \leq 0.01$). On d 3, 4, 5 and 6, Z calves spent more time standing ($P \leq 0.01$) while B calves spent more time laying on their sternum ($P \leq 0.03$). Banded calves had lower Hp concentrations on d 1, 2, 3, 7, and 28 compared to Z calves ($P \leq 0.01$). Total white blood cell concentrations, both proportions and concentrations of neutrophils, and proportion of lymphocytes were greater in Z calves compared to B calves on d 1, 2, and 3 ($P \leq 0.01$). Gene expression of IL1$_\beta$, IL6 and TNFα did not differ between treatments over time ($P \geq 0.83$). Although growth performance was not hindered by the

zinc castration method, it did cause behaviors indicative of pain and resulted in a spike in inflammation and immune function compared to banded calves. Therefore, castrating with zinc would not be a sufficient alternative to conventional methods.

Key words: behavior, growth, health, zinc castration

INTRODUCTION

Castration is performed on approximately 16 million bull calves in the U.S. alone (USDA, 2018). Methods include physical, chemical or hormonal damage to the testicles (Currah et al., 2009). Physical castration is used most commonly with reportedly 57% of producers choosing the surgical castration technique (Coetzee et al., 2010). Regardless of method, castration overall has proven to reduce unwanted sexual and aggressive behaviors (Appleby, 1986) and improve meat quality (Field, 1971) and harvest market value (Faulkner et al., 1992). Unfortunately, the process temporarily induces a period of physiological stress that results in neuroendocrine and behavioral changes indicative of pain (Stafford and Mellor, 2005) and has become a source of public alarm for consumers.

Animal welfare has been a continually voiced concern with regard to painful husbandry practices including branding, dehorning and castration. Castration specifically has resulted in the public demand for the development of pain-relieving remedies (Weary and Fraser, 2004); so much so that European legislation requires local anesthesia administration prior to castration (DEFRA, 2003). Although tactics to reduce pain during castration such as long-lasting nonsteroidal anti-inflammatory drugs or analgesic medications are available (AABP, 2014), a survey reported that very few producers apply these resources (Coetzee et al., 2010).

Despite the underutilization of pain mediating tactics, perhaps it is only a matter of time that the example set by European legislation will become a global requirement, unless castration alternatives can be identified. Therefore, the goal of the current study aimed to investigate a testicular injectable zinc solution as a potential castration alternative compared to traditional banding in weaned beef bulls. The immune response, behavior, hormone production and growth performance were compared between the two castration methods.

MATERIALS AND METHODS

This study in its entirety was conducted in at the University of Arkansas Division of Agriculture Experiment Station (Savoy, AR). All methods and procedures were approved by the University of Arkansas' Institutional Animal Care and Use Committee (approval #19089) prior to the initiation of the study.

Treatments and Diets

Crossbred beef calves (n = 81) were allocated to treatment based on weaning weight (231 ± 4.5 kg) recorded 7 d prior to the initiation of the study and assigned to 1 of 8 pens. Pens were assigned randomly to castration treatment: 1) banded (**B**; n = 41) in which a blood-restrictive rubber band was placed around the dorsal aspect of the scrotum (California Bander, InoSol Co. LLC, El Centro, CA) or 2) a 1 mL intratesticular zinc (100 mg Zn) injection administered into each testicle (**Z**, n = 39). Castration treatments were applied one week post weaning (d 0).

Calves received topical moxidectin on d 7 (Cydectin, Elanco, Greenfield, IN). On days , 84, 113 and 140, calves were treated topically with 0.5% of gamma-cyhalothrin (StandGuard, Elanco). Vaccination against respiratory (Bovashield Gold 5, Zoetis, Florham Park, NJ) and clostridial-tetanus toxoid (Covexin 8, Merck Animal Health, Madison, NJ) were administered on d 7 and boosted on d 28. Calves were housed in 0.4 ha grass traps for the initial 28 d and were supplemented 1.36 kg/hd/d of soybean hulls with ad libitum access to Bermuda grass hay after which they were moved to 10.12 ha mixed Bermuda and fescue pastures for the remainder of the 140-d backgrounding period. Soybean hull pellets were supplemented at a rate of 0.5% of BW/hd/d with access to free choice mineral (4% Beef Mineral, Powell Feed and Milling, Green Forest, AR).

Blood Collection

A subset of 41 calves (B, n = 22; Z, n = 19) were selected for serum haptoglobin (**Hp**), serum testosterone, complete blood count analysis and cytokine gene expression. Intensive sampling was completed the first week post castration for Hp and complete blood count analysis. Blood was collected via jugular venipuncture into vacuum tubes at approximately 0800 hr. Approximately 7 mL of blood was collected for Hp and testosterone analysis. Samples were allowed to clot and were then centrifuged at 3,000 x g for 25 min at 23 °C. Serum was decanted into 1.5 mL microcentrifuge tubes and stored at -20 °C until analysis.

Haptoglobin concentrations for d 0, 1, 2, 3, 7, 28 and every 28 d after until the conclusion of the backgrounding period were determined using a commercial, bovine specific sandwich ELISA kit (Immunology Consultants Laboratory, Portland, OR). Testosterone concentrations for d 0, 7, 28 and every 28 d after were determined by a commercial ^{125}I radioimmunoassay kit (ImmuChem Double Antibody Testosterone, MO Biomedicals, LLC, Solon, OH) at the University of Arkansas Nutrition Laboratory (Fayetteville, AR).

Whole blood was collected into 6 mL vacuum tubes containing EDTA and processed on an automated hemocytometer (HemaVet HV950; Drew Scientific, Miami Lakes, FL) on d 0, 1, 2, 3, 7 and 28 for complete blood count. Whole blood was promptly removed on site from vacuum tubes on d 0, 7 and 28 for IL1$_\beta$, IL6 and TNFα gene expression. Blood was pipetted into 1.5 mL microcentrifuge tubes and placed immediately on dry ice and transported to the laboratory and stored at -80°C until further analysis. Total RNA was extracted (n = 4 per treatment) using TRIzol reagent (Life Technologies, Carlsbad, CA). Integrity and quality of each RNA sample was determined using 1% argarose gel electrophoresis, and concentration purity was measured by Take3 Micro-Volume plate using Synergy HT multimode microplate reader (BioTek, Santa Clara, CA). Samples were RQ1 DNase treated, reverse transcribed using qScript cDNA Synthesis

SuperMix (quanta Biosciences, Gaithersburg, MD) and then amplified by real-time quantitative PCR (Applied Biosystems 7500 Real-Time System, Thermo Fisher Scientific, Waltham, MA) with PowerUp SYBR (Life Technologies, Thermo Fisher Scientific) green master mix (Rajaei-Sharifabadi et al., 2017). Relative gene expression of IL1β, IL6 and TNFα was determined using the $2^{-\Delta\Delta CT}$ method with normalization to ACTB rRNA as a housekeeping gene. Bovine specific primers were used.

