EFFECT OF AGE AND CASTRATION METHOD ON NEUROHORMONAL, AND
ELECTROENCEPHALOGRAPHIC STRESS INDICATORS IN HOLSTEIN CALVES

by

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# Abstract

As public concern for food animal welfare increases, the need for objective pain assessment and methods to alleviate pain associated with production practices such as castration gains attention. The aims of this study were (i) to evaluate the physiological response to pain induced by castration in calves and (ii) to elucidate age-related differences in pain response of calves subjected to different castration methods. Seventy six Holstein bull calves were blocked by age ($\leq$ 6 weeks and $\geq$ 6 months) and randomly assigned to one of four treatment groups: control (n = 20), castration by banding (n = 18), cut and clamp surgical castration (n = 20), and cut and pull surgical castration (n = 18). Measurements included electroencephalogram, heart rate variability, infrared thermography, electrodermal activity, and concentrations of serum cortisol, and plasma substance P prior to, during, and following castration. Electroencephalogram recordings showed desynchronization for all treatments, consistent with increased arousal; yet the magnitude of desynchronization was greatest for 6-month-old calves castrated by cut and clamp. Additionally, older calves in the cut and pull group showed greater desynchronization than younger calves in the same group. Based on the heart rate variability analysis, 6-month-old calves in the control or cut and pull castration groups showed greater sympathetic tone than younger calves in the same treatment groups. Overall, younger calves showed lower electrodermal activity than older calves. Regardless of treatment, concentrations of cortisol and plasma substance P were greater in 6-month-old calves relative to their younger counterparts. In summary, neurohormonal and electroencephalographic stress responses of calves to castration were age-specific. Castration by cut and clamp showed the most pronounced stress response in 6-month-old calves.

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# Acknowledgements

# Dedication

This thesis is dedicated to my friends, family, and fellow graduate students, whose love and support made the rough ride smoother. I would also like to dedicate this thesis to my committee, whose patience and guidance made this feat possible.

# Chapter 1 - Literature Review

## Attitudes toward Food Animal Welfare

The American Veterinary Medical Association defines an adequate state of welfare as "healthy, comfortable, well nourished, safe, able to express innate behavior, and not suffering from unpleasant states such as pain, fear, and distress" (AVMA, http://www.avma.org). Society is becoming increasingly concerned with the way food animals are treated, evidenced by several survey results and ballot initiatives. As interest in animal welfare increases, the livestock industry becomes responsible for meeting the public's expectation of a more humanely-raised food supply (Thornton, 2010).

A survey on the well-being of farm animals conducted by Oklahoma State University asked respondents to agree or disagree with three statements: "low meat prices are more important than the well-being of farm animals"; "I consider the well-being of farm animals when I make decisions about purchasing meat"; and "it is important to me that animals on farms are well cared for" (Lusk and Norwood, 2010). Only 15.6% of those surveyed responded "agree" or "strongly agree" to the first statement, while 48.7% and 95% respectively agreed with the second two statements (Lusk and Norwood, 2010). These results indicate people may be willing to pay an increased price for meat from humanely-raised animals. However, a survey such as this may be limited by respondents' unwillingness to appear insensitive or cruel (Lusk and Norwood, 2010).

Recently several states have begun adopting animal welfare legislation, especially for livestock. A good example is California's Proposition 2, the "Standards for Confining Farm Animals Initiative Statute" (California General Election, http://www.voterguide.sos.ca.gov).

Prop 2 was passed in 2008 with 63% of voters in favor. This law mandates animals must be confined in a way that allows them to stand up, lie down, turn around, and stretch their limbs freely (California General Election, http://www.voterguide.sos.ca.gov). Failure to abide by this law may result in a fine and possible prison sentence (California General Election, http://www.voterguide.sos.ca.gov). Legislative analysts determined this bill could potentially cost the state of California millions of dollars in lost revenue (California General Election, http://www.voterguide.sos.ca.gov). Although the financial impacts were severe, the population chose to vote in favor of what they believed to be improved animal welfare.

As the world becomes concerned about the way food animals are raised, producers and veterinarians must adopt inventive strategies to satisfy the public while still maintaining good animal husbandry. According to the Animal Behavior and Well-Being Symposium, "successful integration of [the] best animal care practices into the farming community begins with a set of well-researched, scientifically and ethically valid, and practical set of standards that meet the approval of producers and the expectations of the public, and it ends with the accurate characterization and reporting of on-farm compliance" (Rushen et al., 2011). Therefore, objective ways to measure pain are becoming more important to help create guidelines and legislation aimed at improving welfare while keeping expenses within reason (Underwood, 2002).

## Pain Assessment in Domestic Animals

Pain is defined as "an aversive feeling or sensation associated with actual or potential tissue damage resulting in physiological, neuroendocrine, and behavioral changes that indicate a stress response" (Molony and Kent, 1997). The study of pain in animals has the potential to improve welfare and accuracy of research, and may have human pain control implications as

well (Mogil, 2009 and Mayer, 2007).  One of the major welfare concerns involving domestic animals is the performance of husbandry procedures such as castration, branding, and dehorning without analgesia (analgesia is defined by the IASP as the "absence of pain in response to stimulation which would normally be painful", IASP, http://www.iasp-pain.org). If it is assumed that animals perceive pain in a similar way to humans, these practices become particularly worrying (Lascelles and Main, 2002).  Pain is often very difficult to assess in animals because exhibiting a behavioral abnormality in nature may result in nonsurvival (Underwood, 2002). However, pain is an inevitable consequence of several food animal husbandry procedures, and thus reliable pain assessment is worthy of investigation.

A common sign of pain in animals is a change in the normal behavioral pattern (Association of Veterinary Teachers and Research Workers, 1989). The main benefit of using behavioral responses to quantify pain is the fact that behavior changes are immediate, unlike physiological and biochemical responses which may take time to rise in response to a stimulus (Mellor et al., 1997; Mellor et al., 2000). In addition, behavioral parameters may be collected without inducing further pain or distress by using a video camera or some other device that minimizes human contact. This may be unavoidable while collecting physiological or biochemical data (Mayer, 2007). A pain scale adapted from the American Society of Anesthesiologists attempts to measure pain in animals more objectively by placing numbers on several variables including behavioral response (1=depressed, 3=normal, 5=apprehensive, 15=aggressive) (Muir and Birchard, 1997). Although the use of this scale is an improvement over simply watching the animal to decipher pain presence and level, it still relies upon a human operator and therefore has some level of observer variation and may be subject to bias (Anil et al., 2002). Behavior observation can be a powerful indicator of pain, but it does not offer

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concrete evidence of a noxious experience and it may not directly correlate with the intensity of the experience (Lester et al., 1996 and Stafford et al., 1993).

Behavior analysis may be conducted more objectively by counting certain movements that may be associated with pain or distress, such as tail-flicks in cattle (Schwartzkopf-Genswein et al., 1997). A study conducted by Stubsjøen et al (2009) measured the frequency of vocalizations, lip-licking, and ear movements in sheep following the application of a forelimb tourniquet with and without pain control. The results of this study showed a marked decrease in lip licking (and therefore stress) in animals treated with flunixine and meglumine (Table 1.1; Stubsjøen et al., 2009).

Yet another way to assess behavior is the use of accelerometers, which have the ability to determine an animal's posture (laying down or standing up) (White et al., 2008). White et al. (2008) determined the accuracy of two-dimensional accelerometers by placing the devices on healthy calves and simultaneously videotaping the animals to observe behavior. The results established that accelerometers have a high degree of accuracy in determining posture (98.3%) (White et al. 2008). This study also examined behavioral differences between calves that underwent castration and controls, and found the castrated group to spend a significantly larger amount of time standing than the control group (White et al., 2008). Therefore, the researchers concluded that the accelerometer is a useful device for measuring behavioral changes in cattle (White et al., 2008).

Pain causes activation of the hypothalamo-pituitary-adrenal axis, an important neuroendocrine stress pathway (Cardo et al., 2011). The activity of this pathway can be measured by its end product, cortisol (Kent et al., 1997). Cortisol levels in the relaxed animal show a circadian rhythm with a peak in the early morning followed by a decline throughout the

day (Bosch et al., 2009). Stressful situations augment this basal secretion and cause episodic

peaks in cortisol concentrations (Bosch et al., 2009), making cortisol levels in the saliva, plasma,

serum, feces, or hair a useful stress indicator. Kent and colleagues (1993) investigated cortisol

levels following castration in lambs and calves and tail docking in lambs. The results showed

changes in cortisol which closely followed changes in posture and behavior, indicating a positive

correlation between increased cortisol levels and increased discomfort as illustrated in Figure 1.1

(Kent et al., 1993). Cortisol was also analyzed in dehorning studies that showed an increase of

cortisol levels in animals undergoing dehorning versus those submitted to simulated or sham

dehorning procedures (Taschke and Fölsch, 1997). However, since cortisol is related to the stress

response, it is not exclusively sensitive to pain (Herskin et al., 2004). A study conducted by

Herskin et al. (2004) examined plasma cortisol levels in dairy cattle that underwent various

stressful situations such as new neighbors or stalls, fixation by the head in the home stall, and

social isolation in new surroundings (Herskin et al., 2004). The results showed increases in

cortisol compared to controls for all three scenarios, as illustrated in Figure 1.2 (Herskin et al.,

2004). Since none of these instances induced pain it can be inferred that cortisol increases as a

result of stress, not pain. Therefore, increased cortisol levels do not necessarily confirm a

stimulus as painful.

The five cardinal signs of inflammation are rubor (redness), calor (heat), functio laesa

(loss of function), tumor (swelling), and dolor (pain) (Cunningham and Klein, 2007).

Haptoglobin is a prominent acute phase protein stimulated by pro-inflammatory cytokines

(Bence et al., 2008). Acute phase protein levels generally rise more than 25% in response to

inflammation, making haptoglobin an important indicator of inflammation (Bence et al., 2008).

Since inflammation is painful, measuring serum haptoglobin levels is indicative of both

inflammation and pain (Petherick et al., 2011). A study performed on pigs attempted to determine whether or not haptoglobin was a reliable measurement of inflammation (Eckersall et al., 1996). Turpentine was injected to stimulate an inflammatory lesion, and haptoglobin levels were subsequently measured (Eckersall et al., 1996). The results showed a more than two-fold increase in serum haptoglobin levels, thus identifying haptoglobin as an appropriate test of inflammation (Eckersall et al., 1996). Another study examined serum acute phase proteins in dairy cattle with hoof disease to attempt to determine serum markers for lameness (Kujala et al., 2010). However, Kujala and colleagues (2010) did not find a significant difference in haptoglobin concentrations between lame and control groups, as shown in Figure 1.3. Kujala et al. (2010) suggest a larger inflammatory response may be required in bovines to elicit a rise in serum haptoglobin concentrations.

Prostaglandin, a prostanoid product of the cyclo-oxygenase pathway of arachidonic acid metabolism, is also involved in the inflammatory response (Narumiya and Furuyashiki, 2011; Narumiya et al., 1999). Prostanoids were first recognized as contributors to inflammation in 1971 when aspirin-like compounds were shown to prevent inflammation by blocking their synthesis (Vane, 1971). Prostaglandin $E_2$ ($PGE_2$) is described as the primary pro-inflammatory prostanoid and an important component in pain processing in the spinal cord and peripheral tissues (Yaksh et al., 1999). In addition to contributing to the five cardinal signs of inflammation, prostaglandin $E_2$ has been implicated in wound healing (Manigrasso and O'Connor, 2010). A study performed on laboratory mice examined the effects of prostaglandin $E_2$ on fracture healing by knocking out the enzyme 5-lipoxygenase, which shunts the common substrate arachidonic acid away from prostaglandin $E_2$ synthesis and toward leukotriene synthesis (Manigrasso and O'Connor, 2010). The results showed excessive leukotriene synthesis to impair fracture healing, and mice with the

knockout gene possessed higher levels of prostaglandin $E_2$ and enhanced fracture repair

(Manigrasso and O'Connor, 2010). Prostaglandin $E_2$ is also thought to be involved in sensitizing

pain receptors, and as a result is the target of many analgesic and anti-inflammatory

pharmaceuticals (Li et. al., 2007). One such drug is meloxicam, a non-steroidal anti-

inflammatory found to inhibit prostaglandin $E_2$ production and thus reduce inflammation and

pain (Rainsford et al., 1997). Prostaglandin $E_2$ may be used as an indicator of not only pain and

inflammation, but also of the body's attempts to heal wounds.

Substance P (SP) is a modulator of nociception, and is involved in processing painful

stimuli in the central nervous system (DeVane, 2001). This neuropeptide is a member of the

tachykinin family and is able to act on the neurokinin-1 receptor (Suzuki et al., 2002). These

receptors in the spinal cord increase the sensitivity of the dorsal horn, whose neurons then

become excited following a painful stimulus (Suzuki et al., 2002). Substance P may also be

involved in nociception at the supra-spinal level, and could contribute to hyper sensitization

(Budai et al., 2007). Coetzee et al. (2008) looked at changes in bovine plasma SP after castration

(Coetzee et al., 2008). The results indicated significantly higher concentrations of SP in calves

that underwent actual castration versus simulated castration. In addition, calves that vocalized

during the procedure had significantly higher plasma SP levels, further supporting the hypothesis

that SP may be used to quantify pain induced by castration (Figure 1.4). Research on human

carpal tunnel syndrome showed similar results, with increased subjective pain directly correlated

with increased levels of SP (Östürk et al., 2010). This study related SP with chronic

inflammation (Östürk et al., 2010), a condition induced by several animal husbandry procedures.