Behavior

An additional subset was selected from the remaining pens (B, n = 11; Z, n = 10) for behavior activity 1 wk post castration. During the first week, 2 loggers from the Z group did not sufficiently measure motion and were thus removed from analysis. Measurements were obtained using similar methods described by Brown and associates (2015). Accelerometers (HOBOware Pendant G; Onset Computer Corp., Bourne, MA) were attached to the right rear metatarsus with a zip tie through the top loop of the accelerometer and further secured with Vetrap (3M, St. Paul, MN) around the leg and accelerometer with the oval end of the datalogger facing downward. The 3-axis accelerometers were programmed to record the date, time, and both x and y axis positions for 20-s intervals.

Data were downloaded using the HOBOware Lite software (Onset Computer Corp.). Data points were graphed according to metatarsus position do indicate calf positions (lying or standing) and were categorized into 3 cluster positions using the clara function of the cluster package for R (R Project for Statistical Computing, www.r-project.org). Graphed data points within cluster 1 (C1) represented the animal lying on their sternum, cluster 2 (C2) represented an animal standing while cluster 3 (C3) was indicative of an animal lying flat on their side. Data were averaged to represent the average X and Y axis points for each minute for statistical analysis.

Procedures

The same subset selected for blood sampling was used for intensive sampling during the initial week of the trial for BW and testicular thickness; from the subset, testis thickness measurements were recorded on the Z calves (n = 19) only. Testicular width was determined by measuring the right testicle using a digital caliper (Model W80152, Performance Tool, Tukwila, WA). Intensive measurements were collected from the subset only on days 2 and 3. Weights were collected on all animals on days 0, 7, 28 and every 28 days thereafter until the conclusion of the investigation. Body weights were used to determine change in BW within the first week and ADG. Testicular thickness measurements were collected on all Z calves (n = 39) during the same time BW were recorded on all calves.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) as a completely randomized design. Data were tested for normality using PROC UNIVARIATE; if improved, nonparametric data were log-transformed prior to analysis and reported as the back-transformed data. Pen served as the experimental unit for all variables except testicular thickness. Treatment served as the fixed effect for growth performance. Testicular thickness was analyzed with day as the lone fixed effect. Serum testosterone concentrations, complete blood analysis, gene expression and Hp included day as a repeated measure where treatment, day and treatment by day were included in the model. Least square means were separated using the PDIFF option in SAS. Movement data was categorized into cluster proportions representing the total daily counts for each animal within each category. The aggregate data was analyzed using the GLIMMIX procedure of SAS for binomial distribution as a proportion of total activity. The model included treatment, day and treatment by day interaction. A random statement was used to model day of

study as a repeated measure with animal as the subject and default variance component's structure. Least square means were estimated and logit values were converted to proportions using the ilink option. If behavior interaction response differed, differences within day were examined using the slicediff option. Significance was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Growth Performance

Growth performance was measured and calculated for change in BW within the first week after castration (Table 2) to determine the acute impact of castration on growth. Body weights did not differ between castration methods from weaning (d -7) to the first week post castration ($P \geq 0.11$) which would suggest that castrating with zinc would not hinder growth performance more so than banding. However, when accounted for change in BW within the same time period, calves that were banded had greater change in BW from d 0 to 1 ($P < 0.01$) and d 2 to 3 ($P = 0.03$) compared to zinc castrated calves; and therefore, had an overall greater increase in BW by roughly 9 kg ($P = 0.04$). While it is well documented that castration temporarily induces a period in which BW and ADG are negatively impacted (Bretschneider, 2005; Fisher et al., 1996), it is apparent that chemically castrating with zinc heightens these symptoms.

The impact of castration method during the backgrounding period was also measured and reported (Table 3). Similar to the intensive sampling, there were no differences in BW between castration type for the duration of the 140-d backgrounding period ($P \geq 0.39$); yet, differences in ADG were observed. Calves chemically castrated with zinc had greater ADG compared to banded calves from d 0 to 28 ($P = 0.03$) and at the conclusion of the backgrounding period from d 113 to 140 ($P = 0.05$) and therefore had an overall greater ADG from castration through the backgrounding period ($P = 0.05$). The difference discovered one month post castration might, in

part, be explained by compensatory gain. Calves chemically castrated with zinc lost more weight during the first week of the trial and therefore stood to gain more.

A similar study utilizing zinc castration in bull calves upon arrival to the feedlot found that there were no differences in BW in zinc castrated calves compared to bulls or banded calves until d 140 in which bulls and zinc castrated cattle were heavier than banded calves (Ball et al., 2018b) and continued this trend until the conclusion of the finishing phase. Despite the differences found on d 140 in the feedlot study and those thereafter, the lack of variation in BW until that point is similar to the findings in the current study and might suggest that calves chemically castrated with zinc could potentially enter the feedlot at a heavier weight. Moreover, in support of our findings, the finishing study determined that zinc castrated calves and bulls had greater ADG compared to those that were banded throughout the finishing phase. The results from the finishing phase and the supporting evidence from the current study would implicate that castrating calves with an injectable zinc solution might improve growth performance.

Acute Phase Response

Haptoglobin was measured to depict the inflammatory response associated with castration method (Figure 1). There was an interaction between treatment and time ($P < 0.01$) where Z calves had greater levels of serum Hp compared to B calves on days 1, 2, 3, 7 and 28. Serum Hp levels in Z calves were elevated by d 1 ($P < 0.01$) and peaked on d 2 and 3 (8,400,066 and 6,465,119 mg/dL, respectively) and began to decline through d 28 until d 56 in which basal concentrations were reached ($P = 0.67$). Concentrations for B calves did not differ at any time point ($P \geq 0.41$).

Haptoglobin is an acute phase protein that elevates rapidly during tissue injury (Conner and Eckersall, 1988) and is therefore a favorable indication of pain and stress. The findings in this study indicate a sizable inflammatory response in zinc castrated calves; one resulting in a 56-d

recovery period. Bull calves injected with zinc upon arrival to the feedlot had greater Hp levels compared to intact males and banded bulls (Ball et al., 2018b), however, the concentrations in the feedlot study were drastically numerically lower compared to Z haptoglobin concentrations in the current study. Specifically, it was reported that peak concentrations in zinc injected calves (d 3) was 1,404,047 mg/dL. Together, the studies contradict most findings claiming that castrating at a younger age negates the issues regarding inflammatory stress (Fisher et al., 2001).