Infrared thermography (IT) is a non-invasive method used to measure the temperature of

surfaces, including body areas. Infrared thermography has been used to measure corneal

temperature in animals, which increases over time with increased stress (Anon, 2005). Anon (2005) measured the eye temperatures of dairy cows during stimulation of the stress axis either by injected hormones or social isolation. The results indicated an increase in corneal temperature in cows whose stress axis activation was confirmed by an increase in adrenocorticotropic hormone, cortisol, and non-esterified fatty acids (Anon, 2005). Stewart and colleagues (2008) measured changes in eye temperature in response to disbudding in calves in four treatment groups (sham control, sham without local anesthetic, sham with local anesthetic, and disbudding with local anesthetic) and found a rapid decrease in corneal temperature up to five minutes post-procedure in the disbudded treatment group with a subsequent rise in corneal temperature which could not be explained by physical activity (Figure 1.5). A study on pain response to hot-iron dehorning also measured corneal temperature in six treatment groups as follows: control with no pain mitigation, dehorning with no pain control, dehorning with local anesthetic only, local anesthetic with sham or simulated dehorning, local anesthetic and non-steroidal anti-inflammatory administration with sham dehorning, and local anesthetic and non-steroidal anti-inflammatory administration with dehorning (Stewart et al., 2009). Infrared thermography results revealed no significant differences in average eye temperature between treatment groups for 2-3 hours post-procedure (Stewart et al., 2009). However, a significant decrease in corneal temperature in the dehorning with local anesthetic group was evident 3 hours post-procedure (Stewart et al., 2009). This drop in eye temperature is similar to the initial decrease reported by Stewart et al. in 2008, and suggests an emergence of pain when the effects of the local anesthetic are diminishing (Stewart et al., 2009). A study on pain due to castration examined infrared thermography in four treatment groups: control, surgical castration with no pain treatment, local anesthetic with no castration, and local anesthetic with castration (Stewart et al., 2010). The

results showed an initial decrease in temperature in the castration without anesthetic group followed by an increase in corneal temperature (Stewart et al., 2010). All treatment groups showed an eventual increase in eye temperature, with the non-treated castration group showing the highest peak temperature and the control group showing the lowest peak temperature (Stewart et al., 2010). Thermography has proven to be a consistent and simple way to measure pain in cattle.

Electrodermal activity (EDA) measures skin electrical conductance, which changes in response to sympathetic stimulation (Critchley, 2005). The reticular activating system is a brain structure which projects from the brainstem through the thalamus to the cerebral cortex, where it relays excitability (Walshe, 1957; Moruzzi, 1964; Steriade and McCarley, 1990). Electrodermal activity is considered to represent reticular activation, and therefore may indicate the complex modalities of behavior and emotions in animals (Sequeira et al., 2009). A study conducted in 2002 examined the relationship between human joint pain and EDA (Fujita et al., 2002). The researchers discovered the percentage change (negative) of skin impedance was directly correlated with the patients' subjective assessment of decreased pain, as illustrated in Figure 1.6 (Fujita et al., 2002). However, EDA was unable to detect differences in arousal between cattle with induced lameness and controls (Kotschwar et al., 2009). In addition, a pain study conducted on laboratory rats found the EDA measuring device ineffective due to several confounding factors such as ambient humidity, area of skin that sensors were applied to, and pressure applied to the device (Richardson et al., 2007). The investigators deduced that skin impedance measures various other sensations besides pain (Richardson et al., 2007). A study performed by Baldridge et al. (2011) found EDA to be affected by xylazine administration. Specifically, the α2-adrenergic receptor agonist effects of xylazine stimulated eccrine sweat glands on the nasal

planum to secrete water, electrolytes, and mucin (Baldridge et al., 2011). These alterations changed the skin conductance and interfered with the EDA readings (Baldridge et al., 2011). Although it holds promise, skin conductance requires further research to determine its validity for pain detection.

Heart rate variability is another non-invasive method used to measure stress and emotional states in animals (von Borell et al., 2007). This technique assesses the function of the autonomic nervous system, especially the balance between sympathetic and vagal tone (von Borell et al., 2007). Some common variables assessed in heart rate variability include mean heart rate (HR), mean R-R interval (RR), low frequency power (LF), high frequency power (HF), low frequency to high frequency ratio (LF/HF), number of successive R-R interval differences greater the 50ms (NN50), and the square root of the mean squared successive differences between adjacent R-R intervals (RMSSD). Heart rate is determined by both sympathetic and parasympathetic (vagal) tone (Saul, 1990). Increased sympathetic tone or decreased vagal tone increases heart rate, while increased vagal tone or decreased sympathetic tone decreases heart rate (Acharya, 2006). The heart rate is a result of the constant balance between these tones, which may be shifted in favor of sympathetic or vagal tone by many stimuli (Acharya, 2006). The R-R interval represents the time between each peak of the QRS complex (ventricular depolarization) on the electrocardiogram (Zigel et al., 2011). Therefore, the mean R-R interval offers information on ventricular rate, with a short interval corresponding to a rapid rate and high sympathetic tone. Sympathetic activity is associated with low frequency power, while parasympathetic (vagal) activity is associated with high frequency power (Acharya, 2006). The ratio of low frequency power to high frequency power (LF/HF) illustrates the balance between these two branches of the autonomic nervous system (Acharya, 2006). A decrease in NN50 is

indicative of increased sympathetic tone and therefore increased heart rate (Nussinovitch et al., 2011). RMSSD (square root of the mean of the sum of the squares of the differences in successive inter-beat intervals, an indication of vagal regulatory activity) represents the average change in R-R interval between beats, and is also negatively correlated with increased sympathetic tone (Nussinovitch et al., 2011). A study examining disbudding in calves found the procedure without local anesthetic decreased the high-frequency power, indicating a reduction in vagal tone (Stewart et al., 2008). This study also found an increased LF/HF, which signifies a shift toward sympathetic (fight or flight) dominance (Stewart et al., 2008). Lastly, the HR was higher in calves disbudded with or without local anesthetic (Figure 1.7; Stewart et al., 2008). Another study on the effects of castration showed an increase in HR for calves castrated with and without local anesthetic, but no change for calves in the control groups (Figure 1.8; Stewart et al., 2010). The RMSSD was greater in castrated groups compared to controls, suggesting increased vagal tone (Stewart et al., 2010). This study also found a decreased LF/HF, further supporting evidence of increased vagal tone (Stewart et al., 2010). This increase in parasympathetic activity may be associated with the deep visceral pain likely experienced when the spermatic cords are torn (Stewart et al., 2010).

While the above examples may be used to search for pain or stress in animals, all of the techniques described measure the outcome of various peripheral body systems. Another option is to look directly at brain electrical activity in response to noxious stimuli, made possible with the use of the electroencephalogram.

## The Electroencephalogram as a Pain Indicator

The electroencephalogram (EEG) records the electrical activity of the cerebral cortical neurons which are believed to play an important role in pain perception (Johnson, 2007). In

particular, the EEG records the current sinks (positive charges transported from areas they can be detected to areas in which they cannot) and current sources (positive charges transported from areas they cannot be detected to areas in which they can) (Murrell and Johnson, 2006). Excitatory and inhibitory post-synaptic action potentials contribute to the formation of current sinks and sources while glial cells are involved in the maintenance of current sinks and sources (Murrell and Johnson, 2006).

Electroencephalogram recordings are often analyzed by exploring the rhythms (spectral characteristics) of EEG signals (Srinivasan, 2006). Spectral analysis provides information on different EEG frequencies, which are associated with differing states of arousal in humans (Srinivasan, 2006). Alpha, a high-frequency rhythm (band), has the most activity when a person is awake but relaxed (Srinivasan, 2006). Beta, another high-frequency band, is associated with sensory processing and increased alertness (Srinivasan, 2006). Delta, a low-frequency band, predominates during sleep (Srinivasan, 2006). Theta, another low-frequency band, is most commonly found in neonates but has also been implicated in memory retrieval (Srinivasan, 2006).

Fast Fourier transform (FFT) is an algorithm which is performed on EEG recordings to detect the Fourier transform coefficients (Murali and Kulish, 2007). These values are then used to find the energy (power) associated with each frequency band (Murali and Kulish, 2007). The power spectrum is calculated using the sum of the squares of the amplitudes of the Fourier transform coefficients in each band (Murali and Kulish, 2007). Power may either be expressed as absolute power or relative power (as compared to a reference electrode) (Bergamasco et al., 2011).

In general, the electroencephalogram response to pain involves a decrease in total power, an increase in 95% spectral edge (the 95th percentile of the power spectrum; F50), and an increase in median frequency (the statistical median of the power spectrum) (Murrell and Johnson, 2006). This is due to a decrease in low frequency activity and an increase in high frequency activity (Murrell and Johnson, 2006). This phenomenon is termed "desynchronization", and is associated with an increased level of arousal (Murrell and Johnson, 2006). The type of noxious stimuli experienced may also be differentiated using the EEG, with somatic pain inducing changes in total power or spectral edge and visceral pain associated with fluctuations in median power (Johnson, 2007). A study performed on the castration of cattle associated EEG responses with cortisol responses found a correlation between the two, thereby suggesting the EEG is useful in monitoring pain (Bergamasco, 2011).

Electroencephalogram recordings are most often performed while an animal is anesthetized or sedated, which has a dampening effect on the degree of response to noxious stimuli (Murrell et al., 2008). A study on the effect of propofol on the EEG found anesthetizing doses to produce progressive slowing of the EEG and sedating doses to increase total power, which is the opposite effect a painful stimulus has (Seifert et al., 1993). Since animals are often anesthetized during experimental procedures for ease of handling, interpretation of EEG recordings may be somewhat limited. A dehorning study on cattle with and without a lidocaine block found a decrease in total power (TP) and increases in the F50 and 95% spectral edge in the non-lidocaine group (Gibson et al., 2007). No significant EEG changes were observed in the group that did receive lidocaine (Gibson et al., 2007). These results are consistent with the previously-determined responses to noxious stimuli, but the cattle in this study were minimally anesthetized (Gibson et al., 2007). Therefore, the magnitude of the increases and decreases of the

EEG parameters are most likely less than would be found in a fully-conscious animal (Murrell et al., 2008).

The EEG is a very precise measurement, which may be disrupted by as little movement as the blink of an eye (Kaiser, 2005). A study in humans attempted to determine other factors which need to be controlled to achieve an accurate EEG pain reading and found a wincing facial expression to interfere with the results (Dowman et al., 2008). A study on mice found the EEG to be sensitive to a nod of the head barely visible to the human eye (McColl et al., 2006). Therefore, it can be concluded that EEG is sensitive to artifact and therefore animal movement must be kept to an absolute minimum in order to attain exact results. Since it can be exceptionally difficult to restrain animals, this is an obstacle to overcome when using the EEG to evaluate pain (Suzuki et al., 1990).

A study in sheep examined the EEG response to castration in different age groups (Johnson, 2005). The results showed greater increase in the relative F50 of the two-week-old group versus the one-month-old group, as illustrated in figure 1.9 (Johnson, 2005). This finding challenges the commonly-accepted notion that the welfare implications of processing procedures without analgesia are smaller in younger animals. However, a similar study found relatively little increase in F50 in lambs that were only a few days old versus one-week-old lambs, as shown in figure 1.10 (Johnson, 2004). These results may be due to young animals experiencing less intense pain or it may be that the cortical structures are not fully mature at this early developmental stage, affecting the EEG recording (Johnson, 2004).

The EEG has shown potential to be a reliable method to evaluate pain in animals. Several positive and negative aspects of EEG measurements versus behavioral measurements are

outlined in Table 1.2. Overall, the EEG requires more study to become fully validated in animals but shows potential for the study of pain.

## Castration Methods

There are currently three castration techniques which may be used on domestic animals worldwide, two of which are not widely used (Stafford and Mellor, 2005). These methods include chemical, hormonal, and physical castration (Stafford and Mellor, 2005). In the United States, physical castration using manual twisting of the spermatic cord or a clamp to crush the spermatic cord is the most prevalent method (Coetzee et al., 2010). Physical castration is also the most popular practice throughout the world, and different physical methods and their prevalence are shown in table 1.3 (Stafford and Mellor, 2005).

Chemical castration involves injection of toxic substances such as lactic acid into the testes in order to halt the production of gametes and hormones (Fordyce et al., 1989). Although this substance may leak into the scrotum and cause swelling and pain, a study on sheep found the peak cortisol response to chemical castration was lower than to surgical castration, suggesting it was overall less painful than physical methods (Cohen et al., 1990). In addition, this study found the average daily gain of chemically castrated sheep to be higher than physically castrated sheep (Cohen et al., 1990). However, Hill and colleagues (1985) found chemical castration to be unsuccessful in 25% of cases, a failure rate too high to make it a useful castration method. Anderson (2007) determined that repeated injections are likely required to maintain sterility (Anderson, 2007). As technology continues to improve the industry may see a rise in the practice of chemical castration, but at present it is not a technique that is commercially used (Cohen et al., 1990).

Hormonal castration involves injection of sex hormones such as gonadotropin-releasing hormone in order to desensitize the body to its effects, thus rendering the animal sterile (Stafford and Mellor, 2005). A study performed on the sterilization of dogs found that an implant which slowly released gonadotropin-releasing hormone into the bloodstream down-regulated the hormone's receptors, causing all implanted dogs to become aspermic (Ludwig et al., 2009). This study also found the process to be reversible; testicular size, prostate size, and the availability of estradiol and testosterone returned to pre-treatment values within twenty-nine weeks (Ludwig et al., 2009). This reversibility could prove problematic in a production situation, where meticulous observation of animals is not always feasible (Ahmed and Ahmed, 2011). Another issue with hormonal castration is public perception, which tends to view hormonal remnants in meat and milk as dangerous (Verbeke and Viaene, 2000). Although residues may not persist in food products with this castration technique, more research is required to determine its safety and efficacy (Stafford and Mellor, 2005).

A popular method, used especially in younger animals, involves applying a tight elastic band around the scrotum and testicular cords, with care taken to keep the band close to the testes and as far as possible from the body wall (Chase et al., 1995). For smaller animals, the band may be stretched over the testicles and released onto the scrotum (Chase et al., 1995). For larger animals, the band may need to be ratcheted down (Chase et al., 1995). An example of an elastrator tool and bands is found in Figure 1.11. In both cases, the aim is to eliminate blood supply to the testes, causing ischemia of sperm-producing tissues (Anderson, 2007). A study performed on lambs found the cortisol response to band castration to be less severe than surgical castration (suggesting less acute pain), but abnormal behaviors persisted longer in the banded group than the surgical group (Lester et al., 1996). Another study on banding found evidence of

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chronic pain lasting as many as forty-two days post-procedure (Molony et al., 1995). However a third study comparing surgical castration and banding found the average daily gain to be the same between the two groups, suggesting the long-term effects are comparable for both treatments (Chase et al., 1995).