In addition to Hp, the expression of proinflammatory cytokines IL1$_\beta$, IL6 and TNFα were measured (Table 4). These cytokines initiate the acute phase response and aid in the synthesis of anti-inflammatory proteins. Interleukin-1 is responsible for activating the pituitary-adrenal system (Gruys et al., 2005) and promotes lymphocyte proliferation (Kampschmidt and Pulliam, 1975). Tumor necrosis factor α signal lymphocytes to the site of infection aid in cell death (Zelová and Hošek, 2013). Finally, IL-6 plays a major role in the mediation of hepatocytic secretion of a majority of the acute phase proteins (Heinrich et al., 1998).

There was no effect for treatment ($P \geq 0.56$) for any of the cytokine expressions, nor was there an effect for day ($P \geq 0.71$) or treatment x day interaction ($P \geq 0.83$). The lack of difference in gene expression might be due to sample size (n = 4 per treatment) and the sampling dates. Samples were collected on d 0, 7 and 28. Although levels of serum Hp were elevated on d 28, they were near basal levels. Differences in cytokine expression might have differed during peak Hp response times such as d3. While additional studies did not find differences in cytokine response to various castration methods (Moya et al., 2008; Pang et al., 2011), it is difficult to compare the findings in this study to previous research due to the long time periods between sampling. Most relevant research collected samples over the course of a few days post castration.

Castrated associated immune response was further analyzed by measuring the concentration and proportions of leukocytes (Table 5). With the exception of monocyte concentrations, there was a treatment x time interaction detected for all blood cell parameters ($P \leq 0.05$). Concentrations and proportions of leukocytes were calculated and reported.

Total white blood cell concentrations (WBC) were similar on d 0 ($P = 0.82$). Post castration, Z calves had elevated WBC on d 1, 2 and 3 ($P \leq 0.02$) compared to B calves until d 7 ($P = 0.36$) and remained similar by d 28 ($P = 0.33$). Concentrations in banded calves were similar to basal measurements prior to castration until d 28 ($P < 0.01$). Likewise, WBC also fell below base line concentrations by d 28 ($P = 0.05$); however, unlike banded calves, Z calves had elevated WBC from d 0 on d 1 and 2 ($P < 0.01$) and began to decline on d 3 through d 7 ($P < 0.01$).

The concentration of neutrophils followed a similar pattern observed for total WBC. Concentrations did not differ at castration ($P = 0.75$). After castration, Z calves had elevated concentrations of neutrophils on d 1, 2 and 3 ($P \leq 0.02$) compared to B calves until d 7 ($P = 0.37$). Banded calves experienced a small spike in neutrophil concentration on d 1 ($P = 0.04$) but quickly declined to d 0 measurements by d 2 ($P = 0.26$) and fell below basal measurements on d 28 ($P < 0.01$). Calves injected with zinc had increased levels of neutrophils on d 1, 2 and 3 ($P < 0.01$) where concentrations peaked on d 1 and 2 and began to decline on d 3 until basal concentrations were met on d 7 ($P = 0.41$). Proportions of neutrophils followed the same trend for differences in castration over time that was observed for neutrophil concentrations where B calves had lower concentrations of neutrophils on d 1, 2 and 3 compared to Z calves ($P \leq 0.01$) and were similar 1 wk post castration ($P = 0.78$).

A treatment x d interaction for lymphocyte concentrations ($P < 0.01$) was detected due to a decrease in Z calves compared to B calves on d 1 ($P < 0.01$). The concentrations of lymphocytes

for B calves did not differ throughout the first week post castration ($P \geq 0.54$) until d 28 in which concentrations fell below d 0 values ($P < 0.01$). Castrating with injectable zinc caused a reduction in the concentration of lymphocytes 1 d post castration ($P < 0.01$) but returned to basal values by d 2 and did not change ($P \geq 0.12$) until d 28 ($P < 0.01$) where again, concentrations were less than d 0. Despite the lymphocyte concentration similarities on d 2 and every sample date after, the proportion of lymphocytes differed between treatments not only on d 1, but on d 2 and 3 ($P \leq 0.01$).

A neutrophil to lymphocyte ratio (**NLR**) is a calculated measurement of animal stress (Kulberg et al., 2002) where a higher NLR value is an indication of an animal experiencing inflammation. Zinc castrated calves had greater NLR ratios ($P \leq 0.01$) the first 3 d post castration but were similar after 1 wk ($P = 0.55$). Ratios for the 1 mo period post castration did not differ for B calves ($P \geq 0.14$) which was different from ratios observed for Z calves where NLR increased on d 1 ($P < 0.01$) and continually increased until d 3 in which NLR values peaked ($P < 0.01$). After 1 wk, NLR values were similar to pre-castration values ($P = 0.59$).

Although monocytes, eosinophils and basophils make up a small percentage of total white blood cells, they play an important role in proinflammatory signaling and serve as inflammatory mediators (Schwartz et al., 2016) and are therefore worth recognizing. There was no interaction between castration method over time for monocyte concentrations ($P = 0.12$); however, there was an interaction ($P < 0.01$) for monocyte proportions where B calves had greater proportion on d 1 compared to Z calves ($P = 0.02$).

Eosinophil concentrations were greater in Z calves compared to B calves the first two days after castration ($P \leq 0.04$). Concentrations in B calves did not differ from pre-castration values until d 7 in which fewer eosinophils were detected. Concentrations reached d 0 values by d 28 ($P = 0.77$).

Eosinophil concentration trend for Z calves during the first month post castration varied from what was observed for B calves. A surge in eosinophil concentration occurred on d 1 and 2 ($P < 0.01$) and declined to initial concentrations by d 3 ($P = 0.60$). However, a drop in eosinophils was observed on d 7 ($P = 0.04$) and were similar to d 0 finding by d 28 ($P = 0.22$).

Proportion and concentrations of basophils were similar to results described for eosinophils where the percentage of basophils was greater for Z calves compared to B calves on d 1 ($P < 0.01$). Furthermore, concentrations on d 1 and 2 were greater for Z calves compared to B calves ($P \leq 0.01$). Basophil concentrations fell below initial measurements on d 7 for banded calves ($P = 0.05$) and remained lower through d 28 ($P = 0.04$). Concentration values for Z calves during the first month post castration was elevated on d 1 and 2 compared to pre-castration concentrations ($P < 0.01$) and declined to basal levels by d 3 ($P = 0.83$) and remained similar through d 28 ($P \geq 0.06$).