A similar method is known as Burdizzo clamping, which involves using a tool to crush the spermatic cords from outside the scrotum (see figure 1.12) (Anderson, 2007). Just as in banding, the aim of the tool is to remove the blood vessels feeding the testes (Anderson, 2007). Although it is related to the banding method, Burdizzo castration has been shown to produce fewer long-term behavioral signs of pain and distress than banding (Melches et al., 2007). A study performed on lambs found the cortisol peak to be smaller and less enduring when using the Burdizzo versus the banding method (Thür et al., 2007). Conversely another study comparing the two techniques found more pro-inflammatory cytokine gene expression with Burdizzo, indicating more severe inflammation with this method (Pang et al., 2009). In addition, a study on lambs and calves found evidence of steriodogenesis and spermatogenesis in calves aged one to four weeks castrated using the Burdizzo method, indicating incomplete castration (Stoffel et al., 2009). Burdizzo castration was always successful in calves aged twelve to sixteen weeks (Stoffel et al., 2009). This study also found evidence of incomplete involution of the testicular parenchyma in almost all lambs, regardless of age at castration (Stoffel et al., 2009). This information questions the reliability and usefulness of the Burdizzo method (Stoffel et al., 2009).

Surgical castration involves cutting the scrotum with a scalpel and removing the testicles either by cutting the spermatic cords or by pulling them out of the body (Anderson, 2007). This method is best performed out of fly season, since the open incision welcomes pests and can cause infection (Anderson, 2007). Surgical castration tends to cause a higher and faster peak in

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cortisol levels, suggesting it is acutely painful (Chase et al., 2005). However, cortisol levels usually fall faster than with other castration methods (Chase et al., 2005). A study on the vocalizations of piglets subjected to surgical castration found the animals called out the most during pulling and severing of the spermatic cord, implicating this process as the most painful aspect of the castration procedure (Taylor and Weary, 2000). This study suggests non-surgical castration techniques may benefit welfare due to their apparently less-painful nature (Taylor and Weary, 2000). Another study conducted on pigs found barrows to display painful postures and behaviors for as many as six days post-procedure (Van Beirendonck et al., 2011). Stewart and colleagues (2010) found increased eye temperature, heart rate, epinephrine levels, and cortisol levels in surgically castrated calves compared to controls. The application of local anesthetic to the scrotum and testes reduced but did not eliminate these responses, suggesting the pain is deeper than can be alleviated with lidocaine (Stewart et al., 2010). Surgical castration is overall the most reliable method due to the certainty of testicular removal and also seems to have the fewest long-term effects, making it the most popular method in production systems (Anderson, 2007).

## Conclusion

Different castration methods have varying effects in animals, which may be measured using validated, non-invasive pain assessment techniques such as infrared thermography, heart rate variability, and cortisol measurement. Measuring the pain reaction to castration using the EEG in combination with validated pain assessment techniques will provide a scientific basis for future study of noxious stimuli.

# Figures and Tables

Figure 1.1– Responses of groups of 5 to 6 day old Dorset crossbred lambs (n=7) to the following treatments of decreasing severity: castration and tail docking (C+TD), bilateral castration (C*2), unilateral castration (C*1), short scrotum castration (C*0), tail docking (TD), short scrotum castration and local anesthesia (C*0+LA), or handled controls (H). Values are the mean (±SD) peak cortisol concentrations (nmol/L) reached during the 180 minutes after treatment. The median values are indicated by the full-width horizontal line. Mean values for treatment groups labeled with the same letter are not significantly different (P>0.05).

## Cortisol response
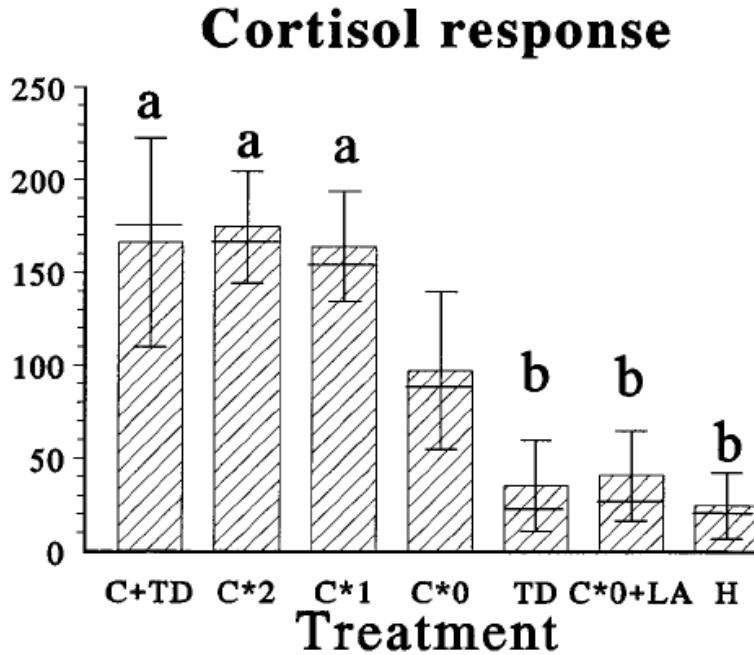


Figure from Molony and Kent, 1997

Figure 1.2 – The plasma concentration of cortisol (ng/mL) obtained by puncture of the jugular vein before and after exposure to the four treatments (control-CON, novel neighbors/stall-NEIGH, fixation by the head in the home stall-FIX, and social isolation in novel surroundings-ISOL). The black bar indicates the duration of treatment.
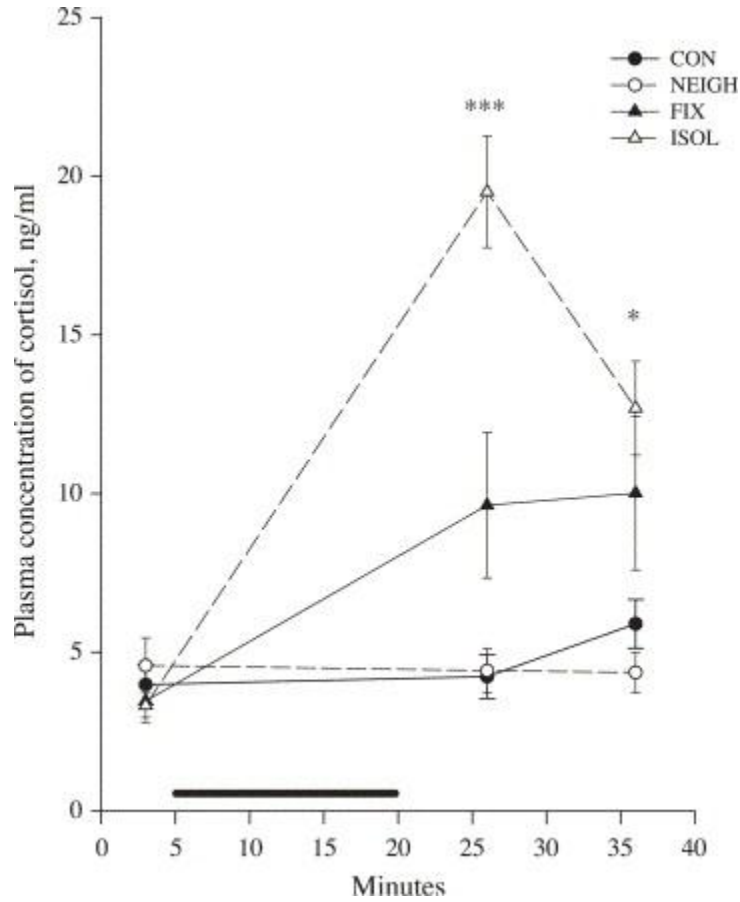


Figure from Herskin et al., 2004

Figure 1.3 – Serum concentrations of haptoglobin in healthy cows sampled on the day of clinical examination (day 0) and in lame cows sampled on days 0, 4, 7 or 8, and 14. Significant differences between healthy and lame cows are indicated: *P=0.05, **P=0.01
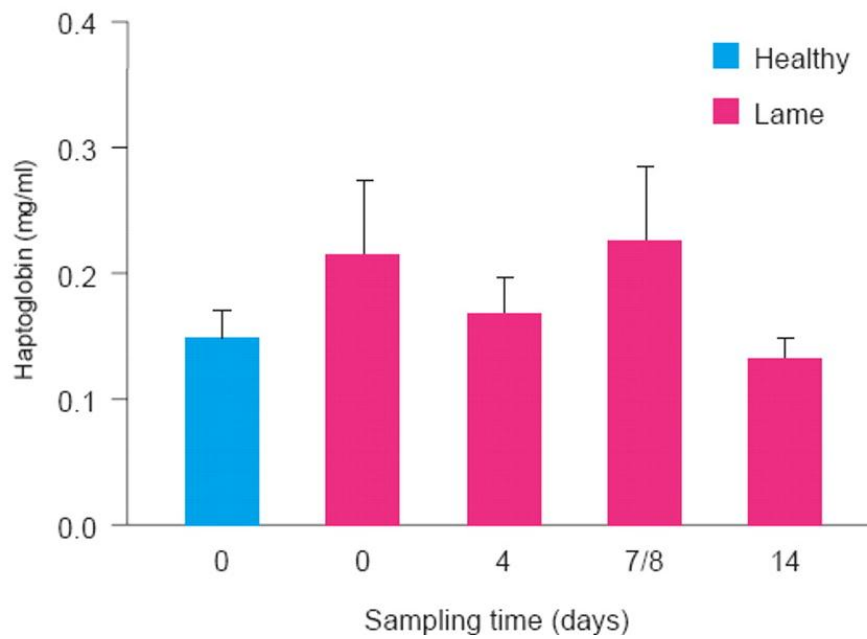


Figure from Kujala et al., 2010

Figure 1.4 – Mean ± SEM plasma SP concentration in beef calves (n = 5 calves/group) after surgical castration (black triangles) or simulated castration (white squares). Time of castration or simulated castration was designated as time 0. Concentrations differed significantly ($P = 0.042$; repeated-measures ANOVA) between groups.
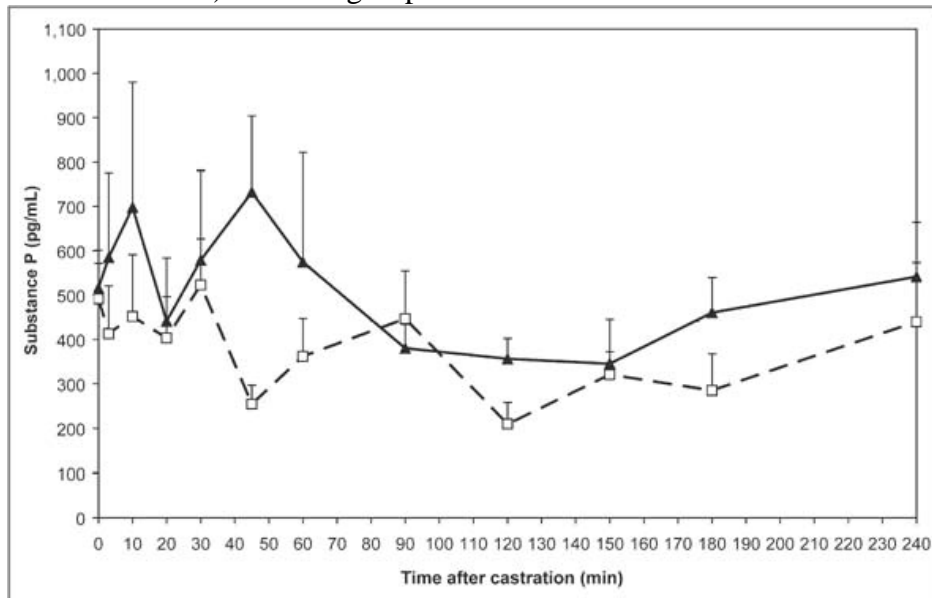


Figure from Coetzee et al., 2008

Figure 1.5  - Maximum eye temperature (°C) during the 40 min sampling period for control (■, n=8), local anesthetic control (▲, n=8), disbudded with local anesthetic (□, n=8) and disbudded without local anesthetic (●, n=6). Lines were smoothed using a loess smoother separately for each animal pre and post disbudding. The dashed vertical line indicates the time that local anesthetic or the sham procedure was administered and 0 min indicates the time of treatment.
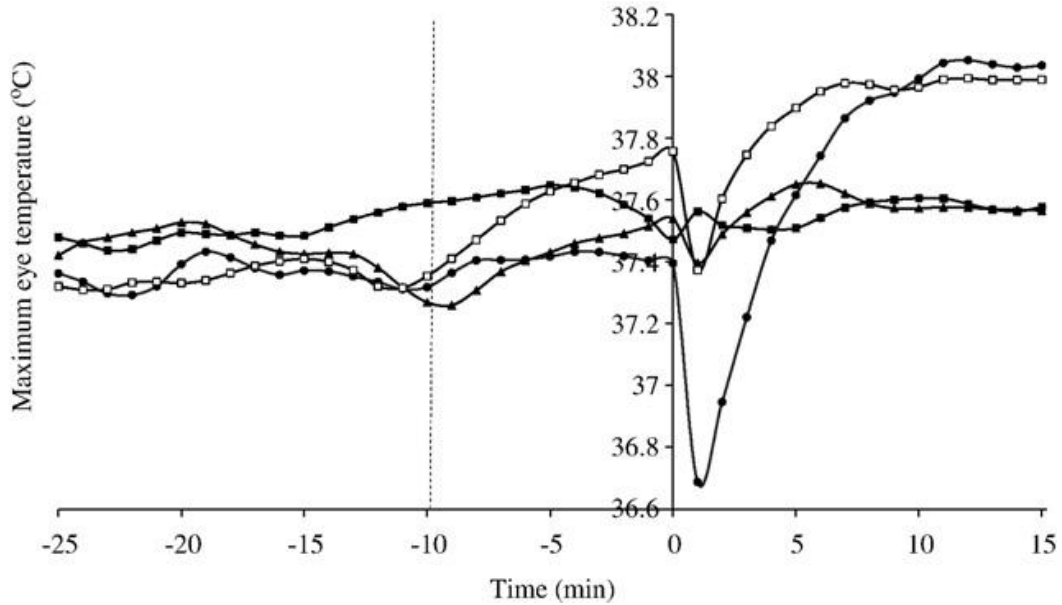


Figure from Stewart et al., 2008.

Figure 1.6 – Fall of skin impedance (percentage) from the basal value, in parallel with subjective pain (effect by face scale) in response to physical loading on the joint (i.e., standing, squatting, walking, and ascending and descending). A highly significant alleviation of subjective pain on the face scale and in terms of fall of skin impedance, was noted in the supplementation group (closed bars) compared with the control group (open bars). Values are means ± SEM.
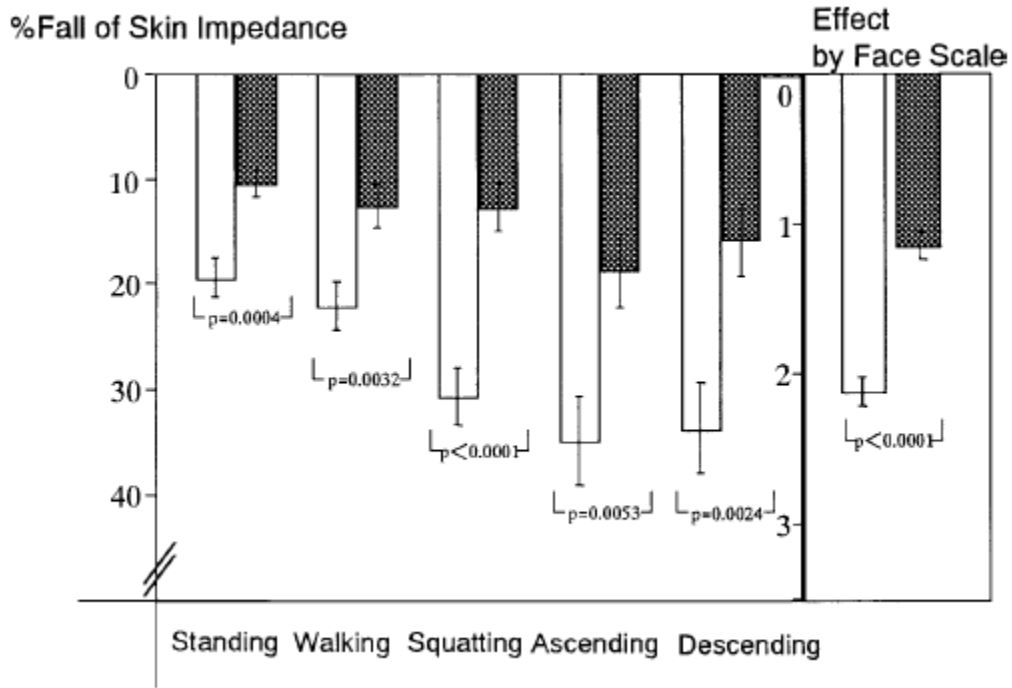


Figure from Fujita et al., 2002

Figure 1.7 – Mean heart rate (bpm) during the 40 min sampling period for control (■, n=8), local anesthetic control (▲, n=8), disbudded with local anesthetic (□, n=8) and disbudded without local anesthetic (●, n=5). The dashed line indicates the time that local anesthetic or the sham procedure was administered and 0 min indicates the time of treatment.
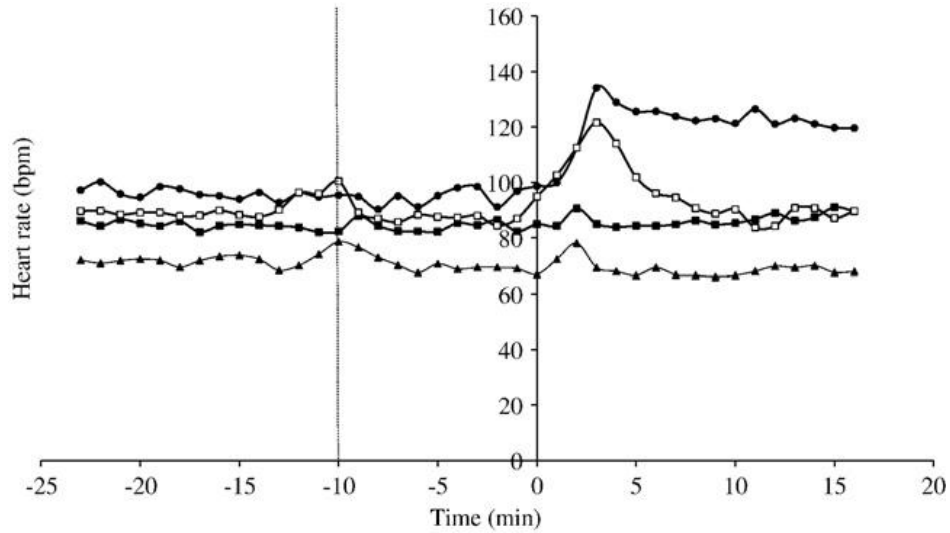


Figure from Stewart et al., 2008

Figure 1.8 – Mean heart rate (beats/min) for control (C, n = 8), castrated without local anesthetic (SC, n = 6), local anesthetic control (LAC, n = 8), and castrated with local anesthetic (LASC, n = 7) treatment groups. The dashed line indicates the time that local anesthetic or the sham procedure was administered and 0 min indicates the time of treatment.
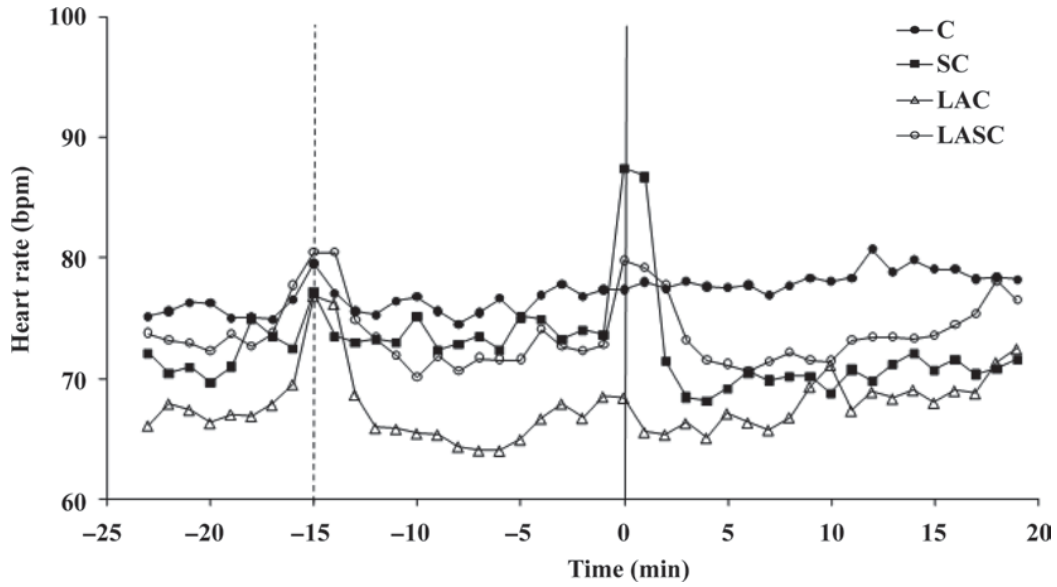


Figure from Stewart et al., 2010

Figure 1.9 – Relative response in median frequency following castration in lambs of differing ages. Young lambs ~14 days old, old lambs ~30 days old.
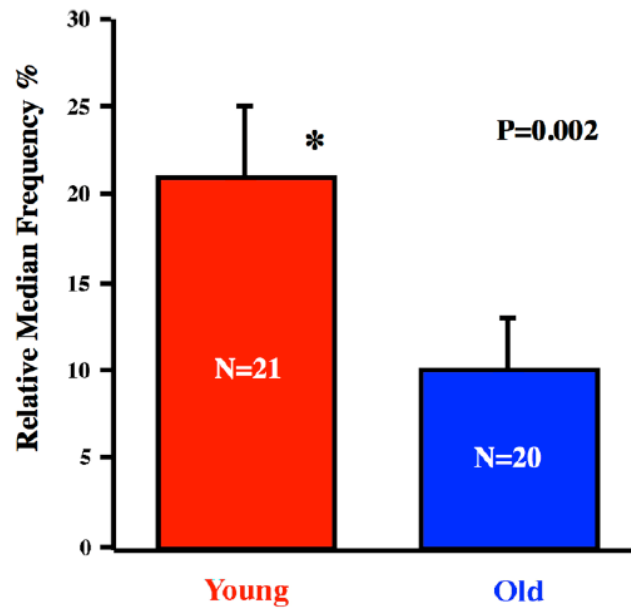


Figure from Johnson et al., 2005

Figure 1.10 – Relative response in median frequency following castration in lambs during first week of life. Each data point represents a rolling mean of 10 animals.
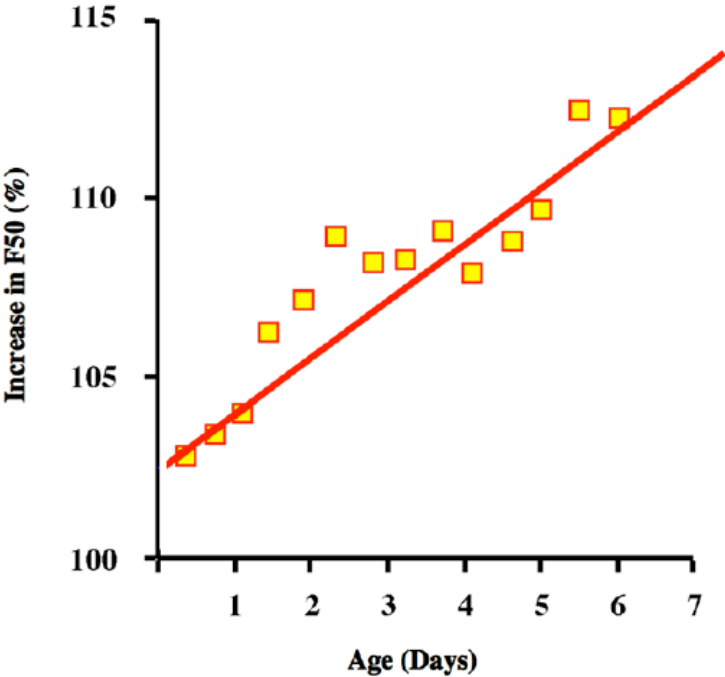


Figure from Johnson et al., 2004

Figure 1.11 – Elastrator tool with bands.
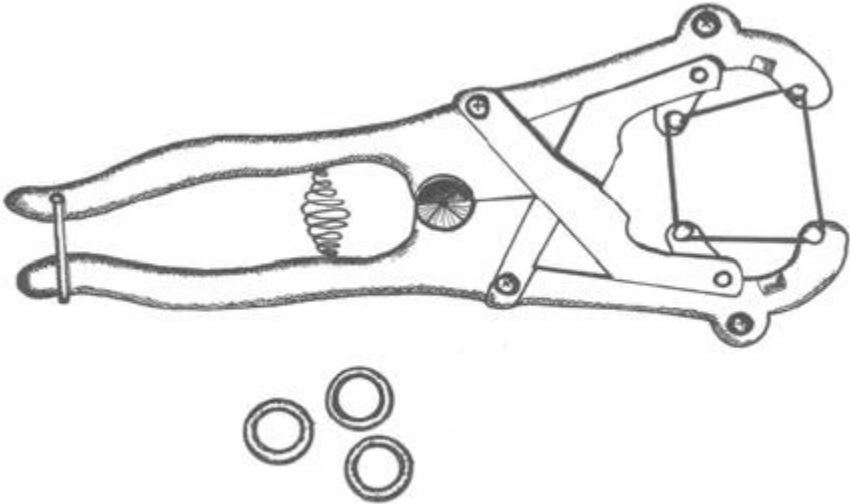


Figure from Anderson, 2007

Figure 1.12 – Burdizzo clamp.
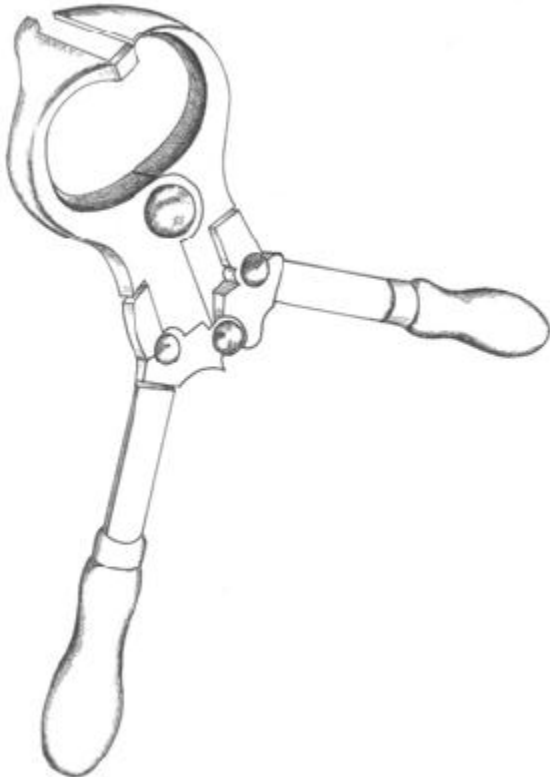


Figure from Anderson, 2007

Table 1.1 – Mean frequency of vocalization (number of observations/duration of test period in minutes), lip licking (number of observations/duration of test period in minutes) and eat movements for the treatments noxious ischaemic stimulus (S), noxious ischaemic stimulus and flunixin meglumin (S+F), flunixin meglumine (F) and test days 1-3.

| | Vocalization | | | Lip licking | | | Frequency of ear movements | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coefficient | Standard Error | $p$ | Coefficient | Standard Error | $p$ | Coefficient | Standard Error | $p$ |
| Test Day 1 | 0.7 | 5.7 | <0.001 | 0.5 | 3.88 | <0.001 | -1.2 | 0.1 | <0.001 |
| Test Day 2 | 0.01 | 0.1 | 0.929 | 0.004 | 0.04 | 0.966 | -0.3 | 0.1 | 0.007 |
| Test Day 3 | 0 | . | . | 0 | . | . | 0 | . | . |
| Treatment S | 0.2 | 1.8 | 0.1 | 0.07 | 1.36 | 0.174 | 0.2 | 0.1 | 0.124 |
| Treatment S+F | 0.3 | 5.2 | <0.001 | -0.02 | -0.14 | 0.887 | 0.5 | 0.1 | <0.001 |
| Treatment F | 0 | . | . | 0 | . | . | 0 | . | . |
| Intercept | 0.1 | 0.9 | 0.388 | 0.4 | 3.3 | 0.001 | -0.2 | 0.2 | <0.001 |

Table adapted from Stubsjøen et al., 2009

Table 1.2 – Advantages and disadvantages of electroencephalogram and behavioral analysis in the study of pain in animals.

| EEG Analysis | Behavioral Analysis |
|---|---|
| Animals may need to be anesthetized | Animals must be conscious |
| Statistical differences apparent with small numbers | Larger numbers required for statistical differences |
| Mathematical concepts complex | No complex mathematical concepts |
| Rapid analysis of data | Data analysis laborious |
| Suited to rapidly-applied stimuli | Suited to prolonged perception of pain |
| Consistent responses in many mammals | Behavior specific to species, type, etc. |
| Pain research without causing pain to study animals | Research animals must suffer pain in order to measure pain-related behavior |
| Differentiation of visceral and somatic pain | Behavior specific to noxious stimuli |

Table adapted from Johnson, 2007

Table 1.3 – The percentage of farmers using various methods to castrate calves, and the age of calves at castration, in New Zealand (NZ) and the United Kingdom (UK).

| Method | NZ | UK |
|---|---|---|
| Rubber Ring | 85% (up to 3 months old) | 32% (1 week old) |
| Clamp (Burdizzo) | 1% | 43% |
| Surgery | 18% | 39% |
| Less than 12 weeks of age | 60% | 62% |
| Local anesthesia used | 3% | 15% |
| Performed by veterinarian | 3% | 21% |
| Failure rate of castration | 0.4% | 0.7% |

Table adapted from Stafford and Mellor, 2005

# Chapter 2 - Effect of Age and Castration Method on Neurohormonal and Electroencephalographic Stress Indicators in Holstein Calves

## Introduction

Castration is a standard husbandry procedure commonly used to decrease aggressive behavior, prevent unwanted matings, and modify carcass characteristics in cattle (Stafford, 2007). Banding and surgical methods of castration are the two most common methods and both are known to produce pain regardless of calf age (Robertson, 1994; Coetzee et al., 2010). As the public's concern for food animal welfare increases, objective and non-invasive approaches to measure pain become more important. This study was designed to assess the physiological response of growing calves to castration-induced pain by measuring their electroencephalogram signal, heart rate variability, infrared thermography, electrodermal activity, and plasma concentrations of cortisol and substance P.