The complete blood analysis revealed elevated concentrations and proportions of leukocytes sometime within the first 3 d post castration. Especially within the zinc castrated calves. Although the duration was not as chronic as Hp, the spike in leukocyte concentrations within the first 3 d coincides with the elevation and peak values described for Hp on d 2 and 3. Since white blood cells play numerous roles in inflammation as both proinflammatory and anti-inflammatory mediators, it is not surprising that all variables described for complete blood analysis followed a similar pattern found in Hp concentrations. The differences found between castration methods and the changes in physiological responses shortly after castration is well understood (Coetzee, 2011). Previous studies have also reported leukocyte concentrations peaking around d 2, regardless of castration method (Chase et al., 1995; Pang et al., 2011) which were similar to our findings and would suggest that the response to castration in the current study, regardless of whether calves were banded or chemically castrated with zinc, would be a normal and expected response.

However, it is important to note that the differences detected between treatments within the first 3 d post castration were due to elevated levels in Z calves compared to B calves, which would suggest that not only does castrating induce inflammation, but that castrating with an injectable zinc solution exacerbates the response compared to conventional methods of castration. Furthermore, several studies have shown that the inclusion of an analgesic at the time of castration has subsided the castration associated inflammatory response (Brown et al., 2015; Chase et al., 1995; Pang et al., 2011). Although the inflammatory response in zinc injected cattle followed the trend reported for conventionally castrated calves, the elevated inflammatory response compared to banding might warrant the inclusion of a pain mediating tactic.

Motion Index

Behavior for the current study was defined as the proportions of time that the animal spent either lying flat on its side (Figure 2), standing (Figure 3) or lying on its sternum (Figure 4). There was a treatment x time interaction for all behavioral assessments ($P < 0.01$). One day post castration, Z calves spent a greater proportion of time ($P = 0.03$) lying flat on their side compared to B calves until days 3 and 4 in which B calves spent more time on their side than Z calves ($P \leq 0.02$). Proportions of time standing and lying on sternum appeared to be inversely related. On days 1 and 2, Z calves tended to spend more time on their sternum ($P = 0.07$ and 0.08, respectively) while B calves spent more time standing ($P \leq 0.01$). By days 3, 4, 5, and 6, B calves spent more time lying on their sternum ($P \leq 0.03$) while Z calves spent more time standing ($P \leq 0.01$) until d 7 in which no difference was detected between castration treatments for time spent lying on sternum ($P = 0.73$) or standing ($P = 0.88$).

Calves lying flat on their side with their legs extended has been described as a possible behavior response to pain (Robertson et al., 1994). The spike in proportion of time lying flat on

their side exhibited by Z calves might explain the 7 kg drop in BW detected 1 d post castration. Although there were no differences due to castration method ($P = 0.11$), an effect of time was detected ($P < 0.01$) where more time was spent lying laterally on d 1 and declined through d 3 ($P < 0.01$) until d 4 which was similar to the amount of time spent lying laterally on d 1 ($P = 0.09$). The increase in time lying laterally on d 4 might be explained by the increase in B calves lying laterally on that day. However, the trend over time illustrates the similarity between castration methods. Neither was really advantageous over the other in regards to time spent lying laterally in pain.

It is difficult to determine whether standing or lying time was due to pain rather than content. Standing might be an indication of grazing time or time spent motionless due to discomfort. Behaviors assessed after traditional methods of castration (banding or surgical) are well established and report that standing time post castration is generally increased due to pain (Coetzee, 2011). The time spent standing on days 1 and 2 by B calves coincides with past reports. The time spent standing on days 3 through 6 for Z calves might be a delayed response to pain since standing and lying laterally appear to be inversely proportionate on d 1 and 2; meaning that the acute pain response from chemically castrating with zinc resulted in the animal lying flat on their side while the period following that was spent standing and was more indicative of chronic pain. Additionally, the proportion of time that Z calves spent standing peaked numerically on d 3 which was also a period in which BW decreased by 0.1 kg and when serum Hp concentrations were numerically greatest.

Testicular Thickness

Due to testicular loss in banded calves, testicular thickness was measured on zinc injected calves only throughout the backgrounding period (Figure 5). Testicular width in Z calves differed

over time ($P < 0.01$). One d post castration, testicular measurements increased ($P < 0.01$) and continued to increase on d 2 ($P = 0.04$). Testicular thickness peaked on d 2 and 3 ($P = 0.30$) where values on d 3 were numerically greatest on d 3. Width values began to decline one week post castration, plateaued on d 28 and declined again until d 84 ($P < 0.01$). Measurements were similar to those taken at the time of castration on d 113 ($P = 0.67$) and remained unchanged through d 140.

The changes in testicular thickness over time are directly related to the inflammatory response described for Z calves. The greatest width values were recorded on d 2 and 3 which was also described in the leukocyte response. Haptoglobin concentrations were also greatest on d 2 and 3 where d 3 was numerically greatest compared to all other time points. While the concentrations in white blood cells subsided much quicker than testicular thickness, Hp was elevated for an extended period (d 56). The prolonged period of Hp levels coupled with the extended period in which testicular width was greater than castration levels, would suggest that injecting calves intratesticularly with zinc induces chronic tissue inflammation.

Similar results were described for bull calves that were injected with zinc at feed yard entry (Ball et al., 2018b). The study compared injected calves to calves that were banded and bulls. The study found that injecting with zinc caused and increase in testicular thickness compared to either treatment for the first month post castration (Ball et al., 2018b). While testicular thickness was smaller than bulls by d 56, the study did not report when baseline measurements were met in injected calves only. Other studies have used canines and felines as a model to test the efficacy of zinc gluconate. The findings reported in these investigations claimed that intratesticular injections atrophied the testis; however, the use of zinc gluconate reportedly caused a decrease in testicular

thickness (Fagundes et al., 2014; Fahim et al., 1993a; Oliveira et al., 2013; Oliveira et al., 2007) which cannot be said for the current study.

Serum Testosterone

Testosterone was measured throughout the backgrounding period (Figure 6) to compare castration methods and their impacts on hormone concentrations over time. There was an effect of treatment ($P < 0.01$), time ($P < 0.01$), and an interaction between treatments over the backgrounding period ($P < 0.01$). Although it cannot be explained, there was a difference in testosterone concentrations at the time of castration ($P = 0.02$) where Z castrated calves had greater serum testosterone levels compared to banded ones (0.66 vs. 1.09 ng/mL). At one week post castration, serum testosterone levels for B calves were undetectable and by d 28, all testis had been removed as a result of banding; therefore, differences between treatments were greater in Z calves compared to B calves at every time point throughout the backgrounding period ($P < 0.01$). Concentrations for Z calves were numerically lower 1 wk post castration but did not differ ($P = 0.23$). Serum testosterone levels by d 28 had fallen below levels measured on d 0 ($P < 0.01$) but increased to similar levels by d 56 ($P = 0.08$). Concentrations did not differ between initial measurements again until d 140 in which testosterone had dropped below d 0 measurements ($P < 0.01$).