The electroencephalogram (EEG) records the electrical activity of the cerebral cortical neurons which are believed to play a role in pain perception (Johnson, 2007). Activity is measured by four bands, namely low frequency delta and theta and high frequency alpha and beta. In general terms, a typical EEG response to pain involves a decrease in total power, an increase in 95% spectral edge (the 95th percentile of the power spectrum), and an increase in median frequency (the statistical median of the power spectrum) (Murrell and Johnson, 2006). This response is attributed to a decrease in low frequency activity and an increase in high frequency activity. This phenomenon is termed "desynchronization", and has been associated with increased arousal (Murrell and Johnson, 2006). A study performed on cattle castration

showed an association between EEG responses and cortisol concentrations, thereby suggesting that EEG may be useful in monitoring pain (Bergamasco, 2011).

Heart rate variability (HRV) has also been used to measure stress in animals (von Borell et al., 2007). Heart rate variability assesses the function of the autonomic nervous system, especially the balance between sympathetic and vagal tone (von Borell et al., 2007; Saul, 1990). Some common variables assessed in HRV analysis include mean heart rate (HR), low frequency power (LF), high frequency power (HF), low frequency to high frequency ratio (LF/HF), and number of successive R-R interval differences greater the 50ms (NN50). Increased sympathetic tone (or decreased vagal tone) increases heart rate, while increased vagal tone (or decreased sympathetic tone) decreases heart rate (Acharya, 2006). Heart rate is a result of the constant balance between these tones, which may be shifted in favor of sympathetic or vagal tone by many stimuli (Acharya, 2006). The NN50 is one of the most commonly used measures derived from R-R interval differences as it measures short-term variation in HRV and estimates high frequency variation in HR. Sympathetic activity is usually associated with LF, while parasympathetic (vagal) activity is most frequently associated with HF (Acharya, 2006). Thus, the LF/HF ratio can be used to illustrate the balance between these two branches of the autonomic nervous system (Acharya, 2006).

Infrared thermography (IT) is a technique used to measure the temperature of body areas. In particular, ocular temperature has been shown to respond to castration-induced pain in a stimulus-dependent manner (Stewart et al., 2010). Electrodermal activity (EDA) is the measurable change in skin electrical conductance in response to sympathetic stimulation (Critchley, 2005). Electrodermal activity is considered to represent brain stem reticular

activation, and therefore may indicate the complex modalities of behavior and emotions in animals (Sequeira et al., 2009).

Pain causes activation of the hypothalamo-pituitary-adrenal axis, which is the neuroendocrine stress pathway (Cardo et al., 2011). The activity of this pathway can be measured by its end product, cortisol (Kent et al., 1993). Cortisol concentrations in the relaxed animal show a circadian rhythm with a peak in the early morning followed by a decline throughout the day (Bosch et al., 2009). Stressful situations augment this basal secretion and cause episodic peaks in cortisol concentrations, making it a useful stress indicator (Minton, 1994; Grandin, 1997; Bosch et al., 2009).

Substance P (SP) has been described as a modulator of nociception, and has also been involved with transmission of painful stimuli to the central nervous system (DeVane, 2001). This neuropeptide is a member of the tachykinin family and is able to act on the neurokinin-1 receptor (Suzuki et al., 2002). These receptors in the spinal cord increase the sensitivity of the dorsal horn, whose neurons then become excited following a painful stimulus (Suzuki et al., 2002). Substance P may also be involved in nociception at the supra-spinal level, and may contribute to hyper sensitization (Budai et al., 2007). Further, previous studies have reported elevated SP concentrations in calves following castration (Coetzee et al., 2008).

In the present study, we examine physiological indicators of stress and pain induced by one of two surgical castration methods or a non-surgical castration method applied to either young ($\leq$ 6 weeks) or older ($\geq$ 6 month) calves. These indicators may then be used to assess stress or pain responses to different castration methods. The aims of the present study were (i) to evaluate the physiological response to pain induced by castration in calves and (ii) to elucidate age-related differences in pain response of calves subjected to different castration methods.

# Materials and Methods

## *Animals and Housing*

Forty Holstein bull calves aged approximately 6 weeks and forty Holstein bull calves aged approximately 6 months were enrolled in the study. Six-week-old calves arrived from Boulder, CO on May 24, 2011. Six-month-old calves arrived from Conway Springs, KS on June 1, 2011. Processing procedures occurred one day after arrival. All calves received a single subcutaneous dose of oxytetracycline 300 mg/mL (Noromycin 300 LA, Norbrook Inc., Lenexa, KS), a single subcutaneous dose of an eight-way clostridial vaccine (Covexin 8, Intervet/Schering-Plough Animal Health, Summit, NJ), and a single subcutaneous dose of a bovine rhinopneumonitis vaccine (Bovi-Shield GOLD 5, Pfizer Animal Health, New York, NY) to prevent common bovine disease. A topical pour-on comprised of 5% permethrin and 5% piperonyl butoxide (Ultra Boss Pour-On Insecticide, Intervet/Schering-Plough Animal Health, Summit, NJ) was applied to all calves upon arrival and repeated as needed for fly control. Six-week-old calves were given 14 days for acclimation and 6-month-old calves were given 6 days for acclimation before study commencement. Calves were weighed on arrival, at treatment, and 4 days post-procedure on an in-chute scale (Smartscale 500 USA, Gallagher Group Ltd., Hamilton, New Zealand). Weights at arrival were later used in the randomization process.

Six-week-old calves were fed daily approximately at 0500 hours and at 1700 hours. Each feeding consisted of 340 g of unmedicated milk replacer powder (Land O'Lakes Instant Amplifier Max, Land O'Lakes Inc., Shoreview, MN) mixed with 2.18 L of water in calf bottles. In addition, all calves were fed 0.68 kg of a calf starter diet per day composed of rolled corn, soybean hulls, dry distiller's grains, and soybean meal. Water was offered in buckets ad libitum.

Calves were individually housed in two identical barns composed of twenty 1.5 m by 1.5 m stalls with concrete floors, per industry standard with one-half of each stall shaded by a tin roof.

Six-month-old calves were fed daily at 1300 hours each day approximately 10.10 kg/head of a growth diet composed of brome hay, dried distillers grains with solids, steep-monocalcium phosphate, soybean hulls, and dry rolled corn. Water was offered ad libitum via an automatic waterer. Calves were housed in identical 4.3 m by 8.5 m pens with concrete floors in groups of four with one half of each pen shaded by mesh shade cloth.

This protocol was approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol #2831). All calves were housed at the Kansas State University Beef Cattle Research Center, Manhattan, KS. Calves were continuously monitored for pain for 8 h following surgery, then twice daily for 7 days. Rescue analgesia (flunixin meglumine, 2.2 mg/kg IV BID) was available for animals exhibiting signs of excessive pain including hunched posture, excessive vocalization, and anorexia.

### *Group Assignment and Randomization Procedures*

At arrival, calves were blocked by age and weight and were then randomly assigned to treatment groups: control (CONT; n=20), banding (BAND; n=18), cut and clamp (CC; n=20), or cut and pull (CP; n=18). All treatments took place in a hydraulic, double-alley squeeze chute with a belly bar to prevent collapse (Daniels Manufacturing Co., Ainsworth, NE). The CONT treatment consisted of scrotal manipulation only with no pulling. The BAND treatment consisted of scrotal manipulation followed by the application of a latex band around the scrotum using an elastrator tool. The CC treatment consisted of scrotal manipulation followed by a scrotal incision with a scalpel and clamping of the spermatic cords with an emasculator. Lastly, the CP treatment consisted of scrotal manipulation followed by a scrotal incision with a scalpel and pulling of the

testicles until rupture of the spermatic cords. Prior to castration, the scrotum was washed with an iodine solution (Triadine Povidone-Iodine USP Prep Solution, Triad Disposables Inc., Brookfield, WI) and any tools were thoroughly cleaned with 70% isopropyl alcohol (Isopropyl Rubbing Alcohol 70%, Vi-Jon Inc., St. Louis, MO). For consistency, all treatments were performed by the same experienced operator. A total of 8 calves were processed on each study day such that all treatment and age group combinations (arranged in random order) were represented in a given day.

Jugular catheters (14 G x 140 mm; Abbocath-T (305mL/min), Abbott Ireland, Sligo, Republic of Ireland) with 76 cm extension sets (Extension Set with Slip Luer Slide Clamp, Abbott Ireland, Sligo, Republic of Ireland) were placed on the left side of the neck one day prior to each calf's assigned study day. Before catheter placement the neck was shaved and cleaned with iodine and 70% isopropyl alcohol (Isopropyl Rubbing Alcohol 70%, Vi-Jon Inc., St. Louis, MO). Local anesthesia was provided (0.5mL Lidocaine HCl 2% 20 mg/mL, Hospira Inc., Lake Forest, IL) followed by a stab incision with a #22 scalpel blade and catheter insertion. Catheters remained in place for approximately 24 h.

### *EEG Data Recording and Management*

During each experimental session, calves were restrained in the chute and electrodes were placed transcutaneously in a 12-channel montage (F3, F4, T3, C3, Cz, C4, T4, P3, Pz, P4, O1, O2; odd number = left hemisphere, even number = right hemisphere) to record EEG (Sandman Spyder, Tyco Healthcare, Puritan Bennet Ltd., Kanata, ON, Canada) as previously described (Bergamasco et al., 2011).

EEG recording started when electrode placement was completed, approximately 10 min after calves were restrained.  The total recording time was 30 min for each calf, including

calibration and the initial impedance check. EEG data were then stored in the acquisition station for later processing. The timeline of the EEG recording included: 1. Baseline (8-10 min prior to treatment; Base), 2. Immediate recovery (0-5 min post-treatment; R05), 3. Middle recovery (5-10 min post-treatment; R510), and 4. Late recovery (10-20 min post-treatment; R1020). Visual inspection of the electroencephalogram was performed in order to evaluate background activity by an experienced operator masked to treatment and age classification. Data from one CC calf and one CONT calf were excluded from further analysis due to unacceptable levels of artifact contamination of the EEG.

Spectral analysis of the EEG was performed using Fast Fourier Transformation (FFT) (Sandman Spyder, Tyco Healthcare, Puritan Bennet Ltd., Kanata, ON, Canada). For all calves, at least 30 replications of 2-second long artifact-free epochs were selected from each EEG time point. Fast Fourier Transformation was calculated and averaged for each channel. The spectral bands of delta (0.50-4.00 Hz), theta (4.10-8.00 Hz), alpha (8.10-12.00 Hz), and beta (12.10-30.00 Hz) were calculated and expressed as absolute power (AP; $\mu V^2$) and relative power (RP; %). Total power (Ptot; $\mu V^2$) of the entire spectrum (0.5-30.0Hz) was also calculated.

### *Heart Rate Variability*

Heart rate and HRV were continuously recorded with commercially-available heart rate monitors (Polar Equine, Polar Electro Oy, Kempele, Finland), which consisted of a transmitter and receiver. Monitors were fitted 5 min before calves were placed into the chute and removed at the end of the sampling period. The left and right sides of the thorax over the cardiac silhouette were shaved and ultrasound gel (Ultrasound Gel, Medline Industries Inc., Mundelein, IL) was applied to facilitate electrode contact with the thorax. Transmitter and receiver were fixed to the animal using an elastic belt and cohesive flexible bandages (Fisherbrand Cohesive Flexible

Bandage, Fisher Scientific, Pittsburgh, PA. The timeline of HRV analysis included: 1. Baseline (5 min pre-treatment; Base), 2. Immediate recovery (0-5 min post-treatment; R05), 3. Middle recovery (5-10 min post-treatment; R510), and 4. Late recovery (10-20 min post-treatment; R1020). Time periods of equal length (5 min) were analyzed to fulfill recommendations for analysis of HRV (Task force, 1996). Variables analyzed included mean heart rate (HR), number of R-R intervals greater than 50 ms (NN50), normalized low-frequency (LF), normalized high-frequency (HF), and low-frequency to high-frequency ratio (LF/HF). These data were then evaluated using research grade HRV analysis software (Kubios HRV version 2.0, Biosignal Analysis and Medical Imaging Group, Joensuu, Finland). The detrending method used was smoothing priors (lambda = 500) and the frequency bands were specified as follows: very low-frequency 0.003-0.04 Hz, low-frequency 0.04-0.3 Hz, and high-frequency 0.3-0.8 Hz. The interpolation rate was 4 Hz and there were 256 points/Hz in the frequency domain. Fast Fourier transformation spectrum options were set to window width 256 s and window overlap 50%.