The initial fall in serum testosterone concentrations observed on d 28 might be due to the chronic state of inflammation and tissue swelling which might have hindered testosterone production. However, the drop on d 140 could not be explained by testicular inflammation and might allude to testicular damage and possibly atrophy; however, this is clearly speculation and cannot be determined conclusively in the current study. The feedlot study also observed a drop in testosterone in injected calves, but similar to our findings, testosterone was restored and were

similar to levels found in bulls (Ball et al., 2018b). A preliminary study that chemically castrated near birth indicated that serum testosterone levels were similar to those that were surgically castrated which would suggest that castrating with zinc is more efficacious in younger calves (Ball et al., 2018a) Like other chemical castration studies, the results described in the current investigation for testosterone are not favorable to conventional castration methods (Cohen et al., 1990) or decline at a much slower rate (Feher et al., 1985).

IMPLICATIONS

There were no advantages or adverse effects overall of a novel zinc injectable castration method on growth performance. However, the process caused changes in behavior as exhibited by longer standing times which can be an indication of discomfort. Although this behavior is typical post castration, the amount of time spent standing within the first week after castration was longer than banding. Additionally, the acute inflammatory response was substantial with high levels of serum Hp and leukocyte concentrations. The acute inflammation became chronic as Hp levels did not subside until d 56 and testicular width remained above initial measurements until d 113. Finally, testosterone was not eradicated by injecting zinc. In this investigation, the combined results for the backgrounding period indicate that chemically castrating with zinc was not a suitable alternative to commonly used castration methods around weaning. The process resulted in greater inflammation and stress compared to conventional methods.

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Table 1. Analyzed nutrient composition of diets (DM basis) in the backgrounding phase.

Item	SBH[1]	Forage	Hay[2]
NDF, %	67.99	68.06	68.99
ADF, %	48.83	31.38	31.43
CP, %	10.69	15.38	13.13
Ash, %	5.13	7.93	6.85
Ca, %	0.64	0.47	0.49
P, %	0.10	0.37	0.39
Mg, %	0.23	0.20	0.36
K, %	1.17	1.52	1.91
S, %	0.09	0.26	0.25
Fe, ppm	393	237	123
Mn, ppm	26	96	97
Z, ppm	44	206	94
Cu, ppm	7	16	9

[1]Soybean hull pellets were supplemented 1.36 kg/hd/d while housed in grass traps and supplemented at 0.5% of BW for the remainder of the backgrounding phase.

Table 2. Effects of castration method at one week post weaning on growth performance in beef calves one week after castration.

	Treatment[1]			
Item	B	Z	SEM[2]	*P* - Value
BW, kg				
Day -7, Weaning	236	229	7.0	0.51
Day 0	243	239	7.3	0.71
Day 1	246	232	7.1	0.17
Day 2	251	236	8.0	0.18
Day 3	253	236	7.7	0.11
Day 7	254	240	7.4	0.22
Chang in BW, kg				
Day -7 to 0	7.4	10.3	1.9	0.31
Day 0 to 1	2.9[a]	-7.3[b]	2.6	<0.01
Day 1 to 2	4.8	4.6	2.2	0.31
Day 2 to 3	2.2[a]	-0.1[b]	0.7	0.03
Day 3 to 7	0.8	4.2	2.0	0.26
Overall	10.6[a]	1.5[b]	2.1	0.04

[1]B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis.
[2]Pooled standard error of the mean.
[a-b] Means within a row without common superscript differ ($P \leq 0.05$).

Table 3. Effects of castration method one week post weaning on growth performance during a 140-d backgrounding phase in beef calves.

	Treatment[1]			
Item	B	Z	SEM[2]	*P* - Value
BW, kg				
Day -7, Weaning	234	231	4.5	0.60
Day 0	245	244	4.7	0.81
Day 28	263	267	6.6	0.60
Day 56	269	272	4.9	0.61
Day 84	289	293	5.7	0.55
Day 113	297	300	5.7	0.64
Day 140	297	303	5.2	0.39
ADG, kg				
Day -7 to 0	1.6	1.8	0.19	0.36
Day 0 to 28	0.63[b]	0.81[a]	0.09	0.03
Day 28 to 56	0.21	0.18	0.07	0.81
Day 56 to 84	0.71	0.75	0.05	0.76
Day 84 to 113	0.30	0.26	0.04	0.56
Day 113 to 140	-0.04[b]	0.10[a]	0.05	0.05
Overall	0.37[b]	0.42[a]	0.02	0.05

[1]B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis.

[2]Pooled standard error of the mean.

[a-b] Means within a row without common superscript differ ($P \leq 0.05$).

Table 4. Effects of castration method one week post weaning on acute phase gene expression.

	Treatment[1]							*P* - Value		
	B			Z						
Item[3]	D0	D7	D28	D0	D7	D28	SEM[2]	TRT	Day	TRT x Day
$IL1_{\beta}$	1.03	1.02	1.07	1.07	1.14	1.19	0.25	0.65	0.95	0.98
IL6	1.07	1.25	1.03	1.05	1.54	1.35	0.40	0.56	0.71	0.90
TNF_{α}	1.17	1.22	1.01	1.03	1.09	1.19	0.29	0.89	0.97	0.83

[1] B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis.

[2]Pooled standard error of the mean for treatment by day interaction.

[3]Gene expression was measured using real-time q-PCR and the relative expression was determined by $2^{-\Delta\Delta Ct}$ method.

Table 5. Effects of in injectable zinc solution post weaning on the innate immune response.