### *Infrared Thermography*

Changes in ocular temperature were measured using a commercially-available infrared inspection system (ThermaCAM® S65, FLIR Systems, Wilsonville, OR). Pictures were taken at 180 s, 120 s, and 60 s before treatment, at the time of treatment (0 s) and 60 s, 90 s, 120 s, 150 s, and 180 s after treatment. Images were analyzed for changes in temperature using research grade software (Thermacam Researcher Pro 2.8 SR-1, FLIR Systems, Wilsonville, OR) by selecting a circular area around the eye and calculating the maximum eye temperature, minimum eye temperature, and average eye temperature (˚C). Ambient temperature and relative humidity in the barn were recorded and entered into the infrared camera to ensure calibration for atmospheric conditions.

### *Electrodermal Activity*

Electrodermal activity was measured using a commercially-available pain device (Pain Gauge, PHIS Inc., Dublin, OH). Possible values recorded by this device ranged from 0 ("calm/no pain") to 9.9 ("tense/severe pain"). Electrodes were placed across the nasal planum and measurements taken 10 min before treatment, 5 min before treatment, at treatment, 5 min after treatment, and 10 min after treatment as previously described (Kotschwar et al., 2009; Baldridge et al., 2011).

### *Cortisol*

Blood samples were drawn at baseline, 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 120 min, 240 min, 480 min, and 5760 min relative to treatment for cortisol analysis. Blood was collected from catheters into serum clot activator tubes (Vacuette 6mL Z Tubes, Greiner Bio-One, Kremsmünster, Austria) and was centrifuged at 1500 g for 10 min within 30 min of collection. The serum was removed with transfer pipettes (Graduated 3mL Transfer Pipettes Large Bulb, Samco Scientific, San Fernando, CA) and stored in 2 mL cryogenic vials (Fisherbrand Cryogenic Storage Vials, Fisher Scientific, Pittsburgh, PA) and frozen at -80 ˚C prior to analysis. Serum cortisol concentrations were determined as previously described (Coetzee et al., 2007). This method has been cross-validated between serum and plasma in the Kansas State University Veterinary Diagnostic Laboratory (Baldridge et al., 2011). In addition, the area under the plasma concentration curve (AUC), maximum cortisol concentration (Cmax), and time to maximum cortisol concentration (Tmax) were calculated.

### *Substance P*

Blood samples were drawn at baseline, 60 min, 120 min, 240 min, 480 min, and 5760 min relative to castration for SP analysis. Blood was collected from catheters into EDTA K3

tubes (Vacuette 6mL K3E Tubes, Greiner Bio-One, Kremsmünster, Austria). A 20 mM solution

of benzamidine was prepared in water and 300 μL was added to each EDTA K3 tube for a final

concentration of 1 mM benzamidine in whole blood to act as a protease inhibitor. These tubes

were centrifuged at 1500 g for 10 min. The plasma was removed with 3 mL transfer pipettes and

stored in 2 mL cryogenic vials and stored at -80 ˚C until analysis. Samples were analyzed for SP

concentrations using a validated analytical method and in the same laboratory as previously

described (Coetzee et al., 2008).

## Statistical Analysis

A general linear mixed model was fitted to each of the following response variables: EEG

single frequency AP and RP for each of delta, theta, alpha, and beta bands, EEG Ptot, mean HR,

NN50, LF/HF ratio, maximum eye temperature, minimum eye temperature, average eye

temperature, electrodermal activity, cortisol concentrations, cortisol Tmax, cortisol Cmax,

cortisol AUC, and SP concentrations. The following responses were log-transformed prior to

analysis in order to stabilize variances and meet model assumptions. In particular, EEG single

frequency AP for each of the bands and RP for alpha, beta, and theta bands, EEG Ptot, LF/HF

ratio, cortisol Tmax, and SP concentrations were log transformed, whereas delta band relative

power was transformed by squaring (i.e. power of 2).

In turn, a generalized linear mixed model was fitted to each of the responses LF and HF

assuming a beta distribution and using a logit link function to connect to the linear predictor.

The linear predictor in the statistical models used for analysis included the fixed effects

of treatment (CONT, BAND, CC, or CP), age group (6 wks or 6 mo), and time relative to

castration, as well as all 2- and 3-way interactions.

For all EEG responses (i.e. AP, RP, and Ptot), a random effect of calf nested within age-and-treatment combination was specified to recognize potential correlations between repeated observations on a given calf and also identify the calf as the appropriate experimental unit for the corresponding factors. In addition, the residual covariance structure for each calf measured at each timepoint was fitted using a spatial power structure to accommodate the spatial arrangement of EEG equipment. A blocking effect of pen was fitted as a random component when possible; however for HR, NN50, LF, HF, LF/HF, EDA and cortisol concentration, the corresponding variance component estimate converged to zero, thus the effect of pen was removed from the model. For EDA, an additional random effect of animal-by-timepoint combination was fitted to recognize unbalanced subsampling in the data collection process. For IT and SP, the variance components for the random effects of pen nested within age group and also of pen crossed with treatment were included in the final model in order to recognize the appropriate experimental units for each of these factors.

Satterthwaite's method was used to estimate degrees of freedom and Kenward Roger's procedure was used for the corresponding adjustments in estimated standard errors. When general linear mixed models were fitted, assumptions were evaluated using externally studentized residuals; inference followed upon indication that those assumptions were reasonably met. Data points that were considered to be subject to technical recording errors were excluded from analyses. Also, significant outliers detected using a Bonferroni-corrected test on studentized residuals were excluded from analyses (HR and cortisol concentration). All statistical models were fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC), implemented using Newton-Raphson with ridging as the optimization technique. Relevant pairwise comparisons were conducted using Bonferroni or Tukey-Kramer adjustments, as

deemed appropriate in each case, to avoid inflation of Type I error rate due to multiple comparisons. A p-value of $P \leq 0.05$ was considered statistically significant while a p-value of $P \leq 0.10$ was considered marginally significant.

## Results and Discussion

The primary objective of this study was to assess the physiological response to pain in calves, using castration as a model to elucidate the differences in pain response between age groups and castration methods. Rescue analgesia was not deemed necessary for any animals in this study.

### *Electroencephalogram*

On AP, a 2-way interaction between age and treatment was noted for delta, theta, alpha, and beta bands ($P = 0.01$, $P = 0.07$, $P = 0.06$, and $P = 0.07$ respectively). In particular, delta band AP was greater for 6-month-old CONT calves relative to those castrated by CC ($P = 0.01$; Figure 2.1i), whereas castration by CP and BAND showed intermediate delta band AP that was not significantly different from either. In contrast, amongst 6-week-old calves, delta band AP was greater under castration by CP than under CONT or CC ($P = 0.05$ and $P = 0.05$, respectively; Figure 2.1i); castration of 6-week-old calves by BAND showed intermediate AP of the delta band and was not significantly different from any other treatment. Also, younger calves castrated by CP had greater AP for theta ($P = 0.03$) and alpha ($P = 0.03$) bands than those castrated by CC (Figure 2.1ii and 2.1iii); whereas castration by CONT and BAND showed intermediate AP for both bands. For the beta band, castration of 6-week-old calves by CP showed greater AP than castration by BAND ($P = 0.02$; Figure 2.1iv). In contrast, amongst 6-month-old calves, there was no evidence for differences between castration treatments for any of the theta, alpha, or beta frequency bands. Additionally, when castrated by CP, 6-week-old calves had greater AP of delta,

theta, alpha, and beta bands than their 6-month-old counterparts ($P = 0.004$, $P = 0.002$, $P = 0.001$, and $P = 0.04$, respectively); these age-related differences were not apparent under other castration methods.

Regarding RP, a 2-way interaction between age and treatment was also evident for delta and beta bands ($P = 0.01$ and $P = 0.05$ respectively; Figure 2.2). In particular, 6-month-old calves in the CONT group had greater delta RP ($P = 0.004$) and lower beta RP ($P = 0.03$) than those castrated by CC. In contrast, no significant differences between castration methods were noted for RP in 6-week-old calves. Additionally, under CONT conditions, delta RP was greater for 6-month-old than for 6-week-old calves ($P = 0.01$).

On Ptot, there was also evidence for a 2-way interaction between age and treatment ($P = 0.03$; Figure 2.3) whereby 6-month-old calves under CONT showed greater Ptot than calves of the same age castrated by CC ($P = 0.05$). In turn, amongst 6-week-old calves, the CP group showed greater Ptot than castration by CC ($P = 0.05$) and CONT ($P = 0.06$). Moreover, age-specific differences in Ptot were apparent only when castration was conducted by CP, whereby 6-week-old calves had greater Ptot than 6-month-old calves ($P = 0.004$); no other castration method showed evidence for age difference in Ptot.

A time effect was also noted for several EEG results. Specifically, the AP of theta and alpha bands decreased significantly from Base through R1020 ($P = 0.0001$) for all ages and treatments. In contrast, RP of the delta band decreased from Base through R1020 ($P < 0.0001$) whereas RP of the theta and beta bands increased from Base through R1020 for all ages and castration methods ($P = 0.0039$ and $P = 0.02$, respectively). For RP of the alpha band, power increased from Base through R510 for all treatments and age groups ($P < 0.02$). Total power

decreased after Base and stayed low throughout the evaluation period for all ages and treatments ($P < 0.0001$).

A desynchronization signal in the EEG typically involves a decrease in total power, given by a decrease in low frequency activity (delta and theta bands) and an increase in high frequency activity (alpha and beta bands) (Murrell and Johnson, 2006). This phenomenon has been associated with an increased level of arousal, and possibly an increased level of pain (Murrell and Johnson, 2006). Frequency activity is more often reported in terms of RP than of AP, as RP is intended to standardize AP across calves thus decreasing sensitivity to errors created by differences in amplifier gain between channels. As a result, the effects of amplitude differences of non-cerebral origin, such as those due to varying skull thickness or asymmetrical inter-electrode distances are minimized (Bromm and Lorenz, 1998).

According to EEG results, 6-week-old animals showed a more pronounced response to CONT than CP, as evidenced by the lower Ptot of the CONT group. No other clear pattern of desynchronization was apparent across castration methods. This unexpected result may be partially explained by lack of maturity of neural connections in younger calves (Holmes and Ben-Ari, 2001). Other studies have succeeded in recording electroencephalogram signals from young calves (1-10 weeks and 12 weeks and older) (Takeuchi et al., 1998; Bergamasco et al., 2011). It has been suggested that in calves, the brain stem function matures before birth but cortical functions continue to develop for at least the first 10 weeks of life (Takeuchi et al., 1998). In humans, developing brains have been reported to be more prone to excitability (Holmes and Ben-Ari, 2001). To the author's knowledge, no data on age-related EEG responses to different castration techniques in calves has been reported thus far. Further studies are

required to evaluate whether the EEG response observed in younger animals may be due to increased distress or hyperexcitability of younger animals.

In contrast, the physiological response to castration-induced pain seemed to be more clearly displayed in 6-month-old calves, which likely have more mature neural networks than their 6-week-old counterparts. In our study, 6-month-old calves showed the most pronounced response to castration by CC compared to CONT, as evidenced by lower Ptot, greater high frequency RP (beta band) and lower low frequency RP (delta band) of the former. While all groups (CONT included) showed evidence for EEG desynchronization over time during the procedure, desynchronization was particularly apparent in 6-month-old calves castrated by CC relative to other castration methods as described above. In addition, 6-month-old CP calves had decreased low frequency power (AP of delta and theta bands) and lower total power than 6-week-old CP calves when castrated by CP. This is also evidence for age-specific EEG desynchronization, suggesting older animals may be more distressed by the CP treatment than younger animals.

### *Heart Rate Variability*

For mean HR, we found evidence of a 2-way interaction between age and time ($P = 0.01$; Figure 2.4) but no significant effect of castration method was apparent. More specifically, regardless of castration method, younger calves experienced an increase in HR after castration through 10-20 min post-castration ($P = 0.01$); this increase was no apparent in older calves. This may be related to a transient decrease of sympathetic tone during treatment, which has been shown to decrease when visceral pain is induced by the tearing of the spermatic cords (Stewart et al., 2010). A study on the effects of castration in cattle showed an increase in HR for calves castrated with and without local anesthetic compared to controls, suggesting increased

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sympathetic tone (Stewart et al., 2010). These age-specific changes over time in mean HR suggest greater sympathetic tone in 6-week-old animals compared to 6-month-old animals. In fact, younger calves have a physiologically higher basal heart rate than older calves due to increased metabolic rate (Schmidt-Nielsen, 1970).

Regarding NN50, a main effect of age was evident whereby younger calves showed a greater NN50 than older calves ($P = 0.03$). This indicates a greater number of R-R intervals greater than 50 ms in length, which may be associated with slower ventricular rates (and increased vagal tone) or irregular cardiac rhythm (Nussinovitch et al., 2011). No evidence for any effect of castration method was apparent on this response.

For LF, we found evidence for a 2-way interaction between treatment and age ($P = 0.04$), whereby 6-month-old calves in CONT ($P = 0.02$) or castrated by CP ($P = 0.01$) showed greater LF than 6-week-old calves in the same treatment groups (Figure 2.5); no evidence for age differences in LF was apparent amongst calves castrated by BAND or CC. Increased LF is indicative of increased sympathetic activity (Acharya, 2006), which may be associated with increased distress. This result suggests 6-month-old calves may have experienced increased distress compared to their younger counterparts when under CONT or when castrated by CP.

Results for HF showed evidence for a 2-way interaction between treatment and age ($P = 0.04$). In particular, 6-month-old calves in CONT ($P = 0.02$) or castrated by CP ($P = 0.01$) showed lower HF compared to 6-week-old animals in the same groups. Decreased HF is indicative of decreased parasympathetic activity (Acharya, 2006), suggesting older calves experienced more distress than younger calves under CONT or castrated by CP. As expected, the results for HF mirrored the results for LF as the two variables are natural complements of one another in defining the balance between sympathetic and vagal tones.