	Treatment[1]													*P* - Value		
	Banded						Zinc									
Item	D0	D1	D2	D3	D7	D28	D0	D1	D2	D3	D7	D28	SEM	TRT	Time	TRT x Time
Concentration, K/µl																
White Blood Cells	9.58	10.60	10.37	10.12	9.47	6.80	9.37	16.06	15.55	12.55	8.60	7.75	0.64	0.08	<0.01	<0.01
Neutrophil	3.59	4.58	4.13	4.06	3.93	2.53	3.36	10.95	9.83	7.30	3.09	2.86	0.63	0.18	<0.01	<0.01
Lymphocyte	5.38	5.40	5.58	5.68	5.22	3.70	5.46	3.89	4.79	4.78	5.10	4.37	0.30	0.31	<0.01	0.01
Monocyte	0.32	0.30	0.30	0.21	0.19	0.29	0.31	0.24	0.29	0.27	0.25	0.25	0.04	0.79	0.02	0.12
Eosinophil	0.25	0.29	0.32	0.15	0.11	0.27	0.21	0.85	0.55	0.17	0.13	0.25	0.05	0.26	<0.01	0.01
Basophil	0.03	0.04	0.04	0.02	0.01	0.01	0.03	0.13	0.09	0.03	0.03	0.01	0.01	0.21	<0.01	0.01
N:L Ratio	0.69	0.91	0.79	0.76	0.77	0.74	0.63	3.03	2.08	1.58	0.62	0.65	0.17	0.18	<0.01	<0.01
Proportions, %																
Neutrophil	37.39	42.12	39.18	40.12	39.88	37.38	35.77	67.18	62.26	57.83	36.08	35.90	3.61	0.17	<0.01	<0.01
Lymphocyte	56.43	51.96	54.63	56.07	56.84	54.36	58.25	25.25	31.69	38.46	59.67	57.48	3.92	0.19	<0.01	<0.01
Monocyte	3.33	2.91	2.91	2.09	1.99	4.23	3.27	1.54	1.96	2.18	2.72	3.32	0.34	0.32	<0.01	<0.01
Eosinophil	2.49	2.63	2.93	1.53	1.15	3.83	2.22	5.21	3.46	1.33	1.32	3.17	0.41	0.40	<0.01	0.02
Basophil	0.35	0.35	0.36	0.18	0.14	0.20	0.33	0.82	0.61	0.21	0.21	0.13	0.87	0.24	<0.01	0.05

[1] B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis.

[2]SEM = pooled standard error of the mean for treatment x time interaction.

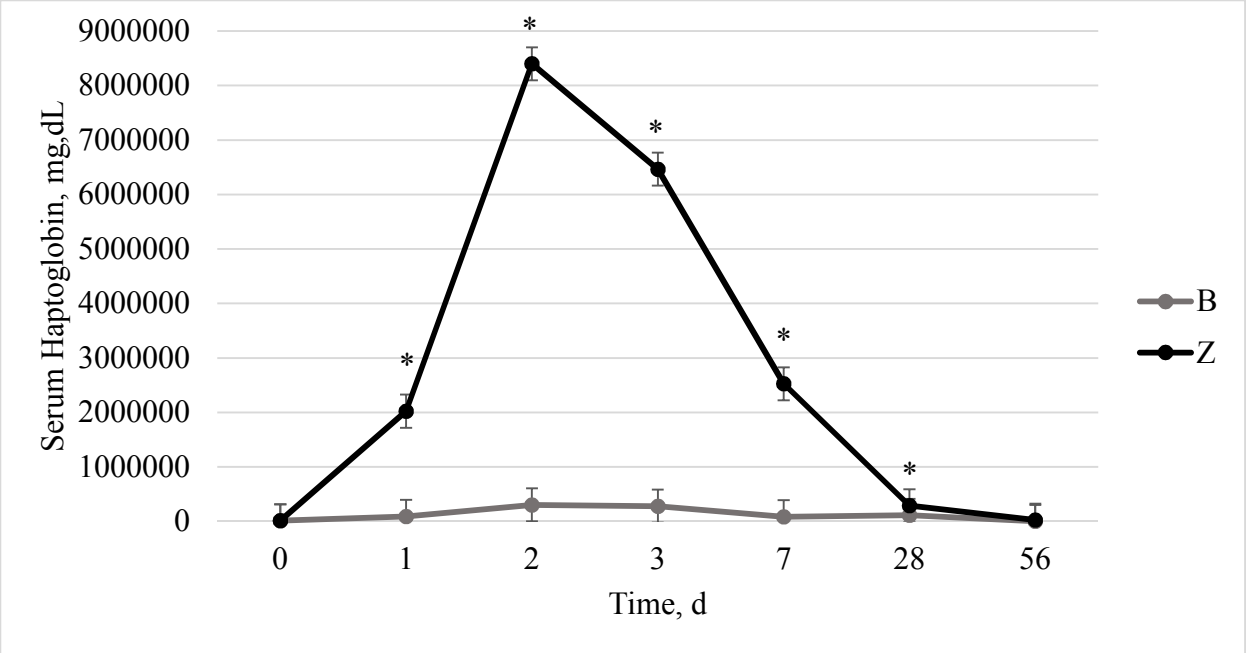

Figure 1. Effects of castration method one week post weaning on serum haptoglobin concentrations. B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of treatment ($P < 0.01$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from B calves.

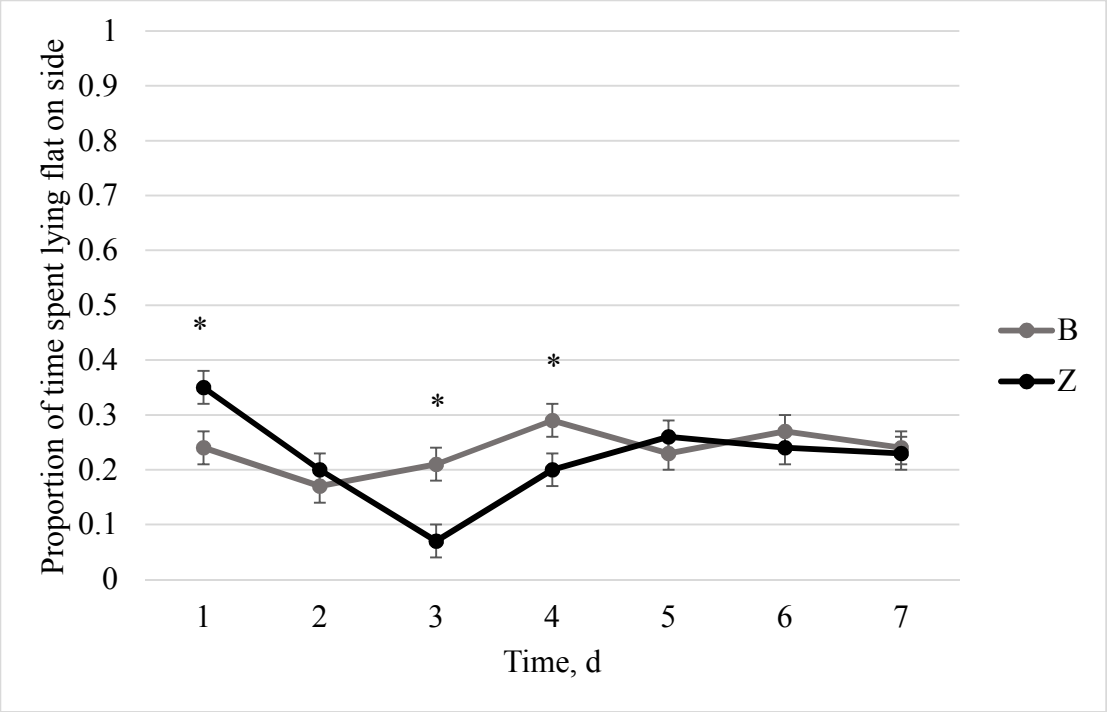

Figure 2. Effects of castration method one week post weaning on proportions of time lying flat on side. B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of treatment ($P = 0.11$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from B calves.

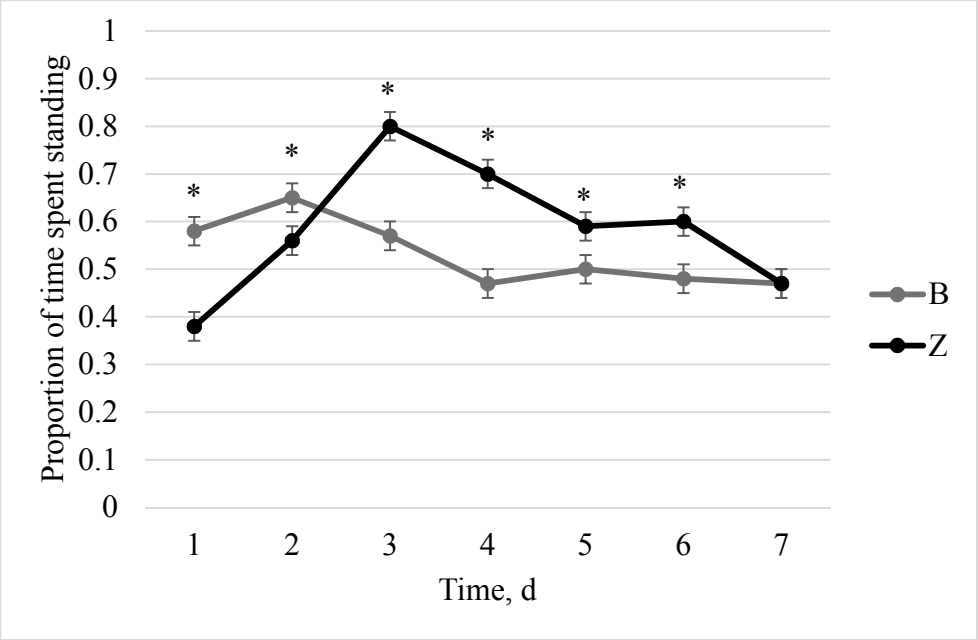

Figure 3. Effects of castration method one week post weaning on proportions of time standing. B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of treatment ($P < 0.01$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from B calves.

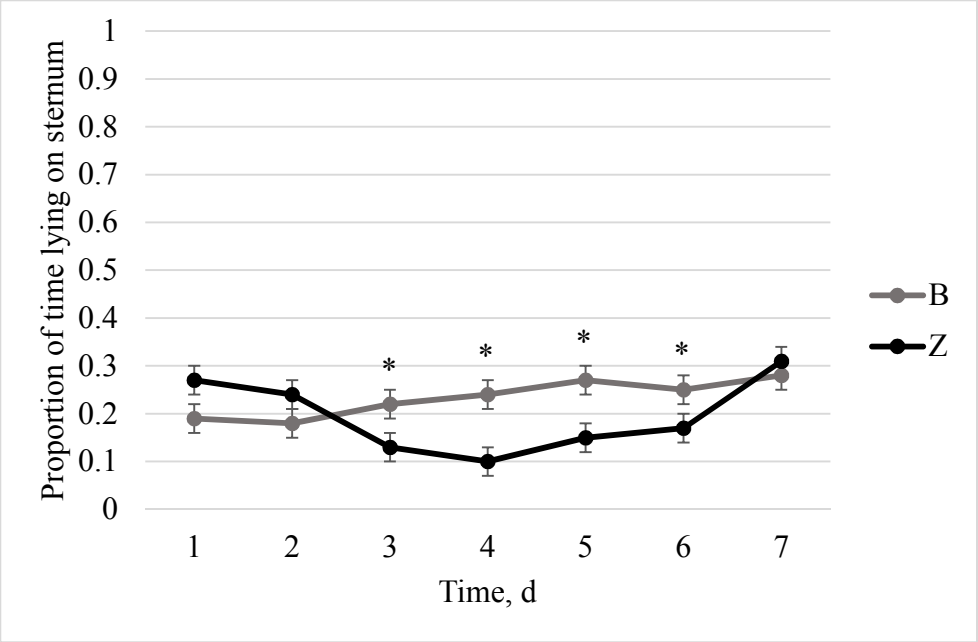

Figure 4. Effects of castration method one week post weaning on proportions of time spent lying on sternum. B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of treatment ($P < 0.01$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from B calves.