Similarly for LF/HF ratio, a 2-way interaction between treatment and age ($P = 0.05$) was noted whereby 6-month-old calves in CONT ($P = 0.06$) or castrated by CP ($P = 0.01$) showed greater LF/HF ratio than younger calves undergoing the same treatments. An increase in the LF/HF ratio indicates a shift toward increased sympathetic tone, which is frequently associated with increased distress (Acharya, 2006). According to these results, older animals in the CONT and CP groups displayed increased sympathetic tone and possibly experienced increased distress compared to younger animals. Normalized LF/HF ratio followed a similar pattern as LF. This is anticipated because LF is a contributing factor to LF/HF ratio.

### *Infrared Thermography*

Regarding average eye temperature, we found marginal evidence for a main effect of age ($P = 0.06$). Regardless of castration method, 6-month-old calves showed marginally greater average eye temperature than 6-week-old calves over the period of chute restraint.

For minimum eye temperature, marginal evidence for a 2-way interaction between age and time ($P = 0.08$) was noted. More specifically, 6-week-old calves showed evidence for an increase in minimum eye temperature from -180 s to treatment (time 0) and later, regardless of treatment. In contrast, 6-month-old calves showed no evidence of changes in minimum eye temperature over time for any castration method.

No evidence was found for differences in maximum eye temperature between treatment or age groups. However, this response showed evidence for a time effect ($P < 0.0001$) whereby, regardless of age and castration method, an increase in maximum eye temperature was apparent from prior to after treatment with a small temporary peak at the time of castration.

Taken together, these results may seem contradictory with physiological expectations as sympathetic stimulation associated with pain causes vasoconstriction, which would be

anticipated to decrease temperature in the eye. However, the observed increase in temperature could also be explained by activation of the autonomic nervous system, as several articles have hypothesized the release of vasodilators, such as nitric oxide, in response to pain or distress (Mellor et al., 2000; Stewart et al., 2010). A study on pain due to castration examined infrared thermography in four treatment groups: control, surgical castration with no pain treatment, local anesthetic with no castration, and local anesthetic with castration (Stewart et al., 2010). Results showed an initial decrease in maximum eye temperature followed by an increase in maximum eye temperature amongst calves castrated without anesthetic (Stewart et al., 2010). In fact, for all castration treatments, maximum eye temperature was eventually increased after treatment, with the non-treated castration group showing the highest peak temperature and the control group showing the lowest peak temperature (Stewart et al., 2010).

### *Electrodermal Activity*

No evidence for any effect of castration treatment on EDA was apparent, but a 2-way interaction between age and time was evident ($P = 0.02$). Regardless of castration method, 6-week-old calves showed lower EDA at treatment and through 10 min post-treatment than older calves ($P = 0.03$, $P = 0.0003$, and $P = 0.01$, respectively; Figure 2.6). Lower EDA suggests decreased distress or less severe pain (Richardson et al., 2007) amongst younger calves. It should be noted, however, that several articles have reported poor reliability of the Pain Gauge for measuring nociception in large animals (Richardson et al., 2007; Kotschwar et al., 2009). In particular, Baldridge et al. (2011) hypothesized that mucin, electrolytes, and water secreted by eccrine sweat glands of the nasal planum may interfere with the Pain Gauge. It is also possible that the observed EDA differences may be simply due to physiological differences between the two age groups (Coetzee, 2011).

## *Cortisol*

For cortisol concentrations in plasma, we noted evidence for a 2-way interaction between age and time ($P < 0.001$). In all castration treatments (including CONT), 6-week-old calves showed lower cortisol concentrations than older calves during the first 60 min following treatment. No further evidence for age differences was apparent past this point. This suggests that younger calves may be less acutely distressed by handling and castration procedures than their older counterparts. No evidence for differences between castration methods was noted.

We also found evidence for a main effect of age on cortisol AUC ($P = 0.002$) and on Cmax ($P = 0.001$) such that both were increased in 6-month-old calves relative to their 6-week-old counterparts, regardless of castration method (Table 2.1). We interpret cortisol AUC as total exposure to cortisol, such that increased AUC in 6-month-old animals may be indicative of increased distress compared to 6-week-old animals, which is consistent with previous results (Mellor et al., 2000). In turn, lower Cmax in 6-week-old calves suggests that this group of animals experienced less distress (Mellor et al., 2000).

Finally, for cortisol Tmax, a 2-way interaction between age and treatment ($P = 0.04$) was noted (Table 1). Amongst controls, 6-month-old calves showed lower Tmax compared to 6-week-old calves ($P = 0.02$); however, no significant age differences were apparent under the remaining castration methods. Furthermore, for 6-month-old calves, castration by BAND or CC induced greater Tmax than CONT ($P = 0.02$ and $P = 0.02$, respectively). No treatment differences were evident in 6-week-old calves. The longer duration of the cortisol peak in older calves is consistent with increased distress, as previously reported (Mellor et al., 2000).

*Substance P*

Concentrations of SP differed by age ($P = 0.01$) whereby 6-month-old calves had greater concentrations of SP (177.05 nmol/L, 95% CI [148.92, 210.48]) relative to 6-week-old calves (137.26 nmol/L, 95% CI [124.81, 150.95]) regardless of castration method. Released by sensory nerves and by a variety of nonneural sources such as endothelial cells, macrophages, and eosinophils, SP has an important function in the modulation of nociception (Rameshwar et al., 1993). Results for SP suggest that 6-month-old calves experienced more stress or pain than their 6-week-old counterparts. Alternatively, these age-specific levels of SP may be indicative of other physiological differences between the two age groups.

## Conclusion

In conclusion, these physiological measurements indicate 6-week-old calves reacted differently than 6-month-old calves to handling and castration procedures. While all treated and control calves showed an increased level of arousal after the time of treatment, EEG evidence suggests that 6-month-old calves had the most pronounced response to the CC treatment (lower delta power, higher beta power, and lower Ptot compared to control). Although no clear pattern of desynchronization was evident in 6-week-old calves in any castration treatment, younger calves showed significant differences in EEG response when treated with CP compared to 6-month-old calves. Taken together, these physiological measurements might then be used to provide data on varying responses of different ages and castration methods on stress and pain.

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# Figures and Tables

Figure 2.1 – Least square mean estimates (and 95% confidence intervals) for absolute power $(\mu V^2)$ of the delta (i), theta (ii), alpha (iii), and beta bands (iv) of 6-week-old and 6-month-old calves subjected to control (CONT) or castration by banding (BAND), cut and clamp (CC), or cut and pull (CP) procedure. (A, B) indicate significant differences between castration treatments for 6-month-old animals; (a, b) indicate significant differences between castration treatments for 6-week-old animals ($P \leq 0.05$).



i)



ii)
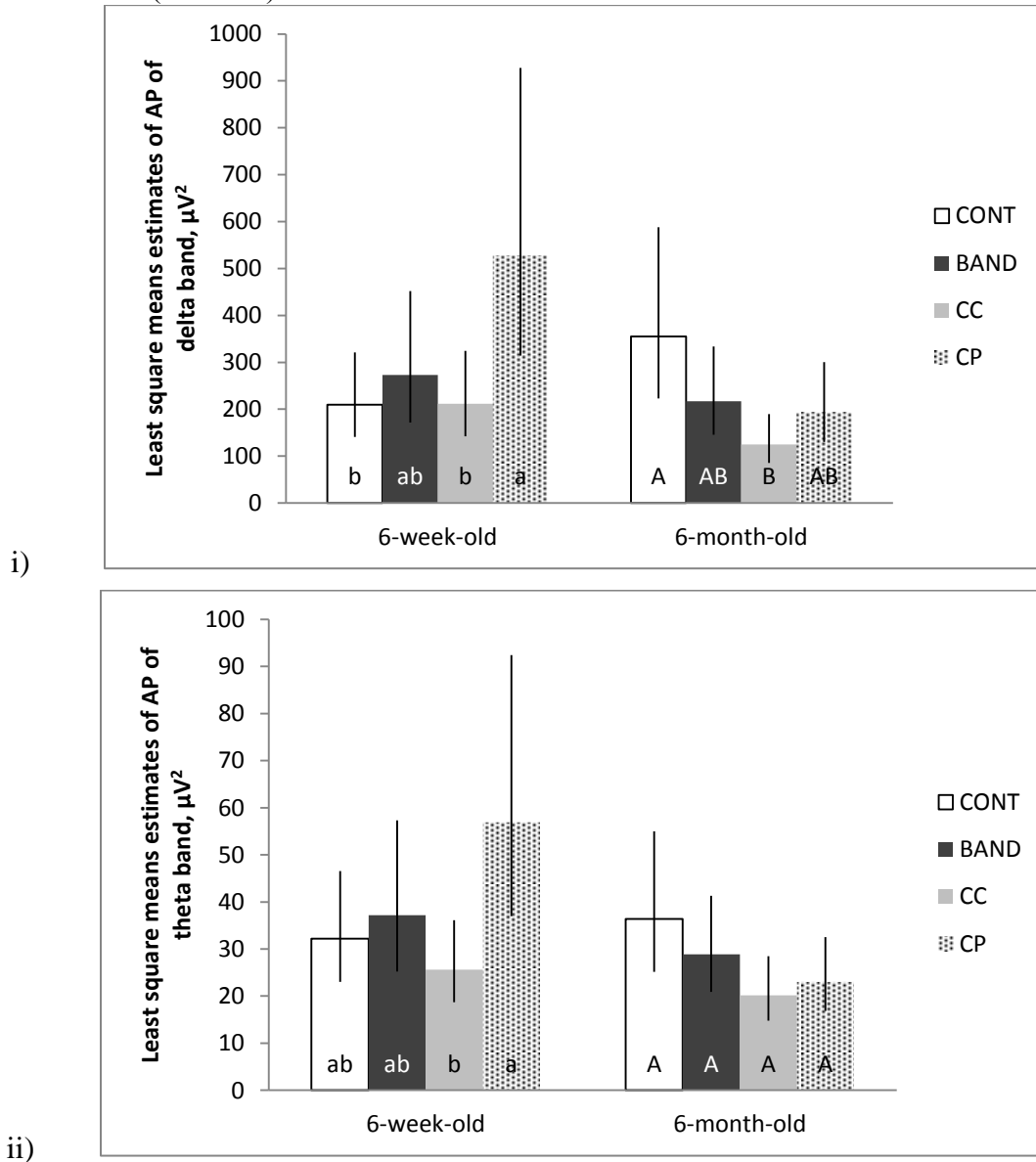
iii)
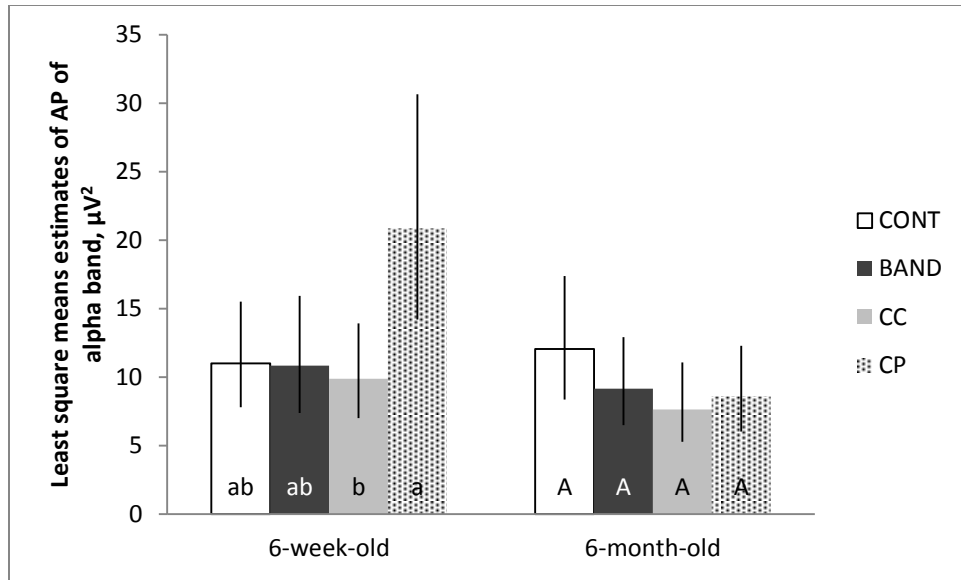


iv)

Figure 2.2 – Least square mean estimates (and 95% confidence intervals) for relative power (%) of the delta (i) and beta (ii) bands of 6-week-old and 6-month-old calves subjected to control (CONT) or castration by banding (BAND), cut and clamp (CC), or cut and pull (CP) procedure. (A, B) indicate significant differences between castration treatments in 6-month-old animals; (a, b) indicate significant differences between castration treatments for 6-week-old animals (P ≤ 0.05).



i)



ii)
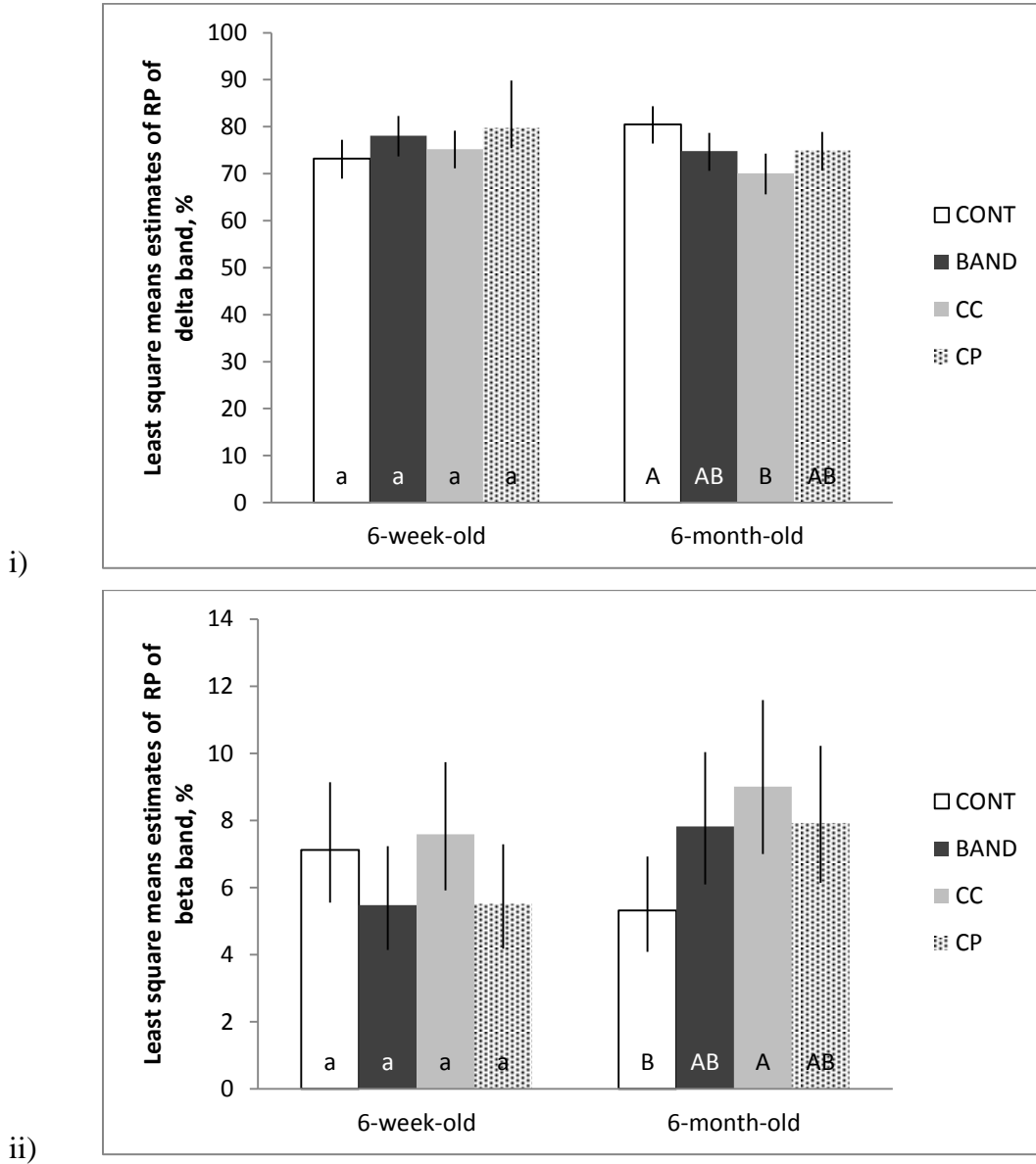
Figure 2.3 – Least square mean estimates (and 95% confidence intervals) for total power ($\mu V^2$) of 6-week-old and 6-month-old calves subjected to control (CONT) or castration by banding (BAND), cut and clamp (CC), or cut and pull (CP) procedure. (A, B) indicate differences between castration treatments in 6-month-old animals; (a, b) indicate significant differences between castration treatments in 6-week-old animals ($P \leq 0.05$).

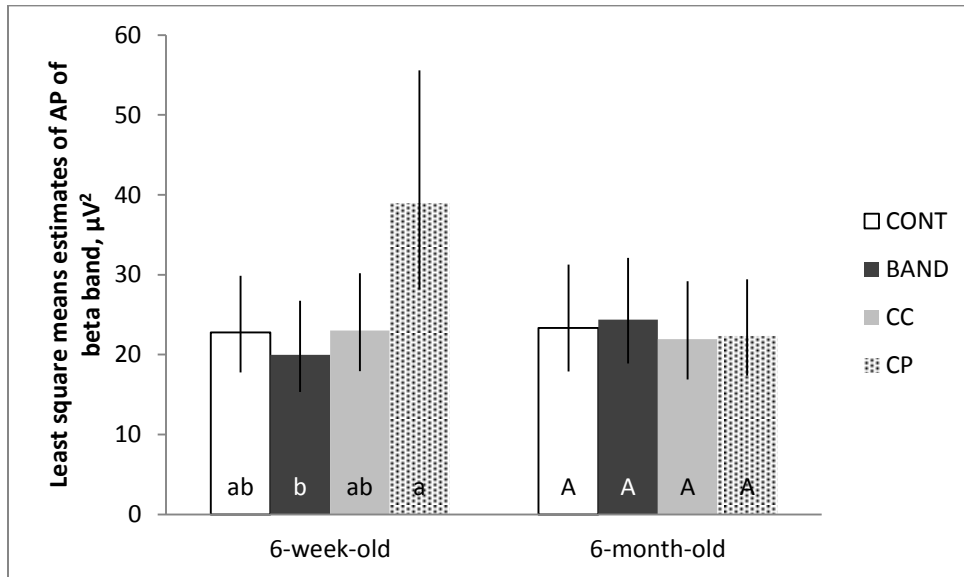Figure 2.4 – Least square mean estimates (and standard errors) for mean heart rate (bpm) of 6-week-old and 6-month-old calves. * significant difference between time points after castration within a given age group (P ≤ 0.05).
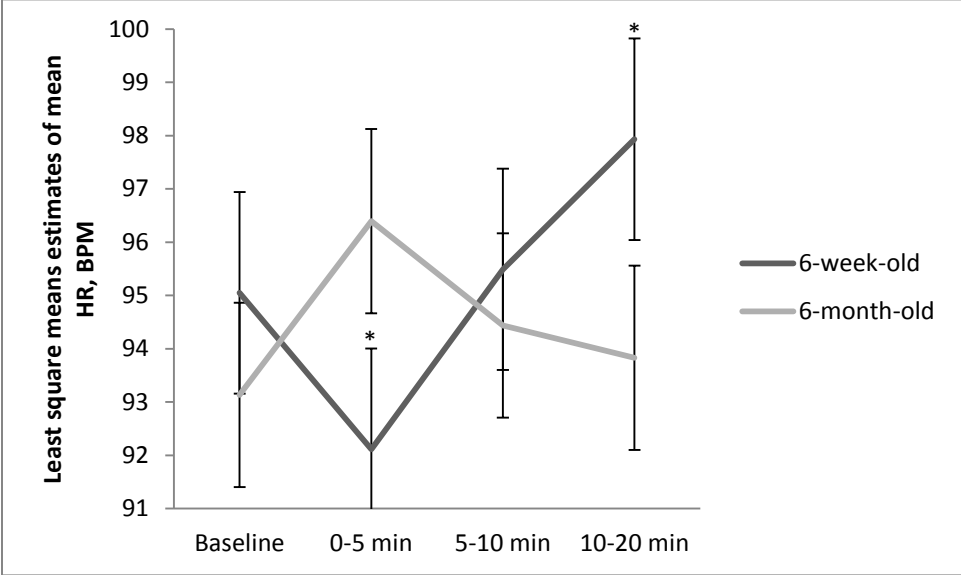
Figure 2.5 – Least square mean estimates (and standard errors) for heart rate variability normalized low frequency power (%) of 6-week-old and 6-month-old calves subjected to control (CONT) or castration by banding (BAND), cut and clamp (CC), or cut and pull (CP) procedure. * significant difference between age groups within a given castration treatment (P ≤ 0.05).
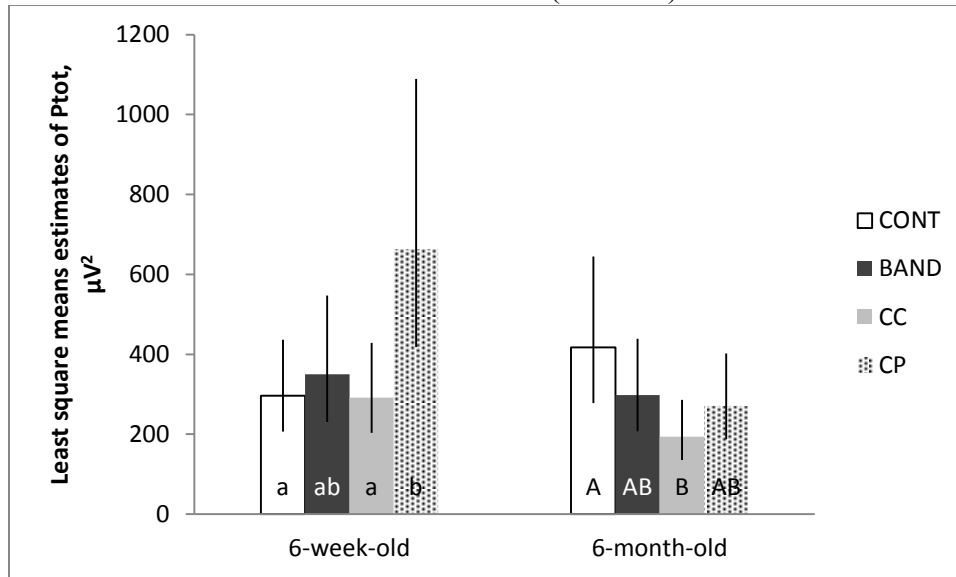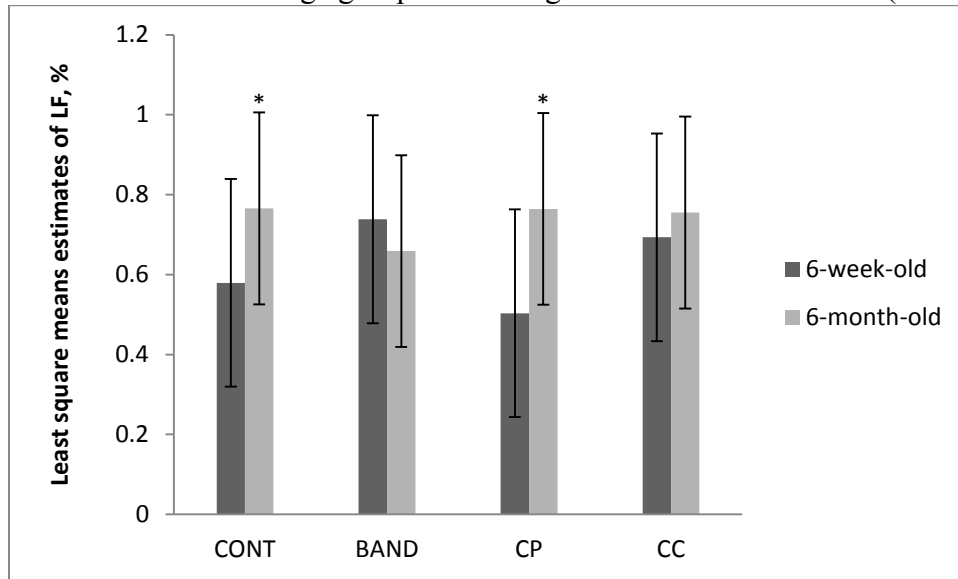
Figure 2.6 – Least square mean estimates (and standard errors) for electrodermal activity of 6-week-old and 6-month-old calves. * significant difference between age groups within a given time interval after castration (P ≤ 0.05).
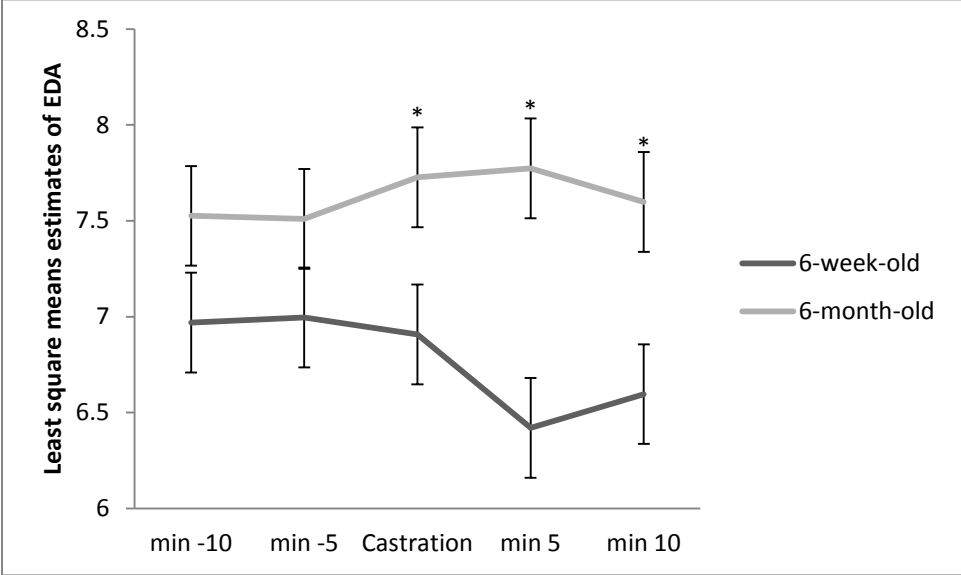
Table 2.1 – Least square mean estimates (and standard error or 95% confidence intervals) for area under the plasma cortisol curve (nmol/L)min, maximum cortisol concentration (nmol/L), and time to maximum cortisol concentration (min). (A, B) indicate significant differences between age groups for a given treatment ($P \leq 0.05$).

| Pharmacokinetics | Treatment | 6-week-old | 6-month-old |
|---|---|---|---|
| AUC (nmol/L)min | CONT | 106753.33 [72732.07, 140774.58][A] | 144967.49 [84736.67, 205198.31][B] |
| | BAND | 69681.72 [31644.80, 107718.64][A] | 156430.73 [99221.98, 213639.47][B] |
| | CP | 102308.16 [64271.24, 140345.07][A] | 182457.68 [125248.93, 239666.42][B] |
| | CC | 103747.1 [69725.85, 137768.35][A] | 199291.4 [142082.66, 256500.14][B] |
| Cmax (nmol/L) | CONT | $104.32 \pm 8.5$[A] | $137.23 \pm 8.5$[B] |
| | BAND | $81.98 \pm 9.5$[A] | $121.91 \pm 8.5$[B] |
| | CP | $104.99 \pm 9.5$[A] | $127.84 \pm 8.5$[B] |
| | CC | $104.54 \pm 8.5$[A] | $124.75 \pm 8.5$[B] |
| Tmax (min) | CONT | 30.5 [17.38, 52.99][A] | 8.37 [4.13, 16.12][B] |
| | BAND | 25.42 [13.47, 47.26] | 31.25 [17.27, 55.90] |
| | CP | 26.82 [14.23, 49.81] | 17.82 [9.98, 31.25] |
| | CC | 26.00 [14.75, 45.27] | 33.55 [18.58, 59.97] |

# Chapter 3 - Implications for Further Research

The results of this study indicate younger calves have less of a physiological response to the pain induced by castration than older animals. Further research is required to determine if this difference is truly due to variability in pain perception or if the tools used to measure response to pain are not sensitive to younger calves' physiology.

In addition, this study identified CC as the most reactive castration procedure for older calves. The next step toward analgesic approval by the United States Food and Drug Administration is to show attenuation of pain when drugs are administered. Since this group showed the most robust response, cut and clamp should be the method used to test various pharmaceuticals.

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