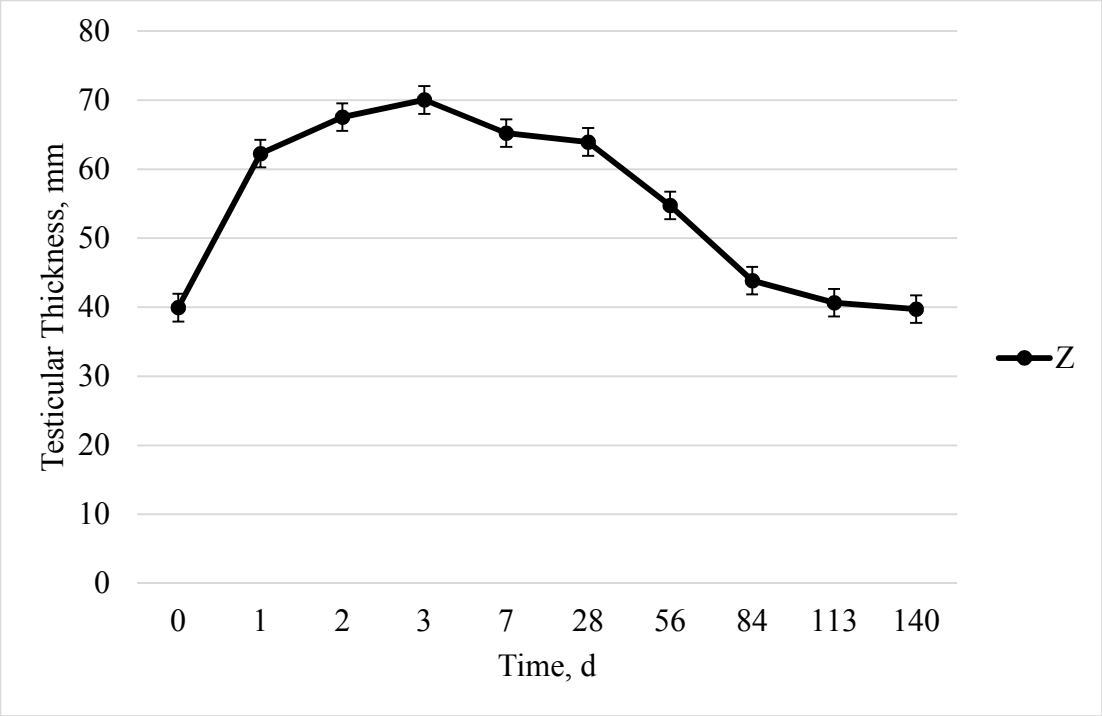

Figure 5. Effects of castration method one week post weaning on proportions on testicular thickness of Z calves. Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of day ($P < 0.01$).

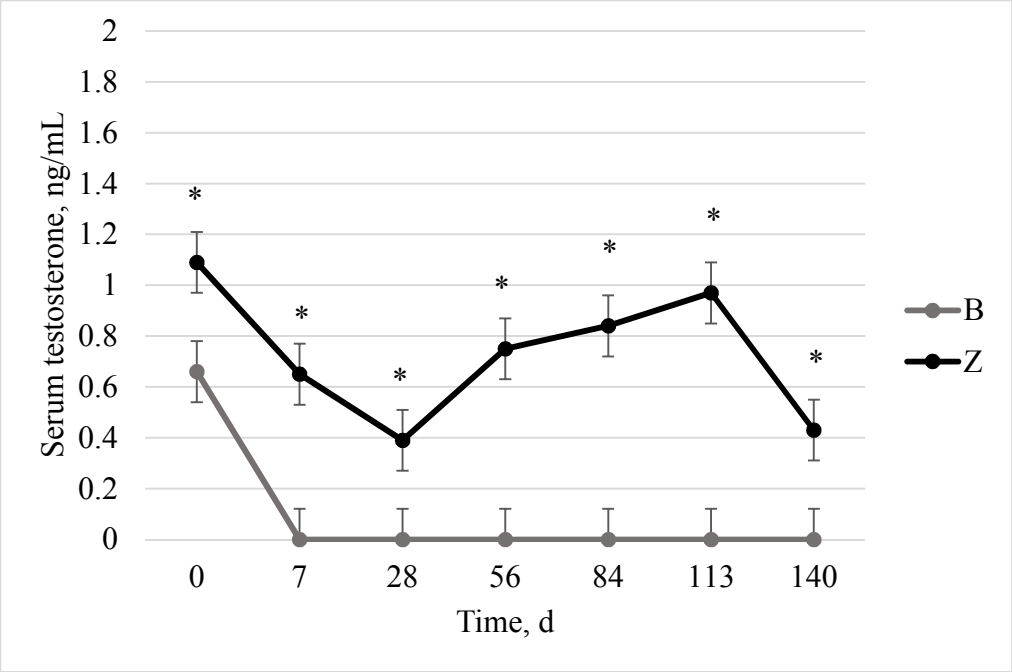

Figure 6. Effects of castration method one week post weaning on serum testosterone concentrations. B = blood-restrictive rubber band was placed around the dorsal aspect of the scrotum, Z = calves that received a 1 mL intratesticular injection of a zinc solution (100 mg Zn) in both testis. Effect of treatment ($P < 0.01$), day ($P < 0.01$), and treatment x day ($P < 0.01$) were detected. *Indicates Z differ from B calves.

CHAPTER VI

CONCLUSIONS

Castrating with an intratesticular injection of zinc did not impact intensive BW measurements when compared to opposing treatments in either trail. There was a difference between the change in BW within the first week when zinc castrated calves were compared to banded ones where banded calves gained more one week post castration. The similarity in intensive BW measurements and the change in weight in the first experiment might be explained by weaning behavior. More specifically, it is possible that BW changes in those surgically castrated at birth did not differ from calves castrated with zinc at weaning because surgically castrated calves exhibited feeding behaviors indicative of weaning stress while the second study castrated one week post weaning which would have mitigated those behaviors by the time calves were castrated. While there were no differences during the backgrounding period in either trail until the end, when measured throughout the finishing phase, those castrated with zinc had heavier BW and subsequently produced heavier carcasses with a larger area of lean muscle.

Although castrating with zinc did not negatively impact BW in either trial proved to be advantageous during a finishing phase, both investigations indicated an increased inflammatory response as indicated by elevated concentrations of leukocytes and haptoglobin when compared to either surgically castrated calves at birth or those banded at weaning, along with an acute increase in testicular thickness. Additionally, both studies found that castrating with zinc resulted in behaviors indicative of pain.

Finally, castrating with zinc did not sterilize calves. Testosterone was temporarily reduced in both studies in calves that received and injection of zinc, but once the inflammation subsided,

testosterone concentrations were elevated while the opposing treatments seized to produce detectable concentrations. Therefore, the findings in the current studies would not suggest that castrating with and intratesticular injection of zinc would not only be ineffective in the age of calves tested, but possibly induces more pain and stress compared to traditional castration methods.

CHAPTER VII

APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Jeremy Powell
Fr:	Craig Coon
Date:	August 10th, 2018
Subject:	IACUC Approval
Expiration Date:	August 3rd, 2020

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **19009:** *THE EFFECTS OF AN INJECTABLE ZINC SOLUTION AS AN ALTERNATIVE CASTRATION METHOD IN BEEF BULLS AT WEANING.*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond August 3rd, 2020 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Jermemy Powell, Beth Kegley, Brandon Stewart, Cody Shelton, and Reagan Cauble. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Jeremy Powell
Fr:	Craig Coon
Date:	May 6th, 2019
Subject:	IACUC Approval
Expiration Date:	June 3rd, 2020

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol #**19089:** *THE EFFECTS OF AN INJECTABLE ZINC CASTRATION SOLUTION COMPARED TO BANDING CASTRATION IN BEEF BULLS AT WEANING.*

In granting its approval, the IACUC has approved onlythe information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond June 3rd, 2020 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Jeremy Powell, Beth Kegley, Toby Lester, Pete Hornsby, and Regan Cauble. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp