Investigating strategies to reduce pain in piglets undergoing surgical castration and tail docking

by

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B.S., University of Florida, 2017
M.S., University of Minnesota, 2020

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Within their first week of life, millions of piglets raised each year on commercial swine farms undergo routine husbandry procedures including tail docking and surgical castration. Extensive research has demonstrated that these procedures elicit behavioral and physiological responses associated with stress and pain in piglets. However, these procedures are commonly performed without the use of analgesia or anesthesia for pain relief. This constitutes a significant animal welfare concern as these procedures negatively affect the quality of life of piglets both in the short-and-long term. Therefore, research focused on assessing pain in piglets to determine effective pain mitigation strategies and processing tools that cause less pain are needed.

Research on this topic proves challenging as pigs naturally mask signs of pain and weakness being a prey species. Additionally, animal-based indicators that are objective and can reliably detect pain in piglets over time are essential in gaining drug approval from the U.S. Food and Drug Administration for pain control in swine. As there are currently no drugs with FDA-approval for this purpose, veterinarians and producers are faced with a major obstacle in implementing piglet pain management protocols. The aim of this research is to improve the welfare of piglets on-farm. In this dissertation, study objectives were to evaluate analgesic strategies and the utility of CO_2 surgical laser in reducing pain in piglets after tail docking and surgical castration. The utility of a CO_2 surgical laser was evaluated on reducing behavioral and physiological indicators of pain and on the improvement of wound healing in piglets after tail docking. Tail docking with a CO_2 surgical laser caused less inflammation and tissue damage immediately after the procedure was performed compared to side pliers. Tail docking with a CO_2 surgical laser caused piglets to grimace less than piglets tail docked using side pliers. Additionally, there were no differences in pain

behavior between laser and conventionally tail docked piglets. Results suggest that a CO_2 surgical laser may have the potential to refine the tail docking procedure as opposed to side pliers.

The analgesic effects of transmammary delivered firocoxib to piglets was evaluated on relieving pain after tail docking and castration. Tail docking and castration caused piglets to elicit vocalizations of higher energy and peak amplitude, place more force and pressure on their front limbs than their hind limbs, and have a higher cortisol response. Pain behavior was observed more in male piglets than in female piglets after processing. However, immediately after processing, pain behavior was reduced after the administration of 3.0 and 5.0 mg/kg transmammary delivered firocoxib. The same dose of 3.0 mg/kg firocoxib reduced piglet cortisol concentrations. Results suggest that tail docking and castration cause a heightened stress and pain response in piglets as evidenced by vocalizations, pressure mat gait analysis, and cortisol. However, transmammary delivered firocoxib at a dose of 3.0 mg/kg may be effective at attenuating piglet stress and pain responses.

The analgesic effects of a multimodal approach of firocoxib (oral and transmammary) and bupivacaine liposome suspension were evaluated in reducing pain associated with tail docking and surgical castration using a CO_2 surgical laser. Bleeding was reduced by using the CO_2 surgical laser for piglet tail docking only immediately after the procedure was performed. However, this tool caused more inflammation at the tail docking site than side pliers. Benefits of using a CO_2 surgical laser for surgical castration in piglets were not observed. The multimodal approach of oral firocoxib and bupivacaine liposome suspension resulted in lower cortisol concentrations and reduced changes in gait compared to transmammary delivered firocoxib and bupivacaine liposome suspension or only the bupivacaine liposome suspension block. Results suggest that oral firocoxib in combination with bupivacaine liposome suspension may provide an effective analgesic strategy

at relieving pain in piglets after tail docking and castration as evidenced by cortisol and pressure mat gait outcomes. Further research is needed to assess whether a CO_2 surgical laser can refine piglet processing procedures.

Lastly, the analgesic effects of a multimodal approach of firocoxib and buprenorphine were evaluated in reducing pain associated with surgical castration in male piglets. Castration caused piglets to take longer strides and produce higher concentrations of prostaglandin E_2 metabolites. However, the multimodal approach of 1.0 mg/kg IM firocoxib and buprenorphine attenuated stress and pain responses post-castration by reducing prostaglandin E_2 metabolites concentrations and alterations in weight distribution onto the front limbs. Results suggest 1.0 mg/kg firocoxib and buprenorphine may also be an effective analgesic strategy at reducing piglet pain responses after castration.

In conclusion, the results presented in this dissertation indicate that tail docking and castration induce responses associated with stress and pain in piglets such as high, intense vocalizations, pain-specific-behavior, facial grimacing, inflammation, increased concentrations of cortisol and prostaglandin E_2 metabolite, and weight redistribution across the limbs. The tail docking procedure may be refined using a CO_2 surgical laser. However, evidence suggests otherwise for surgical castration. Studies also demonstrated the successful transfer of firocoxib to piglets via the sow's milk after oral administration of firocoxib to the sow. A multimodal approach of firocoxib and bupivacaine liposome suspension or firocoxib and buprenorphine may be effective options of providing analgesia to piglets in reducing pain after tail docking and castration.

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Dr. Abbie V. Viscardi, PhD

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Dedication

I would like to dedicate this dissertation to my husband, Anthony Carew, for always supporting my dreams and passions and for his daily words of encouragement. To our dogs, Lucas and Max, for their unconditional love and always being my side to put a smile on my face. To my brother, Joshua Toledo, who watches over me and my family from heaven. Joshua, your light will always shine bright. To the piglets that have allowed me to learn and grow as an animal welfare scientist, thank you for making the ultimate sacrifice for the improvement of piglet welfare everywhere.

Chapter 1 - Literature Review

Introduction

According to the most recent report by the United States Department of Agriculture's (USDA) National Agriculture Statistics Service (NASS), the U.S. markets over 100 million pigs each year (National Agriculture Statistics Service, 2023). The life cycle of a pig raised on a commercial farm can range between 25-28 weeks from birth to slaughter. Within the first week after birth, a pig will undergo routine husbandry procedures to facilitate large-scale animal management and maintain an acceptable pork quality standard (Federation of Animal Science Societies, 2020). These procedures include surgical castration, tail docking, ear notching, and teeth clipping, all of which cause clear signs of pain and distress. More research is needed to better understand how to identify, assess, and mitigate pain associated with piglet processing procedures to improve on-farm pig welfare.

While teeth clipping is becoming less common as its negative implications on piglet welfare outweigh its potential benefits, the elimination of castration, tail docking, and ear notching is not practical at present. Alternatives to castration have been examined such as immunocastration or raising intact males, however; risks towards human and animal health and safety have not been fully evaluated (Rault et al., 2011). Therefore, the adoption of pain management protocols is the most practical option in improving on-farm piglet welfare if these routine procedures are to continue.

There are multiple barriers that exist which prevent the implementation of pain management practices in swine production. Currently, there are no drugs approved by the U.S. Food and Drug Administration (FDA) with the specific label indication of pain control for swine. To obtain approval for use in animals, the FDA requires that a drug be tested for safety and

effectiveness using animal-based outcomes that are: 1) objective, 2) repeatable, and 3) clearly defined (US Food and Drug Administration, 2006). From a farm perspective, the added cost, time, and labor to administer pain management drugs are factors requiring significant consideration. Additionally, pain assessment in pigs is challenging given their innate and stoic nature to conceal signs of pain and weakness (Goldberg, 2018). Pain management protocols applicable at the farm-level are dependent on prospective studies with objectives to accurately evaluate pain in piglets and determine the efficacy of mitigation strategies.

Animal Welfare

Animal welfare is centered around animals having a good quality of life from birth until end of life (Fraser et al., 1997). As the factors that constitute a good life for an animal differ among people based on their moral code and perception of animals, animal welfare can be defined in various ways. The World Organization for Animal Health defines animal welfare as "the physical and mental state of an animal in relation to the conditions in which it lives and dies" (Escobar et al., 2018 ;World Organization for Animal Health, 2019). Similarly, the American College of Animal Welfare refers to animal welfare as 'the state of the animal" which can be assessed through the "animal's health, behavior, and biological function" (American College of Animal Welfare, 2010). Regardless of how animal welfare is defined, it fundamentally distinguishes between aspects that positively or negatively impact to the life of an animal.

Public awareness about the welfare of livestock sparked attention over 50 years ago. In 1964, ethical concerns about animals raised for agricultural purposes began after the publication of Animal Machines by Ruth Harrison, where the conditions in which the animals were raised and the practices used were brought to light (Brown and Winnicker, 2015). The following year, the Brambell Committee was appointed by the British government to assess the welfare of farmed

animals resulting in the first published animal welfare assessment framework: the Five Freedoms (Brown and Winnicker, 2015). The Five Freedoms are as follows: 1) freedom from hunger and thirst 2) freedom from discomfort, 3) freedom from pain, injury, and disease, 4) freedom to express normal behavior, and 5) freedom from fear and distress (Mellor, 2016).

The Five Freedoms set a precedent in establishing principles for the humane care and treatment of animals in research, teaching, and testing (Brown and Winnicker, 2015). Animal welfare was recognized as an independent scientific discipline focused on measuring how an animal, a sentient being, perceives and responds to its surroundings. A second animal welfare assessment framework was later established applying three main value-based concepts: biological function, natural living, and affective states (Fraser et al., 1997). Together these three concepts form a well-known framework to assess animal welfare termed the "Three Circles Model" or the "Three Spheres Model" (Fraser et al., 1997).

Biological functioning describes the physical state of an animal (e.g., health and physiology). Natural living emphasizes the need for an animal to live its life in an environment freely expressing its natural and instinctual behaviors. Lastly, affective states are the positive (e.g., happiness, pleasure, and content) and negative emotions (e.g., pain, fear, and anxiety) felt by an animal. Decisions made to improve animal welfare require a "sound scientific understanding of animals and how they are affected" by factors within its environment based on an in-depth assessment of animal welfare (Fraser, 2008). In using the Three Circles Model, a thorough scientific evaluation of animal welfare is possible, using various indicators (e.g., behavior, physiology, nutrition, and housing), with respect to an animal and its environment as a whole.

Animal welfare indicators have been categorized into the following three categories: resource-based, management-based, and animal-based indicators (EFSA Panel on Animal Health

and Welfare, 2012). Piglet processing procedures fall under management-based indicators, while pig responses to these procedures such as pain are assessed through animal-based indicators. Pain research in pigs is integral to improving their health and well-being on-farm. Animal-based indicators are tools animal scientists and producers can utilize to make science-based decisions regarding proper animal care.

Animal welfare in research

Ethical and humane use of animal models for scientific research requires adherence to the 3Rs Replacement, Reduction, and Refinement to consider all means of alleviating animal pain and suffering (Guatteo et al., 2012). The basis of the 3Rs framework is to use other models or technology in place of animals (Replace), use the smallest number of animals necessary to obtain reliable results (Reduce), and modify experimental procedures to inflict minimal pain (Refine). The American Veterinary Medical Association (AVMA) and the American Association of Swine Veterinarians (AASV) have both made statements in support of research objectives similar to the 3Rs in directing efforts towards the approval of analgesic and/or anesthetic protocols and the development of techniques that minimize the adverse effects caused by piglet processing procedures (American Veterinary Medical Association, 2014).

Pain

An understanding of the pain pathway is essential for pain recognition and evaluating effective pain mitigation strategies. Pain feels a certain way, but the challenge lies in determining how it makes an individual feel. The International Association for the Study of Pain (IASP) currently defines pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja et al., 2020).

Nociception is the process by which the peripheral and central nervous systems work together to process the sensation of pain (Osterweis et al., 1987). Transduction, transmission, modulation, and perception are the four main processes that make up the pain pathway in the mammalian species (Osterweis et al., 1987). Transduction is initiated via noxious stimuli in the form of physical pressure, extreme temperatures, or chemicals that cause tissue damage. Pain receptors called nociceptors in the periphery (e.g., skin and muscle) transmit information about the noxious stimuli as a nerve signal to the central nervous system from the dorsal horn of the spinal cord through the spinothalamic tract to the brain. Myelinated A-delta and unmyelinated C-fibers are the primary nerve fibers that transmit the nerve signals to their central location in the brain. Once the signal reaches the thalamus and somatosensory cortex, pain can be modulated (i.e., amplified or suppressed) and perceived.

Pain has an evolutionary function to aid in recovery and survival (Bateson, 1991). Acute pain signals risk and is temporary. Animals learn to adapt by optimizing their conditions to heal from injury or disease and avoid a harmful threat (Mathews et al., 2014). In contrast, chronic pain is maladaptive causing increased sensitivity and abnormal responses (Mathews et al., 2014). Chronic pain can persist for several months to years, a period well-beyond what is considered a normal healing time depending on the injury. Piglet processing procedures are primarily associated with acute pain, but there is evidence suggesting pigs may endure chronic painful conditions (Goldberg, 2018).

Sex, age, health status, temperament, and degree of tissue damage are a few individual differences that determine the level of pain an animal can feel and how it reacts in response to that pain (Reid et al., 2013). Self-reporting is considered the gold-standard of pain assessment in

humans, however; as this is not possible in animals only inferences about their pain experiences can be made (Ison et al., 2016).

Piglet Processing Procedures

Castration

In the U.S., male piglets raised for food production are typically castrated within the first 7 days of life (Rault et al., 2011). Castration is routinely performed on commercial farms to prevent unwanted breeding, reduce aggressive behavior among conspecifics, and inhibit the production of boar taint (Sutherland, 2015). Boar taint is produced by androsterone and skatole stored in the fat, tissue, and gut of intact males, which causes an unpleasant smell and taste in the meat (Sutherland, 2015). The technique most commonly used to castrate piglets involves the use of a sharp scalpel to make a single vertical incision over each testicle (Rault et al., 2011). Following the incisions, the testicles are removed from the body by either cutting or tearing out the spermatic cord (Rault et al., 2011). In some cases, iodine is applied onto the surgical site to reduce infection.

Castration is a painful procedure causing both behavioral and physiological changes associated with pain and distress (Ison et al., 2016). In comparison to other routine piglet processing procedures, surgical castration is more painful as it involves causing tissue damage in highly innervated regions (Schmid and Steinhoff-Wagner, 2022). The removal of the spermatic cord is considered the most painful step (Taylor and Weary, 2000). Despite this, it is a common practice to not provide piglets any form of analgesia or anesthesia to manage castration pain. The Guide for the Care and Use of Agricultural Animals in Research and Teaching (Ag Guide) states that an anesthetic and/or anesthesia should be administered when castration occurs after 14 days of age (Federation of Animal Science Societies, 2020); as piglets are often castrated before they are 7 days old, this requirement for pain management is not applicable.

During castration, piglets evoke high frequency screams and attempt to escape (e.g., leg kicks and body flailing) (Lou et al., 2021). Pain-specific behavior (e.g., prostration, posterior scooting, and scratching) and facial grimacing, increased heart and respiration rates, elevated levels of blood and salivary cortisol, swelling and inflammation at the wound site, and greater wound sensitivity have all been reported in the hours to days after castration (Schmid and Steinhoff-Wagner, 2022).

Tail docking

Tail docking is performed within the first days of life on both male and female piglets to reduce the incidence of tail biting (Valros, 2018). Tail biting can inflict wounds ranging from minor to severe wounds (Sutherland and Tucker, 2011). Pigs are highly motivated to explore their environment through rooting and chewing, so the inability to perform these behaviors within a barren environment may cause them to redirect their stress and frustration onto their pen mates (Valros, 2018). Stocking density, ventilation, temperature, nutrition, and genetics are other factors that have been considered to prompt tail biting (Sutherland and Tucker, 2011).

Tail docking removes about one-third to one-half of the tail (Sutherland and Tucker, 2011). Several tools exist that can be used to tail dock pigs including scissors, a cautery iron, or, most commonly cutting pliers (Sutherland and Tucker, 2011). Studies indicate that tail docking can cause acute and chronic pain (Valros, 2018). Tail docked piglets scream and grunt for longer and at higher intensities, and display more intense body movements at the time of the tail docking procedure compared to sham-handled piglets (Schmid and Steinhoff-Wagner, 2022). In the period following the procedure, tail docked piglets are observed frequently tail jamming (i.e., tail tucking) and tail wagging (Sutherland and Tucker, 2011). Long-term consequences of this procedure, including neuroma formation at the tail tip, hypersensitivity, and alterations in gene expression

associated with inflammatory and neuropathic pain pathways, have also been reported, suggesting that tail docking may lead to chronic pain in pigs (Sutherland, 2015; Schmid and Steinhoff-Wagner, 2022).

Ear notching

Swine identification is a requirement by law for traceability and interstate travel in the U.S to protect public health and safety (USDA Animal and Plant Health Inspection Services, 2020). The unique ID given to a pig allows individuals the ability to locate pigs and maintain accurate records (e.g., source of origin, genetic lineage, health status, and drug administration and withdrawal periods) (Prunier et al., 2020). Ear notching or tagging are methods of pig identification (Schmid and Steinhoff-Wagner, 2022). Tattoos and implanted transponders are other forms of pig identification used in U.S. and other countries (Prunier et al., 2020).

Ear notching is performed shortly after birth before or at the same time as tail docking and castration (Schmid and Steinhoff-Wagner, 2022). Following the universal ear-notching system, V-shaped ear notching pliers are used to remove sections of the auricle up to a certain to depth (4.8-6.35 mm) to avoid tearing of the ears (The Humane Society of the United States, 2010). The right and left ear correspond to the litter and individual pig number, respectively. Ear notching appears to cause acute pain, as piglets have higher cortisol levels, more tissue damage, and are observed intensely head shaking and ear flapping compared to sham-handled and ear-tagged piglets (Schmid and Steinhoff-Wagner, 2022).

Teeth clipping

From birth, piglets are equipped with fully erupted and sharp deciduous canines and third incisors, which are referred to as “needle teeth”. Teeth clipping aims to limit wounds caused by the needle teeth onto the udder of the sow at nursing and face of littermates when competing for

milk access (Sutherland, 2015). Similar to ear notching, teeth clipping is usually done at the time of tail docking and castration. Cutting pliers or electrical rotating grinders are used to remove the teeth up the gum line or to grind down the sharp tips of the teeth. In only removing the tip (top one-third) of the tooth, damage to the pulp cavity and gum line can be prevented and is recommended (Sutherland, 2015).

Despite select studies reporting a reduction in face and teat injuries, the abnormal behavior of teeth chomping and presence of open pulp cavities, tooth factures, hemorrhaging, and abscesses have also been observed after teeth clipping (Herskin and Di Giminiani, 2018). As a result of this, piglets are at a higher risk of developing infections and suffering from long-term pain and inflammation. Given these reported procedural consequences significantly compromising piglet welfare, teeth clipping is gradually being phased out or only performed when necessary (American Veterinary Medical Association, 2014).

Animal-based outcomes for pain assessment

Animal-based indicators are valuable tools used for benchmarking, allowing comparisons across studies and farms with regard to animal welfare (Mench, 2018). Veterinarians, scientists, and producers can use animal-based indicators to assess pain in animals and determine the effectiveness of potential solutions (EFSA Panel on Animal Health and Welfare, 2012).

Body weight

Body weight is an indicator of healthy growth and functioning (Morton and Griffiths, 1985). Thus, body weight, or maintenance of normal growth and functioning in food-producing animals, can be used as measurement of animal welfare. For experimental design and enrollment purposes, piglet body weight is typically collected at the start and end of a study. Animals that fail

to maintain a normal body weight or gain weight can be indication of reduced health and well-being (Morton and Griffiths, 1985).

The body has naturally evolved behavioral and physiological mechanisms to help an animal cope with stressors (Moberg, G.P. and Mench, 2000) Under stress or pain, the body will divert energy and resources (e.g., glucose) away from other biological functions to focus on healing (Moberg, G.P. and Mench, 2000). For example, if an animal has sustained tissue damage, time and energy are needed to heal and recover. Following a painful procedure, animals are observed having reduced feed intake and weight gain (Anil et al., 2005).

The effect of piglet processing procedures on piglet growth has been assessed and the results are varied. Tail docking and ear notching had no effect on average daily gain (ADG) compared to tail manipulation alone (Torrey et al., 2009). Likewise, no differences in body weight were found after tail docking with or without pain control (local or general anesthesia) (Sutherland et al., 2011). With respect to castration, growth performance was not different between castrated and sham-castrated piglets (Hay et al., 2003). In contrast, another study found that castration had negative effects on ADG and pre-weaning mortality based on piglet weight at birth when compared to intact males despite being given meloxicam before castration (Morales et al., 2017).

Behavior

In a pain state, animals will deviate from their normal behavioral patterns and activity levels, where certain behaviors will ether increase or decrease in magnitude. A “deviation from normal behavior is the most important single indicator of pain” (Anil et al., 2005). Pain-specific behavior occurs only or more frequently after a painful event. A multitude of pain-specific behaviors associated with tail docking and surgical castration have been identified including tail jamming, posterior scooting, spasms, trembling, stiffness, and prostration. Previous work had

found that after tail docking, piglets were observed tail jamming (Torrey et al., 2009) and posterior scooting (Sutherland et al., 2008) more than non-tailed docked piglets. However, there were no differences in pain-specific behavior after tail docking with or without pain control (Sutherland et al., 2011).

During the tail docking and castration procedures, piglets overtly display behaviors that have been broadly referred to as either reaction (Walker et al., 2004), escape (Marchant-Forde et al., 2009), defense (Leidig et al., 2009), resistance (Hansson et al., 2011), agitation (Rault and Lay, 2011), or struggle (Lou et al., 2021) behaviors. In comparison to sham-handling, piglet behavioral responses were significantly amplified at the time of castration without pain control (Lou et al., 2021). When castration was performed in combination with pain control, piglet behavioral responses at the time of castration was reduced (Walker et al., 2004 ; Leidig et al., 2009 ;Hansson et al., 2011;Rault and Lay, 2011).

With regards to surgical castration, previous work has described castrated piglets were engaged in the following behaviors more than non-castrated piglets: prostration, stiffness, trembling, posterior scooting, huddling, isolation, desynchronization, and inactive (Hay et al., 2003; Llamas Moya et al., 2008). Pain-specific behaviors were reduced after castration with meloxicam and/or lidocaine (Hansson et al., 2011), while others reported no differences (Burkemper et al., 2020).

With respect to overall activity after castration, behavioral responses are mixed. One study found that castrated piglets spent more time sitting, standing, and in contact with the udder and less time lying compared to sham-castrated piglets (Taylor et al., 2001). Conversely, another study found that castrated piglets spent less time walking, sitting, and in contact with the udder compared to sham-castrated piglets (Llamas Moya et al., 2008). When given a potent analgesic

(buprenorphine), castrated piglets were more active (standing and walking) compared to castrated piglets given no pain control (Viscardi and Turner, 2018a).

Facial grimace

Facial expressions in both humans and animals can provide information about their pain state (McLennan et al., 2019). For the assessment of pain in non-verbal subjects, facial expressions are particularly useful as a form of communicating whether they are in pain and to what extent. In response to a painful stimulus, the following facial expressions increase in intensity and duration in humans: eye tightening, nose wrinkling, and lip raising (Williams, 2002).

In identifying facial expressions associated with pain, scientists have developed species-specific grimace scales to non-invasively detect acute pain in animals undergoing painful procedures including mice (Langford et al., 2010), rats (Sotocina et al., 2011), rabbits (Keating et al., 2012), horses (Dalla Costa et al., 2014) lambs (Guesgen et al., 2016), and piglets (Di Giminiani et al., 2016) . Grimace scales assess changes in facial expressions controlled by muscle movements of the four major regions of the face – eyes, nose and cheek, mouth and jaw, and ears (Descovich, 2017). The changes in facial expression are termed "facial action units" (FAUs) that were developed in part with the Facial Action Coding System (FACS) and are scored using an ordinal scale from 0 (not present) to 2 (obviously present) (McLennan et al., 2019).

Orbital tightening, ear position, nose bulging/cheek tension, temporal tension, and lip contraction are some of the FAUs of the Piglet Grimace Scale (PGS) used to assess pain in piglets after tail docking and castration. The effectiveness of the PGS to detect pain associated with surgical castration and tail docking has been variable. One study did not find differences in PGS scores between castrated and non-castrated piglets (Maria E Lou et al., 2022). However, others have reported the opposite with piglets undergoing surgical castration grimacing more than sham

piglets (Viscardi and Turner, 2018b). Additionally, piglets were reported to have the highest PGS scores within the first 5h after tail docking and castration, but changes were unrelated to treatment (Viscardi et al., 2017). Additional research is needed to validate the PGS and determine its correlation with other validated pain measures such as behavior.

Vocalizations

Pigs are social creatures that primarily communicate using vocalizations. Over 20 unique pig vocalizations have been identified including grunts, squeals, and screams, each with a specific meaning dependent on the acoustic characteristics (e.g., frequency, rate, amplitude, and duration) of the call type (Manteuffel et al., 2004). As neonates, piglets vocalize to communicate with their mother signaling when they are hungry, cold, or mother-seeking (Puppe et al., 2005). Calls characterized by a low pitch and tonality (e.g., grunts) tend to indicate a positive welfare state (e.g., nursing) while calls of a louder and longer pitch (e.g., squeals and screams) may indicate a negative welfare state (e.g., social isolation) (Manteuffel et al., 2004).

Piglet vocal responses to painful stimuli, such as tail docking and castration have been extensively studied at the time of the procedure. Tail docking (and ear notching) elicited more calls of greater frequency than non-processed piglets (Torrey et al., 2009). Conversely, another study found that there were no vocal differences between docked piglets and non-docked piglets (Marchant-Forde et al., 2009). When examining the difference in lidocaine formulation on pain vocalizations, the injectable form of lidocaine reduced the percentage of stress vocalizations during tail docking compared to lidocaine in spray and gel form (Sutherland et al., 2011).

In comparing handling alone to castration, vocalizations were more pronounced during castration with significantly higher duration, peak frequency, and entropy (Puppe et al., 2005). Piglet vocalizations were greatest during the removal of the spermatic cord compared to all other

steps in the castration process, suggesting this step caused the most pain (Taylor and Weary, 2000). The method of spermatic cord removal (cutting or tearing) did not result in a difference in piglet vocalizations (Taylor and Weary, 2000). Castration with lidocaine reduced piglet heart rate and call frequency and intensity compared to castration without lidocaine (White et al., 1995;Hansson et al., 2011). However, an NSAID only (meloxicam), did not reduce piglet vocalization at the time of surgical castration (Kluivers-Poodt et al., 2012;Tenbergen et al., 2014).

Wound scoring

The assessment of tail docking and surgical castration wounds using a quantitative scoring system can provide insight into the wound healing process as well as determine which techniques lead to faster healing.

Hansson et al. (2011) assessed the level of swollenness of the castration site using a scale from 1 (least swollen) to 4 (most swollen). Castrated piglets given no pain control had more swelling at the surgical site than piglets given pain control (meloxicam and/or lidocaine) (Hansson et al., 2011). In another study, castration wounds were evaluated based on three main characteristics: wound closure, level of exudate, and inflammation (calor, dolor, rubor, and tumor) (Kluivers-Poodt et al., 2013). Overall, there were no differences in wound healing, level of exudate, or inflammation in castrated piglets, regardless of whether or not they were provided with pain management (Kluivers-Poodt et al., 2013).

Tail docking (Maria E. Lou et al., 2022) and castration (Sutherland et al., 2010) wound scoring using a quantitative scale have been reported. To our knowledge, (Maria E. Lou et al., 2022) developed the first scale created to assess wounds after tail docking piglets. Tail docking wounds were scored from 0 (fully healed intact skin with no scab) to 5 (raw, open wound with presence of fresh, red blood) (Maria E. Lou et al., 2022). This tail docking wound scoring system

was adapted from the first visual castration wound scoring system, where wounds were scored from 1 (completely healed (no scab)) to 6 (wound is still open and still looks raw) (Sutherland et al., 2010).

Infrared thermography

Infrared thermography (IRT) imaging has been used to non-invasively evaluate physiological changes in body temperature due to stress, pain, and inflammation. The onset of pain after tissue injury is accompanied by systemic and local thermal responses (Whittaker et al., 2023). The activation of the sympathetic nervous system (SNS) leads to peripheral and core changes in temperature as an increase in cardiovascular function prioritizes transporting oxygenated blood to critical organs over peripheral regions (Whittaker et al., 2023). At the site of tissue injury, pro-inflammatory mediators (e.g., prostaglandins and cytokines) cause vasodilation to promote healing for immune cells to easily enter and prevent infection (Whittaker et al., 2023). This vasodilation produces local heat, which radiates from the skin and raises the surface temperature of the injury site (Whittaker et al., 2023).

The IRT imaging technology measures temperature by obtaining the infrared radiation energy emitted by the target object (Zheng et al., 2022). Images of piglets before and after tail docking and surgical castration have targeted anatomical locations including the eyes, ears, snout, cranium, and surgical sites (i.e., tail and scrotum) (Hansson et al., 2011; Bates et al., 2014; Coetzee, 2019; Whittaker et al., 2023). After tail docking, piglets exhibited higher ocular temperatures than at baseline, indicating increased blood to an area critical for pain perception (Whittaker et al., 2023). Moreover, studies showing that tail docked and castrated piglets receiving analgesia (meloxicam or firocoxib) may have an attenuated SNS response, with higher cranial skin temperatures compared to controls (Bates et al., 2014 ;Coetzee, 2019).

Pressure mat gait analysis

An abnormal gait has been identified as a potential indicator of pain or discomfort (Mathews et al., 2014). Animals with an abnormal gait may intentionally be shifting their weight or positioning themselves to remove pressure off an injured area (Mathews et al., 2014). Hence, gait scoring is a "well-studied animal-based measure of pain felt by an individual animal when walking", and be used to assess pain leading to proper treatment (EFSA Panel on Animal Health and Welfare, 2012). Technological advancements have led to commercially available floor-based force plate systems as a method to objectively assess gait (Rushen et al., 2012).

A pressure mat has advantages over visual gait scoring systems, as it has with the ability to calculate stride length, contact pressure, contact area, and stance phase duration (Baysinger et al., 2021). Pressure mat gait analysis in conjunction with behavior scoring resulted in the approval of the first NSAID (Banamine Transdermal) for pain control in a food-producing animal (i.e., cattle with foot rot) (Baysinger et al., 2021).

Regarding castration, pressure mat gait analysis has been used to assess calf responses to transdermal flunixin meglumine (Kleinhenz et al., 2018) and lidocaine, meloxicam, and bupivacaine liposome suspension (Martin et al., 2022). Both studies found that castrated cattle shifted more of their weight onto their front limbs compared to non-castrated cattle (Kleinhenz et al., 2018;Martin et al., 2022). However, pressure mat gait analysis for piglets after tail docking and castration pain piglets has only reported once (Coetzee, 2019). Similar to cattle, Coetzee (2019) found that piglets receiving placebo also applied more force onto their front limbs after castration compared to piglets medicated with firocoxib (Coetzee, 2019). Thus, animals applying more force and pressure onto their front limbs after castration and away from the surgical site may be indicative of pain.

Cortisol

Cortisol, the primary glucocorticoid of most mammals, is a biomarker that has long been used to measure stress and pain in animals (Moberg, G.P. and Mench, 2000). Stress is defined as "the biological response an animal exhibits in an attempt to cope with threats to its homeostasis" (Brown and Winnicker, 2015). In a stressful or painful state, the biological response of the body is to activate the autonomic, neuroendocrine, and immune systems (Moberg, G.P. and Mench, 2000).

As a component of the neuroendocrine systems, the hypothalamic-pituitary-adrenal (HPA) axis has been of primary interest in animal pain assessment studies given its measurable physiological effects, for example, the production and release of cortisol. Cortisol is necessary for the process of gluconeogenesis, which produces glucose as a source of energy for the body during a time of "fight or flight".

Peak cortisol levels have been reported to peak between 20-60 min after tail docking (Sutherland et al., 2011) and castration (Sutherland et al., 2012 ;Lonardi et al., 2015; Merenda et al., 2022) with no differences after 120 min or 24h, indicating a cortisol stress response may be short lasting. Conversely, studies have reported no changes in cortisol after the same two procedures (Sutherland et al., 2008 ;Marchant-Forde et al., 2009). Cortisol results should be interpreted with caution, as stress can present itself in other forms such as eustress and distress (Brown and Winnicker, 2015). Moreover, confounding factors can influence cortisol levels including time of day, season, inter-animal variability, and previous human-animal interactions (Moberg, G.P. and Mench, 2000).

Analgesia for use in swine

The use of analgesia in livestock raised for food and fiber, such as swine, is regulated under the FDA Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) (US Food and Drug Administration, 1994). Under AMDUCA, the provision of analgesia to swine must be under the direct order of a veterinarian in conjunction with a valid veterinarian-client-patient relationship (VCPR) and is designated as "extra-label drug use" (ELDU) (US Food and Drug Administration, 1994). In addition, ELDU requires the use of an FDA-approved animal or human drug for only therapeutic purposes only, where the health of an animal is comprised. It is prohibited to use ELDU in feed or if results in any level of violative residues in food for human consumption that may threaten public health and safety (US Food and Drug Administration, 1994).

Local anesthetics

Lidocaine

Pain-specific sodium channels receive nerve signals from painful stimuli (Meintjes, 2012). Lidocaine targets and blocks the conductance of these sodium channels making them unresponsive, causing a loss of pain sensation locally (Meintjes, 2012). Within 5-10 mins. of administration, lidocaine provides pain relief and has a duration about of about 1h (Burkemper et al., 2020).

For tail docking, lidocaine has been injected into the base of the tail (Sutherland et al., 2011) or applied topically on the entire tail (Viscardi and Turner, 2019). For castration, lidocaine has been applied in spray and gel form (Sutherland et al., 2010). The efficacy of several techniques of lidocaine injection into the scrotum, testes, and/or spermatic cord have been tested (White et al., 1995; Kluivers-Poodt et al., 2012; Maršálek et al., 2015; Bonastre et al., 2016). Successful subcutaneous lidocaine administration enables the drug to diffuse from the testicles toward the

inguinal ring and spermatic cord, allowing the drug to distribute through the surgical site and reduce pain more effectively (White et al., 1995; Kluivers-Poodt et al., 2012).

Different lidocaine formulations (e.g., percentage of lidocaine or inclusion of epinephrine or sodium bicarbonate) and multi-modal approaches combining lidocaine with an NSAID have also been tested (Hansson et al., 2011 ;Kluivers-Poodt et al., 2012; Burkemper et al., 2020). Study results are mixed, with some demonstrating that lidocaine was able to reduce piglet processing pain (White et al., 1995; Hansson et al., 2011; Kluivers-Poodt et al., 2012; Bonastre et al., 2016), while others found no effect (Sutherland et al., 2010; Sutherland et al., 2011; Maršálek et al., 2015; Burkemper et al., 2020). Differences regarding study methodology used across studies may explain why results are mixed. Farm practical guidelines for lidocaine are not possible without clear evidence indicating which formulation, route, and technique all together are most effective and safe for both the piglet and administrator.

Bupivacaine liposome injectable suspension

Given the expectation that post-operative pain and inflammation are most serve within the first 72 hours and the need for long-acting local anesthesia for use in veterinary medicine, the FDA approved a prolonged-release formulation of bupivacaine called bupivacaine liposomal injectable suspension (NOCITA®) for use in dogs and cats (US Food and Drug Administration, 2016a ;US Food and Drug Administration, 2018). Bupivacaine has the same mechanism of action as lidocaine. However, its onset is slower than lidocaine, ranging between 10-20 min, with a longer duration of action of up to 8 h (Bonastre et al., 2016). In the case of bupivacaine liposomal injectable suspension, multi-vesicular liposomes containing bupivacaine slowly release bupivacaine and distribute it from local tissues into systemic circulation over a period of 96 h (Lascelles and Kirkby Shaw, 2016).

Similar results were found in dogs after cranial cruciate ligament surgery, with bupivacaine liposomal injectable suspension-treated dogs having lower pain scores than placebo-treated dogs (US Food and Drug Administration, 2016a). A higher percentage of placebo-treated dogs also required rescue analgesia within the first 24h post-surgery (US Food and Drug Administration, 2016a).

Furthermore, Martin et al. (2022) discovered that bupivacaine liposome suspension may be as effective as the multi-modal approach of lidocaine and meloxicam in reducing castration pain in dairy calves. To date, there are no studies reporting the novel use of bupivacaine liposomal injectable suspension for tail docking and castration pain in piglets, but positive outcomes in other species may justify its exploration.

Non-steroidal anti-inflammatory drugs (NSAIDs)

Firocoxib

Damage to tissues initiates a cascade of pain-inducing and inflammatory events. Firocoxib decreased the acitivty of the COX-2 enzyme, which is a component of the arachidonic acid pathway that synthesizes prostaglandins (Flecknell, 2023). As a COX-2 selective inhibitor, firocoxib primarily inhibits the production of prostaglandins involved with nociceptor sensitization and inflammation, which leads to reduced pain (Meintjes, 2012). The half-life of firocoxib is species-dependent ranging from 6.7 h in calves to 40 h in adult horses (Papich, 2021). Firocoxib also has a longer half-life and larger volume of distribution in sows when compared to meloxicam, flunixin, and ketoprofen administration in pigs (Coetzee et al., 2019).

Like other NSAIDs, firocoxib has anti-inflammatory, antipyretic, and analgesic effects, which are all highly beneficial for pain relief. Firocoxib in the form of chewable tablets received FDA approval for dogs (Previcox®) (US Food and Drug Administration, 2004) and horses

(Equioxx®) (US Food and Drug Administration, 2016b) to relieve pain and inflammation associated with osteoarthritis. To our knowledge, there are two reported studies on the effects of firocoxib on piglet processing pain. Coetzee et al. (2019) found that the administration of transmammary delivered firocoxib to piglets undergoing tail docking, castration, and teeth clipping resulted in lower cortisol concentrations and increased weight gain. In a follow-up study, the transmammary delivery of firocoxib was evaluated for its ability to relieve pain associated with piglet tail docking, castration, and ear notching, where results showed a reduced effect on skin temperature and gait suggesting a reduce stress and pain response (Coetzee, 2019).

At present, the liquid formulation of firocoxib is no longer available and literature on oral firocoxib administration strategies for sows is very limited (Kleinhenz et al., 2020). There is opportunity for future studies to evaluate the oral formulation of firocoxib to determine if similar conclusions are attainable.

Novel drug delivery methods

Transmammary drug delivery

Transmammary drug delivery refers to administering a drug to a neonate via the mother's milk. This is possible by administering the drug to the mother first and having the drug transfer through the milk during lactation. The transmammary delivery of an NSAID has been demonstrated and evaluated in humans (Knoppert et al., 2003; Gardiner et al., 2005; Gardiner et al., 2006) as well as cows (Malreddy et al., 2013) to gain a better understanding of drug concentrations in milk and safe drug transfer to neonates when nursing.

There are a number of studies that have successfully demonstrated the transmammary delivery of an NSAID in alleviating pain in piglets after tail docking and castration, where positive impacts on piglet welfare were found (Bates et al., 2014; Coetzee et al., 2019; Coetzee, 2019).

Tail docked and castrated piglets had lower cortisol concentrations and an attenuated thermal stress response after the transmammary delivery of meloxicam compared to controls receiving transmammary delivery of a placebo (Bates et al., 2014). The transmammary delivery of firocoxib has also been studied for its ability to reduce piglet pain associated with tail docking, castration, ear notching, and teeth clipping. Comparably, the transmammary delivery of firocoxib also led to lower cortisol concentrations, attenuated thermal response and gait change, and higher weaning weights (Coetzee et al., 2019; Coetzee, 2019).

Given reluctance by producers to adopt pain mitigation strategies due to extra time, labor, and cost, associated with analgesic drug administration, transmammary drug delivery may be a practical option, as an NSAID can be administered to the sow once rather than individually administered to each piglet. Furthermore, transmammary delivery may have beneficial effects on sow welfare in providing her relief from pain due to parturition and reduce the risk of feed intake and weight fluctuations associated with parturition. As a result, milk production and composition may be enhanced possibly resulting in increased piglet performance (Coetzee et al., 2019).

Novel processing techniques

CO_2 surgical laser

To enhance animal welfare and minimize pain associated with piglet processing procedures, the application of alterative tools to process piglets is being explored. With a concerted effort to refine piglet processing, scientists have proposed the use of a CO_2 surgical laser for tail docking and castration, given its potential benefits to reduce pain and improve wound healing (Viscardi et al., 2020). Surgical CO_2 lasers have been used in veterinary medicine for over 30 years and are widely accepted (Gans, 2007).

In comparison to a traditional scalpel blade, a CO_2 surgical laser has certain advantages including the capability of making precise incisions, increased surgical speed, decreased bleeding and inflammation through sealed blood and lymphatic vessels, anti-septic effects in destroying harmful microorganisms, and a reduction in post-operative pain in severing nerve endings (Carreira and Azevedo, 2016).

Veterinary practitioners readily use surgical lasers for elective procedures such as castration, tail docking, ovariohysterectomy, and onychectomy (Lopez, 2002). To our knowledge, there has only been one study investigating the utility of a CO_2 surgical laser for piglet castration (Viscardi et al., 2020). Results indicated that while laser-castrated piglets may have experienced less inflammation at the surgical site, they had evidence of thermal tissue damage and displayed more pain behaviors than scalpel-castrated piglets (Viscardi et al., 2020). Additional research is necessary regarding the practicality and effectiveness of a CO_2 surgical laser for other on-farm procedures, such as tail docking, with more focused on improved restraining methods and local blocks as piglets are conscious during the procedure and safety measures (e.g., non-alcoholic aseptic preparation) (Viscardi et al., 2020).

Conclusion

Piglet processing procedures constitute a significant welfare concern for millions of piglets raised for food each year. Tail docking and castration are painful as they inflict varying degrees of tissue damage and have short- and long-term negative implications on piglet health and welfare. As the removal of these routine procedures is currently not practical on large-scale commercial farms, research focused on determining effective pain mitigation strategies appropriate within a farm setting are warranted. With the use of animal-based indicators, animal responses to painful procedures and potential pain mitigation strategies or refined techniques can be observed over time

for a complete assessment. These methods are therefore exceedingly useful in meeting the FDA requirements for analgesic drug approval for use in swine.

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Chapter 2 - Evaluating the utility of a CO_2 surgical laser for piglet tail docking to reduce behavioral and physiological indicators of pain and to improve wound healing

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Abstract

In the U.S. millions of commercially raised piglets are routinely tail docked. Human and veterinary medical patients experienced reduced inflammation, healing time and post-procedural pain using a CO_2 surgical laser compared to standard surgical instruments. Refinement of the tail docking procedure using a laser may improve piglet welfare by reducing tail docking-associated pain compared to cutting pliers. The objectives of this pilot study were to evaluate the ability of a CO_2 surgical laser to 1) reduce pain and 2) improve wound healing of piglets after tail docking. Thirty piglets (male and female; 2 days old) were randomly allocated to one of three treatments (n = 10 piglets/treatment): tail docking with side pliers (SP), tail docking with a CO_2 surgical laser (LA) or sham-tail docking (control; CON). Piglet vocalizations were recorded during the tail docking procedure and the maximum frequency, amplitude and energy were quantified. Piglet behavior was recorded for 30 min at baseline and at 0, 1, 2, 6, 7, 8, and 24 h post-procedure. Facial images were collected at the same time points to assess facial grimacing using the Piglet Grimace Scale (PGS). Digital images of the tail tip were taken at baseline and up to 168 h post-procedure for wound healing assessment. Infrared thermography (IRT) images of the tail tip and its surrounding tissues were taken at baseline, 0, 0.5, 8, and 24 h post-procedure to assess inflammation. Blood samples were collected from each piglet at baseline and 0.5 h and saliva samples at baseline, 0.5 and 8 h post-procedure for cortisol analysis. The LA and CON groups elicited calls of higher energy than the SP group ($P<0.0001$). Across the assessment period, the SP group had higher grimace scores than the LA group ($P=0.03$). Additionally, male piglets grimaced more than female piglets ($P=0.009$). A treatment effect was observed for both wound scores and IRT ($P<0.0001$). At 0 h the SP group had significantly higher wound scores than the LA and CON group ($P<0.0001$). At 0 h, the SP group had a greater temperature difference between the tail tip

and surrounding tissue compared to the LA group (P=0.02). Behavioral indicators of pain were not different between the SP and LA piglets. In conclusion, LA piglets had less tail damage immediately post-procedure and lower facial grimace scores throughout the assessment period, suggesting it may have the utility as a less painful alternative to traditional tail docking with cutting pliers in piglets.

Keywords: animal welfare, tail docking, CO_2 surgical laser, piglet, pain, refinement

Introduction

Piglets are routinely tail docked on commercial swine farms in North America to reduce the prevalence of tail biting (Sutherland, 2015). Tail biting negatively impacts pig welfare causing painful tissue damage, abscess formation, infection and even mortality in severe cases with repeated biting (Pedersen, 2018). Management strategies such as maintaining low stocking densities (Valros, 2018) and environmental enrichment in the form of straw, hay, or wood are widely used in the E.U. to reduce tail biting. However, tail docking has remained a viable solution in North American systems given the complexity of tail biting and economic feasibility (Bracke, 2017).

Tail docking is commonly performed within the first week of life using a variety of techniques (e.g., scalpel, knife, cautery iron or side-cutting pliers) to remove the distal portion of the tail, often without provision of an analgesic or anesthetic for pain management (Sutherland and Tucker, 2011). Tail docking is a painful procedure causing tissue damage as a portion of the tail is surgically removed (Herskin and Di Giminiani, 2018). Tail-docked piglets have shown an increase in escape attempts, high frequency vocalizations, changes in behavior due to pain (e.g., tail jamming) and elevated blood cortisol concentrations (Ison et al., 2016). In addition to acute post-procedural pain, chronic discomfort and dysesthesia may be present in tail docked piglets,

evidenced by the formation of traumatic neuromas through injured peripheral nerves at the tail tip (Sandercock et al., 2016).

The American Veterinary Medical Association (AVMA) considers tail docking along with other common processing procedures to be painful and recommends the development of refinements to improve piglet welfare (American Veterinary Medical Association, 2014). Refined techniques that cause less tissue damage and stress in conjunction with pain management for the overall net benefit of the animal are highly desired. The CO_2 surgical laser was found ideal for soft tissue surgeries providing the ability to make precise incisions resulting in reduced hemorrhaging and inflammation at the surgical site, leading to improved healing time and decreased pain (Gans, 2007; Carreira and Azevedo, 2016). These advantages have led to the widespread application of a CO_2 surgical laser in a variety of human and veterinary medical procedures on numerous tissue types (Vitruk, 2013). Additionally, intra- and post-operative complications have shown to be reduced in studies comparing the alternative method of a CO_2 surgical laser to conventional surgical techniques such as the use of a scalpel or knife (Haytac and Ozcelik, 2006; Tuncer et al., 2010; Lopez-Jornet and Camacho-Alonso, 2013). Thus, a CO_2 surgical laser may be a better alternative for improving the welfare of tail docked piglets. The objectives of this pilot study were to evaluate the ability of a CO_2 surgical laser to 1) reduce pain and 2) improve wound healing of piglets after tail docking. We hypothesized that piglets tail docked using a CO_2 surgical laser would show less post-procedural pain, reduced inflammation at the surgical site and improved wound healing compared to piglets tail docked using side pliers.

Materials and Methods

Study Approval

All animal use and procedures were approved by the Institutional Animal Care and Use Committee at Midwest Veterinary Services (MVS) prior to study commencement (Protocol #MCL-20077)

Animals and Husbandry

For this study, three sows (Yorkshire x Landrace) nursing two-day old piglets were sourced from MVS Klitz Farm (Oakland, NE) after examination and selection by a veterinarian. Sow selection was based on non-aggressive behavior, body condition, health and weaning history. The selected sows had weaned at least one litter of piglets (gilts were excluded) and were in a good physical health (body condition score of 3±0.5 out of 5). A total of 30 healthy piglets (both male and female) with normal tails were selected from the three sow litters with 12 piglets each.

The day prior to study commencement, sows and piglets were loaded onto a livestock trailer and transported to the Central States Research Center (CSRC; MVS facility, Oakland, NE). Sows were transported separately with their litters and a partition on the trailer provided piglets protection from the sow. The distance between MVS Klitz Farm and CSRC was approximately 17 miles and animal transportation took less than 30 minutes. Farrowing crates equipped with a heat lap, water nipples and elevated Tenderfoot® flooring (Tandem Products, Inc., Minneapolis, MN) were used to house sows and their litters. Sows and piglets had ad libitum access to water through the water nipples. Sows were fed a diet that met or exceeded National Research Council (National Research Council, 2012) nutrient requirements for lactating sows. All animals were exposed to approximately 12 h of light per day.

Experimental Design

On the day of study commencement, piglets were weighed (mean BW = 1.8 ± 0.8 kg; 2 days old) and an identification number (1 – 30) was randomly assigned and written on their forehead and back using a black permanent marker. Piglets 1-10, 11-20, and 21-30 were housed in pens A, B, and C, respectively. Piglets were then randomly allocated to 1 of 3 treatment groups (n=10 piglets/treatment group): tail docking using pliers (SP), tail docking using a CO_2 surgical laser (LA) (VetScalpel; Aesculight, LLC, Bothell, WA), and sham tail docking (undocked control; CON). Randomization of treatment assignments was performed based on sow and piglet ID using Excel (Excel, Microsoft, Redmond, WA). All three treatments were represented in each litter.

To perform the tail docking procedure, piglets were removed as a litter from their pen and placed into a large bin. Prior to the start of the procedure, tails were disinfected using Chlorhexidine wipes (Chlorhexidine 4%; Vet One, Boise, ID) and left to fully dry for 2-3 minutes. Piglets were then handled to restrict leg movement by one individual, while a second individual held the piglet tail in place and removed one-third of the tail using the technique as per treatment. Treatments were applied in order of piglet identification number (1-30). Tails were docked either by making 1 horizontal pass along the tail using the CO_2 surgical laser (set to 30 W, continuous) or by clipping the tail off using side pliers. The CO_2 laser was calibrated following each litter to ensure proper functionality (mean calibration yielded 74.3 ± 1.9%); therefore, the actual power of the CO_2 laser at the level of the piglet was 22 W. This is a normal deviation for this surgical laser unit.

Piglets in the sham treatment group were handled and restrained in the same manner as piglets in the tail docked groups and two fingers were used in a "scissor-cutting" motion to simulate the tail docking procedure. All treatments were administered without analgesia between 08:00-

09:00 h and the individuals handling and tail docking piglets remained consistent through the study. Piglets were then returned to their home pen.

One piglet in the CON group was laid on by the sow and died shortly after enrollment. All other piglets (n=29) remained through the duration of the study with 19 males and 10 females. The number of male and female piglets in the SP, LA and CON groups were 6 and 4, 7 and 3, and 6 and 3, respectively.

Data Collection

Outcomes evaluated in this study were the following: behavior, facial grimacing, vocalizations, wound healing, inflammation, and blood and salivary cortisol.

Behavior Recording and Scoring

A high-definition video camera (Sony Handycam HDR-CX405, Sony USA Inc., New York, NY) mounted on a tripod was placed outside of each farrowing crate. Piglet behavior was recorded for 30 min, at 24 h pre-procedure (baseline; -24 h) and then at 0, 1, 2, 6, 7 and 8 h post-procedure. Finally, 24 h post-procedure, behavior was recorded again for 30 min producing a total of 4 h of video data on each litter. The videos were randomized across pen and time point using Excel (Excel, Microsoft, Redmond, WA). Prior to scoring, inter-observer reliability was evaluated until observers achieved an intra-rater correlation coefficient (ICC) of 0.80 indicating good reliability. Behavioral Observation Research Interactive Software (BORIS, University of Torino, Torino, Italy, version 7.10.2) was used by two trained observers to continuously score the behavior of each piglet for 30 min. using an ethogram (**Table 2.1**).

Piglet activity level and total proportion of pain indicative behaviors displayed were assessed across the observation period. Behaviors were initially analyzed individually and then grouped into 3 categories: active, inactive and behaviors due to pain. Walking, playing, nursing,

nosing and standing were categorized as active behaviors. Sleeping, lying and sitting were categorized as inactive behaviors. Tail wagging, trembling, scratching, tail jamming and spasms were categorized as behaviors indicative of pain.

Piglet Grimace Scale (PGS) Recording and Scoring

After behavior data was collected, all piglets within a litter were removed from their home pen and placed together in a plastic tote. Piglets were then removed from the tote individually and placed in a custom-built wooden cradle (29.2 cm x 2.5 cm x 17.8 cm) to facilitate image capture for facial grimace analysis. A total of three still images of each piglet face was taken using a point-and-shoot camera (Olympus Stylus Tough TG-4; Olympus Corporation, Tokyo, Japan) at the same time points (-24, 0, 1, 2, 6, 7, 8, and 24 h post-procedure) as described for behavior recording. The individual capturing the images stood approximately 60 cm away from the piglet to ensure consistent data collection. In total, 696 images were collected of which 232 were selected for final facial grimace assessment based on optimal lighting, angling, and quality. The individual who selected these images ensured one image per piglet per timepoint was included in the data set, while remaining blinded to treatment. The selected images were then edited to remove piglet ID and timepoint to only include the piglet face and minimize observer bias.

The final images were labeled with a randomized number using Excel (Excel, Microsoft, Redmond, WA), which rearranged images in a new numerical order and ensured raters were blinded to timepoint. Images were scored by four raters using three facial action units (FAUs): orbital tightening, ear position and nose bulge/cheek tension (**Figure 2.1**) according to Viscardi et al. (2017) and Lou et al. (2022). Each FAU was scored on a 3-point scale: 0 (not present), 1 (moderately present) and 2 (obviously present). Prior to scoring, raters underwent two training

sessions of 1 h each to discuss the scoring guide and score practice images. Both intra- and inter-rater reliability were assessed.

Vocalizations

Piglet vocalizations were recorded during the tail docking procedure for each piglet. A high-definition video camera (Sony Handycam HDR-CX405, Sony USA Inc., New York, NY) mounted on a tripod was placed close to the focal piglet's face and was used to record vocalizations as piglets were tail docked.

The maximum frequency (Hz), amplitude (μ) and energy (dB) were quantified using Raven Pro: Interactive Sound Analysis Software (Version 1.5, Cornell Lab of Ornithology, Ithaca, NY).

Wound Healing

Still-images of each piglet's tail tip were taken using a point-and-shoot camera (Olympus Stylus Tough TG-4; Olympus Corporation, Tokyo, Japan). Images were taken at baseline and at 0, 8, 24, 48, 72, 96, 120, 144 and 168 h post-procedure. Images were randomized using Excel (Excel, Microsoft, Redmond, WA) prior to being scored. Four individuals blinded to treatment and time point scored the images using a 6-point scale, ranging from 0 to 5 adapted from Sutherland et al. (2010) (**Table 2.2**) for wound scoring in castrated piglets, as a scale for wound assessment after piglet tail docking currently does not exist. Inter-rater reliability was assessed.

Inflammation

A research-grade infrared camera (FLUKE TiX580; FLUKE Corporation, Everett, WA) was used to take a single infrared thermography (IRT) image of each piglet's tail at baseline, and at 0, 0.5, 8, and 24 h post-procedure for the assessment of inflammation associated with the tail docking procedure. Prior to taking images, the camera automatically calibrated to the ambient temperature and relative humidity of the room before the start of every timepoint. The average

temperature (℃) of the tail tip and surrounding tissues of the tail were recorded and analyzed using a research-grade software (SmartView 4.3; FLUKE Corporation, Everett, WA), where a line was drawn across the tip of the tail and surrounding tissues. Further analysis was performed by subtracting the temperature of the surrounding tissues from the temperature of the tail tip to determine the difference between the two tail sites.

Blood and Salivary Cortisol

Once all non-invasive measures (behavior, facial grimacing, wound healing, and IRT images) were collected, all piglets were individually restrained for salivary and blood sample collection.

Salivette® Cortisol tubes (SARSTEDT Ag & Co., Germany) equipped with a synthetic cotton swab designed for cortisol determination were used to collect salivary samples at baseline and at 0.5 and 8 h post-procedure. To stimulate salivation, 2-3 drops of a citric acid solution (Citric acid anhydrous crystalline; Fisher Bioreagents, Ottawa, ON) were placed on the tongue of the piglet. Approximately 2-3 min after the citric acid drops, the individual handling the piglets inserted a cotton swab into the mouth of the animal and gently held it closed for 2 minutes. Then the cotton swab with the absorbed saliva was removed and returned to the tube. Saliva was then centrifuged at 1500 x g for 15 min and pipetted into wells. Saliva samples were stored at -20℃ prior to analysis.

For blood collection, piglets were restrained in the supine position and samples (3.0 mL each) were collected from the jugular vein at baseline and at 0.5 h post-procedure using a 20-gauge x 1 inch blood collection needle (TycoHealth Care, Mansfield, MA) and vacutainer serum separator tube (BD Vacutainer, Franklin Lakes, NJ). Blood sample tubes were placed on ice immediately after collection (BD Vacutainer, Franklin Lakes, NJ). The samples were then

centrifuged at 3000 x g for 10 min and serum was pipetted into cryovials. Blood samples were stored at -80℃ prior to analysis.

All salivary and blood samples were analyzed by laboratory personnel at Kansas State University blinded to treatment and time point. Saliva cortisol concentrations were determined using a commercially available enzyme immunoassay (EIA) kit (Salimetrics, State College, PA) following manufacturer specifications. Blood serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals cat. no. 07-221106-R) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. A 500 ng/mL standard was created by diluting the supplied 1,000 ng/mL kit standard. The standard curve ranged from 1 to 1,000 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a PerkinElmer Wizard2 gamma counter for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation over 18% were re-analyzed.

Statistical Analysis

Prior to analysis, the total duration of time piglets spent performing each behavior was converted into a proportion to account for time the piglet was not in view. Behavior data were analyzed using a generalized linear mixed model (GLIMMIX) in SAS (Statistical Analysis System

9.4, SAS Institute Inc., NC) including treatment, time, litter and time x treatment interactions. Piglet was the experimental unit, with litter included as a random effect and time as a repeated measure. The Tukey-Kramer adjustment was used to conduct a post hoc test with statistical significance set a $P \leq 0.05$.

A mixed model was used to analyze wound scores, IRT temperatures, PGS scores, vocalization parameters and salivary and blood cortisol. The mixed model included litter, time, treatment and time x treatment interactions. Piglet, litter and time were designated as the experimental unit, random effect and repeated measure, respectively. The Tukey-Kramer adjustment was used to conduct post hoc tests. Statistical significance was set at $P \leq 0.05$.

Results

Behavior

There were no treatment differences for any of the analyzed behaviors; however, there were time differences ($P < 0.0001$). Irrespective of treatment, piglets spent the least amount of time lying and sleeping and the most amount of time nursing immediately post-procedure (0 h) ($P < 0.0001$) (**Table 2.3**). Activity levels and the proportion of time piglets expressed behaviors indicative of pain (tail wagging, scratching, trembling, spams, and tail jamming) did not differ between the tail docked groups ($P = 0.83$ and 0.15, respectively). Although not significant, SP and LA piglets spent 5.4% and 5.6% of the time isolated, respectively, compared to CON piglets spending 1.3% of the time isolated.

Piglet Grimace Scale

The ICCs for inter-rater reliability among the four raters were 0.62, 0.80, and 0.37 for orbital tightening, ear position and nose bulge/cheek tension, respectively, with an overall value

of 0.64 (moderate reliability). The ICCs for intra-rater reliability for each rater were greater than or equal to 0.80, indicating good reliability.

Across the assessment period, piglets in the side plier group had higher grimace scores (2.3 ± 0.1) than the laser group (1.9 ± 0.1; P = 0.03). Piglets in the side plier group also exhibited more orbital tightening than piglets in the laser group (P < 0.0001). Irrespective of treatment, piglets grimaced more at baseline (24 h pre-procedure; 2.9 ± 0.2; P = 0.0007) compared to 0 (1.9 ± 0.2; P = 0.0005), 1 (2.0 ± 0.2; P = 0.0026), 2 (2.0 ± 0.2; P = 0.0031), and 24 h post-procedure (2.1 ± 0.2; P = 0.03). Nose bulging/cheek tension was the FAU expressed the most at baseline (P < 0.0001).

The males (2.4 ± 0.07) grimaced more than females (2.0 ± 0.1) across the assessment period (P = 0.009; **Figure 2.2**). Specifically, males exhibited more nose bulging/cheek tension than females (P = 0.0002).

Vocalizations

There were no treatment differences for maximum frequency and amplitude. However, piglets in the LA and CON groups elicited calls of higher sound energy compared to piglets in the SP group (P < 0.0001).

Wound Healing

The ICC for inter-rater reliability among the four raters was 0.89, indicating excellent reliability. There were significant treatment and treatment x time effects on wound scores (P < 0.0001 for both) (**Figure 2.3**). Both tail-docked groups (LA and SP) had higher wound scores than the CON group (P < 0.0001). At 0 h (immediately post-procedure) the LA and SP groups had greater wound scores compared to the CON group, with SP having significantly higher wound scores than the LA group (P < 0.0001). From 8-168 h post-procedure, both the LA and SP groups

had higher wound scores compared to the CON group ($P < 0.0001$). Irrespective of treatment, piglets had the highest wound scores at 0 and 8 h compared to all other time points ($P \leq 0.05$).

Inflammation

For the average temperature of the tail tip, there were significant time and treatment x time interactions ($P < 0.0001$ for both; **Figure 2.4a**). At 0.5 h, the average temperature of the tail tip was significantly lower than all other time points ($P < 0.0001$). At 0 h, the LA group had higher average tail tip temperatures (33.7 ± 0.7 °C) than the SP group (29.3 ± 0.7°C; $P = 0.003$). At 0.5 h, the CON group had higher average tail tip temperatures (31.4 ± 0.8 °C) than both the tail-docked groups (LA: 27.4 ± 0.7 °C, SP: 27.3 ± 0.7 °C; $P < 0.01$). The average temperature of the tail's surrounding tissues was lowest at 0.5 h compared to all other time points, irrespective of treatment ($P \leq 0.05$).

There were treatment, time and treatment x time interactions for the difference in temperature between the surrounding tissue and tail tip ($P < 0.0001$; **Figure 2.4b**). Both tail-docked groups had significantly higher average temperature differences than the CON group ($P < 0.0001$). At 0 h, the SP group had higher temperature differences compared to the LA group ($P = 0.02$). At 0.5 h, both tail-docked groups had a higher temperature difference between the two focal sites on the tail compared to the CON group ($P < 0.0001$). Irrespective of treatment, piglets had the highest temperature difference at 0.5 h post-procedure compared to all other time points ($P \leq 0.01$).

Salivary Cortisol

There were no treatment or treatment x time interactions for salivary cortisol concentrations. However, irrespective of treatment, at 8 h post-procedure, piglets had higher salivary cortisol concentrations compared to baseline ($P = 0.02$).

Blood Cortisol

There were no treatment, time or treatment x time interactions found for blood plasma cortisol concentrations.

Discussion

To our knowledge, this is the first study to investigate the utility of a CO_2 surgical laser towards refining the tail docking procedure. Refinement of this procedure aims to improve wound healing and reduce pain. Pain is commonly associated with inactivity, such as an increase in lying bouts or sleeping, but reports have also indicated that discomfort and protection of the surgical site (e.g., tail jamming) are indicative of pain (Torrey et al., 2009; Mathews et al., 2014). In this present study, activity levels and pain-associated behaviors of piglets resulting from the tail docking procedure did not differ among the treatment groups suggesting that the CO_2 surgical laser and side pliers caused a similar pain response. A similar finding was reported in disbudded dairy calves using a CO_2 surgical laser compared to calves disbudded by cautery iron (Kleinhenz et al., 2021). These results suggest that the CO_2 laser technique requires further testing to determine whether in fact it reduces animal pain responses. Larger studies may help confirm these findings. This also indicates that procedural pain is unlikely reduced without the provision of pain management regardless of the technique.

Piglets tail docked using the pliers expressed more facial grimacing throughout the assessment period than piglets tail docked using the surgical laser, suggesting side pliers may result in more acute pain. Specifically, it has been reported that tail docked piglets had significant changes in orbital tightening compared to castrated piglets, which may indicate specificity of this FAU to the tail docking procedure (Di Giminiani et al., 2016). Control piglets grimaced and similar outcomes suggest that pigs undergoing sham procedures of lesser weight may grimace more

(Viscardi and Turner, 2018). A sex difference in facial grimacing was also observed, with males grimacing more than females throughout the study period. Ample evidence suggests that sex hormone differences contribute to higher pain sensitivity in females (i.e., high estrogen and low testosterone) compared to males leading to a greater proinflammatory response, which is contradictory to our study results (Sorge and Totsch, 2017; Cleve et al., 2013). However, greater facial grimace scores in males compared to females was previously reported in a study involving tail-docked piglets (Viscardi and Turner, 2019). Miller and Leach (2015) also reported greater grimace scores in male mice than female mice possibly due to phenotypic variations among mouse strains modifying factors such as nociception, behavior, and analgesic efficacy. Nonetheless, results from this pilot study may be interpreted cautiously in lieu of the small sample size and disproportionate number of males and females. Unexpectedly, piglets grimaced more prior to the procedure than at all other time points. This may have resulted from piglets being transported 24 h prior to the start of the study and restrained for collection procedures for the first time, particularly for blood sampling. All of these events are known to cause significant stress (Grandin, 2014). Moreover, piglets were placed in a cradle when PGS images were being taken and the novelty of the object may have elicited a fearful response (Mellor and Beausoleil, 2015). Future studies may, therefore, acclimate piglets to the novel cradle prior to baseline data collection and allow a longer period of time for piglets to rest after a transportation event to reduce these potentially distressing situations from impacting grimace behavior.

Across the mammal species, changes in facial expression caused by painful procedures have led to the development of non-invasive species-specific grimace scales (Descovich, 2017). The PGS has demonstrated reliability among raters and a correlation between facial grimacing and behavior, predominately focusing on orbital tightening, ear position, cheek tension, and temporal

tension (Gottardo et al., 2016; Di Giminiani et al., 2016; Viscardi et al., 2017). Temporal tension was not taken into consideration in this study due to its poor reliability among raters, suggesting it may not be a good PGS indicator of stress or pain. Further PGS modifications were made to provide raters with clear, piglet-specific facial descriptions for each score and symbols to direct attention to distinct features in our PGS scoring guide. Reliability for orbital tightening (ICC = 0.62) and ear position (ICC = 0.80) in this study are lower than values reported in current literature across species (rat orbital tightening ICC = 0.96; rabbit orbital tightening ICC = 0.86; horse orbital tightening and ear position ICC = 0.83 and 0.97, respectively) (Sotocina et al., 2011; Keating et al., 2012; Dalla Costa et al., 2014). The inter-rater reliability for total PGS scores in this present study was moderate indicating the need for further rater training or collection of higher quality images.

Vocalizations have long been considered a reliable indicator of pain in pigs (Weary et al., 1998). During the tail docking procedure, piglets in the laser and control groups produced vocalizations of higher energy compared to piglets in the side plier group. On average, the duration for the laser, control and side plier tail docking procedures were 8, 7, and 3 seconds, respectively. Therefore, piglets in the laser and control groups were handled significantly longer than piglets in the side plier group. The increase in vocal energy may have resulted from a longer period of handling, which can cause significant stress to piglets (Manteuffel et al., 2004; Grandin and Shivley, 2015). To balance treatment differences of technique and duration between a laser and side pliers, a midpoint duration of approximately 5 sec would have been a more appropriate estimate for the control procedure. Future studies are recommended to ensure control treatments are executed in the same manner and duration as other treatments to facilitate interpretation of outcomes (Lou et al., 2021). Additionally, the laser-tail docking technique required restraint of

piglets for almost 3 times longer than the side pliers, likely resulting in an increased stress response, which should also be considered in future studies.

To the best of our knowledge, the tail wound scale developed in this study is the first scale on tail docking wound assessment. Immediately after tail docking (0 h), the laser group had lower wound scores than the side plier group (4 vs. 5). This is consistent with Mison et al. (2003) who reported that an advantage of using a CO_2 surgical laser is the ability to instantly ablate and cauterize blood vessels to decrease bleeding. Conversely, using side pliers results in an open wound, increasing bleeding and susceptibility to infection. This further supports the need to refine current tail docking techniques to improve piglet welfare. Irrespective of treatment, the highest wound scores were observed at 0 and 8 h post-procedure, which is reasonable given the proximity of these time points following the tail docking procedure.

Cutaneous temperature and inflammation of a surgical site have been used to assess post-procedural pain, measured through non-invasive IRT imaging (Stewart et al., 2005). The IRT imaging in this current study showed that at 0 h, piglets in the laser group had a lower temperature difference between surrounding tissues and the tail as compared to the side plier group, but at later time points these differences were not discernable. These results along with the findings on wound scores indicate that using a CO_2 surgical laser to tail dock piglets may have a short-term benefit. At 0.5 h post-procedure, both the average temperature of the tail tip and the average temperature of the surrounding tissues were significantly lower than at all other time points. More specifically at this time point, the average temperature of the tail tip was lower than that of surrounding tissues for both tail-docked groups compared to the sham group. These results are consistent with a previous study using a CO_2 surgical laser for piglet castration (Viscardi et al., 2020). At 0.5 h post-procedure, piglets had been removed from the sow and tail docked all within a 30 min time frame,

which may have elicited a heightened stress response. The overall drop in temperature from 0 to 0.5 h may have resulted from activation of the sympathetic nervous system (SNS) categorized by movement of blood from the periphery (e.g., tail and limbs) to the core of the body, which has been observed in animals under stress or in pain (Bates et al., 2014). Conversely, sham piglets had higher tail tip temperatures than both tail-docked groups at 0.5 h likely due to intact tails (i.e., skin) emitting higher temperatures compared to docked tails (i.e., open wound with presence of blood or a scab).

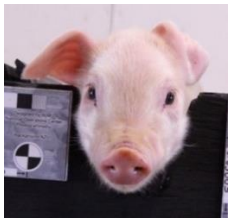
Cortisol is physiological parameter that is widely used to assess stress and acute pain in farm animals, but can be vastly influenced by a variety of factors such as individual variation, diurnal changes, transportation or by restraint (Molony and Kent, 1997). In this study, blood and salivary cortisol concentrations were assessed in part to compare collection methods and work towards optimization of less invasive biomarker collection. To our knowledge, this is the first study to quantify salivary cortisol in pigs less than 5 days old (14 days old, Rodarte et al., 2004; 10 weeks old, Ott et al., 2014). As a less intrusive procedure than blood sampling, salivary cortisol may be advantageous in providing better outcomes eliciting reduced stress or pain responses. Based on the treatment differences found in vocal response and procedure (i.e., handling) duration a difference in cortisol was also expected. Our study found that cortisol extracted from plasma yielded higher values than cortisol extracted from saliva, however; both resulted in no differences based on treatment or time.

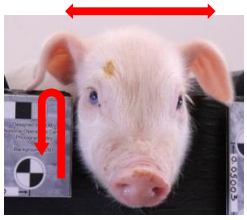
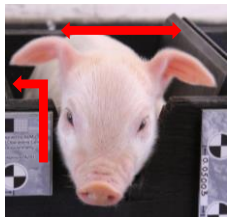
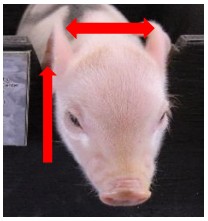
Cortisol concentrations have shown to be influenced by the tail docking technique with blunt trauma cutters resulting in higher concentrations than cautery iron 60 min after the procedure (Sutherland et al., 2008). Even when performed with pain management (i.e., topical and injectable local anesthesia and CO_2 gas), tail docking produced greater cortisol concentrations 30 min post-

procedure compared to the sham procedure (Sutherland et al., 2011). For this study, an increase in salivary cortisol at 8 h post-procedure was observed potentially due to an increase stress response caused by repeated handling for PGS and tail image collection beforehand. In contrast, (Prunier et al., 2005) found tail docking had no effect on plasma cortisol.

Conclusion

A CO_2 surgical laser reduced inflammation and caused less tissue damage immediately after the tail docking procedure. Piglets tail docked using this technique also grimaced less than piglets tail docked using side pliers. Furthermore, the CO_2 surgical laser did not elicit more behavioral indicators of pain in piglets compared to those that were docked using side pliers. The results of this pilot study suggest that a CO_2 surgical laser has the potential to refine the tail docking procedure and improve on-farm piglet welfare. Further research with a larger cohort of animals is needed to investigate the full utility of this technique.

Orbital Tightening		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Eyes wide open, with a round, circular shape No tightening of or around the eyelid Whites of eyes may be visible **Both eyes should be scored 0**	Eyes are closed halfway, with a teardrop shape Eyelids are partially tightened (i.e., "eye squeezing") "Eyebrow" furrowing is present Whites of eyes may be partly visible **Features may be seen only or more strongly in 1 eye**	Eyes are closed more than halfway (may see full eye closure) Eyelids are fully tightened, with obvious "eye squeezing" Significant "eyebrow" furrowing Whites of the eyes are not visible **Features may be seen only or more strongly in 1 eye**

Ear Position		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Ears are forward and relaxed Normal distance between ears Inner pinna is partly visible Base of ear has the following shape:  **Both ears should be scored 0**	Ears are upward and drawn back Distance between ears is reduced (Length of red arrow is less than in Score 0) Inner pinna may be more visible Base of ear has the following shape:  **Features may be seen only or more strongly in 1 ear**	Ears are fully drawn back, flush against head Distance between ears is further reduced (Length of red arrow is less than in Score 1) Base of ear has the following shape: Inner pinna not visible Base of ear has the following shape: **Features may be seen only or more strongly in 1 ear**

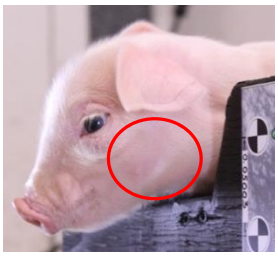
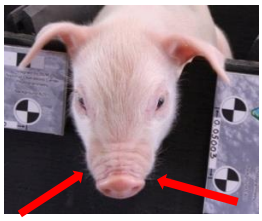
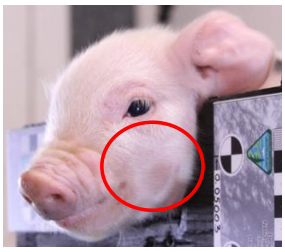
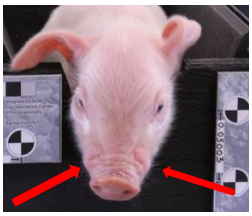
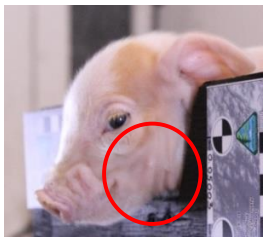
Nose Bulge/Cheek tension		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
 	 	 – 
Nose Pigs naturally have some wrinkles/skin folds on nose **Cheeks** Skin gradient is flat, smooth, and relaxed No tightening **Note:** Nose muscles move congruently with cheek muscles	**Nose** Increase in bulging Moderate "bulging" appearance with skin folds Nose moves inward **Cheeks** Moderate tightening/tension with few indentations/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**	**Nose** Significant increase in bulging Significant "bulging" with several skin folds Nose moves inward **Cheeks** Significant tightening/tension with several indentions/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**

Figure 2.1. The Piglet Grimace Scale (PGS) with the description of the facial action units (FAUs) assessed. (Modified from Viscardi et al., 2017 and Lou et al., 2022)

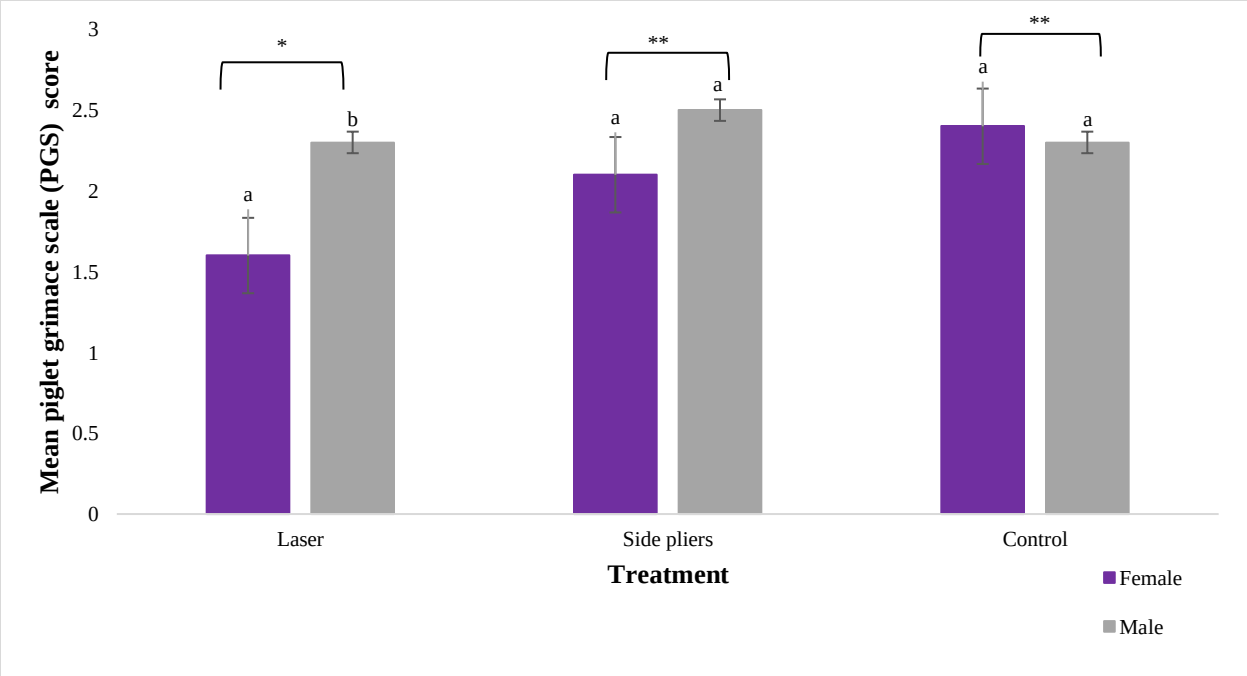


Figure 2.2. Mean (± SE) piglet grimace scale (PGS) scores in each treatment group after tail-docking.

Asterisks indicate significant differences between treatment groups ($P < 0.05$). Different superscripts indicate significant differences between sex within treatment groups ($P < 0.05$).

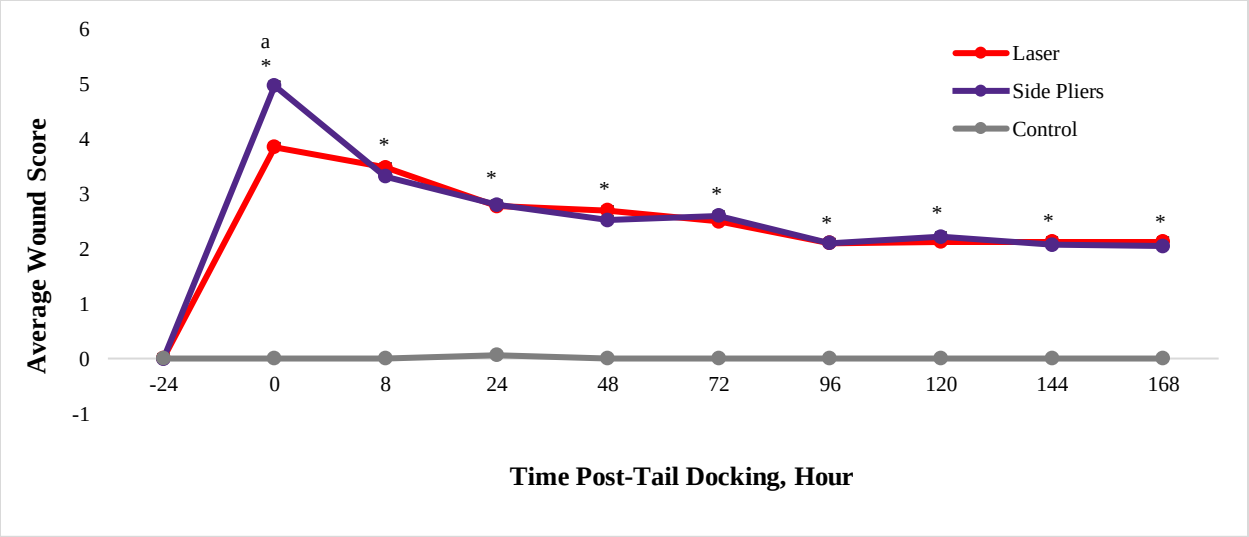
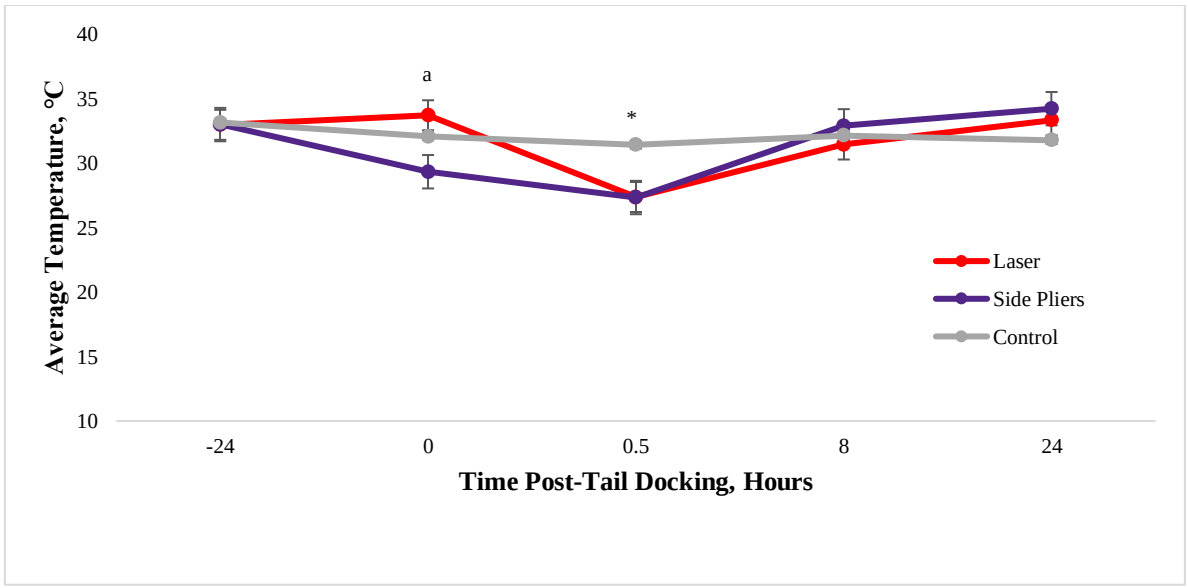
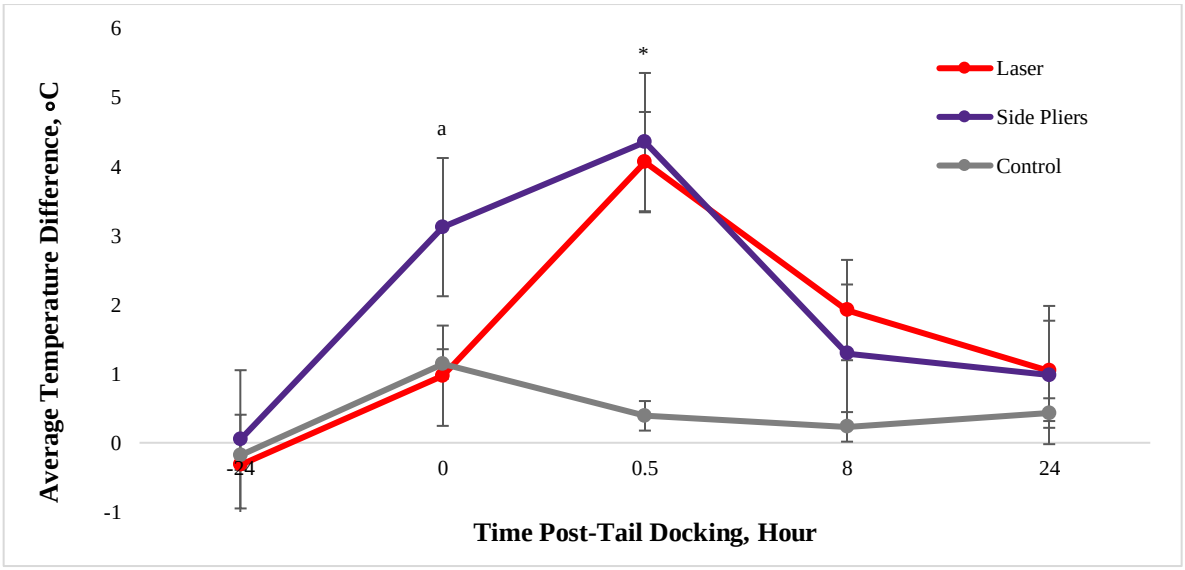


Figure 2.3. Average wound score (± SE) of piglets in each treatment group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between both tail-docked groups and the control group. Letter represents a significant difference ($P < 0.0001$) between the tail-docked piglets.



a)



b)

Figure 2.4. Average temperature (℃) a) of the tail tip (± SE) and b) temperature difference (± SE) between the tail tip and surrounding tissues of the tail of piglets in each treatment group across the assessment period.

Asterisks represent a significant difference ($P < 0.05$) between both tail-docked groups and the control group; letter represents a significant difference ($P < 0.05$) between the laser and side plier groups.

Table 2.1. Ethogram used to score piglet behavior grouped into the following categories: posture, nursing, location, pain, and social cohesion. (Modified from Viscardi et al., 2020)

Behavior	Description
Posture	
Lying	Body weight supported by side or belly; all four limbs in non-direct contact with floor
Sitting	Body weight supported by hindquarters and front legs
Standing	Body weight supported by four legs; all four limbs in direct contact with floor
Nursing	
Nursing	Piglet snout is in contact with the udder; nosing or suckling teat
Locomotion	
Playing	Energetic interaction (springing, bouncy, twirling movements) alone or with littermates
Agonistic	Biting or fighting other littermates
Walking	Moving forward or backward at a normal pace
Sleeping	Lying down motionless with eyes closed for at least 30 seconds
Nosing	Snout in contact with a substrate, pen surface, or littermate
Behavioral indicators of pain	
Trembling	Body shivering or shaking
Spasms	Quick and involuntary contractions of the muscles (typically involving the whole body)
Scratching	Rubbing the rump against the floor, pen walls, or littermates
Tail wagging	Tail movement from side to side (or up and down)
Tail jamming	Tucking the tail between the hind legs
Social cohesion	
Isolated	Alone, or with one littermate, distance of 40 cm separates the piglet(s) from the closest group of littermates. Piglet(s) may be lying, sitting, or standing alone

Table 2.2. The 6-point wound healing scale with descriptions of each score used to assess tail wounds in docked piglets. (Adapted from Sutherland et al., 2010)

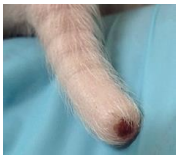
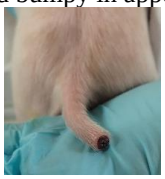
Score	Description
0	 Fully healed intact skin with no scab
1	 Intact skin with a thin, light-colored scab
2	 Fully formed scab (browner in color) with granulation (thin and bumpy in appearance)
3	 Partially opened with a thick, dark scab with presence of dried, black blood
4	 Open wound with presence of older, darker red blood
5	 Raw, open wound with presence of fresh, red blood. Bone may be visible

Table 2.3. **Proportion of time piglets (n=29) were engaged in specific behaviors pre and post tail docking. Values represent the proportional means (±SE)**

	Pre-tail docking	Post-tail docking							
Behavior	**Baseline**	**0h**	**1h**	**2h**	**6h**	**7h**	**8h**	**24h**	**P-value**
Posture									
Lying	0.62 ± 0.08^{bc}	0.34 ± 0.04^{a}	0.78 ±0.04^{b}	0.71 ±0.04^{bd}	0.57 ±0.05^{cd}	0.75 ±0.04^{b}	0.71 ±0.04^{bd}	0.47 ±0.04^{ac}	**<0.0001**
Sitting	0.09 ±0.04	0.1 ±0.03	0.1 ±0.03	0.05 ±0.02	0.05 ±0.02	0.08 ±0.03	0.09 ±0.03	0.1 ±0.03	0.24
Standing	0.30 ±0.17	0.58 ±0.11	0.14 ±0.08	0.24 ±0.09	0.42 ± 0.11	0.20 ± 0.08	0.20 ± 0.09	0.45 ± 0.1	**0.04**[1]
Nursing									
Nursing	0.44 ±0.09^{ac}	0.63 ±0.06^{a}	0.33 ±0.06^{c}	0.30 ±0.06^{bcd}	0.22 ±0.05^{bcd}	0.16 ±0.04^{d}	0.32 ±0.06^{bcd}	0.32 ±0.05^{bc}	**<0.0001**
Locomotion									
Playing	-[2]	0.04 ±0.25	-	0.01 ±0.03	0.02 ±0.08	0.002 ±0.02	0.01 ±0.07	0.01 ±0.03	0.99
Agonistic	0.0004 ±0.002	0.005 ±0.002	0.001 ±0.003	0.003 ±0.002	0.01 ±0.01	0.02 ±0.005	0.004 ±0.002	0.01 ±0.003	**0.01**[1]
Walking	0.03 ±0.02	0.07 ±0.02	0.06 ±0.02	0.04 ±0.01	0.10 ±0.02	0.1 ±0.02	0.04 ±0.01	0.07 ±0.02	**0.006**[1]
Sleeping	0.60 ±0.09^{bc}	0.15 ±0.06^{a}	0.55 ±0.06^{bc}	0.46 ±0.06^{bc}	0.52 ±0.07^{bc}	0.62 ±0.06^{b}	0.47 ±0.06^{bc}	0.37 ±0.07^{ac}	**<0.0001**
Nosing	0.001 ±0.005	0.003 ±0.004	0.005 ±0.005	0.005 ±0.006	0.004 ±0.01	0.07 ±0.02	0.03 ±0.01	0.01 ±0.008	**0.006**[1]
Behavioral indicators of pain									
Trembling	-	-	-	-	-	-	-	-	-
Spasms	0.004 ±0.002	0.002 ±0.001	0.004 ±0.001	0.005 ±0.001	0.003 ±0.001	0.004 ±0.001	0.003 ±0.001	0.03 ±0.001	0.09
Scratching									
Tail wagging	0.01 ±0.01	0.03 ±0.01	0.07 ±0.02	0.02 ±0.01	0.06 ±0.02	0.03 ±0.01	0.04 ±0.1	0.06 ±0.02	**0.006**[1]
Tail jamming	-	-	-	-	-	-	-	-	-
Social cohesion									
Isolated	0.005 ±0.02	-	0.16 ±0.18	0.04 ±0.06	0.05 ±0.05	0.11 ±0.12	0.01 ±0.03	0.02 ±0.03	0.88

[1]Not significant after Tukey-Kramer adjustment
[2]Dash indicated behavior was not observed
[a-d]Values within a row with different superscripts are significantly different ($P < 0.05$)

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Chapter 3 - Evaluation of oral firocoxib, administered to the sow and delivered transmammary to piglets, to alleviate pain associated with tail docking and castration

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Abstract

In the U.S., piglets raised on commercial farms undergo tail docking and surgical castration without the provision of analgesia. Practical and effective forms of on-farm analgesia are needed to improve piglet welfare. The objective of this study was to determine which dose of transmammary-delivered firocoxib was most effective at alleviating surgical castration and tail docking pain in piglets. Sows (n = 40) were randomly assigned to one of four treatment groups of oral firocoxib at the following doses: (1) 3.0 mg/kg (FIRO3), (2) 4.0 mg/kg (FIRO4), (3) 5.0 mg/kg (FIRO5), (4) 6.0 mg/kg (FIRO6), or oral placebo (CON; n = 8 sows/treatment). Piglets (n = 400, 10 piglets/litter) were randomly allocated to undergo 1 of 2 processing procedures: processed (n = 4 male, n = 4 female) or sham (n = 1 male, n = 1 female). Piglet vocalizations were recorded at the time of processing to quantify peak frequency, peak amplitude, and energy. Video recording of piglet behavior and collection of piglet facial images for facial grimace analysis were obtained at baseline (-24h pre-processing) and at 0, 7, 24, and 48h post-processing. Infrared thermography (IRT) images of the tail docking and castration surgical sites were taken to assess inflammation and pressure mat gait analysis parameters were collected at -24h and at 0, 7, 24, 36, and 48h post-processing. Blood was collected at -24, and at 1 and 24h post-processing for cortisol and firocoxib concentration analysis. Castration and tail docking elicited calls of greater amplitude and energy compared to sham-handled piglets ($P < 0.05$). Irrespective of treatment, processed piglets applied more force and pressure on their front limbs and had higher cortisol concentrations compared to sham-handled piglets ($P < 0.05$). Male piglets exhibited more pain-specific behaviors than females ($P = 0.003$). The FIRO3 and FIRO5 groups displayed less pain-specific behaviors immediately after processing than the CON group ($P = 0.03$). Piglets in the FIRO3 group had lower cortisol concentrations than piglets in the FIRO5 group ($P = 0.02$). Results from this study suggest that 3.0

mg/kg oral firocoxib, administered to the sow and delivered transmammary to her nursing piglets, may be effective at reducing stress and pain associated with surgical castration and tail docking.

Introduction

In the United States, commercial swine farms tail dock and castrate millions of piglets annually to facilitate animal management and maintain a high standard of meat quality (Herskin and Di Giminiani, 2018). It is standard practice to perform these procedures without providing piglets a form of analgesia or anesthesia for pain relief. A vast number of studies have shown that tail docking and castration elicit behavioral and physiological responses in piglets associated with pain, including high-pitched vocalizations and escape attempts, reduced body weight, inactivity, facial grimacing, inflammation, changes in gait, and higher cortisol production in comparison to non-processed piglets (Schmid and Steinhoff-Wagner, 2022). Consequently, societal concern over the well-being of piglets undergoing these painful procedures continues to grow. Therefore, research and development of safe and effective pain mitigation strategies for piglets is crucial in addressing this welfare concern.

Unlike Canada and the European Union, there are currently no drugs approved in the U.S. by the Food and Drug Administration (FDA) for pain control in pigs, which stands as a significant barrier for veterinarians and producers attempting to implement analgesic strategies (Burkemper et al., 2020). Animal-based outcomes that are objective, repeatable, and clearly defined are required to meet FDA approval of drug safety and effectiveness (US Food and Drug Administration, 2006). In addition to meeting the criteria required for FDA-approval, a drug must not produce illegal drug residues and be low-cost, time- and labor-efficient to make it favorable for on-farm adoption (Wagner et al., 2020). Transmammary drug delivery to piglets is the process by which a drug is administered to the sow, transfers into her milk, and is ingested by her piglets

when nursing (Coetzee et al., 2019). This method of drug delivery provides a suitable alternative to individually administering a drug to each piglet, which can be highly stressful for both piglets and animal caretakers.

As an FDA-approved non-steroidal anti-inflammatory drug (NSAID) to manage osteoarthritis pain in dogs and horses, firocoxib has been a strong analgesic candidate in studies evaluating transmammary drug delivery to piglets before processing (Coetzee, 2019). The transmammary delivery of firocoxib (sow dose: 0.5-2.0 mg/kg IM) for tail docking and castration pain management showed promising results, with piglets having a reduced stress response, minimal alternations in gait, and improved growth (Coetzee, 2019). However, a secondary study concluded that sows may need to receive doses higher than 2.0 mg/kg for piglet firocoxib concentrations to be clinically effective to alleviate post-procedural pain (Viscardi, 2023).

The objective of this study was to determine which oral dose of firocoxib (3.0, 4.0, 5.0, or 6.0 mg/kg), administered to the sow and delivered transmammary to her nursing piglets, would be most effective at alleviating pain caused by surgical castration and tail docking using animal-based behavioral and physiologic outcome measures. We hypothesized that there would be a dose-dependent reduction in piglet pain, where the highest dose of transmammary-delivered firocoxib would lead to the greatest reduction in pain.

Materials and Methods

All animals use and procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC protocol #4762) prior to study commencement.

Animals

This study was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS from June 2022 to August 2022. A total of 40 sows (Yorkshire x

Landrace; mean body weight = 232.11 ± 34.6 kg; mean parity = 1.98 ± 1.15) across two cohorts (n = 20 sows/cohort) were enrolled onto this study (**Table 3.1**). Sows from two different cohorts were necessary, as each farrowing group had less than the number of required sows for this study, so sows were split equally across cohorts. Sows were selected based on health status and conformation and were identified using ear tags with a unique color-coded identification number. Cross-fostering among the litters occurred within the first 48h after farrowing until each sow had a litter of a minimum of 10 piglets consisting of at least 5 male and 5 female piglets. Piglets that were not cross-fostered and/or did not meet the inclusion criteria for enrollment were allowed to remain with the sow to continue through the production system.

Healthy piglets (n = 400; 3 ± 1-day old; mean body weight = 3.74 ± 0.66 kg) that met the following inclusion criteria were selected for enrollment: a minimum body weight of 1.5 kg, a fully developed tail, and males had to have two descended testicles. On day 1 of age, piglets were administered 1.0 mL of iron (unifern 200, Pharmacosmos, Inc., Watchung, NJ) and 1.0 mL of ceftiofur crystalline free acid (Excede® for Swine Sterile Suspension 100 mg/mL, Zoetis, Kalamazoo, MI) via intramuscular injection into the neck.

Sows and their litters were housed together in individual commercial farrowing crates (152.4 cm x 213.4 cm) inside a temperature-controlled farrowing barn. All farrowing crates were equipped with a heat mat to provide piglets with supplemental heat. All animals had ab libitum access to water via a water nipple. Sows were fed ab libitum via automatic feeders that provided sows with a diet at set times throughout the day meeting or exceeded the National Research Council nutrient requirements for a lactating sow (National Research Council, 2012).

Experimental Design

Sows (n = 40) were randomly assigned to one of four treatment groups of oral firocoxib at the following doses: (1) 3.0 mg/kg (FIRO3), (2) 4.0 mg/kg (FIRO4), (3) 5.0 mg/kg (FIRO5), (4) 6.0 mg/kg (FIRO6), or oral placebo (CON; n = 8 sows/treatment). Firocoxib doses selected for this study were based on rationale from previous work (Kleinhenz et al., 2020 ;Viscardi, 2023). Sow treatment doses were calculated using their post-farrowing weights. Firocoxib 57mg (Equioxx, Boehringer Ingelheim, Duluth, GA) and 227mg (Previcox, Boehringer Ingelheim, Duluth, GA) tablets were combined, and doses for each sow was rounded to the nearest whole capsule. One half of a 57mg tablet (i.e., approximately 28.5 mg) was added to all sow firocoxib doses to account for loss during treatment preparation and administration. A pill grinder (Easy-Grip Pill Crusher, Equate, Bentonville, AR) was used to crush the tablets. Once the tablets were converted into a fine powder, the powder was transferred into a 35 mL syringe (Monojet, Fisher Scientific, Waltham, MA) and mixed vigorously with grape flavored Gatorade (Gatorade, PepsiCo, Chicago, IL) until fully dissolved. The uniform solution was then transferred into a 30 mL nylon syringe with a drench tip (Nylon Syringe, Neogen Corporation, Lexington, KY) and administered to the sows orally 12 h before piglets were processed. Sows assigned to the oral placebo treatment group (CON) were only administered Gatorade (30 mL) using the same technique.

The day before baseline outcomes were collected (Study Day -2), all piglets within each litter were randomly assigned an identification number (1-10) that was written on their back. Each litter was assigned a letter (A-Z) that was written on their right hind leg. Piglet numbers and letters were written using a black permanent marker (Dura-Ink, Markal, India). Within each litter, piglets

(n = 10 piglets/litter, 5 male and 5 female) were randomly allocated to undergo 1 of 2 processing procedures: processed (n = 4 male, n = 4 female) or sham (n = 1 male, n = 1 female).

Each cohort of sows (n = 20 sows/cohort) and their piglets (n = 200 piglets/cohort) were split into groups, with Cohort 1 having 3 groups and Cohort 2 having 4 groups. Groups consisted of 6 or 7 litters, with sow farrowing date determining the group the litter was assigned to (i.e., piglets born first were assigned to Group 1 and processed first; piglets born last were assigned to Group 3 in Cohort 1 and Group 4 in Cohort 2 and processed last). The purpose of these groups was to stagger piglet processing and outcome data collection over a period of a week. All piglets were processed between 07:00-11:00h.

Given adverse events resulting from the technique used to castrate males in Cohort 1 Group 1, all males from this group were removed from the study. To replace the males from Cohort 1 Group 1, Cohort 2 Group 4 consisted of only male piglets receiving the same treatments and procedures.

All piglets were restrained and processed by the same trained individual. The order that piglets were processed was randomized; therefore, the individual numbers (1-10) assigned to piglets did not correspond to the same sex or procedure across litters. At the time of processing, piglets were removed from the farrowing crate as a group and placed into a cart. A disinfectant (Chlorhexidine 4%, Vet One, Boise, ID) was used to disinfect the tail and scrotum before tail docking and castration was performed. For tail docking, ~1/3 of the tail was clipped off using side-cutting pliers. For castration, a scalpel blade was used to make 2 vertical incisions over the skin of each testicle and manual pressure exteriorized the testicles. The spermatic cord was then pulled until tearing and the testicles were removed. Sham-castrated and sham-tail docked piglets were restrained in the same way as processed piglets. The handle of the scalpel and manual pressure

were used to mimic the incisions and manipulation of the scrotum for sham-castration, while two fingers were used to simulate the "scissor-cutting" motion of the side-cutting pliers.

Data Collection

Animal-based outcomes evaluated in this study were the following: body weight, vocalizations, behavior, infrared thermography, pressure mat gait analysis, facial grimace, and plasma cortisol and firocoxib concentrations.

Body weight

Piglet body weights were collected at enrollment (Study Day -2) and at weaning (Day 21). Piglets were placed individually into a cart scale to record their body weights. To determine a difference in weight gain over the study period, piglet enrollment weight was subtracted from their weaning weight.

Vocalizations

Piglet vocalizations were recorded at the time of tail docking and surgical castration) using a high-definition video camera with an internal microphone (Sony Handycam HDR-CX405, Sony USA, Inc., New York, NY). The camera was attached to a tripod and positioned to have the piglet fully in view. Vocal parameters of peak frequency (Hz), peak amplitude (U), and energy (dB) were analyzed using the Raven Pro: Interactive Sound Analysis Software (Version 1.5) (Version 1.6, Cornell Lab of Ornithology, Ithaca, NY).

Behavior

High-definition cameras (Sony Handycam HDR-CX405, Sony USA, Inc., New York, NY) connected to tripods were placed outside of to the farrowing crates to record piglet behavior. Piglet behavior was recorded at baseline (-24h pre-processing) and at 0, 7, 24, and 48h post-processing for 30 mins. Continuous behavior scoring for the first 15 min of each video was completed using

an ethogram (**Table 3.2**) and BORIS (Behavioral Observation Research Interactive Software v 7.7.3, Torino, Italy) by 2 trained observers. Prior to behavior scoring, observers were required to achieve a minimum of 0.90 in their inter-class correlation coefficient (ICC) analysis to indicate an excellent level of reliability.

The random number generator in Excel (Excel, Microsoft, Redmond, WA) was used to relabel the behavior videos and keep observers masked to treatment and time point. From the 400 study piglets, a subset of 120 piglets were selected for the final behavior analysis, including 24 piglets from each treatment group (FIRO3, FIRO4, FIRO5, FIRO6, and CON). Within each group of 24 piglets the following procedural treatments were included: processed (n = 10 male, n = 10 female) and sham (n = 4 male, n = 4 female). Piglets in the subset excluded piglets bled for cortisol and firocoxib analysis as to remove potential confound from jugular venipuncture.

A total of over 9,000 mins (150 h) of piglet behavior was analyzed to determine the total proportion of time spent performing each behavior across the assessment period. Behaviors were also analyzed after being grouped into one of three categories: active, inactive, and pain-specific behaviors. Active behaviors included walking, playing, nursing, nosing, and standing. Inactive behaviors were sitting, lying, and sleeping. Pain-specific behaviors were trembling, scratching, tail wagging, tail jamming, and spasms.

Infrared thermography

Infrared thermography (IRT) images were taken of the tail docking and castration surgical sites using a research-grade infrared camera (FLUKE TiX580, Fluke Corporation, Everett, WA). The camera was manually calibrated to account for the ambient temperature and relative humidity of the farrowing barn at the start of each study day. Images were taken at -24h and at 1, 7, 24, 36 and 48h post-processing. The IRT images were analyzed using research-grade software

(SmartView V4.3, Fluke Thermography, Everett, WA) to determine the degree of inflammation. A single image was effective in capturing both the tail docking and castration sites.

Tail temperatures were obtained by drawing a line over the tip and mid-portion of the tail. Scrotal temperatures were obtained by drawing a circle around the both incisions, to assess inflammation of the castration site, and by drawing a line drawn on the left and right side of each incision, to assess inflammation of the scrotal tissue. Further analysis was performed to calculate the difference in temperature between the focal surgical sites and their surrounding tissues. The temperature of the tissue surrounding the tail was subtracted from the temperature of the tail tip. The temperature of the tissues surrounding the castration incisions was subtracted from temperature of the incision site.

Pressure mat gait analysis

A commercially available floormat-based pressure mat (Strideway, Tekscan, Inc., South Boston, MA) was used to record piglet gait at -24h and at 1, 7, 24, 36 and 48h post-processing. Weights of a known mass were used to calibrate the pressure mat at the start of each study day and ensure accurate measurements. A total of 3 attempts to complete a successful walk across the pressure mat was given to each piglet. An acceptable walk consisted of three continuous, full strides at a normal pace with no running, stops, slips, or falls.

The following gait parameters were analyzed using research grade software (Strideway 7.80, Tekscan, Inc., South Boston, MA): stance time (sec), stride length (cm), peak vertical force (kg), vertical impulse (kg*sec), peak pressure (kg/cm^2), gait distance (cm), and gait velocity (cm/sec).

A laptop (ThinkPad W541, Lenovo, Morrisville, NC) and web camera (Logitech HD 720P, Suzhou, China) were connected and synced to the pressure mat. The webcam video recorded

piglets walking across the pressure mat and video synchronization allowed analyzers to ensure piglets exhibited a consistent gait.

Piglet Grimace Scale

The webcam positioned at the end of pressure mat walkway was used to capture piglet facial images for facial grimace analysis. Piglet facial images were extracted from the pressure mat videos using VLC Media Player (Version 3.0.20, VideoLAN, Paris, France) at -24h and at 1, 7, 24, and 48h post-processing. A single image per piglet per time point was selected based on optimal lightening and angle; ideal images were clear with piglets facing forward. Facial images were only obtained from the same subset of piglets included in the final behavior analysis (n = 120). Thus, a total of 600 images were collected of which 593 were selected for facial grimace assessment.

The random number generator in Excel (Excel, Microsoft, Redmond, WA) was used to relabel the facial images to keep observers masked to treatment and time point. The piglet facial images were assessed using the Piglet Grimace Scale (PGS) from Lou et al. (2022) (**Figure 3.1**). Two raters with extensive experience using the PGS scored the images and evaluated 3 facial action units (FAUs): orbital tightening, ear position, and nose bulge/cheek tension. Each FAU was scored on a 3-point scale: 0 (Not Present), 1 (Moderately Present), and 2 (Obviously Present). Inter-rater reliability was assessed among the two raters.

Plasma cortisol concentrations

Piglet blood was collected at -24h and at 1 and 24h post-processing to determine cortisol concentrations. Blood was collected from a subset of piglets, specifically 3 piglets from each litter (n = 120). The three piglets were randomly selected using the random number generator in Excel (Excel, Microsoft, Redmond, WA) and included a tail docked and castrated male, a tail docked

female, and a sham-handled piglet (male or female). An alternative sorting method was used to determine whether a sham-handled male or female was selected, to ensure an equal number of sham males and females were represented in the final data set.

A veterinarian experienced with blood collection in neonatal piglets collected 3.0 mL of whole blood from each piglet via jugular venipuncture as piglets were restrained in the supine position. A 21-gauge x 1 in. blood collection needle (TycoHealth Care, Mansfield, MA) and vacutainer serum separator tube (BD Vacutainer, Franklin Lakes, NJ) were used to collect piglet blood. After collection, blood sample tubes were placed immediately on ice. Then, the samples were centrifuged at 3000 x g for 10 min and serum was pipetted into cryovials. Blood samples were stored at -80℃ prior to analysis.

All blood samples were analyzed by laboratory personnel at Kansas State University blinded to treatment, procedure, and time point. Blood serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals cat. no. 07-221106-R) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. A 500 ng/mL standard was created by diluting the supplied 1,000 ng/mL kit standard. The standard curve ranged from 1 to 1,000 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a PerkinElmer Wizard2 gamma counter for 1 minute. The raw data file was then uploaded onto MyAssays Desktop

software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation over 18% were re-analyzed.

Plasma firocoxib concentrations

Blood (3.0 mL of whole blood per piglet) for firocoxib analysis was collected simultaneously with blood for cortisol analysis from the same subset of piglets using the same methods. After collection, blood sample tubes were placed immediately on ice. Then, the samples were centrifuged at 3000 x g for 10 min and serum was pipetted into cryovials. Blood samples were stored at -80℃ prior to analysis. All blood samples were analyzed by laboratory personnel at Kansas State University blinded to treatment, procedure, and time point.

The analytical standards, firocoxib and firocoxib-d6, were purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and Alfa Chemistry (Holbrook, NY, USA), respectively. Optima LC-MS grade methanol, acetonitrile, formic acid, ammonium hydroxide, ammonium formate, water, and HPLC grade phosphoric acid were purchased from Fisher Chemical (Ottawa, Ontario, CA). Untreated, blank porcine plasma in K_2EDTA was supplied by Lampire Biological Laboratories (Pipersville, PA, USA). Fisher Scientific (Pittsburg, PA, USA) provided 96-well non-tissue culture treated plates. The Oasis PRiME HLB 96-well µElution plates were supplied by Waters Corporation (Milford, MA, USA).

Stock standard solutions of firocoxib (FRC) and firocoxib-d6 -internal standard (IS)- were prepared independently at 1.00 mg/mL in methanol and stored at -20°C. Serial dilutions of the FRC stock preparation were performed with aqueous phosphoric acid (4%, v/v) to establish daily working standard solution sets at the following concentrations: 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 1.00E+02, 2.50E+02, 5.00E+02, 1.00E+03, 1.50E+03, and 2.00E+03

ng/mL. The IS stock preparation was attenuated with aqueous phosphoric acid (4%, v/v) to yield daily working standard solutions at 1.00E+02 ng/mL.

Samples (300.0 µL) were prepared in 96-well preparatory plates. To compose daily calibrator and process quality control (QC) samples, the FRC and IS working standard solutions (100.0 µL each) were mixed with untreated, blank porcine plasma (100.0 µL). The daily calibrator samples were equal in concentration to the FRC and IS working standard solutions. The daily process QC samples were arranged at 5.00, 10.0, 1.00E+02, 5.00E+02, and 1.00E+03 ng/mL of FRC with 1.00E+02 ng/mL of IS. The porcine plasma samples - 4% v/v aqueous phosphoric acid (200.0 µL; 1:1, v/v) were spiked with the IS working standard solution (100.0 µL) for analytical sampling. Insoluble matrix particulates were removed by centrifugation at 1300 g for 20 mins at 20°C with Thermo Scientific's Sorvall ST 16R Centrifuge (Waltham, MA, USA) to prevent solid phase extraction (SPE) sorbent obstructions. To execute reversed-phase extraction via positive pressure, the supernatants were processed through Oasis PRiME HLB µElution plates (Waters Corporation). Elimination of hydrophilic matrix interferences was completed with a 300.0 µL water - methanol (90:10, v/v) wash. A 50.00 µL introduction of acetonitrile - methanol (90:10, v/v) to induce eluate extraction disrupted the nonpolar van der Waals and pi-pi interactions between the copolymer sorbent's lipophilic divinylbenzene moiety and FRC's cyclopropyl and aryl group. The clarified samples were acidified with 50.00 µL of 2 mM ammonium formate in 0.1% v/v aqueous formic acid -prepared at pH 4 with ammonium hydroxide-.

FRC separation, detection, and quantitation were fulfilled on a Waters Acquity Ultra Performance Liquid Chromatography H-class PLUS-tandem Xevo TQ-S Mass Spectrometry unit (UPLC-MS/MS) and instrumentation was facilitated through MassLynx software. Samples, housed in the 5°C autosampler, were injected (2.00 µL) onto an Acquity UPLC C18 HSS T3

column -100 Å, 1.8 µm, 2.1 x 50 mm- at 40°C; an Acquity UPLC VanGuard precolumn -100 Å, 1.8 µm, 2.1 x 5 mm- of identical chemistry and particle technology was installed for column longevity. Directed at a 0.400 mL/min flow rate, the binary mobile phase was comprised of 2 mM ammonium formate in 0.1% v/v aqueous formic acid at pH 4 (A) and 100% acetonitrile (B). Chromatographic separation with a 7.00 min gradient elution program commenced at 5% B and transitioned to 95% B by 4.00 mins. The solvent system constitution was preserved for an additional minute, adjusted at 5.01 mins to 5% B, and maintained until the termination of the re-equilibration period. The mass spectrometer parameters were adopted from an established protocol (Kleinhenz et al. 2020) and optimized to achieve maximum sensitivity. Electrospray ionization in the positive polarity mode (ESI+) instigated solvent oxidation and analyte charging with a 3.60 kV capillary voltage and a 150°C source temperature. The pneumatically nebulized species were subjected to solvent disintegration, Coulombic fission, and ion evaporation with a desolvation gas (nitrogen) flow rate and temperature of 1000 L/hr and 600°C, respectively. The cone gas (nitrogen) flow rate was set at 150 L/hr to support optimal ion transmission and the minimization of solvent ion cluster formations. Thereafter, the charged gaseous species were fragmented by collision-induced dissociation (CID) with a 0.15 mL/min collision gas (argon) flow rate to conduct multiple reaction monitoring (MRM) detection within the triple quadruple mass spectrometer. Specifically, delivered through the mass analyzer with 50 V of cone energy, firocoxib's parent ion, m/z 337.10, generated daughter ions of m/z 282.96 (quantifier; collision energy at 10 V) and m/z 129.81 (confirmer; collision energy at 28 V). Likewise, with a cone energy of 34 V, firocoxib-d6's parent ion, m/z 343.16, generated daughter ions of m/z 289.03 (quantifier; collision energy at 8 V) and m/z 135.74 (confirmer; collision energy at 28 V). The assay concluded with quantitation through TargetLynx software.

The UPLC-MS/MS method development for the quantitation of FRC in porcine plasma was congruent with the FDA's 2018 *Bioanalytical Method Validation Guidance for Industry* report (U.S. Food and Drug Administration, 2018). The method produced linear, log-log calibration curves from 1.00 to 2.00E+03 ng/mL of FRC with coefficients of determination (R^2) greater than 0.999. An extrapolation of the calibration curves demonstrated that the limit of detection (LOD) was 0.124 ng/mL (S/N = 3). An analysis of process QC reproducibility indicated that the method's limit of quantitation (LOQ) was 5.00 ng/mL. An evaluation of the process QC set triplicates at 5.00, 10.0, 1.00E+02, 5.00E+02, and 1.00E+03 ng/mL of FRC revealed throughout method validation the following average percent recoveries: 105, 98.4, 98.8, 106, and 108 %, respectively. An intra-assay precision analysis of process QC set triplicates established the following percent relative standard deviations (% RSD): 5.64, 8.36, 8.12, 5.32, and 2.81%, respectively. An inter-assay precision analysis of the process QC set triplicates for three separate validation runs demonstrated the following % RSDs: 6.08, 6.40, 6.47, 5.85, and 6.30 %, respectively.

Statistical Analysis

All data were analyzed using SAS (Statistical Analysis System 9.4, SAS Institute Inc., NC). Body weight, vocalizations, IRT temperatures, pressure mat gait analysis, facial grimace scores, and plasma cortisol and firocoxib concentrations were analyzed using a mixed model. Cortisol was log-transformed for normality prior to analysis. Within the mixed model, pig was set as the experimental unit. Pen and time were set as a random effect and repeated measure, respectively. The model analyzed the following interactions: treatment, procedure, time, time x treatment, time x procedure, and treatment x procedure. The Tukey-Kramer adjustment was included to conduct post hoc tests. Statistical significance was set at $P \leq 0.05$, with a trend toward significance set at $P \leq 0.10$.

For behavior analysis, a generalized linear mixed model (GLIMMIX) with a beta distribution was used incorporating the following interactions: treatment, procedure, time, time x treatment, and time x procedure. To account for observation periods where piglets were not in view and could not be scored, the total duration of time piglets spent displaying each behavior was converted into a proportion before the final analysis. The Tukey-Kramer adjustment was included to conduct post hoc tests. Statistical significance was set at $P \leq 0.05$, with a trend toward significance set at $P \leq 0.10$.

Results

Body weight

There were no significant treatment, procedure, time, treatment x time, or procedure x time differences for piglet body weight ($P > 0.05$).

Vocalizations

There were significant procedure and sex differences for energy and peak amplitude ($P < 0.0001$ for all). Processed males produced calls of higher energy than sham-handled piglets ($P < 0.0001$). Sham-handled females produced calls of higher peak amplitude than processed females ($P = 0.05$). Male piglets produced calls of higher energy and peak amplitude than females ($P < 0.0001$). Additionally, there were significant piglet procedure differences for peak frequency ($P < 0.0001$). Processed male and female piglets and sham-handled males produced calls of higher peak frequency than sham-handled females ($P < 0.05$).

Behavior

There were significant procedure x time and sex differences for tail wagging ($P < 0.05$ for all). At 0h post-processing, sham-handled piglets tail wagged more than processed piglets ($P = 0.0004$). Male piglets tail wagged more than female piglets ($P = 0.0003$). Scratching trended

toward significance based on procedure, where processed piglets tended to scratch more than sham-handled piglets ($P = 0.07$). There was a significant sex difference for scratching ($P = 0.05$), as males scratched more than females ($P = 0.05$). There were also significant treatment and time differences for itching ($P < 0.05$ for both). Piglets in the FIRO6 group itched more than piglets in the FIRO5 group ($P = 0.008$). Piglets itched more at 48h post-processing than at baseline and 7, and 24h ($P < 0.05$).

Prostration trended toward significance based on treatment ($P = 0.08$), where piglets in the CON group tended to display more prostration than piglets in the FIRO5 group. Piglets prostrated more at 0h post-processing than at 7 and 24h ($P < 0.05$). Males prostrated more than females ($P = 0.008$). There was a significant time difference for spasms ($P = 0.008$). Piglets had significantly more spasms at baseline and 7h post-processing compared to immediately post-processing ($P < 0.05$).

There were significant procedure x time, treatment x time, and sex differences for pain behavior ($P < 0.05$ for all). At 0h post-processing, sham-handled piglets exhibited more pain-specific behaviors than processed piglets ($P < 0.0001$). Additionally, at 0h, piglets in the CON group exhibited more pain-specific behaviors than piglets in the FIRO3 and FIRO5 groups ($P = 0.03$). Piglets in the CON group also tended to exhibit more pain-specific behaviors than piglets in the FIRO4 group immediately after processing ($P = 0.09$). Male piglets exhibited more pain-specific behaviors than females ($P = 0.003$).

Infrared Thermography

There were significant procedure, time, procedure x time, and treatment x time differences for the average temperature of the tail tip ($P < 0.0001$ for all) (**Figure 3.2**). Processed piglets had higher average tail tip temperatures than sham-handled piglets ($P \leq 0.0001$). Average tail tip

temperatures were lower at baseline than at 7, 36, and 48h post-processing ($P < 0.0001$). Average tail tip temperatures were lower at 1h post-processing than at baseline and at 7, 24, 36, and 48h ($P < 0.0001$). Average tail tip temperatures were also lower at 24h post-processing than at 7, 36, and 48h ($P < 0.05$). Average tail tip temperatures were lower at 48h than at 36h post-processing ($P = 0.02$). At 1h post-processing, sham-handled females had greater average tail tip temperatures compared to processed females ($P = 0.02$). At 24 and 48h post-processing, processed piglets had greater average tail tip temperatures compared sham-handled piglets ($P < 0.0001$). At 36h post-processing, piglets in the CON group had higher average tail tip temperatures compared to piglets in the FIRO6 group ($P = 0.04$).

There were significant procedure, time, and procedure x time differences for the average temperature of the tissue surrounding the tail ($P < 0.0001$ for all). Processed piglets had higher average tail tissue temperatures than sham-handled piglets ($P < 0.0001$). The average tail tissue temperatures were lower at baseline than at 7, 24, 36, and 48h post-processing ($P < 0.05$). The average tail tissue temperatures were lower at 1h post-processing than at 7, 24, 36, and 48h ($P < 0.05$). Additionally, the average tail tissue temperatures were lower at 24 and 48h post-processing compared to 7 and 36h ($P < 0.0001$). A 1, 7, 24, 36, and 48h post-processing, processed females had greater average tail tissue temperatures compared to sham-handled females ($P < 0.0001$). At 1, 24, 36, and 48h post-processing, processed males greater average tail tissue temperatures compared to sham-handled males ($P < 0.0001$).

There were significant procedure, time, and procedure x time differences for the average temperature difference between the tip of the tail and its surrounding tissues ($P < 0.0001$ for all).

Sham-handled piglets had a lower difference in tail temperatures than processed piglets ($P < 0.0001$). There was a lower difference in tail temperature at baseline than at 1, 7, 24, 36, and 48h

post-processing ($P < 0.0001$). There was also a lower difference in tail temperatures at 7, 24, 36, and 48h post-processing than at 1h ($P < 0.0001$). At 1h post-processing, sham-handled males had a lower difference in tail temperatures compared to processed females ($P = 0.04$). At 1, 7, and 36h post-processing, sham-handled females had a lower difference in tail temperatures compared to processed females ($P < 0.05$). At 1 and 7h post-processing, sham-handled males had a lower difference in tail temperatures than processed males ($P < 0.0001$).

There were significant procedure, time, and procedure x time differences for the average temperature of the castration incision site ($P < 0.0001$ for all) (**Figure 3.3**). Sham-handled males (i.e., no actual incision) (37.5 ± 0.08 °C) had higher average incision site temperature than processed males (36.6 ± 0.06 °C; $P < 0.0001$). The average incision site temperature was higher at baseline than at 1, 7, 24, 36, and 48h post-processing ($P < 0.0001$). The average incision site temperature was higher at 7 and 36h post-processing than at 1, 24, and 48h ($P < 0.05$). At 1, 7, 24, and 36h post-processing, sham-handled males had higher average incision site temperatures than processed males ($P < 0.0001$). There was a significant treatment x time difference for the average temperature of the incision site ($P = 0.0006$); however, this was not significant after the Tukey-Kramer adjustment. At 7h post-processing, piglets in the FIRO6 group tended to have a greater average temperature at the incision site compared to piglets in the FIRO4 group ($P = 0.06$). In addition, piglets in the FIRO6 group tended to have a greater average temperature at the incision site compared to piglets in the FIRO3 group at 24h post-processing ($P = 0.09$).

There were significant treatment, procedure, time, treatment x time, and procedure x time differences for the average temperature of the tissue surrounding the incision site ($P < 0.0001$ for all) (**Figure 3.4**). Piglets in the FIRO6 group (37.3 ± 0.13 °C) had higher average temperatures around the incision site than piglets in the FIRO4 group (36.7 ± 0.14 °C; $P = 0.04$). Sham-handled

males (37.2 ± 0.08 °C) had higher average temperatures around the incision site than processed males (36.7 ± 0.06 °C; $P < 0.0001$). The average temperatures around the incision site were higher at baseline compared to all other time points post-processing ($P < 0.05$). The average temperatures around the incision site were also higher at 1h post-processing than at 7h ($P < 0.0001$). The average temperatures around the incision site were higher at 7 and 36h post-processing than at 24h ($P < 0.05$), with temperatures higher at 7h than at 36h post-processing ($P = 0.01$). The average temperatures around the incision site were lower at 48h post-processing than at 1, 7, 24, and 36h post-processing ($P < 0.05$). At 24h post-processing, piglets in the FIRO6 group had greater average temperatures around the incision site than piglets in the FIRO3 and FIRO4 group ($P < 0.05$) (**Figure 3.5**). At 1 and 7h post-processing, sham-handled males had greater average temperatures around the incision site than processed males ($P < 0.0001$).

There were significant procedure, time, and procedure x time differences for the average temperature difference between the castration incision site and its surrounding tissues ($P < 0.0001$ for all). Sham-handled males had a 0.32 ± 0.03 °C increase in temperature at the incision site compared with the surrounding tissues, while processed male piglets had a 0.13 ± 0.02 °C decrease in temperature at the incision site compared with the surrounding tissues ($P < 0.0001$). The average temperature of the incision site was lower than the average temperature of its surrounding tissues at 1 and 7h post-processing compared to baseline and 24, 36, and 48h post-processing ($P < 0.05$). Across all post-processing time points, there was a significant temperature difference between the average temperature of the incision site and the average temperature of its surrounding tissues between processed and sham-handled males ($P < 0.05$). At 1, 7, and 24h post-processing, temperatures of the tissue surrounding the incision site were higher than temperatures at the incision site for processed males compared to sham-handled males ($P < 0.0001$). At 36 and 48h

post-processing, temperatures of the tissue surrounding the incision site were lower than temperatures at the incision site for processed males compared to sham-handled males ($P < 0.05$).

Pressure mat gait analysis

Stance Time (sec)

There were no significant treatment, procedure, time, treatment x time, or procedure x time differences for stance time ($P > 0.05$ for all).

Stride Length (cm)

There were significant procedure and time differences for mean front stride length ($P < 0.05$) (**Table 3.3**). Processed males (17.6 ± 0.30 sec) and females (17.3 ± 0.24 sec), and sham-handled females (17.5 ± 0.37 sec) had a longer mean front stride length than sham-handled males (15.8 ± 0.44 sec) ($P < 0.05$). Mean front stride length was longer at 48h post-processing than at baseline and 7h post-processing ($P < 0.05$).

There were significant procedure and time differences for mean hind stride length ($P = 0.02$). Processed males (17.4 ± 0.37 sec) and females (17.3 ± 0.31 sec) had a longer mean hind stride length than sham-handled males (16.0 ± 0.51sec; $P < 0.05$). Mean hind stride length was longer at 48h post-processing than at 7h ($P = 0.01$).

Peak Force (kg)

There were significant procedure, time, and sex differences for mean front peak force ($P < 0.05$). Processed males (2.06 ± 0.11 kg) had a greater mean front peak force than processed females (1.97 ± 0.10 kg) and sham-handled females (1.85 ± 0.11 kg) ($P < 0.05$). Processed females also had a greater mean front peak force than sham-handled females ($P = 0.01$). Mean front peak force was lower at baseline compared to all other time points (1, 7, 24, 36, and 48h post-processing) ($P < 0.05$). Mean front peak force was lower at 1h post-processing than at 24, 36, and 48h ($P < 0.05$).

Mean front peak force was lower at 7 and 24h post-processing than at 36 and 48h ($P < 0.05$). Males had a greater mean front peak force than females ($P = 0.003$).

There was a significant time difference for mean hind peak force ($P < 0.0001$). Mean hind peak force was lower at baseline compared to all other time points (1, 7, 24, 36, and 48h post-processing) ($P < 0.05$). Mean hind peak force was also lower at 1h post-processing compared to 24, 36, and 48h ($P < 0.05$). Additionally, mean hind peak force was lower at 7h post-processing than at 48h ($P = 0.001$).

Vertical Impulse (kg*sec)

There were significant procedure and time differences for mean front impulse ($P < 0.05$). Processed males (0.78 ± 0.05 kg*sec) produced a greater mean front impulse than sham-handled females (0.70 ± 0.05 kg*sec) ($P = 0.03$). Processed females tended to produce a greater mean front impulse than sham-handled females ($P = 0.08$). Mean front impulse was lower at baseline than at 7, 24, 36, and 48h post-processing ($P < 0.05$). Mean front impulse was also lower at 1h post-processing than at 24, 36, and 48h ($P < 0.05$).

There were no significant treatment, procedure, time, treatment x time, or procedure x time differences for hind vertical impulse ($P > 0.05$ for all).

Peak Contact Pressure (kg/cm^2)

There were significant piglet procedure, time, and sex differences for mean front peak contact pressure ($P < 0.05$). Processed males (1.54 ± 0.06 kg/cm^2) distributed more contact pressure on their front limbs than processed females (1.48 ± 0.06 kg/cm^2) and sham-handled females (1.43 ± 0.07 kg/cm^2) ($P < 0.05$). Mean front peak contact pressure was lower at baseline compared to all other time points (1, 7, 24, 36, and 48h post-processing) ($P < 0.05$). Mean front peak contact pressure was also lower at 1h post-processing compared to 24, 36, and 48h ($P < 0.05$).

Additionally, front peak contact pressure was lower at 7h post-processing than at 48h ($P = 0.004$). Males had greater mean front peak contact force than females ($P = 0.004$).

There were significant time differences for mean hind peak contact pressure ($P < 0.01$). Mean hind peak contact pressure was lower at baseline compared to all other time points (1, 7, 24, 36, and 48h post-processing) ($P < 0.05$). Mean hind peak contact pressure was lower at 1 and 7h post-processing compared to 24 and 48h ($P < 0.05$).

Gait Distance (cm)

There were no significant treatment, procedure, time, treatment x time, or procedure x time differences for gait distance ($P > 0.05$ for all).

Gait Velocity (cm/sec)

There was a significant piglet procedure difference for gait velocity ($P = 0.009$). Processed piglets had a faster gait velocity than sham-handled males ($P < 0.05$).

Piglet Grimace Scale

The ICCs for inter-rater reliability among the two raters were 0.07, 0.58, 0, and 0.43 for orbital tightening, ear position, nose bulge/cheek tension, and total PGS score, respectively. Intra-class correlation coefficient values less than 0.50 are indicative of poor reliability and values between 0.50 and 0.75 are indicative of moderate reliability (Koo and Li, 2016). Given poor reliability, orbital tightening and nose bulge/cheek tension were removed from the final PGS analysis. There was a significant time difference for ear position ($P = 0.0004$). Ear position scores were higher at 7, 24, and 48h post-processing compared to baseline ($P < 0.05$).

Plasma cortisol concentrations

There was significant treatment, procedure, time, treatment x time, and sex differences for cortisol concentrations ($P < 0.05$ for all) (**Figure 3.6**). Piglets in the FIRO5 group had higher

cortisol concentrations than piglets in the FIRO3 group (P = 0.02) (**Figure 3.7**). Processed males had higher cortisol concentrations than all other procedural groups (P < 0.05). Cortisol concentrations were higher at baseline compared to 1 and 24h post-processing (P < 0.0001). Cortisol concentrations were also higher at 1h post-processing compared to 24h (P < 0.0001). At 1h post-processing, processed males had higher cortisol concentrations than all other procedural groups (P < 0.0001). Males had higher cortisol concentrations than females (P = 0.006).

Plasma firocoxib concentrations

There were significant treatment, time, and treatment x time differences for firocoxib concentrations (P < 0.05) (**Figure 3.8**). The mean plasma firocoxib concentrations for CON, FIRO3, FIRO4, FIRO5, and FIRO6 piglets were the following: 15.13 ± 45.7, 119.03 ± 47.8, 190.87 ± 54.0, 222.36 ± 42.83, and 206.24 ± 56.0 ng/ml. Firocoxib concentrations were higher in piglets in the FIRO4 (190.87 ± 54.01 ng/ml), FIRO5 (222.36 ± 42.83 ng/ml), and FIRO6 groups (206.24 ± 55 ng/ml) compared to piglets in the CON group (15.13 ± 45.7 ng/ml) (P < 0.05). Firocoxib concentrations were higher at 1 and 24h post-processing compared to baseline, with no differences between 1 and 24h (P < 0.0001). At 1 and 24h post-processing, firocoxib concentrations were higher in piglets in the FIRO4, FIRO5, and FIRO6 groups compared to piglets in the CON group (P < 0.05) (**Figure 3.9**).

Discussion

Firocoxib may be a more effective NSAID at providing pain relief to piglets undergoing tail docking and surgical castration than meloxicam, ketoprofen and flunixin. A pharmacokinetic analysis found that a single injection of firocoxib in sows had a longer half-life and volume of distribution than meloxicam, ketoprofen and flunixin (Coetzee et al., 2019). This suggests firocoxib can provide analgesia for a longer period and has a greater distribution throughout the

body. It was demonstrated that transmammary delivered firocoxib at a dose of 2.0 mg/kg was not clinically effective at reducing pain associated with tail docking and castration, as there were no differences in pain-specific behavior, cortisol, or gait score when compared to placebo (Viscardi, 2023). Researchers recommended future work evaluate higher doses and administer the drug more than 7h before piglets are processed for improved drug transfer across the blood-milk barrier (Viscardi, 2023).

Previous work used the injectable formulation of firocoxib, which is no longer in production. To the best of our knowledge, this is the first study to report the use of the oral formulation of firocoxib for pain relief in piglets via transmammary delivery. Transmammary delivery would be beneficial to piglets in having an analgesic on-board prior to processing, administered through nursing rather than individual handling and dosing, which can be stressful. It would also be less time consuming and cost-effective for producers to administer a drug at the sow level and have fewer negative human-animal interactions. Sow welfare may also be enhanced from analgesic effects if experiencing pain associated with farrowing.

Body weight has been used as indicator of piglet welfare regarding normal growth and functioning (Morton and Griffiths, 1985). Although not a direct measure of pain, there is evidence to suggest piglet processing can depress piglet growth (McGlone et al., 1993; Kielly et al., 1999). Treatments in this study had no effect on piglet growth after processing. Similarly, other NSAIDS, such as meloxicam and ketoprofen, did not have an effect on piglet growth after tail docking and castration when administered orally either before or after processing (Hansson et al., 2011; Tenbergen, Friendship, Cassar, Amezcua, & Haley, 2014; Cassar et al., 2014). Transmammary delivered firocoxib, however; improved piglet average daily gain after tail docking and castration (Coetzee et al., 2019), but was not repeatable (Viscardi, 2023). Researchers suspect the provision

of an NSAID to the sow reduced negative impacts that can result from parturition (e.g., reduced feed intake) leading to enhanced milk production and composition (e.g., added fat and protein), which may have had a positive effect on piglet performance (Coetzee et al., 2019). A main difference in our study was the use of the oral formulation of firocoxib for transmammary delivery. This necessitates the need for future studies to evaluate the effects of oral firocoxib on sow performance (e.g., behavior, feed intake, and weight) post-farrowing, and milk composition. A decrease in nursing behavior post-processing has been reported and is likely to affect piglet growth (Taylor et al., 2001); however, nursing behavior pre-and post-processing were consistent in this study.

Pigs use vocalizations as a primary method of communication, where key differences in vocal parameters may signal unpleasant experiences (Dawkins, 2004). Scientists have discovered that there are significant differences between piglet vocalizations at the time of processing compared to handling alone, which align with our results (Schmid and Steinhoff-Wagner, 2022). Vocalizations produced by male piglets that were tail docked and castrated were of greater energy and peak amplitude compared to tail-docked only females and sham-handled piglets. These vocalizations were louder and more intense, which have been referred to as a "screaming" call type (Marx et al., 2003).

The castration procedure is considered to be more painful than other piglet processing procedures given the level of tissue damage it causes with multiple invasive steps (Schmid and Steinhoff-Wagner, 2022). The castration procedure also requires piglets to be handled for longer. Longer procedures have shown to cause higher stress responses in piglets (Marchant-Forde et al., 2009). Thus, it is reasonable that males overall produced calls with these higher vocal characteristics than females.

Treatment had no effect on piglet vocalizations at the time of processing. The transmammary delivery of an oral NSAID may have a slow onset. Castration can involve several incisions and the removal of the spermatic cord, which are processes likely to cause acute pain not mitigated by an NSAID (Coetzee et al., 2019). It is also notable that sham-handled piglets produced calls of high energy, peak amplitude, and frequency, providing additional evidence that handling alone induces a stress response in piglets. However, significant differences can still be discerned between handling and processing indicating vocalization is a reliable outcome to measure pain in piglets.

Animal behavior is recognized as a reliable and direct indicator of animal welfare (Fraser et al., 1997). In assessing animal responses to painful events, scientists have focused on identifying deviations from their normal behavior (Anil et al., 2005). After tail docking and castration, piglets are observed displaying certain behaviors more frequently or behaviors that were not present prior to processing. Trembling, spasms, posterior scooting, tail jamming, and prostration are some pain-specific behaviors observed in piglets post-processing (Herskin and Di Giminiani, 2018).

Tail wagging has been reported to increase in piglets after tail docking (Noonan et al., 1994). Tail wagging also increased after castration in comparison to sham-handling (Viscardi and Turner, 2019). Hay et al. (2003) found that castrated piglets tail wagged more than non-castrated piglets for up to 2 days after processing. Yet, the opposite was observed in this current study, as the time spent tail wagging was significantly greater in sham-handled piglets compared to processed piglets. This was also observed immediately after processing than at all other assessment time points. This may be attributed to difficultly in viewing the tail in tail-docked piglets compared to sham-handled piglet with intact tails, which has been described by others (Viscardi and Turner, 2019).

Scratching tended to be greater in processed piglets compared to sham-handled piglets, which aligns well with other studies. Llamas Moya et al. (2008) reported the same results when comparing castrates to non-castrates. Additionally, castrated piglets have been observed scratching their rump more than non-castrates for up to 24h post-procedure (Hay et al., 2003). Researchers have suggested that scratching is a behavior performed by piglets possibly signaling a feeling of discomfort after a painful procedure (Llamas Moya et al., 2008).

Like scratching, itching is similar as both behaviors may be performed temporarily to help alleviate pain from a localized location on the body. Itching of a location near a surgical site may help alleviate irritation or pain during the healing process, which is commonly seen in humans (Hay et al., 2003). Furthermore, severe itching can be caused by inflammatory mediators (Meintjes, 2012). In this study, piglets itched more at 48h than at baseline, 7, and 24h post-processing, a point in time where inflammation may have been more severe. Itching was scored less in FIRO5 piglets than FIRO6 piglets. Piglets in the FIRO5 group also tended to prostrate less than piglets in the CON group. It may be that 0.5 mg/kg firocoxib reduced the inflammatory response, lessening piglet discomfort or pain post-procedure.

An unexpected finding was that sham-handled piglets exhibited more pain behaviors than processed piglets across the entire assessment period. However, further analysis determined that when tail wagging was removed from the subset of pain behaviors, this was no longer the case. Additionally, male piglets expressed more pain behaviors than females likely due to do tail wagging and scratching being higher in males than females. A positive outcome was discovered with CON piglets displaying more pain behaviors than piglets in the FIRO3 and FIRO5 group immediately after processing. Behavior results in this study seem to indicate a positive effect of

FIRO3 and FIRO5 on piglet behavior by reducing pain-specific behaviors, itching, and prostrating post-processing.

Physiological responses caused by painful stimuli, such as inflammation, as well as thermal responses mediated by the sympathetic nervous system (SNS) can be assessed using thermal imaging (Whittaker et al., 2023). The temperatures of certain anatomical locations, such as the eyes, cranium, ears, and snout, have been of great interest when using IRT to assess animal responses to castration. In castrated calves, ocular temperatures have been of major focus in detecting SNS activation (Kleinhenz et al., 2018; Martin et al., 2022). Similarly, ocular (Coetzee, 2019) and cranium, ear, and snout (Bates et al., 2014) temperatures were assessed after tail docking and castration in piglets. Literature assessing the degree of inflammation at the tail docking and castration site in piglets post-processing is very limited. Following tissue injury, inflammation causes an increase in vasodilation to facilitate the healing process leading to an increase in skin temperature as blood flows towards the affected area.

In this study, piglet procedures affected skin temperatures and therefore possibly inflammation. For tail docking, tail docked piglets had higher temperatures at both tail docking sites (tail tip and surrounding tissues) than sham-handled piglets across the assessment period. This was an expected finding, as no tissue damage was present in sham-handled piglets. However, at 1h post-processing tail docked piglets had lower tail temperatures than sham-handled piglets. This coincides with (Lou et al., 2022) who reported lower tail tip temperatures in tail docked piglets than sham-handled piglets at 0.5h post-processing. This rapid drop in temperature has been linked to an SNS response as blood is directed towards critical organs over peripheral regions (Whittaker et al., 2023). Despite this sudden drop in temperature within the first hour post-processing, tail

docked piglets maintained higher tail tissue temperatures for up to 48h post-processing, indicating the presence of inflammation after tissue injury.

The opposite was observed for temperatures at the castration site, where sham-castrated piglets had higher temperatures at the incision site and its surrounding tissues than processed piglets across the assessment period. Sham-castrated piglets also had a higher temperature difference than castrated piglets between the two castration sites. This same difference in temperature at the incision site between sham-castrated and castrated piglets was reported in another study, but only up to 7h post-castration (Viscardi et al., 2020). These differences may have been attributed to using a CO_2 surgical laser to castrate piglets, as it functions to reduce inflammation (Viscardi et al., 2020).

Pain and inflammation are observed after castration as it is a highly invasive procedure involving manipulation of soft tissues (Whittaker et al., 2023). In calves, scrotal temperatures after castration using IRT have been assessed. Moya et al. (2014) found that scrotal temperatures were higher 48h post-castration. A second study found that scrotal temperatures in castrated calves were higher on day 6 post-castration than on day 1 and 2 (Van Der Saag et al., 2018). Similarly, others reported scrotal temperatures were highest from day 21-34 post-castration with inflammation peaking on day 2-3 (Mintline et al., 2014). Results from other studies on calves indicate that that inflammation is present at the castration site post-procedure (Moya et al., 2014; Mintline et al., 2014; Van Der Saag et al., 2018). Unlike the calf related studies, this study assessed scrotal temperatures up to 48h post-procedure. The authors hypothesized that temperatures increase at a later stage in the healing process given revascularization of the tissue and scab loss (Mintline et al., 2014; Van Der Saag et al., 2018).

The use of NSAIDs to reduce the inflammatory response after piglet processing using IRT have been investigated. One study recorded the ear and castration site temperature of piglets post-castration (Hansson et al., 2011). Researchers found no differences in temperature at either location between piglets castrated without analgesia and with analgesia (lidocaine, meloxicam, or lidocaine and meloxicam) (Hansson et al., 2011). In our study, FIRO6 piglets had lower tail tip temperatures than CON piglets 36h post-tail docking, suggesting reduced inflammation. However, temperatures of tissues around the incision site were higher for FIRO6 piglets compared to FIRO4 piglets. Castration incision site temperatures also tended to be higher for FIRO6 piglets than FIRO4 piglets at 7h post-castration, and FIRO3 piglets at 24h post-castration, which may suggest lower doses were more effective at reducing inflammation at the castration site.

The results of this study suggest that tail docking causes inflammation. However, the onset of inflammation at the castration site may be delayed, so scrotal temperatures should be assessed beyond 48h post-procedure. Results also suggest doses lower than 6.0 mg/kg may be effective at reducing the inflammatory response post-processing. Additional research focused on the development of inflammation at the tail docking and castration sites in piglets using IRT is needed.

According to FDA standards, pressure mat gait analysis is considered a reliable method of pain assessment (Baysinger et al., 2021). This system has been able to detect gait abnormalities exhibited in lame cows (Rushen et al., 2012), post-partum cows (Kleinhenz et al., 2019), and castrated calves (Martin et al., 2022). While detecting gait abnormalities, animals have been observed redistributing their body weight to put less force and pressure onto the painful area. In comparison to sham-castrated calves, castrated calves applied more force and pressure onto their front limbs than their hind limbs (Kleinhenz et al., 2018). Researchers implied this may be an indicator of calves experiencing pain associated with castration and retributing their weight away

from the castration site. However, literature on the use of pressure mat outcomes to assess pain in piglets after tail docking and castration is lacking.

Similar results were reported in this study regarding peak force, vertical impulse, and peak contact pressure based on piglet procedure. Pressure mat gait parameters describe gait in terms of the amount of force and pressure placed onto the limbs and limb contact time on the pressure mat. Castrated piglets exhibited a significantly greater peak force, vertical impulse, and peak contact pressure onto their front limbs than sham-handled piglets. These results demonstrate males applied more force and pressure onto their front limbs than females. Stride length, which is the distance traveled between two consecutive foot falls was also longer in castrated piglets than sham-handled piglets for both front and hind limbs. However, Currah et al. (2009), reported the opposite with calves having a shorter stride length post-castration. Differences in animal sizes and the fixed length of a pressure mat have been reported to affect stride length and whether or not complete strides are recorded (Kleinhenz et al., 2018).

Our results are consistent with others, demonstrating an altered gait in castrated piglets post-procedure, compared to baseline. Like calves, it appears castrated piglets shift their weight onto their front limbs, attempting to relieve added pressure and force off of the castration site. Interestingly, tail docked females also exhibited a longer stride length and applied more force onto their front limbs than sham-handled piglets. This may indicate that tail docking can cause an abnormal gait post-procedure with similar shifts in weight away from their hind limbs and off of the tail docking site.

A second interesting finding was that processed piglets had a faster gait velocity (i.e., walked faster across the pressure mat) than sham-handled piglets. This is contrary to the results of previous work, showing that sham-handled calves walked across the pressure mat faster than

castrated calves due to them being in a less painful state (Kleinhenz et al., 2018). Gait velocity differences between calves and piglets may be linked to differences in overall body size, limb length, and ease of walking across the pressure mat. Study personnel in this study experienced challenges in walking piglets across the pressure mat with reluctance from piglets to walk and complete full strides.

Gait abnormalities post-castration have been improved with the provision of analgesia in calves (Currah et al., 2009 ;Martin et al., 2022). Calves administered flunixin and lidocaine displayed a longer front stride length post-castration compared to calves administered lidocaine or placebo (Currah et al., 2009). Castrated calves given lidocaine and meloxicam or only a local block (bupivacaine liposomal injectable suspension) had a shorter front stance time than castrated calves given placebo (Martin et al., 2022). Additionally, piglets receiving transmammary delivered firocoxib applied more force onto their front limbs unlike piglets receiving placebo (Coetzee, 2019). Firocoxib treatments administered in this study were likely not effective at reducing change in weight distribution associated with pain post-processing in piglets.

Facial expressions induced by painful stimuli have been identified and assessed in piglets after tail docking and castration using the PGS (Gottardo et al., 2016). The FAUs used to in this study to assess piglet grimacing were orbital tightening, ear position, and nose bulge/cheek tension, which have shown acceptable inter-rater reliability across raters (Di Giminiani et al., 2016 ;Viscardi et al., 2017). After a painful event, humans appear to express a "prototypical facial configuration" signaling a negative affective state through similar FAUs (Descovich, 2017). Like humans, these FAUs have also been observed in animals after a painful procedure such as mice (Langford et al., 2010), rats (Sotocina et al., 2011), and horses (Dalla Costa et al., 2014).

In this study, reliability for orbital tightening and nose bulge/cheek tension were poor, while ear position had moderate reliability. A common method used to obtain facial images from animals for grimace analysis is taking still digital images during animal restraint (McLennan et al., 2019). The quality of the piglet facial images used in this study may have been affected by methods used to collect images, making FAUs difficult to score. Images were of piglets actively walking across a pressure mat, which may have caused low resolution or added pixilation impairing the visibility of the FAUs. This novel method was chosen attempting to reduce the stress response in piglets from physical restraint.

Ear position scores were higher from 7-48h post-processing when compared to the period before piglets were processed. Comparably, in previous work, total PGS scores followed a similar pattern with higher scores notes from 0-5h post-processing (Viscardi et al., 2017). However, ear position was only significant based on time, so further interpretation regarding the effects of procedure or treatment on ear position cannot be inferred. It is possible that ear position scores may have increased from responses to due to handling, actual processing, or other environmental factors. Ear position's moderate reliability and reported changes over time may also suggest it is the most readily observed. Ears that are backward facing or flattened are more frequently observed in animals (e.g., dogs, horses, and sheep) during negative situations (Descovich, 2017).

Stress and pain responses in animals have been measured using cortisol as a biomarker (Brown and Winnicker, 2015). In response to tail docking and surgical castration, piglet cortisol levels have demonstrated to reach peak levels 15-90 min after processing compared to sham-processing (Prunier et al., 2006;Sutherland et al., 2008;von Borell et al., 2009). Likewise, in this study cortisol levels were higher in tail docked and castrated piglets than in sham-handled piglets and were highest within the first hour post-processing compared to 24.

Cortisol levels were also higher 24h before piglet processing compared to 1 and 24h post-processing. It is reasonable to suggest that piglets found the collection of baseline outcomes highly stressful, with first time exposure to novel events and extensive manipulation. Thus, cortisol presents limitations as a direct measure of pain, as levels can fluctuate due to various factors other than painful stimuli and should be interpreted cautiously (Brown and Winnicker, 2015).

Treatment had an effect on cortisol production, where FIRO3 piglets produced less cortisol than FIRO5 piglets, suggesting dose a of 3.0 mg/kg of firocoxib delivered transmammary may be effective at reducing the stress response in piglets after processing. The transmammary delivery of meloxicam (30 mg/kg) (Bates et al., 2014) and firocoxib (2.0 mg/kg) (Coetzee et al., 2019) after tail docking and castration have both resulted in lower cortisol concentrations compared to piglets receiving placebo. In comparing the doses used in our study and earlier work focused on transmammary delivery of firocoxib that led to lower cortisol, these doses (3.0 and 2.0 mg/kg) are close in range and may imply lower doses can provide some benefit.

As this may be the first reported use of oral firocoxib for transmammary drug delivery to piglets, there is no direct comparable data. The mean plasma firocoxib concentrations for CON, FIRO3, FIRO4, FIRO5, and FIRO6 piglets in this study were the following: 15.13 ± 45.7, 119.03 ± 47.8, 190.87 ± 54.0, 222.36 ± 42.83, and 206.24 ± 56.0 ng/ml, indicating a dose-dependent increase. This demonstrates that firocoxib was successfully transferred to piglets via sow milk after oral administered of firocoxib to sows approximately 12h prior to piglet processing.

Previous work demonstrated the transmammary delivery of firocoxib using lower doses (0.5-2.0 mg/kg) via intramuscular injection to the sow approximately 7h pre-processing (Coetzee et al., 2019). The pharmacokinetic (PK) analysis reported a dose-dependent increased with the following peak firocoxib plasma concentrations (Cmax) in piglets at 24h post-processing: 9.53,

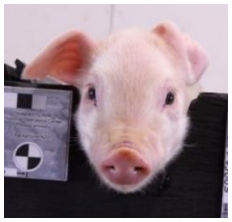
31.04, 53.30, and 44.03 ng/ml after 0.5, 1.0, 1.5, and 2.0 mg/kg firocoxib (Coetzee et al., 2019). The PK analysis also reported that piglet firocoxib concentrations seem to not increase linearly, as concentrations from the 2.0 mg/kg group plateaued after 48h post-processing, suggesting higher doses may not lead to added drug transfer into the milk (Coetzee et al., 2019). Results from this study indicate otherwise and may imply that the oral formulation of firocoxib may be more effective at crossing the blood-milk barrier. However, our samples were only collected up to 24h post-processing. It would be useful for future studies to collect blood or milk samples beyond 24h post-processing to interpret whether the oral formulation of firocoxib shows a similar pattern.

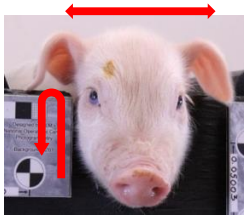
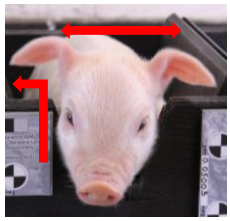
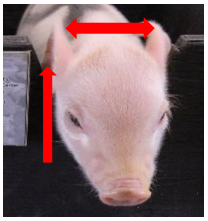
A second positive outcome of our results were that firocoxib concentrations were consistent from 1 to 24h piglet post-processing. In piglets, firocoxib has a plasma elimination half-life of 26-31h (Coetzee et al., 2019). A stable concentration of firocoxib for up to 24h may be attributed to its long half-life. Future studies performing a PK analysis evaluating the oral formulation of firocoxib would provide insight on these findings and be beneficial in making further comparisons.

Conclusion

Tail docking and castration caused piglets to display more intense, louder vocalizations, inflammation, a greater redistribution of weight onto their front limbs, and higher cortisol concentrations than handling alone. Pain-specific behavior was observed more in males than in females. Transmammary delivered firocoxib at a dose of 3.0 and 5.0 mg/kg reduced pain-specific behaviors in piglets immediately after processing compared to placebo. Additionally, 3.0 mg/kg firocoxib reduced cortisol levels more than 5.0 mg/kg. This study demonstrated that tail docking and castration may induce pain and inflammation. It also demonstrated that as a dose of 3.0 mg/kg may be beneficial, as it reduced some behavioral and physiological indicators of pain. Further

research would prove beneficial in evaluating the efficacy of these doses in reducing pain in piglets post-processing.

Orbital Tightening		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Eyes wide open, with a round, circular shape No tightening of or around the eyelid Whites of eyes may be visible **Both eyes should be scored 0**	Eyes are closed halfway, with a teardrop shape Eyelids are partially tightened (i.e., "eye squeezing") "Eyebrow" furrowing is present Whites of eyes may be partly visible **Features may be seen only or more strongly in 1 eye**	Eyes are closed more than halfway (may see full eye closure) Eyelids are fully tightened, with obvious "eye squeezing" Significant "eyebrow" furrowing Whites of the eyes are not visible **Features may be seen only or more strongly in 1 eye**

Ear Position		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Ears are forward and relaxed Normal distance between ears Inner pinna is partly visible Base of ear has the following shape:  **Both ears should be scored 0**	Ears are upward and drawn back Distance between ears is reduced (Length of red arrow is less than in Score 0) Inner pinna may be more visible Base of ear has the following shape:  **Features may be seen only or more strongly in 1 ear**	Ears are fully drawn back, flush against head Distance between ears is further reduced (Length of red arrow is less than in Score 1) Base of ear has the following shape: Inner pinna not visible Base of ear has the following shape: **Features may be seen only or more strongly in 1 ear**

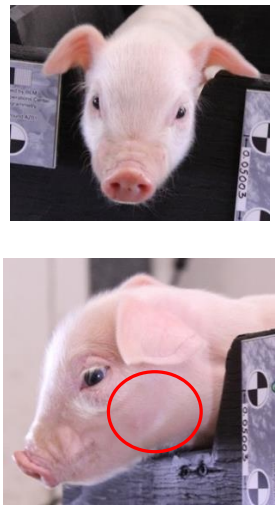
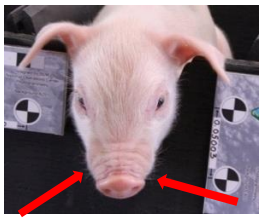
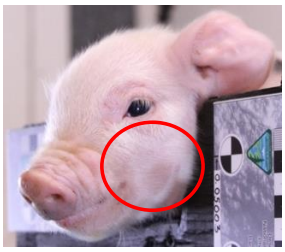
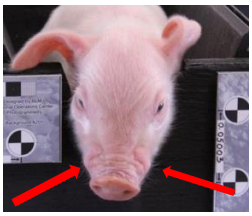
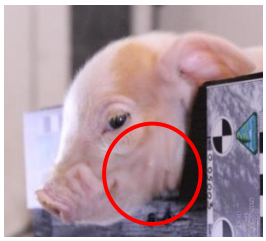
Nose Bulge/Cheek tension		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
	 	 – 
Nose Pigs naturally have some wrinkles/skin folds on nose **Cheeks** Skin gradient is flat, smooth, and relaxed No tightening **Note:** Nose muscles move congruently with cheek muscles	**Nose** Increase in bulging Moderate "bulging" appearance with skin folds Nose moves inward **Cheeks** Moderate tightening/tension with few indentations/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**	**Nose** Significant increase in bulging Significant "bulging" with several skin folds Nose moves inward **Cheeks** Significant tightening/tension with several indentions/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**

Figure 3.1. The Piglet Grimace Scale (PGS) with the description of the facial action units (FAUs) assessed. (Modified from Viscardi et al., 2017 and Lou et al., 2022).

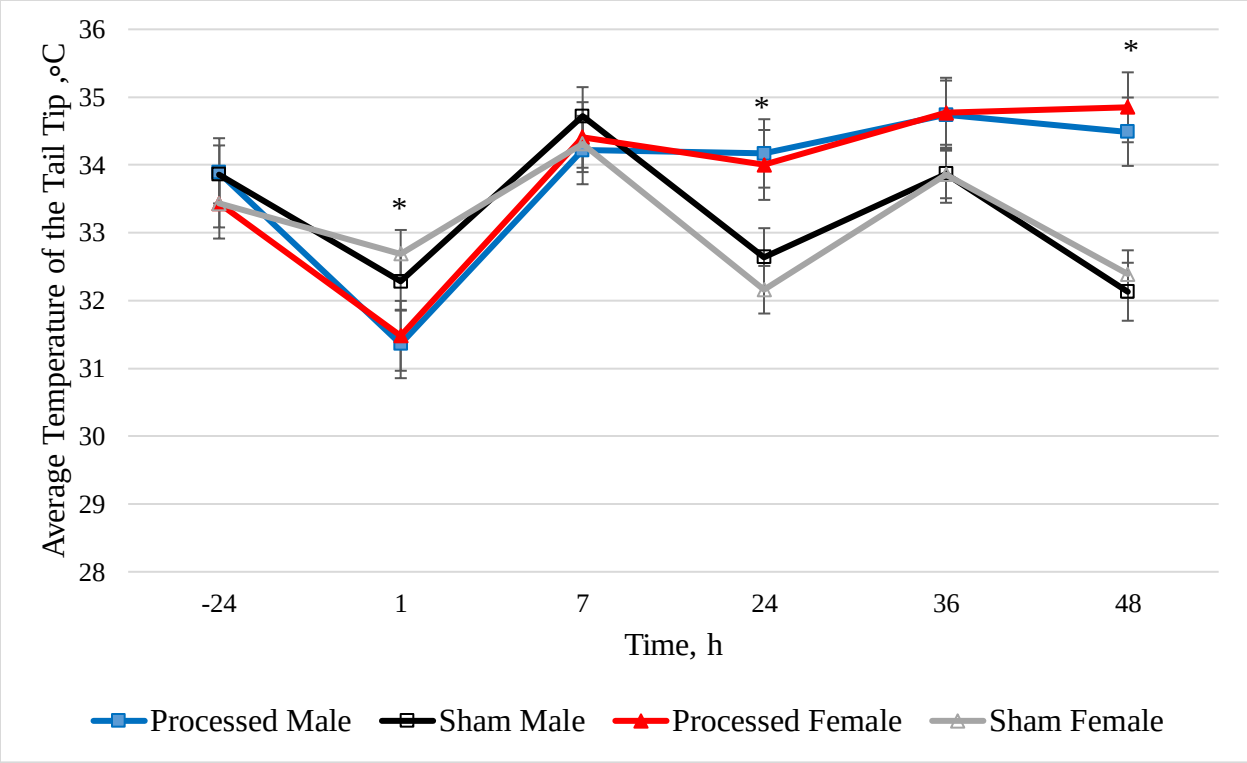


Figure 3.2. Average temperature (°C) of the tail tip (mean ± SE) of piglets in each processing group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between processed and sham-handled piglets.

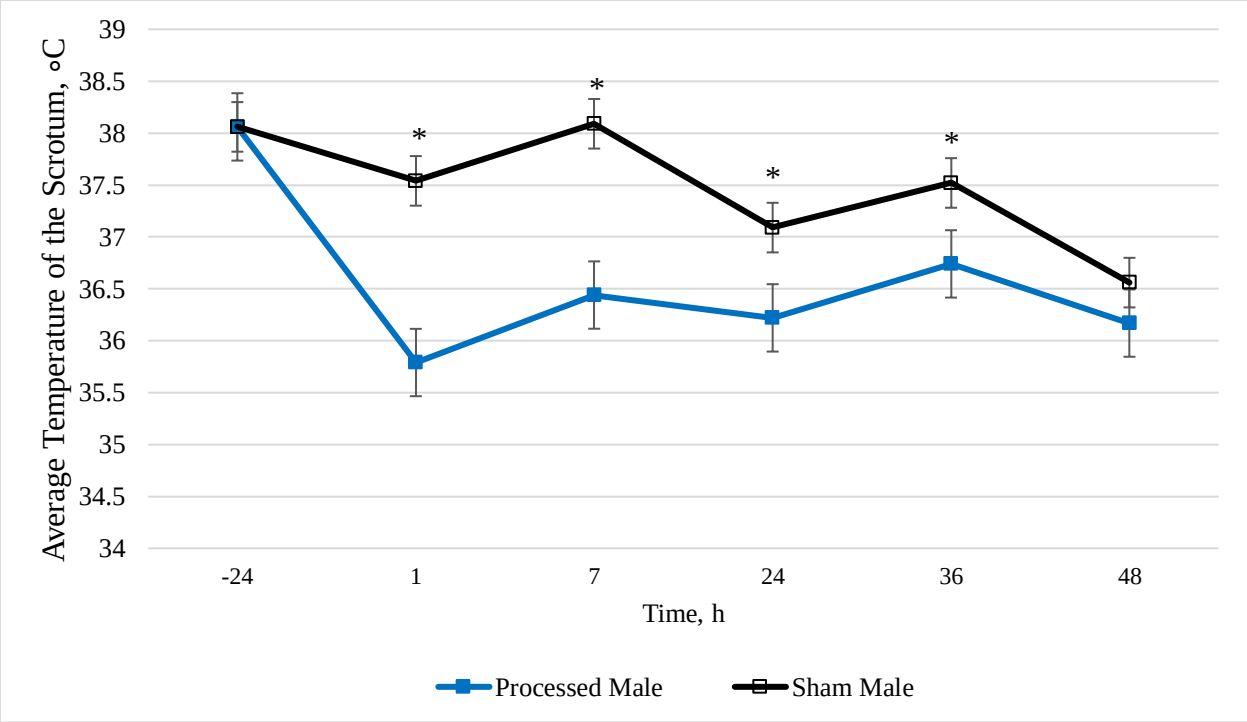


Figure 3.3. Average temperature (°C) of the castration incision site (mean ± SE) of piglets in each processing group across the assessment period.

Asterisks represents a significant difference ($P < 0.0001$) between castrated and sham-handled piglets.

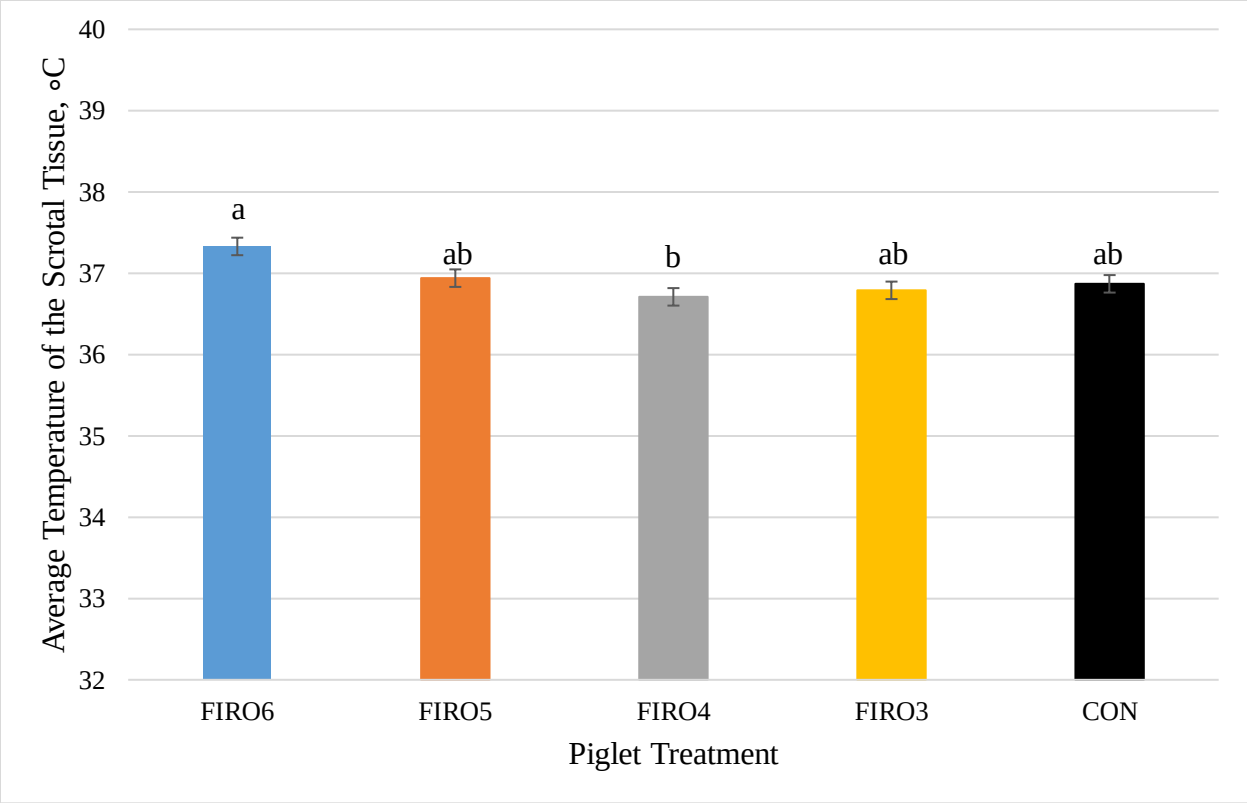


Figure 3.4. Average temperature (°C) of the tissue surrounding the castration incision site (mean ± SE) of piglets in each treatment group.

Different letters represent a significant difference ($P < 0.05$) between treatment groups.

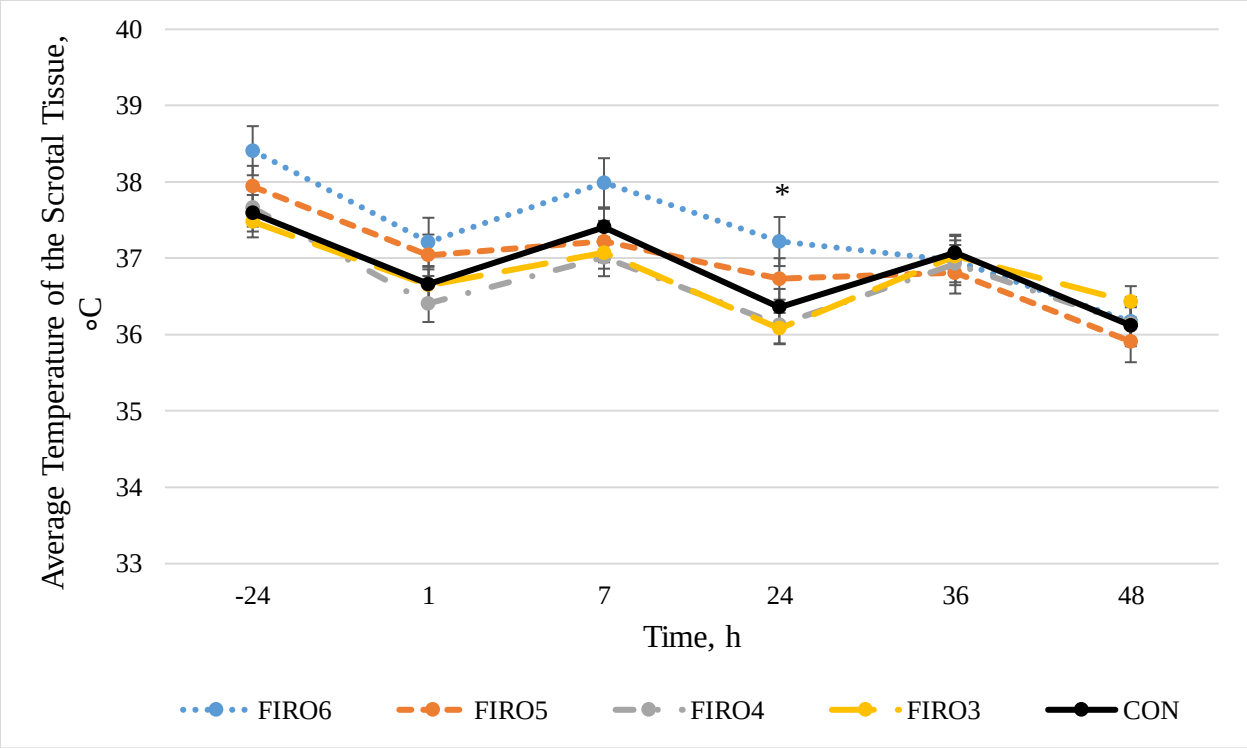


Figure 3.5. Average temperature (°C) of the tissue surrounding the castration incision site (mean ± SE) of piglets in each treatment group across the assessment period.

Asterisks represents a significant difference (P = 0.0004) between treatment groups.

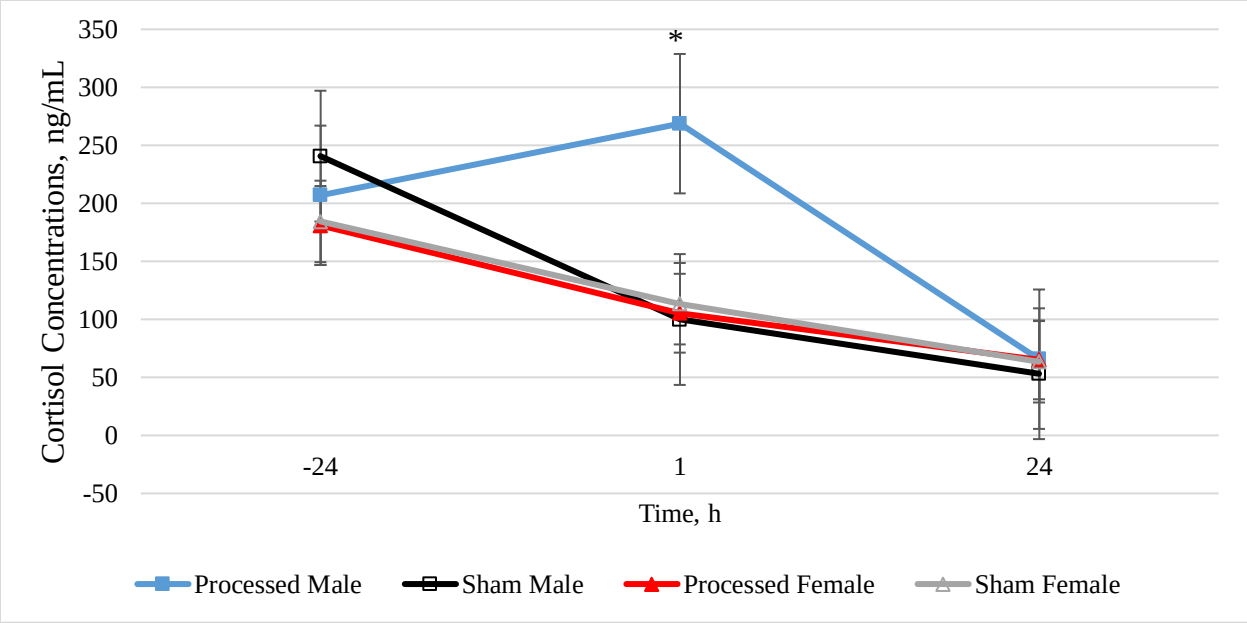


Figure 3.6. Mean cortisol concentrations (mean ± SE) of piglets in each processing group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between processed piglets and sham-handled piglets.

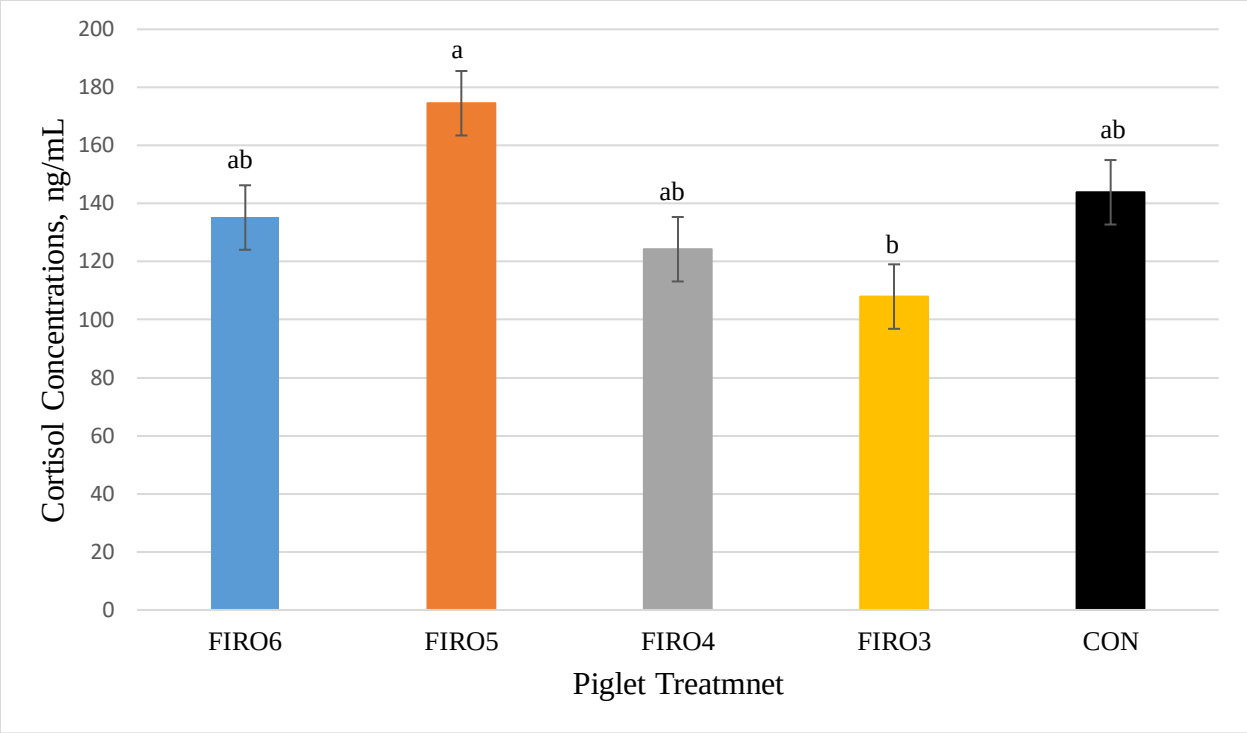


Figure 3.7. Mean cortisol concentrations (mean ± SE) of piglets in each treatment group across the assessment period.

Different letters represent a significant difference (P = 0.04) between treatment groups.

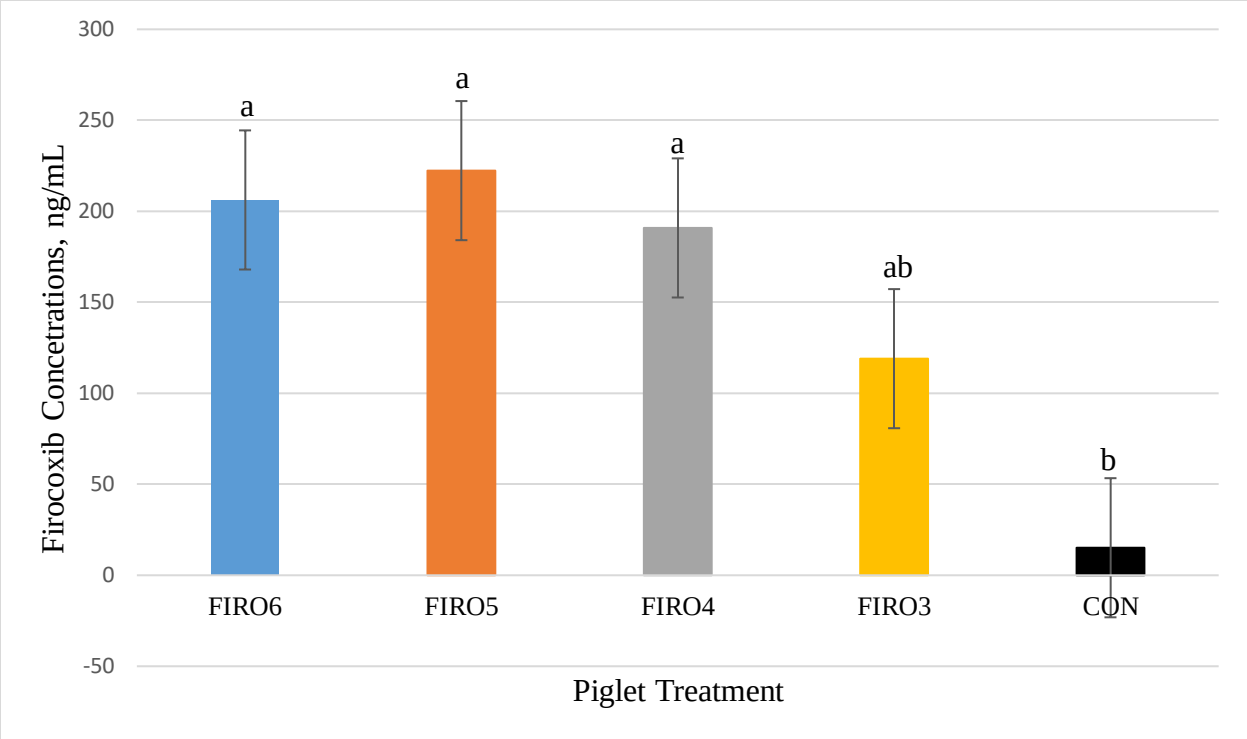


Figure 3.8. Mean firocoxib concentrations (mean ± SE) of piglets in each treatment group. Different letters represent a significant difference (P = 0.004) between treatment groups.

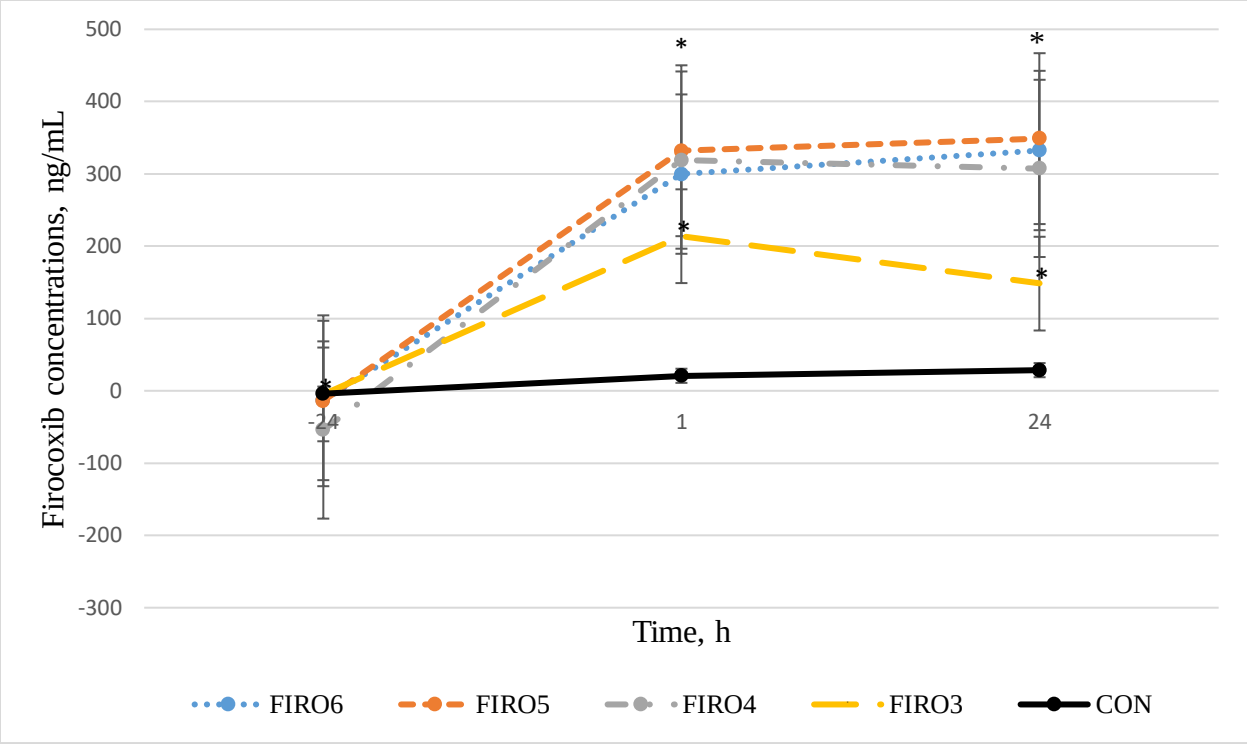


Figure 3.9. Mean firocoxib concentrations (mean ± SE) of piglets in each treatment group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between treatment groups.

Table 3.1. Description of the sow study population and their treatments

Cohort	Group	Sow ID	Parity	Weight (kg) [a]	Tx[b]	Dose (mg)
1	1	B153	2	275.0	CON	0
		B98	4	252.7	4.0	908
		B99	4	309.6	3.0	909
		Y25	1	211.4	CON	0
		Y23	1	200.9	4.0	1079
		Y16	1	208.6	CON	0
	2	Y18	1	190.0	6.0	1192
		Y17	1	224.55	6.0	1476
		B177	2	221.8	6.0	1306
		Y20	1	192.7	6.0	1249
		Y26	5	202.73	6.0	1419
		B33	1	272.7	5.0	1362
		B127	3	258.2	5.0	1306
	3	Y28	1	201.8	4.0	852
		Y8	1	230.0	CON	0
		B178	2	241.8	6.0	1476
		B89	4	277.3	5.0	1419
		Y3	1	204.1	5.0	1022
		B137	3	255.5	3.0	795
		Y30	1	196.4	6.0	1192
2	1	B82	4	274.6	6.0	1646
		B196	2	217.3	CON	0
		B138	3	269.1	3.0	795
		B140	3	242.7	4.0	965
		B152	3	240.0	5.0	1192
		B102	4	267.3	3.0	795
		Y33	1	221.4	5.0	1136
	2	Y40	1	225.5	4.0	908
		B186	2	233.64	CON	0
		Y36	1	190.9	4.0	795
		B195	2	242.7	5.0	1249
		Y14	1	203.6	4.0	852
		Y34	1	165.4	CON	0
		Y31	1	196.4	3.0	625
	3	Y46	1	213.2	CON	0
		Y42	1	207.7	5.0	1079
		B194	2	210.0	3.0	625
		Y38	1	186.4	4.0	738
		B188	2	239.1	3.0	738
		B197	2	234.6	3.0	738
	4[c]	B184	2	271.8.	CON	0
		B159	3	282.7	4.0	1135
		B108	4	325.5	CON	0
		B187	2	246.4	3.0	738
		B174	2	243.6	CON	0
		Y22	1	198.2	4.0	795

[a]Sow post-farrowing weights were used to calculate treatment doses

[b]Sow treatments – 3.0, 4.0, 5.0 or 6.0 mg/kg PO firocoxib and CON (60 mL Gatorade)

[c]Cohort 2 Group 4 –all male piglet replacement litter following the removal of male piglets from Cohort 1 Group 1

Table 3.2. **Ethogram used to score piglet behavior grouped into the following categories: posture, nursing, locomotion, pain-specific, and social cohesion. (Modified from Viscardi et al., 2020 and Lou et al., 2022)**

Behavior	Description
Posture	
Lying	Body weight supported by side or belly; all four limbs in non-direct contact with floor
Sitting	Body weight supported by hindquarters and front legs
Standing	Body weight supported by four legs; all four limbs in direct contact with floor
Nursing	
Nursing	Piglet snout is in contact with the udder; nosing or suckling teat
Locomotion	
Walking	Moving forward or backward at a normal pace
Sleeping	Lying down motionless with eyes closed for at least 30 seconds
Nosing	Snout in contact with a substrate, pen surface, or littermate
Playing	Energetic interaction (springing, bouncy, twirling movements) alone or with littermates
Agonistic	Biting or fighting other littermates
Pain-specific	
Prostration	Sitting or standing with head lower than shoulder level
Trembling	Body shivering or shaking
Spasms	Quick and involuntary contractions of the muscles (typically involving the whole body)
Scratching	Rubbing the rump against the floor, pen walls, or littermates
Itching	Rubbing body against pen and/or with leg
Tail wagging	Tail movement from side to side (or up and down)
Tail jamming	Tucking the tail between the hind legs
Social cohesion	
Isolated	Alone, or with one littermate, distance of 40 cm separates the piglet(s) from the closest group of littermates. Piglet(s) may be lying, sitting, or standing alone.
Desynchronization	Activity different from that of most littermates (at least 75% of littermates)

Table 3.3. Least square means (± SE) of outcome measures form the pressure mat gait analysis.

	Treatment[1]					P-Value		
	FIRO6	**FIRO5**	**FIRO4**	**FIRO3**	**CON**	**Tx**	**Time**	**Tx x Time**
Parameter								
Front limbs								
Stance time (s)	0.57 ± 0.02	0.60 ± 0.03	0.54 ± 0.03	0.55 ± 0.05	0.58 ± 0.03	0.60	0.62	0.04[2]
Stride length (cm)	17.23 ± 0.30	16.37 ± 0.42.	16.88 ± 0.45.	18.00 ± 0.82	16.75 ± 0.41	0.34	0.00014	0.88
Peak vertical force (kg)	2.13 ± 0.14	2.03 ± 0.20	1.72 ± 0.21	1.83 ± 0.36	2.11 ± 0.18	0.54	< 0.0001	0.0024[2]
Peak vertical impulse (kg x s)	0.81 ± 0.07	0.82 ± 0.09	0.63 ± 0.10	0.69 ± 0.16	0.82 ± 0.08	0.53	< 0.0001	0.16
Peak contact pressure (kg/cm^2)	1.62 ± 0.08	1.64 ± 0.12	1.32 ± 0.12	1.37 ± 0.21	1.49 ± 0.10	0.28	< 0.0001	0.04[2]
Hind limbs								
Stance time (s)	0.38 ± 0.01	0.40 ± 0.02	0.37 ± 0.02	0.41 ± 0.04	0.41 ± 0.02	0.62	0.09	0.05[2]
Stride length (cm)	17.00 ± 0.40	16.41 ± 0.56	17.06 ± 0.59	18.02 ± 1.06	16.69 ± 0.53	0.70	0.02	0.90
Peak vertical force (kg)	1.03 ± 0.08	1.00 ± 0.12	0.81 ± 0.12	0.85 ± 0.20	1.00 ± 0.10	0.61	< 0.0001	0.004[2]
Peak vertical impulse (kg x s)	0.28 ± 0.04	0.37 ± 0.05	0.24 ± 0.06	0.28 ± 0.12	0.32 ± 0.06	0.53	0.47	0.73
Peak contact pressure (kg/cm^2)	1.30 ± 0.09	1.26 ± 0.13	1.05 ± 0.13	1.07 ± 0.23	1.19 ± 0.11	0.58	< 0.0001	0.01[2]
Gait distance (cm)	39.09 ± 1.37	42.09 ± 1.90	40.58 ± 2.07	38.66 ± 3.77	38.95 ± 1.90	0.69	0.29	0.04[2]
Gait velocity (cm/sec)	20.87 ± 0.55	19.21 ± 0.76	21.08 ± 0.85	22.30 ± 1.57	20.58 ± 0.79	0.32	0.09	0.61

[1]FIRO6 = 6.0 mg/kg oral firocoxib; FIRO5 = 5.0 mg/kg oral firocoxib; FIRO4 = 4.0 mg/kg oral firocoxib; FIRO3 = 3.0 mg/kg oral firocoxib; CON = placebo.
[2]Not significant after Tukey-Kramer Adjustment.
[a,b] Values within a row with different superscripts differ significantly ($P \leq 0.05$).

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Chapter 4 - Evaluating the utility of firocoxib and bupivacaine liposome injectable suspension to reduce pain associated with surgical castration and tail docking in piglets using a CO_2 surgical laser: A pilot study

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Abstract

The use of a CO_2 surgical laser for soft tissue surgeries in human and veterinary medicine is widely accepted given its benefits over a traditional scalpel blade, including reduced bleeding, inflammation, and post-operative pain. Refinement of common techniques to surgically castrate and tail dock piglets is warranted to improve their welfare on-farm. Therefore, this pilot study had two objectives. First, to evaluate the utility of a CO_2 surgical laser to reduce pain and improve wound healing in piglets after tail docking and surgical castration in comparison to standard side pliers and a scalpel, respectively. Second, to determine the efficacy of firocoxib (oral or transmammary) in combination with bupivacaine liposomal injectable suspension to relieve pain associated with tail docking and surgical castration. A total of 3 sows and 30 piglets (10 piglets/litter/sow; 3 ± 1 day old) were enrolled in this study. Sows were randomly allocated into one of two treatment groups: 1) oral firocoxib (FIRO) or oral placebo (CON). Each piglet within a litter was also provided a treatment, based on the sow's treatment group: 1) transmammary (CON sow) + oral firocoxib (3.0 mg/kg) + bupivacaine liposome suspension (FIROPIG), 2) transmammary firocoxib (FIRO sow) + oral placebo (1 mL Gatorade) + bupivacaine liposome suspension (FIROSOW) or 3) transmammary placebo (CON sow) + oral placebo (1 mL Gatorade) + bupivacaine liposome suspension (PLBO). Lastly, piglets (n = 30) were randomly allocated to one of three processing treatments: 1) laser, 2) conventional (scalpel and side pliers), and 3) sham. During drug administration and processing, piglet vocalizations were recorded to quantify the peak frequency, peak amplitude, and energy. Digital and infrared thermography images of the tail and scrotum were taken at baseline and up to 7 days post-processing, for wound healing and inflammation assessment, respectively. Piglet facial images for facial grimace assessment were taken in conjunction with pressure mat gait analysis at baseline and up to 48h post-processing.

Blood cortisol and drug concentrations were analyzed from piglet blood samples collected at baseline, 1, and 24h post-processing. Piglets in all processed treatment groups elicited calls of higher energy compared to the sham group ($P < 0.05$). Males produced calls of significantly higher energy and peak amplitude compared to females ($P < 0.0001$). Immediately post-procedure, conventionally tail docked piglets had higher wound scores than laser tail docked piglets ($P < 0.0001$); however, laser tail docked piglets had higher tail tip temperatures compared to piglets who had conventionally docked tails ($P < 0.0001$). Weight distribution onto the front and hind limbs was significantly higher for FIROPIG piglets compared to FIROSOW and PLBO piglets ($P < 0.05$). Piglets in the FIROPIG group grimaced more than piglets in the FIROSOW and PLBO groups ($P = 0.0002$). Piglets in the FIROPIG group also had greater firocoxib concentrations and lower cortisol concentrations than piglets in the FIROSOW group ($P < 0.05$). Surgical castration and tail docking with a laser led to no significant differences for wound healing nor inflammation. The multimodal approach of firocoxib and bupivacaine liposomal injectable suspension was effective in reducing cortisol levels and shifts in weight distribution across front and hind limbs associated with tail docking and surgical castration. Oral firocoxib may provide additional benefit to piglets over transmammary with a greater reduction in piglet responses associated with stress and pain.

Introduction

Tail docking and castration are standard agricultural practices performed on millions of piglets each year on commercial production farms to reduce the risk of tail biting and uphold high-quality pork products (Prunier et al., 2020). These painful procedures are commonly performed with side pliers and a scalpel, with no analgesia provision for pain control. Excessive bleeding, edema, intestinal herniation, neuromas, and increased risk of infection and mortality are some

complications that can result from piglet tail docking and castration (Schmid and Steinhoff-Wagner, 2022). However, given the low cost and practicality of these conventional tools for large-scale farms these procedures remain unchanged. In addition, the lack of approved drugs by the U.S. Food and Drug Administration (FDA) for pain control in swine greatly limits the implementation of providing pain relief protocols for piglet processing procedures. The American Veterinary Medical Association (AVMA) and the American Association of Swine Veterinarians (AASV) are aligned in their goal to refine piglet processing procedures and have efforts geared towards evaluating alternative techniques and analgesic protocols that reduce pain and improve piglet welfare outcomes (American Veterinary Medical Association, 2014).

The use of a CO_2 surgical laser for piglet tail docking (Lou et al., 2022) and castration (Viscardi et al., 2020) has been explored, given its advantages over a standard scalpel blade reported in both the human and veterinary medical fields. In comparison to a scalpel blade, the precise incisions made by a CO_2 surgical laser led to minimal tissue damage, bleeding, inflammation, and overall post-operative pain in dogs (Carreira and Azevedo, 2016). These advantages are due to the surgical laser's production of a heated light energy at a specific wavelength that is readily absorbed by intracellular water, allowing the ablation of tissue cells, blood and lymphatic vessels, and potentially harmful microorganisms (Gans, 2007). Previous studies suggest that the laser technique for piglet tail docking and castration needs improvement, such as better piglet restraint during the procedure and analgesia provision for post-procedural pain management, to optimize the potential welfare benefits (Viscardi et al., 2020; Lou et al., 2022).

The Animal Medicinal Drug Use Clarification Act (AMDUCA) permits veterinarians to administer analgesics to swine following strict extra-label guidelines (US Food and Drug Administration, 1994). Firocoxib is an FDA-approved non-steroidal anti-inflammatory drug

(NSAID) for dogs (Previcox®) and horses (Equioxx®) with osteoarthritis-induced pain and inflammation (Papich, 2021). As a highly-selective COX-2 NSAID, firocoxib has less of an impact on gastroprotective mechanisms. In piglets, firocoxib has been shown to reduce pain-related outcomes after tail docking and castration (Coetzee, 2019). Bupivacaine liposome injectable suspension (NOCITA®) is also approved by the FDA for pain control in dogs and cats, but it has yet to be evaluated for piglet processing (Lascelles and Kirkby Shaw, 2016). Unlike other lidocaine and bupivacaine injectable formulations, the extended-release formulation of bupivacaine liposome injectable suspension is indicated for pain relief for up to 72h. In calves, bupivacaine liposome injectable suspension presented the same efficacy in controlling pain as the multimodal approach of lidocaine and meloxicam at the time of castration (Martin et al., 2022).

This pilot study had two main objectives. First, to the evaluate the utility of a CO_2 surgical laser to reduce pain and improve wound healing for piglets after tail docking and surgical castration in comparison to cutting pliers and a scalpel blade, respectively. Second, to determine the efficacy of firocoxib (transmammary or oral) in combination with bupivacaine liposomal injectable suspension to relieve pain associated with surgical castration and tail docking in piglets. We hypothesized that piglets processed using a CO_2 surgical laser and administered firocoxib (either orally or through transmammary delivery) and bupivacaine liposomal injectable suspension would lead to a significant reduction in pain and improved wound healing compared to piglets only given a local block (bupivacaine liposomal injectable suspension) and processed using cutting pliers and a scalpel blade.

Materials and Methods

All animal use and procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC protocol #4860) and Midwest Veterinary Service (protocol #MCL-23017) prior to study commencement.

Animals and housing

A total of 3 sows (Yorkshire x Landrace) (mean parity 8 ± 3.61) nursing 12 (n = 6 male and n = 6 female) piglets were sourced from Wendt Farms in Leigh, NE (**Table 4.1**). A veterinarian selected the sows after reviewing their health and performing a physical examination. The selected sows were determined to be non-aggressive and in good physical health with a body condition score of 3 ± 0.5 out of 5. For identification, sows were given ear tags. Piglets (3 ± 1-day old, mean BW 1.9 ± 0.35kg) enrolled in the study had a minimum body weight of 1.5 kg, had a normal developed tail (both males and females), and two descended testicles (males). An additional 2 piglets (1 male and 1 female) were enrolled in each litter and deemed as "extra" in case an alternate was needed before the start of the study to ensure appropriate study power.

Three days prior to study commencement sows and piglets were transported a distance of 27 mi from the source farm to the study site. The animals were transported using a livestock trailer fashioned with partitions to separate the sows and their piglets. Upon arrival at the study site, sows and piglets were weighed (sow mean BW = 247.3 ± 16.7 kg; piglet mean BW 1.9 ± 0.35 kg) for firocoxib dosage calculation. Both sows and piglets received enrofloxacin (Baytril 100, Elanco, Shawnee, KS) at 20.0 mL IM and 0.3 mL IM, respectively. Piglets also received a 1.0 mL IM iron injection (uniferon 200, Pharmacosmos, Inc., Watchung, NJ).

Each sow along with her piglets were housed in stainless steel farrowing crates (152.4 cm x 213.4 cm) equipped with a heat mat and water nipple providing ab libitum water access. Sows

were fed a diet that met or exceeded the National Research Council nutrient requirements for lactating sows (National Research Council, 2012). Timers within the farrowing barn were set to provide all animals with 12h light: 12h dark cycle.

Experimental Design

Sows (n = 3) were randomly assigned to one of two treatment groups: 1) oral firocoxib (FIRO; 3.0 mg/kg; n = 1) or oral placebo (CON; 60 mL Grape Gatorade, PepsiCo, Chicago, IL; n = 2 sows) (**Figure 4.1**). Sow treatments were administered 12h before piglet processing. Firocoxib (Equioxx, 57mg and Previcox 227mg, Boehringer Ingelheim, Duluth, GA) tablets were used, and each dose was rounded to the nearest whole capsule. The tablets were then ground into a powder using a pill crusher (Easy-Grip Pill Crusher, Equate, Bentonville, AR) and mixed with grape flavored Gatorade inside a 30cc drench tip syringe (Nylon Syringe, Neogen Corporation, Lexington, KY). Once a uniform suspension was present, the treatment was administered orally to the sow pen-side, without restraint. The same treatment administration procedures were used for sows in the control group, except only Gatorade was administered. All sow treatments were administered by the same trained veterinarian using a new syringe for each sow to prevent drug cross-contamination.

The day prior to study commencement, piglets were randomly assigned an identification number (1-10) and a litter letter (A-C). With a black permanent maker, their identification number and litter letter were written on their back and right hind leg, respectively. In addition to enrollment weight collection, piglet body weights were collected 24h pre-dosing to ensure a more accurate treatment dose calculation.

Each piglet litter (n = 3 litters, 10 piglets/litter) was assigned one of three treatments depending on which random treatment the sow received. Piglet treatment groups were as follows:

1) transmammary placebo + oral firocoxib (3.0 mg/kg) + bupivacaine liposome suspension (FIROPIG), 2) transmammary firocoxib + oral placebo (1 mL Gatorade) + bupivacaine liposome suspension (FIROSOW) or 3) transmammary placebo + oral placebo (1 mL Gatorade) + bupivacaine liposome suspension (PLBO). A uniform suspension of 10 mg/mL firocoxib-dosed Gatorade was made, to facilitate administration to piglets. A nasal cannula (Zoetis, Parsippany-Troy Hills, NJ) attached to a luer-lock syringe (1 mL) was used to administer the treatments orally to piglets to reduce drug loss. Piglets in the placebo groups underwent the same treatment administration process, except only Gatorade was administered.

All piglets (n = 30) received a local block of bupivacaine liposome suspension (BUP; Nocita 13.3 mg/mL, Elanco, Greenfield, IN) as a subcutaneous injection using an 18 G, 0.625-0.5" needle to improve the humane aspects of the procedure. For male and female piglets, 0.5 mL BUP was injected as a ring block at the base of the tail. For male piglets, 1.0 mL BUP was also injected inter-testicular (into the spermatic cord) and subcutaneously into the scrotum. All piglet treatments were administered 30 min before piglets were processed (surgically castrated and tail docked) by the same trained veterinarian, with the oral dose administered first and local block second.

Prior to processing, piglets within each litter were randomly allocated into one of three processing options: 1) laser (n = 2 male, n = 2 female), 2) conventional (scalpel and side pliers) (n = 2 male, n = 2 female), and 3) sham (n = 1 male, n = 1 female). All piglets were processed by the same trained veterinarian between 0700-0900 h. The CO_2 surgical laser (VetScalpel Aesculight, LLC, Bothell, WA) was calibrated before each litter was processed to ensure proper functionality (mean calibration yielded 65.7 ± 2.4%). For tail docking and surgical castration, the laser was set to 30 W continuous (actual laser power = 20 W) and 15 W continuous (actual laser power = 10 W), respectively.

Approximately 5 mins. before processing, both the tail and scrotum of piglets were disinfected using a non-alcoholic based disinfectant (Chlorhexidine 4%, Vet One, Boise, ID), allotting time for the disinfectant to fully dry. Then, each piglet was placed into a castration bench (The Schippers Group, Hapert, Netherlands) to restrict piglet movement for both the safety of the piglet and veterinarian operating the CO_2 surgical laser. For tail docking, ~1/2 of the tail was removed by the side pliers or CO_2 surgical laser. For surgical castration, a single horizontal incision was made by a scalpel or CO_2 surgical laser over the scrotum. The testes were removed by cutting or ablating the spermatic cord using the scalpel or CO_2 surgical laser, respectively. Piglets in the sham group underwent the same handling procedures as piglets actually being processed, except no cutting or incisions were made.

Data Collection

Animal-based outcomes evaluated in this study were the following: body weight, vocalizations, wound healing, inflammation, pressure mat gait analysis, facial grimace, and blood plasma cortisol and firocoxib concentrations.

Body weight

Piglet birth weights were collected at enrollment to confirm enrollment eligibility based on the study inclusion criteria. The day prior to processing, piglet body weights were collected a second time to ensure an accurate treatment dosage calculation. Lastly, piglet weaning weights were collected on Day 21. All piglet weights were collected by placing each piglet individually inside a plastic tote onto a scale. The empty tote was used to tare the scale in between each piglet. Piglet weaning weight was subtracted from weight at enrollment to determine how much weight was gained throughout the study period.

Vocalizations

Piglet vocalizations were recorded using a high-definition video camera (Sony Handycam HDR-CX405, Sony USA, Inc., New York, NY) attached to a tripod. The camera was positioned in front of the individual restraining the piglet, close to the piglet's face. Vocalizations were recorded at two time periods: during drug administration and during processing (surgical castration, tail docking, or sham). Piglet vocalizations were analyzed based on peak frequency (Hz), peak amplitude (U), and energy (dB) using the Raven Pro: Interactive Sound Analysis Software (Version 1.6, Cornell Lab of Ornithology, Ithaca, NY).

Wound healing

Tail and scrotal images of each piglet were taken to assess wound healing of the surgical sites using a high-definition digital point-and-shoot camera (Kodak Pixpro AZ528, JK Imaging, Gardena, CA). Images were taken at baseline and at, 0, 8, 24, 48, 72, 96, 120, 144, and 168h post-processing. In total, 450 images were collected. Three individuals scored images using a wound scoring system for tail docking (Score 0-5) (**Table 4.2**) and castration (Score 1-6) (**Table 4.3**) adapted from Lou et al. (2022) and Sutherland et al. (2009), respectively. All individuals were blinded to treatment and time point. Inter-rater reliability was assessed among the scores of all raters.

Infrared thermography

A research-grade infrared camera (FLUKE TiX580; FLUKE Corporation, Everett, WA) was used to take infrared thermography (IRT) images of the tail docking and castration sites to determine cutaneous temperature and, by extension, the degree of inflammation. At the start of each image collection day, the IRT camera was manually calibrated to account for the ambient temperature and relative humidity of the farrowing barn. Images were taken at baseline and at 0,

1, 8, 24, and 48h post-processing. A distance of ~0.5 m was maintained between the IRT camera and the piglet to ensure consistent image collection.

The IRT images were analyzed using a research-grade software (SmartView 4.3; FLUKE Corporation, Everett, WA). To assess the temperatures of the tail docking site, a line was drawn directly over the tip of the tail and middle of the tail. To assess the temperatures of the castration site, a circle was drawn around the incision and a line drawn on the left and right side of the incision. Further analysis was performed where the temperature of the surrounding tissues for both the tail docking and castration sites were subtracted from the temperature of the tail tip and scrotal incision, respectively, to evaluate the difference in temperature between the main surgical sites and surrounding tissue.

Pressure mat gait analysis

A commercially available floormat-based pressure mat system (Strideway, Tekscan, Inc., South Boston, MA) was used to quantify piglet gait and analyze the following parameters: stance time (sec), stride length (cm), peak vertical force (kg), peak vertical impulse (kg*sec), peak contact pressure (kg/cm^2), gait distance (cm), and gait velocity (cm/sec). The pressure mat data was analyzed using a research grade software (Strideway 7.80, Tekscan, Inc., South Boston, MA).

At the start of each study day, the mat was calibrated using certified weights of a known mass to ensure accurate measurements. Gait parameters were collected at baseline and at 0, 1, 8, 24, and 48h post processing. Each piglet was given a total of three attempts to complete an acceptable walk across the pressure mat to obtain quality data. An acceptable walk consisted of three continuous, full strides at a normal pace with no running, stops, slips, or falls. To ensure consistent gait, a laptop (ThinkPad W541, Lenovo, Morrisville, NC) and web camera (Logitech HD 720P, Suzhou, China) were connected and synced to the pressure mat. The webcam was

positioned at the end of the pressure mat walkway with the piglet in view and would record as piglets walked across the pressure mat.

Piglet Grimace Scale (PGS) recording and scoring

Facial images from each piglet were collected in conjunction with the pressure mat analysis. The same web camera that was connected and synced to the pressure mat was also used to collect piglet facial images. From the videos of piglets walking across the pressure mat, piglet facial images were extracted at the same time points as the pressure mat analysis (baseline, and 0, 1, 8, 24, and 48h post processing) using VLC Media Player (Version 3.0.20, VideoLAN, Paris, France). The single best image per piglet per time point was selected based on optimal lighting and angle, with the piglet fully forward-facing. A total of 150 images were collected.

The Piglet Grimace Scale (PGS) (**Figure 4.2**), revised by Lou et al. (2022) was utilized by three individuals, blinded to piglet treatment and time point, to score the images. The PGS consists of scoring three facial action units (FAUs): orbital tightening, ear position, and nose bulge/cheek tension. Each FAU was scored on a 3-point scale: 0 (Not Present), 1 (Moderately Present), and 2 (Obviously Present). Prior to scoring, all raters were required to attend two group training sessions (~30 min each) to learn how to use the PGS scoring guide and practice scoring images. Inter-rater reliability was assessed among the scores of all raters.

Blood cortisol analysis

Blood from each piglet was collected at baseline and at 1 and 24h post-processing. Blood draws were performed by a trained individual experienced in blood collection in neonatal pigs. To collect blood, piglets were restrained in the supine position and samples (2 mL of whole blood/piglet) were collected from the jugular vein using a 20-gauge x 1-inch multiple use drawing needle (VACUETTE, Greiner Bio-One, Monroe, NC) and a 3 mL vacutainer serum separator tube

(BD Vacutainer Lithium Heparin, Becton, Dickinson, and Company, Franklin Lakes, NJ). Blood sample tubes were placed immediately on ice after collection. The samples were then centrifuged at 3000 x g for 10 min and serum was pipetted into cryovials. Blood samples were stored at -80℃ prior to analysis. All blood samples were analyzed by laboratory personnel at Kansas State University blinded to treatment, procedure, and time point.

Blood serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals cat. no. 07-221106-R) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. A 500 ng/mL standard was created by diluting the supplied 1,000 ng/mL kit standard. The standard curve ranged from 1 to 1,000 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were run at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a PerkinElmer Wizard2 gamma counter for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation over 18% were re-analyzed.

Blood firocoxib analysis

The analytical standards, firocoxib and firocoxib-d6, were purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and Alfa Chemistry (Holbrook, NY, USA), respectively. Optima LC-MS grade methanol, acetonitrile, formic acid, ammonium hydroxide,

ammonium formate, water, and HPLC grade phosphoric acid were purchased from Fisher Chemical (Ottawa, Ontario, CA). Untreated, blank porcine plasma in K_2EDTA was supplied by Lampire Biological Laboratories (Pipersville, PA, USA). Fisher Scientific (Pittsburg, PA, USA) provided 96-well non-tissue culture treated plates. The Oasis PRiME HLB 96-well µElution plates were supplied by Waters Corporation (Milford, MA, USA).

Stock standard solutions of firocoxib (FRC) and firocoxib-d6 -internal standard (IS)- were prepared independently at 1.00 mg/mL in methanol and stored at -20°C. Serial dilutions of the FRC stock preparation were performed with aqueous phosphoric acid (4%, v/v) to establish daily working standard solution sets at the following concentrations: 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0, 1.00E+02, 2.50E+02, 5.00E+02, 1.00E+03, 1.50E+03, and 2.00E+03 ng/mL. The IS stock preparation was attenuated with aqueous phosphoric acid (4%, v/v) to yield daily working standard solutions at 1.00E+02 ng/mL.

Samples (300.0 µL) were prepared in 96-well preparatory plates. To compose daily calibrator and process quality control (QC) samples, the FRC and IS working standard solutions (100.0 µL each) were mixed with untreated, blank porcine plasma (100.0 µL). The daily calibrator samples were equal in concentration to the FRC and IS working standard solutions. The daily process QC samples were arranged at 5.00, 10.0, 1.00E+02, 5.00E+02, and 1.00E+03 ng/mL of FRC with 1.00E+02 ng/mL of IS. The porcine plasma samples - 4% v/v aqueous phosphoric acid (200.0 µL; 1:1, v/v) were spiked with the IS working standard solution (100.0 µL) for analytical sampling. Insoluble matrix particulates were removed by centrifugation at 1300 g for 20 mins at 20°C with Thermo Scientific's Sorvall ST 16R Centrifuge (Waltham, MA, USA) to prevent solid phase extraction (SPE) sorbent obstructions. To execute reversed-phase extraction via positive pressure, the supernatants were processed through Oasis PRiME HLB µElution plates (Waters

Corporation). Elimination of hydrophilic matrix interferences was completed with a 300.0 µL water - methanol (90:10, v/v) wash. A 50.00 µL introduction of acetonitrile - methanol (90:10, v/v) to induce eluate extraction disrupted the nonpolar van der Waals and pi-pi interactions between the copolymer sorbent's lipophilic divinylbenzene moiety and FRC's cyclopropyl and aryl group. The clarified samples were acidified with 50.00 µL of 2 mM ammonium formate in 0.1% v/v aqueous formic acid -prepared at pH 4 with ammonium hydroxide-.

FRC separation, detection, and quantitation were fulfilled on a Waters Acquity Ultra Performance Liquid Chromatography H-class PLUS-tandem Xevo TQ-S Mass Spectrometry unit (UPLC-MS/MS) and instrumentation was facilitated through MassLynx software. Samples, housed in the 5°C autosampler, were injected (2.00 µL) onto an Acquity UPLC C18 HSS T3 column -100 Å, 1.8 µm, 2.1 x 50 mm- at 40°C; an Acquity UPLC VanGuard precolumn -100 Å, 1.8 µm, 2.1 x 5 mm- of identical chemistry and particle technology was installed for column longevity. Directed at a 0.400 mL/min flow rate, the binary mobile phase was comprised of 2 mM ammonium formate in 0.1% v/v aqueous formic acid at pH 4 (A) and 100% acetonitrile (B). Chromatographic separation with a 7.00 min gradient elution program commenced at 5% B and transitioned to 95% B by 4.00 mins. The solvent system constitution was preserved for an additional minute, adjusted at 5.01 mins to 5% B, and maintained until the termination of the re-equilibration period. The mass spectrometer parameters were adopted from an established protocol (Kleinhenz et al., 2020) and optimized to achieve maximum sensitivity. Electrospray ionization in the positive polarity mode (ESI+) instigated solvent oxidation and analyte charging with a 3.60 kV capillary voltage and a 150°C source temperature. The pneumatically nebulized species were subjected to solvent disintegration, Coulombic fission, and ion evaporation with a desolvation gas (nitrogen) flow rate and temperature of 1000 L/hr and 600°C, respectively. The cone gas (nitrogen)

flow rate was set at 150 L/hr to support optimal ion transmission and the minimization of solvent ion cluster formations. Thereafter, the charged gaseous species were fragmented by collision-induced dissociation (CID) with a 0.15 mL/min collision gas (argon) flow rate to conduct multiple reaction monitoring (MRM) detection within the triple quadruple mass spectrometer. Specifically, delivered through the mass analyzer with 50 V of cone energy, firocoxib's parent ion, m/z 337.10, generated daughter ions of m/z 282.96 (quantifier; collision energy at 10 V) and m/z 129.81 (confirmer; collision energy at 28 V). Likewise, with a cone energy of 34 V, firocoxib-d6's parent ion, m/z 343.16, generated daughter ions of m/z 289.03 (quantifier; collision energy at 8 V) and m/z 135.74 (confirmer; collision energy at 28 V). The assay concluded with quantitation through TargetLynx software.

The UPLC-MS/MS method development for the quantitation of FRC in porcine plasma was congruent with the FDA's 2018 *Bioanalytical Method Validation Guidance for Industry* report (U.S. Food and Drug Administration, 2018). The method produced linear, log-log calibration curves from 1.00 to 2.00E+03 ng/mL of FRC with coefficients of determination (R^2) greater than 0.999. An extrapolation of the calibration curves demonstrated that the limit of detection (LOD) was 0.124 ng/mL (S/N = 3). An analysis of process QC reproducibility indicated that the method's limit of quantitation (LOQ) was 5.00 ng/mL. An evaluation of the process QC set triplicates at 5.00, 10.0, 1.00E+02, 5.00E+02, and 1.00E+03 ng/mL of FRC revealed throughout method validation the following average percent recoveries: 105, 98.4, 98.8, 106, and 108 %, respectively. An intra-assay precision analysis of process QC set triplicates established the following percent relative standard deviations (% RSD): 5.64, 8.36, 8.12, 5.32, and 2.81%, respectively. An inter-assay precision analysis of the process QC set triplicates for three separate validation runs demonstrated the following % RSDs: 6.08, 6.40, 6.47, 5.85, and 6.30 %, respectively.

Statistical Analysis

All data were analyzed using SAS (Statistical Analysis System 9.4, SAS Institute Inc., NC). Statistical significance was set at $P \leq 0.05$. A mixed model was used to analyze the following outcomes: body weight, vocalizations, wound scores, IRT temperatures, gait, facial grimace scores, and blood cortisol and firocoxib concentrations. Prior to analysis, cortisol values were log-transformed for normality.

In the mixed model, piglet, pen, and time were set as the experimental unit, random effect, and repeated measure, respectively. Treatment, procedure, time, sex, and the following interactions were included in the model: time x treatment, time x procedure, and treatment x procedure. The Tukey-Kramer adjustment was used to conduct post hoc tests. Statistical significance was set at $P \leq 0.05$ with $P \geq 0.05$ and ≤ 0.10 considered a trend toward significance.

Results

Body weight

There were no differences found for piglet weight gain based on treatment, procedure, or time ($P > 0.05$ for all).

Vocalizations

For vocal energy and peak amplitude there were differences based on procedure ($P < 0.05$ for both). Piglets in all processed treatment groups elicited calls of higher energy compared to piglets in the sham group ($P < 0.05$). For peak amplitude, female tail docked piglets produced calls of higher peak amplitude than piglets in the sham group ($P < 0.05$). In conventionally processed piglets, females produced calls of higher peak amplitude than males ($P = 0.05$).

Time differences in vocalization energy, peak amplitude, and peak frequency were also found ($P < 0.05$ for all). Piglets produced calls of increased energy, peak amplitude, and peak

frequency when treatments were being administered compared to at the time of processing ($P < 0.05$). A sex x time interaction for vocalization energy and peak amplitude were also found ($P < 0.05$), with males producing calls of significantly higher energy and peak amplitude compared to females at the time of processing.

Wound healing

The ICCs for inter-rater reliability among the three raters were 0.85 and 0.83 for tail and scrotum wound scores, respectively, indicating good reliability. In cases where two or more raters could not score an image, that image was removed from the data set for the final analysis (< 1%).

For tail docking wound scores, there were procedure, time, and procedure x time differences ($P < 0.0001$ for all) (**Figure 4.3**). Tail docked piglets (laser: 2.56 ± 0.05 and conventional: 2.39 ± 0.05) had higher wound scores than sham piglets (0.005 ± 0.07) across the entirety of the study ($P < 0.0001$). With respect to time, tail wound scores were lowest at baseline compared to all other time points ($P < 0.0001$). Tail wound scores were highest at 0 and 8h post-processing compared to all other time points (24, 48, 72, 96, 120, 144, and 168h), with no differences between 0 and 8h ($P < 0.05$). Tail wound scores were also higher at 24 and 48h post-processing compared to 144 and 168h, with no differences between 24 and 48h ($P < 0.05$). At 0h post-processing, conventionally tail docked piglets had higher wound scores than laser tail docked piglets ($P < 0.0001$). Lastly, at 72 and 168h post-processing, laser tail docked piglets had higher wound scores than conventionally tail docked piglets ($P < 0.05$).

For castration wound scores, there were procedure, time, and procedure x time differences ($P < 0.0001$ for all) (**Figure 4.4**). Castrated piglets (laser: 3.29 ± 0.09 and conventional: 3.18 ± 0.09) had higher wound scores than sham piglets (0.97 ± 0.13; $P < 0.0001$). With respect to time, scrotum wound scores were lowest at baseline compared to all other time points ($P < 0.0001$).

Scrotum wound scores were highest at 0h post-processing compared to 24, 48, 72, 96, 120, 144, and 168h ($P < 0.05$). Additionally, scrotum wound scores were higher at 8h post-processing compared to 72, 96, 144, and 168h ($P < 0.05$). Finally, from 0-120h, castrated piglets (conventional and laser) had higher wound scores than sham piglets ($P < 0.05$). At 144h, laser castrated piglets had higher wound scores than sham piglets ($P = 0.05$).

There were no significant treatment differences in wound healing found in this study.

Infrared thermography

For the tail docked piglets, there were procedure and time differences ($P < 0.0001$ for both) **(Figure 4.5**). The average temperature of the tail tip was higher for tail docked piglets (laser: 31.9 ± 0.27°C and conventional: 30.9 ± 0.27°C) than sham piglets (29.5 ± 0.40°C; $P < 0.05$). Laser tail docked piglets had higher tail tip temperatures than conventionally tail docked piglets ($P = 0.05$). With respect to time, the average tail tip temperature was higher at 0h compared to all other time points (baseline, and 8, 24, and 48h post-processing) ($P < 0.0001$). The average temperature of the tail tip was lower at 24h post-processing compared to baseline and 8h ($P < 0.05$). At 48h post-processing, the average tail tip temperature was higher than at 8 and 24h ($P < 0.05$).

For the tissue surrounding the tail docking site, herein referred to as tail tissue, were also procedure and time differences ($P < 0.0001$ for both)**.** The average temperature of the tail tissue was higher for tail docked piglets (laser: 33.1 ± 0.22°C and conventional: 32.8 ± 0.22°C) than sham piglets (30.6 ± 0.31°C; $P < 0.05$). Regarding time, the average temperature of the tail tissue was higher at immediately post-tail docking compared to all other time points (baseline, and 8, 24, and 48h post-processing; $P < 0.0001$). As well, the average temperature of the tail tissue was lower at 24h post-processing than at baseline and 8, and 48h ($P < 0.0001$). There were also treatment and treatment x procedure differences found in this study ($P < 0.05$ for both) (**Figure 4.6**). Piglets in

the FIROPIG (32.6 ± 0.24°C) and FIROSOW (32.4 ± 0.24°C) groups had higher average tail tissue temperatures compared to piglets in the PLBO group (31.5 ± 0.24°C; $P < 0.05$). Sham-handled piglets in the PLBO group had significantly lower average tail tissue temperatures than sham-handled piglets in the FIROPIG and FIROSOW groups ($P < 0.05$).

For the difference in temperatures between the tail tip and tail tissue, there were procedure and time differences ($P < 0.05$). The tail tissue of the conventionally processed piglets was 1.79 ± 0.18°C higher in temperature than the tip of the tail, compared to a temperature difference of 1.15 ± 0.18°C in laser-processed piglets ($P = 0.03$). Irrespective of treatment, tail tissue-tail tip temperature differences were higher at 8h post-processing than at baseline and, 0, and 48h ($P < 0.05$).

For the castration site and tissue surrounding the castration site, there were differences based on time only ($P < 0.0001$ for both). The average temperature of the castration site was highest at baseline compared to all post-castration time points ($P < 0.0001$). The average temperature of the castration site and the tissue surrounding the castration site were lowest immediately post-castration compared to all other time points ($P < 0.0001$). The average temperature of the tissue surrounding the castration site was higher at baseline compared to 24 and 48h post-castration ($p < 0.05$).

For the difference in temperatures between the castration site and tissue surrounding the castration site, there were procedure and time differences ($P < 0.0001$). Laser and conventionally processed piglets had a 0.68 ± 0.06°C and 0.59 ± 0.06°C increase in temperature, respectively, of the tissue surrounding the castration site compared with the castration site itself, which was significantly higher than sham piglets (0.19 ± 0.09°C; $P = 0.03$). Irrespective of treatment, scrotum and tissue temperature differences were higher at 8 and 48h post-processing than at baseline ($P <$

0.05). Scrotum and tissue temperature differences were also higher at 8, 24, and 48h post-processing than at immediately post-castration ($P < 0.05$). Furthermore, scrotum and tissue temperature differences were higher at 8h than at 24 and 48h post-procedure ($P < 0.05$).

There were no significant treatment differences in temperature of the tail or scrotum between piglets administered firocoxib (FIROPIG and FIROSOW) and PLBO piglets.

Pressure mat gait analysis

For the pressure mat gait analysis, the mean stance time (sec), stride length (cm), peak vertical force (kg), peak vertical impulse (kg*sec), and peak contact pressure (kg/cm^2) were calculated for each limb individually. Then, the pressure mat gait outcomes for the left and right front limbs and left and right hind limbs were combined to produce averages for the front and hind, respectively.

Stance Time (sec)

There were procedure, procedure x sex, and time differences ($P < 0.05$ for all) (**Table 4.4**) for mean front stance time. Sham (0.46 ± 0.02 sec) and conventionally (0.44 ± 0.02 sec) processed piglets had a longer mean front stance time compared to laser processed piglets (0.39 ± 0.02 sec; $P < 0.05$). Sham-handled female piglets exhibited a longer mean front stance time than laser processed female piglets ($P = 0.0021$). Irrespective of treatment and procedure, piglets had a longer mean front stance time at 24h post-processing than at 8h ($P = 0.009$).

There were no treatment, procedure, or time differences for mean hind stance time.

Stride Length (cm)

There were time and treatment x procedure differences for mean front stride length ($P < 0.05$ for both). Irrespective of treatment and procedure, piglets had a longer mean front stride length at 1, 8, and 48h post-processing than at baseline ($P < 0.05$). However, piglet mean front

stride length was longer at 48h post-processing compared to 1, 8, and 24h ($P < 0.05$). Laser processed piglets in the FIROPIG group had a lengthier mean front stance time compared to laser processed piglets in the FIROSOW group ($P = 0.003$).

There were time differences found for mean hind stride length ($P = 0.0003$). Irrespective of treatment and procedure, piglets had a longer mean hind stride length at 8, 24, and 48h post-processing than at baseline ($P < 0.05$). Mean hind stride length was also longer at 48h post-processing than at 1h ($P = 0.04$). There was a trend toward significance for a treatment x procedure difference ($P = 0.07$), where laser processed piglets in the FIROPIG group tended to have longer mean hind stride length compared to laser processed piglets in the FIROSOW group ($P = 0.03$).

Peak Vertical Force (kg)

There were treatment and time differences for mean front peak vertical force ($P < 0.0001$ for both). Piglets in the FIROPIG group applied more force on their front limbs (4.65 ± 0.15 kg) compared to piglets in the FIROSOW (3.49 ± 0.15 kg) and PLBO (3.79 ± 0.17 kg) groups ($P < 0.05$). Irrespective of treatment and procedure, piglets applied less force on their front limbs at baseline compared to all other time points ($P < 0.05$). Piglets also put more force on their front limbs at 48h post-processing compared to 1h ($P = 0.05$).

There were treatment and treatment x procedure differences for mean hind peak vertical force ($P < 0.05$ for both). Piglets in the FIROPIG group applied more force on their hind limbs (1.87 ± 0.08 kg) compared to piglets in the FIROSOW (1.43 ± 0.08 kg) and PLBO (1.51 ± 0.10 kg) groups ($P < 0.05$). Conventionally processed piglets in the FIROPIG group applied more force on their hind limbs compared to conventionally processed piglets in the FIROSOW group ($P < 0.0001$). Conventionally processed piglets in the FIROPIG group also tended to apply more force on their hind limbs than conventionally processed piglets in the PLBO group ($P = 0.09$).

Peak Vertical Impulse (kg*sec)

There were significant treatment and time differences for mean front peak vertical impulse ($P < 0.05$). Piglets in the FIROPIG group produced a higher impulse on their front limbs (1.40 ± 0.07 kg*sec) than piglets in the FIROWSOW group (1.00 ± 0.07 kg*sec; $P = 0.0002$). Piglets in the FIROPIG group also tended to produce a higher impulse on their front limbs than piglets in the PLBO group ($P = 0.09$). Irrespective of treatment and procedure, piglets produced a higher mean front impulse at 24 and 48h post-processing than at baseline ($P < 0.05$).

There was a trend toward significance for a treatment difference in the mean hind peak vertical impulse ($P = 0.09$), where piglets in the FIROPIG group tended to produce a higher impulse on their hind legs (0.39 ± 0.03 kg*sec) compared to piglets in the FIROSOW group (0.29 ± 0.03 kg*sec; $P = 0.06$). Additionally, there was a trend toward significance for a treatment x procedure difference ($P = 0.06$), where conventionally processed piglets in the FIROPIG group tended to produce a higher impulse on their hind legs compared to conventionally processed piglets in the FIROSOW group ($P = 0.08$).

Peak Contact Pressure (kg/cm^2)

There was a treatment, time, and sex difference for mean front peak contact pressure ($P < 0.05$).

Piglets in the FIROPIG group distributed more contact pressure on their front limbs (3.88 ± 0.13 kg/cm^2) than piglets in the FIROSOW (3.02 ± 0.13 kg/cm^2) and PLBO (3.36 ± 0.14 kg/cm^2) groups ($P < 0.05$). Irrespective of treatment and procedure, mean contact pressure was higher at 8, 24, and 48h post-processing than at baseline ($P < 0.05$). Moreover, female piglets made more contact pressure than males ($P = 0.01$).

There was a treatment x procedure difference for hind peak contact pressure (P = 0.0007); however, this was not significant after the Tukey-Kramer test. Therefore, conventionally processed piglets in the FIROPIG group tended to distribute more contact pressure on their hind legs compared to conventionally processed piglets in the FIROSOW group (P = 0.09).

Gait Distance (cm)

There was a time difference for gait distance (P = 0.05), however; after the Tukey-Kramer test there were no significant differences. There were also treatment x procedure difference for gait distance (P = 0.0003). Gait distance was longer in conventionally processed piglets in the FIROPIG group compared to conventionally processed piglets in the PLBO group (P = 0.03). Additionally, gait distance tended to be longer in conventionally processed piglets in the FIROSOW group compared to conventionally processed piglets in the PLBO group (P = 0.06).

Gait Velocity (cm/sec)

There was a difference in time for gait velocity (P = 0.0001). Piglet gait velocity was faster 8 and 48h post-processing than at baseline (P < 0.05). Gait velocity was also faster at 48h post-processing than at 1 and 24h (P < 0.05).

Piglet Grimace Scale

The ICCs for inter-rater reliability across the three raters were 0.33, 0.45, 0.24, and 0.42 for orbital tightening (OT), ear position (EP), nose bulge/cheek tension (NBCT), and total PGS score, respectively, indicating poor reliability.

There were treatment and treatment x procedure differences for ear position (P < 0.05 for both).

Piglets in the FIROPIG group had higher ear position scores than piglets in the FIROSOW and PLBO groups (P < 0.0001). In addition, conventionally and sham-processed piglets in the

FIROPIG group had higher ear position scores than conventionally and sham-processed piglets in the FIROSOW group ($P < 0.05$). Laser processed piglets in the FIROPIG group also had higher ear position scores than laser processed piglets in the PLBO group ($P = 0.03$).

For total PGS scores, there were treatment and treatment x procedure differences ($P < 0.05$) (**Figure 4.7**). Piglets in the FIROPIG group grimaced more (2.2 ± 0.13) than piglets in the FIROSOW (1.4 ± 0.13) and PLBO (1.5 ± 0.13) groups ($P = 0.0002$). Sham-handled piglets in the FIROPIG group also grimaced more than sham-handled piglets in the FIROSOW and PLBO groups ($P = 0.0054$).

Blood cortisol concentrations

For blood cortisol concentrations, there were treatment, time, and treatment x time differences ($P < 0.05$ for all) (**Figure 4.8**). Piglets in the PLBO group had higher cortisol concentrations (221.58 ± 18.5 ng/ml) than piglets in the FIROPIG (100.96 ± 18.5 ng/ml) and FIROSOW (144.48 ng/ml ± 18.5) groups ($P < 0.05$). Furthermore, piglets in the FIROSOW group had higher cortisol concentrations than piglets in the FIROPIG group ($P = 0.03$).

Cortisol concentrations were highest at baseline compared to 1 and 24h ($P < 0.0001$). Cortisol concentrations were higher at 1h post-processing compared to 24h ($P < 0.0001$). Lastly, at 1h post-processing piglets in the PLBO group had higher cortisol concentrations than piglets in the FIROPIG and FIROSOW groups ($P < 0.05$).

Blood firocoxib concentrations

For blood firocoxib concentrations, there were treatment, time, and treatment x time differences ($P < 0.0001$ for all) (**Figure 4.9**). Piglets in the FIROPIG (622.08 ± 18.6 ng/ml) and FIROSOW (80 ± 18.6 ng/ml) groups had higher firocoxib concentrations than piglets in the PLBO (0 ng/ml) group ($P < 0.05$), with piglets in the FIROPIG group having higher firocoxib

concentrations than piglets in the FIROSOW group ($P < 0.0001$). At baseline, trace levels (i.e., non-quantifiable levels) of firocoxib were found across all piglets, compared to at 1 and 24h ($P < 0.0001$). At 1 and 24h, piglets in the FIROPIG group had higher firocoxib concentrations than piglets in the FIROSOW and PLBO groups ($P < 0.0001$).

Discussion

This study aimed to investigate the multimodal approach of firocoxib and bupivacaine liposomal injectable suspension in reducing pain in piglets after tail docking and surgical castration using a CO_2 surgical laser. Previous work using a CO_2 surgical laser for piglet castration found that laser-castrated piglets displayed more pain-specific and agonistic behaviors than conventionally castrated piglets using a scalpel, which was partly attributed to challenges in immobilizing conscious piglets while the laser was in use (Viscardi et al., 2020). There were also no differences in the amount of pain-specific behavior displayed between laser-tailed docked and conventionally tail-docked piglets using side pliers (Maria E. Lou et al., 2022). Consequently, the authors indicated certain factors, such as the provision of pain control, be considered in future studies using a surgical laser in piglets to potentially improve the outcome of this tool (Viscardi et al., 2020; Maria E. Lou et al., 2022).

A multimodal approach of providing analgesia to help mitigate perioperative and postoperative pain is considered best practice for piglet processing (Baysinger et al., 2021). A pharmacokinetic analysis determined that firocoxib has a longer half-life and volume of distribution in pigs compared to other NSAIDS such as meloxicam, ketoprofen, and flunixin, which may result in longer analgesic effects and wider drug distribution across the body (Coetzee et al., 2019). Successful transmammary delivery of firocoxib to piglets was achieved by administering firocoxib to sows via an intramuscular injection (Coetzee, 2019). However, the

injectable formulation of firocoxib has since been discontinued and the neither the oral nor transmammary routes of administration using the oral formulation of firocoxib had been evaluated for piglet processing pain, prior to this study. To our knowledge, this is also the first study to use bupivacaine liposomal injectable suspension to reduce pain in piglets after tail docking and surgical castration.

Like other commonly used local anesthetics (e.g., lidocaine), bupivacaine blocks the conductance of sodium channels and inhibits the transmission of pain signals. Additionally, bupivacaine liposomal injectable suspension can deliver analgesia for up 72h in both humans and animals (dogs and cats), providing an added benefit during the inflammatory phase of the wound healing period, which lasts approximately 72h (Enomoto et al., 2020).

There was no difference in body weight among piglets based on treatment or procedure over the study period prior to weaning. Similarly, a multimodal approach of lidocaine and meloxicam did not have an effect on piglet growth after castration (Hansson et al., 2011; Kluivers-Poodt et al., 2012;Kluivers-Poodt et al., 2013) nor did a combination of lidocaine, bupivacaine, and meloxicam (Bonastre et al., 2016). In contrast, Coetzee et al. (2019) found that the transmammary delivery of firocoxib (2.0 mg/kg) did result in increased weight gain in tail docked and castrated piglets. Piglet growth rate may not be the best indicator of post-procedural pain in piglets as it can vary widely based on initial birth weight, sow parity, and established teat order. Sows in this study were of older parities (5, 7, and 12) which can be associated with differences in farrowing complications and milk production and composition affecting piglet growth (Tenbergen, Friendship, Cassar, Amezcua, & Haley, 2014).

In this present study, piglet vocalizations were recorded at the time of treatment administration. In humans, bupivacaine (Exparael®) has an onset of action time of ~2-5 min.

Piglets were administered bupivacaine 30 min prior to processing, to maximize pain blocking effects prior to surgical castration and tail docking. Interestingly, piglets produced calls of higher energy, peak frequency, and peak amplitude during treatment administration compared to processing, suggesting the injection of bupivacaine at the base of the tail and into the scrotum caused some degree of distress. It is important to note that the placement of piglets in the castration bench may have also muffled their vocalization. Therefore, it is difficult to attribute the reduced vocalizations at the time of castration and tail docking solely to the effectiveness of bupivacaine.

Piglet vocal responses to tail docking and castration in comparison to handling alone have shown to be clearly distinct, as calls are "more extended and more powerful" with regard to duration, frequency, pitch, and energy during the processing procedures (Marx et al., 2003). Our results support this, as both laser and conventionally tail docked and castrated piglets produced calls of higher energy and peak amplitude than sham-handled piglets. Research has shown that regardless of the tool (clippers vs. cautery clippers) used to tail dock and technique (pulling vs. cutting the spermatic cord) used to castrate, the vocal response does not differ, suggesting that all methods are painful (Marchant-Forde et al., 2009).

Males produced calls of higher energy and peak amplitude than females in this study. Unlike female piglets, male piglets underwent castration, which has shown to be more painful than tail docking in having multiple successive steps causing tissue damage (Schmid and Steinhoff-Wagner, 2022). The time needed to execute the tail docking and castration procedure has been observed to influence piglet responses, with procedures that take the least amount of time and cause minimal tissue damage inducing a lower stress and pain response (Marchant-Forde et al., 2009). It is worth noting to present that, on average, laser tail docking and castration (73.0 sec)

and laser tail docking only (40.1 sec) took longer than conventional tail docking and castration (55.9 sec) and tail docking only (38.6 sec).

A sub-optimal laser technique for piglet castration was reported given difficulty in immobilizing conscious piglets and having the need to make sequential passes, which likely lead to the observed thermal burns (Viscardi et al., 2020). In the present study, piglets were restrained in a castration bench in an effort to minimize body movement, which facilitated more precise incisions to be made and caused no tail or scrotal burns. Nonetheless, both laser and conventionally tail docked and castrated piglets had higher wound scores compared to sham-handled piglets over the entire assessment period. This was not expected as laser and conventional tail docking wounds were not different, indicating both techniques may cause similar degrees of tissue injury. The same results were reported when comparing a CO_2 surgical laser to conventional methods (side pliers and scalpel) for both piglet tail docking (Maria E. Lou et al., 2022) and castration (Viscardi et al., 2020).

Our results are also consistent with others across the assessment period, where wound scores were highest within the first 8h after tail docking and castration compared to all other time points (Viscardi et al., 2020 ;Maria E. Lou et al., 2022). Immediately after tail docking, wounds from laser tail-docked piglets were less severe than piglets tail docked by side pliers; however, there were no other differences observed throughout the study period. Lou et al. (2022) reported the same difference immediately after tail docking using the laser and side pliers with no differences thereafter.

In using a CO2 surgical laser, wounds are immediately cauterized, and blood vessels are sealed reducing bleeding, unlike wounds that are left open when using a conventional scalpel or side pliers. This advantage was observed in dogs having less than 1mL of blood loss after laser

surgery (Patel, Niraj, Patil, D.B., Parikh, P.V., Kelawala, Divyesh, and Sale, 2013). Castration (laser and conventional) wound scores were no longer different from sham-handled piglets by the end of the assessment period (168h), which coincides with results from (Viscardi et al., 2020). This suggests there are no differences in wound healing time for either castration tool. Benefits of using a surgical laser were not observed, despite optimizing the laser technique in using a castration bench and improving piglet immobilization. In human and veterinary medicine, it can be common practice for patients to undergo general anesthesia during a laser procedure, allowing practitioners to fully optimize the technique without uncontrolled movement. However, this is not a safe and viable option for piglets on-farm, who may be at risk of being injured by the sow.

Stress responses mediated by the sympathetic nervous system (SNS) (i.e., systemic and local thermal responses) and inflammation can be non-invasively measured using infrared thermography imaging (Sutherland et al., 2015). A decrease in cutaneous temperature at the site of tissue damage suggests a decreased sympathetic, inflammation, or pain response (Whittaker et al., 2023). As expected with wound scores, both laser and conventionally tail docked piglets had higher temperatures at the tail tip and surrounding tissues compared to sham-handled piglets indicating the presence of inflammation following tissue damage. Laser tail docked piglets had higher temperatures throughout the study period compared to conventionally tail docked piglets, suggesting they experienced more inflammation at the tail docking site. This was also observed in a previous study comparing a CO_2 surgical laser to side pliers for piglet tail docking, but only immediately after the procedure (Lou et al., 2022). Others have also observed that thermal tissue damage from a CO_2 surgical laser resulted in an increased inflammatory response in dogs (Mison et al., 2003), pigs (Cochrane et al., 2005), and rats (Hall, 2005).

While the castration bench used in this study improved piglet restraint during laser castration, it did not provide a similar benefit for the laser tail docking procedure. The operator had difficulty immobilizing the tail, which made the laser harder to control and retain within the targeted region. Evidence suggests that wounds caused by a CO_2 surgical laser heal quicker than traditional surgical tools when operators have better light beam control to make precise incisions causing less thermal damage, inflammation and necrosis around the wound edges (Speyer et al., 1996).

Immediately post-tail docking, irrespective of the tool used, temperatures of both the tail tip and surrounding tissues were higher than at all other time points. This is the opposite of what was previously reported when comparing a laser and conventional tools for piglet tail docking (Lou et al., 2022). A key difference in this current study is that piglets were provided with an NSAID that functions to reduce pain, inflammation, and fever. Piglets administered firocoxib (oral or transmammary) had a higher tail tissue temperature than piglets given placebo. In exploring these findings further, it was discovered that this significant increase in temperature was only observed in sham-handled piglets given firocoxib compared to sham-handled piglets given placebo. This may be explained by the provision of firocoxib producing a less pronounced SNS response to handling. This outcome is supported by previous work where piglets given an NSAID (meloxicam) had higher cranium temperatures than placebo treated piglets (Bates et al., 2014). Therefore, the temperature differences captured by the IRT camera may have not measured true inflammation in the tissue surrounding the tail, but rather an attenuated SNS response as sham-handled piglets had no tissue damage.

Piglet treatment had no effect on temperatures of the castration incision site or its surrounding tissues. In contrast to tail docking, castrated piglets exhibited lower temperatures

immediately post-procedure than at all other time points at both the castration site and the scrotal tissue, which is an agreeance with (Viscardi et al., 2020) It is possible that the treatments in this study may be more effective at attenuating pain and stress responses from piglet tail docking, but not castration.

Animals experiencing localized pain are observed redistributing their body weight onto their non-affected limb (i.e., away from the site of pain) (Kleinhenz et al., 2018). The FDA recognizes pressure mat gait analysis as an optimal pain assessment outcome (Kleinhenz, Viscardi and Coetzee, 2021). After castration, calves and piglets put more weight onto their front limbs than hind limbs to redistribute weight off of the castration site, however; reduced changes in gait from animals given pain management have been observed (Kleinhenz et al., 2018; Coetzee, 2019; Martin et al., 2022).

Procedural differences were found for time spent standing on the front the limbs. Front stance time was longer in conventionally and sham processed piglets than laser processed piglets. Martin et al. (2022) found that castrated calves without analgesia had a shorter front stance time than castrated calves administered either lidocaine and meloxicam or bupivacaine liposome injectable suspension alone. Our results combined with results from (Martin et al., 2022) suggest that a shorter front stance time may be a sign of castrated animals experiencing pain, where animals provided effective analgesia or processed using conventional tools may experience less pain.

Treatment differences were also found for the amount of force and pressure placed onto the limbs, specifically front and hind peak vertical force, front peak vertical impulse, and front peak contact pressure. In comparison to piglets in the FIROSOW and PLBO groups, piglets in the FIROPIG applied more force on their front and hind limbs and pressure on their front limbs. Previous work found placebo-treated piglets put more force on their front limbs than firocoxib-

treated piglets (Coetzee, 2019). Together, these results suggest that removing force and pressure from a localized area may indicate a painful state.

There were also treatment differences in gait when comparing across procedures. Conventionally processed piglets in the FIROPIG group applied more force on their hind limbs than FIROSOW piglets. They also tended to apply more force and contact pressure on their hind limbs compared to FIROSOW piglets, after conventional processing. In laser-processed piglets, piglets in the FIROPIG group had a longer stride length than FIROSOW piglets. In previous work, castrated animals exhibited shorter stride lengths (Kleinhenz et al., 2018). Pressure mat outcomes presented in this study suggest that oral firocoxib may have been more effective than transmammary-delivered firocoxib at reducing pain associated with surgical castration and tail docking, as FIROPIG piglets were able to distribute more weight across all limbs.

The PGS has been used to identify and assess changes in facial expressions associated with painful stimuli, such as piglet tail docking and castration (Di Giminiani et al., 2016 ;Viscardi et al., 2017). The following FAUs have been of primary focus when using the PGS - orbital tightening, ear position, and nose bulge/cheek tension, given their acceptable inter-rater reliability among raters and the frequency at which they are observed after a painful event across animals (Di Giminiani et al., 2016; Viscardi et al., 2017; Maria E Lou et al., 2022). This was not the case in this study, as inter-rater reliability among the raters for all FAUs indicated poor reliability. The method used to obtain PGS images analyzed in this study is different from methods other researchers have previously used, as images were extracted from videos of piglets walking across a pressure mat. This method was chosen to avoid physical restraint of piglets by hand or novel devices during image collection, which is known to be highly stressful and could confound piglet facial expression interpretation (Grandin and Shivley, 2015).

Low quality images (poor lighting, angle, and resolution) was a significant factor affecting how raters scored images (Maria E Lou et al., 2022). It is possible FAUs were hard to discern, as using images of piglets in motion may have added pixilation or blurriness. Rater training may have also impacted how well raters scored images. The ICC values reported by (Maria E. Lou et al., 2022) were higher than scores presented in this study possibly attributed to more intensive training (two 1-hour sessions).

After tail docking and castration, piglets have shown to have significantly higher grimace scores compared to non-processed piglets (Di Giminiani et al., 2016 ;Viscardi et al., 2017 ;Viscardi and Turner, 2018). Piglet grimace scores were different based on treatment and procedure in this present study; however, the only significant FAU ear position. After conventional processing and sham-handling, piglets in the FIROPIG group had higher EP scores than piglets in the FIROSOW group. After laser-processing, FIROPIG piglets had higher EP scores than PLBO piglets. These results likely led to the overall (i.e., total PGS scores) outcome of piglets in the FIROPIG group grimacing more than piglets in the FIROSOW and PLBO groups, which is contrary to our hypothesis. Despite low FAU reliability, scores from all raters indicated piglets in the FIROPIG group had higher grimace scores than piglets in the FIROSOW and PLBO groups when analyzed individually.

Like IRT, total PGS score results were further explored, finding that FIROPIG sham-handled piglets grimaced more than FIROSOW and PLBO sham-handled piglets when tissue damage was not present. As pressure mat and PGS outcomes were collected together, pressure mat results may provide insight into PGS results. Piglets in the FIROPIG group were able to distribute more weight across both front and hind limbs. In other words, piglets in the FIROPIG group had their gait least affected post-processing suggesting a reduced pain response. Future studies may

need to consider improved methods of piglet facial image collection having minimal environmental influence and additional rater training.

Cortisol is a common biomarker used as a proxy to measure stress and pain in animals (Baysinger et al., 2021). Several studies have shown that piglet cortisol concentrations peak between 30-90 min after tail docking and castration (Prunier et al., 2005 ;Sutherland et al., 2010 ;Sutherland et al., 2011). Cortisol was highest at baseline compared to 1 and 24h post-processing. Although this was before piglets were processed, it was the first-time piglets were exposed to procedures for baseline outcome measure collection and they were extensively handled. Handling, alone along with novel events, can increase cortisol and fearful emotions and must be interpreted cautiously (Moberg, G.P. and Mench, 2000). Cortisol was also higher within the first hour after processing compared to than 24h post-processing which is consistent with other studies (Sutherland et al., 2011).

There no differences in cortisol concentrations in laser and conventionally processed piglets, suggesting both methods cause distress. This is consistent with previous work comparing laser and conventional methods to tail dock (Maria E. Lou et al., 2022)and castrate (Viscardi et al., 2020) piglets. Piglets in the FIROPIG group had significantly lower cortisol concentrations than piglets in the FIROSOW and PLBO groups, with even lower cortisol concentrations compared to FIROSOW piglets. Tenbergen et al. (2014) found a similar result, with meloxicam-treated piglets having lower cortisol concentrations than placebo-treated piglets after tail docking and castration. Another study showed that both castrated piglets treated with meloxicam or flunixin had a reduce cortisol levels compared to castrated piglets given no pain control (Reiner et al., 2012). Firocoxib led to dose-dependent reduction in cortisol with a dose of 2.0 mg/kg delivered transmammary reducing cortisol more than doses of 0.5 and 1.0 mg/kg after tail docking and

castration (Coetzee et al., 2019). Our results suggest that oral administration of firocoxib in combination with bupivacaine liposome injectable suspension may be more effective as reducing the stress response caused by tail docking and castration than transmammary firocoxib.

As expected, piglets in the FIROPIG and FIROSOW group had higher firocoxib concentrations than piglets in the PLBO group that were not administered firocoxib, with piglets in the FIROPIG group having higher firocoxib concentrations than piglets in the FIROSOW group. Our results demonstrate that both direct (oral) and indirect (transmammary) routes of firocoxib administration to piglets were successful in transferring firocoxib to the piglet. Currently, there no studies with data on piglet firocoxib concentrations comparing direct oral administration and indirect transmammary delivery (from the sow to her nursing piglets) after oral administration to the sow. Firocoxib concentrations in FIROPIG piglets were about 8x higher than FIROSOW piglets.

In an earlier study, piglet firocoxib concentrations were assessed up to 120h post-processing after transmammary delivery of lower doses (0.5-2.0 mg/kg) used in this present study (Coetzee et al., 2019). Results demonstrated that lower doses of transmammary delivered firocoxib were also successful in crossing the blood-milk barrier when administered ~7h before piglet processing via IM injection to the sow (Coetzee et al., 2019). However, a follow up study further investigating the dose of 2.0 mg/kg firocoxib delivered transammary after IM injection to the sow for pain relief in piglets after tail docking and castration found that a higher dose than 2.0 mg/kg may be needed to be clinically effective (Viscardi, 2023). The authors also recommended administering the drug more than 7h prior to piglet processing to provide the drug more time to reach effective concentrations at the level of the piglet (Viscardi, 2023).

Firocoxib concentrations were not different between 1 and 24h post-processing indicating a stable amount of drug for up to 24h which supports the long-lasting effects of firocoxib. Over the 24h period after processing, oral administration of firocoxib resulted in higher drug concentrations than transmammary delivery. This coincides with reported plasma elimination half-life of firocoxib in sows and piglets ranging between 26 -31h and 30-48h, respectively (Coetzee et al., 2019). Future work is needed to understand the pharmacokinetics of the oral formulation of firocoxib in piglets comparing oral and transmammary routes of administration.

Conclusion

While the CO_2 surgical laser caused less bleeding immediately after the tail docking procedure, laser tail docked piglets experienced more inflammation through the study period than piglets that were tail docked with side pliers. There were no benefits found when using the CO_2 surgical laser for piglet castration. Piglets administered oral firocoxib and bupivacaine liposome injectional suspension had significantly higher firocoxib levels and lower plasma cortisol concentrations than piglets provided with firocoxib transmammary and bupivacaine liposome injectional suspension and who were provided the bupivacaine block only. Piglets administered oral firocoxib and bupivacaine liposome injectional suspension also had their gait least affected compared to FIROSOW and PLBO piglets. This study demonstrated that a multi-modal approach of oral firocoxib and bupivacaine liposomal injectable suspension may reduce the stress response and changes to weight distribution in piglets after tail docking and castration, suggesting it is a more effective pain reducing strategy than transmammary firocoxib and bupivacaine liposome injectional suspension and bupivacaine liposome injectional suspension alone. As benefits were not observed when comparing the laser to conventional tools for piglet tail docking and surgical

castration, additional research is warranted to determine if a CO2 surgical laser is a refined alternative for this purpose.

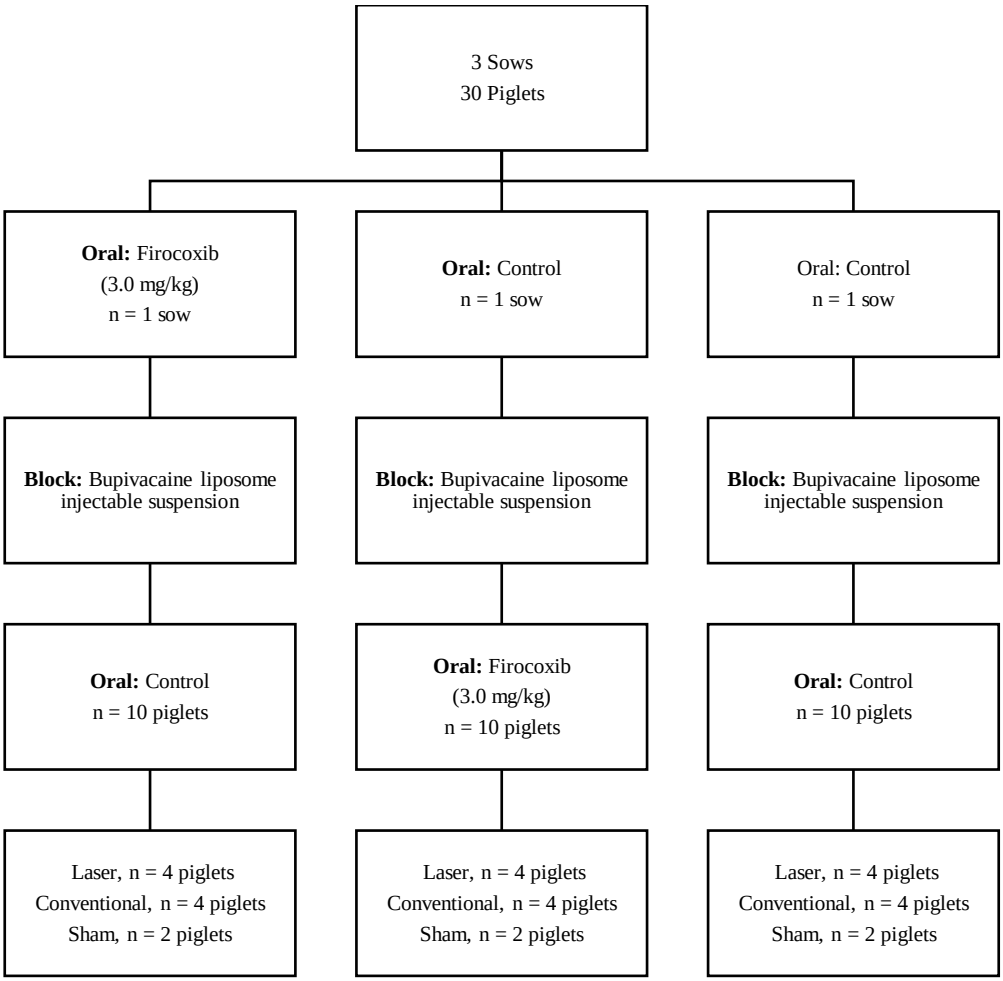
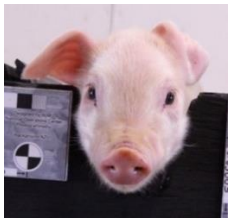
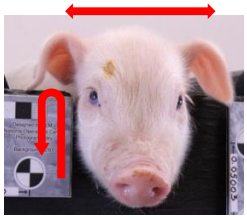
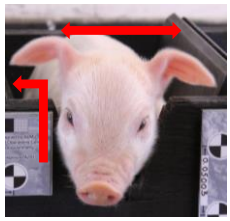
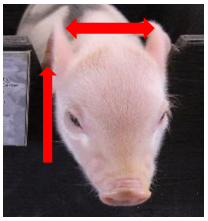


Figure 4.1. Flow chart of sow and piglet treatments

Orbital Tightening		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Eyes wide open, with a round, circular shape No tightening of or around the eyelid Whites of eyes may be visible **Both eyes should be scored 0**	Eyes are closed halfway, with a teardrop shape Eyelids are partially tightened (i.e., "eye squeezing") "Eyebrow" furrowing is present Whites of eyes may be partly visible **Features may be seen only or more strongly in 1 eye**	Eyes are closed more than halfway (may see full eye closure) Eyelids are fully tightened, with obvious "eye squeezing" Significant "eyebrow" furrowing Whites of the eyes are not visible **Features may be seen only or more strongly in 1 eye**

Ear Position		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
		
Ears are forward and relaxed Normal distance between ears Inner pinna is partly visible Base of ear has the following shape:  **Both ears should be scored 0**	Ears are upward and drawn back Distance between ears is reduced (Length of red arrow is less than in Score 0) Inner pinna may be more visible Base of ear has the following shape:  **Features may be seen only or more strongly in 1 ear**	Ears are fully drawn back, flush against head Distance between ears is further reduced (Length of red arrow is less than in Score 1) Base of ear has the following shape: Inner pinna not visible Base of ear has the following shape: **Features may be seen only or more strongly in 1 ear**

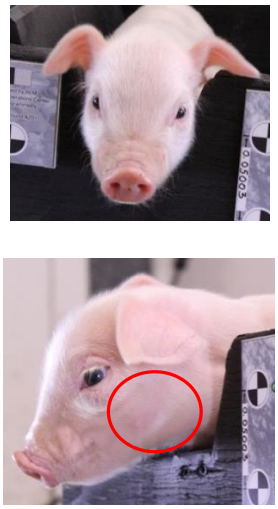
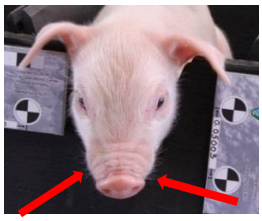
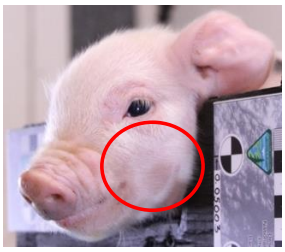
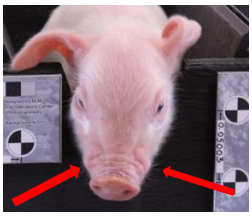
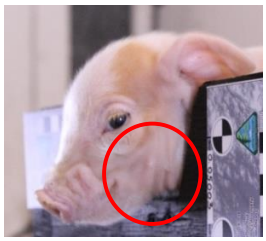
Nose Bulge/Cheek tension		
Not Present (0)	**Moderately Present (1)**	**Obviously Present (2)**
	 	 
Nose Pigs naturally have some wrinkles/skin folds on nose **Cheeks** Skin gradient is flat, smooth, and relaxed No tightening **Note:** Nose muscles move congruently with cheek muscles	**Nose** Increase in bulging Moderate "bulging" appearance with skin folds Nose moves inward **Cheeks** Moderate tightening/tension with few indentations/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**	**Nose** Significant increase in bulging Significant "bulging" with several skin folds Nose moves inward **Cheeks** Significant tightening/tension with several indentions/wrinkles **Features may be seen only or more strongly on 1 side of the cheeks and nose**

Figure 4.2. The Piglet Grimace Scale (PGS) with the description of the facial action units (FAUs) assessed. (Modified from Viscardi et al., 2017 and Lou et al., 2022).

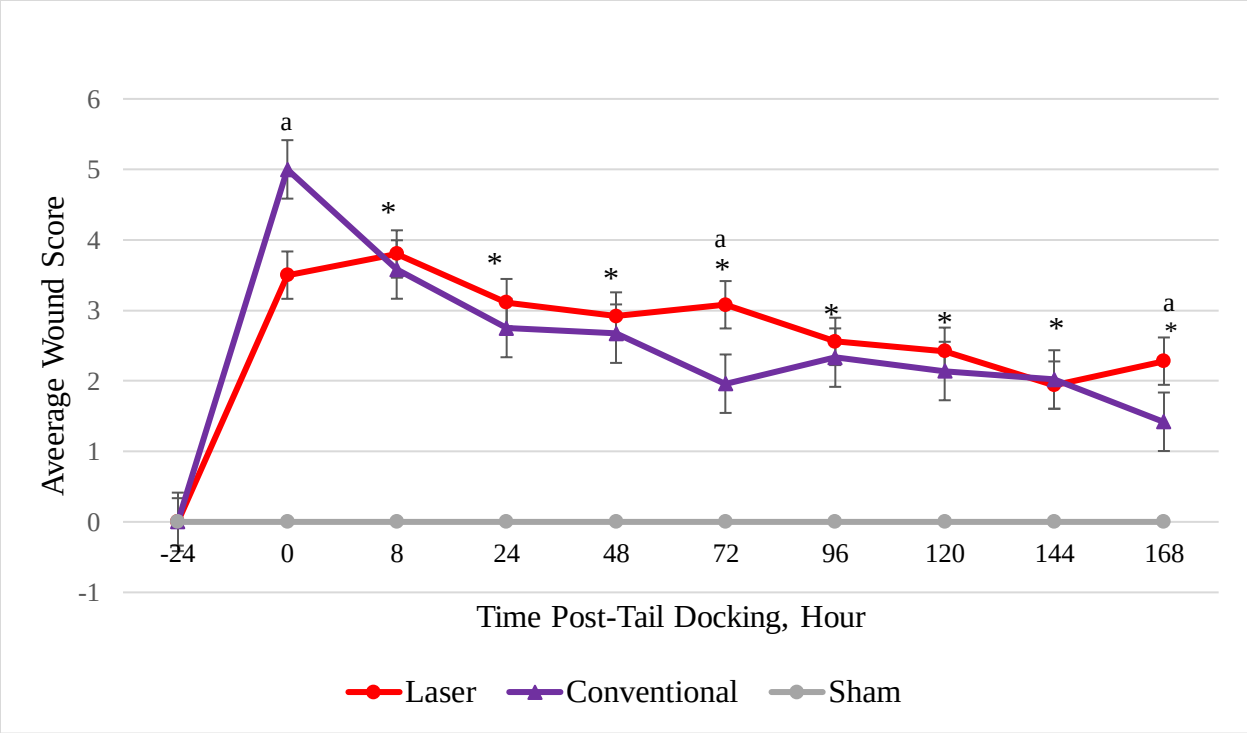


Figure 4.3. Average wound score of the tail docking site (mean ± SE) of piglets in each procedure across the assessment period after tail docking.

Asterisks represent a significant difference ($P < 0.0001$) between processed and sham-handled piglets. Letter represents a significant difference ($P < 0.0001$) between laser and conventional castrated piglets.

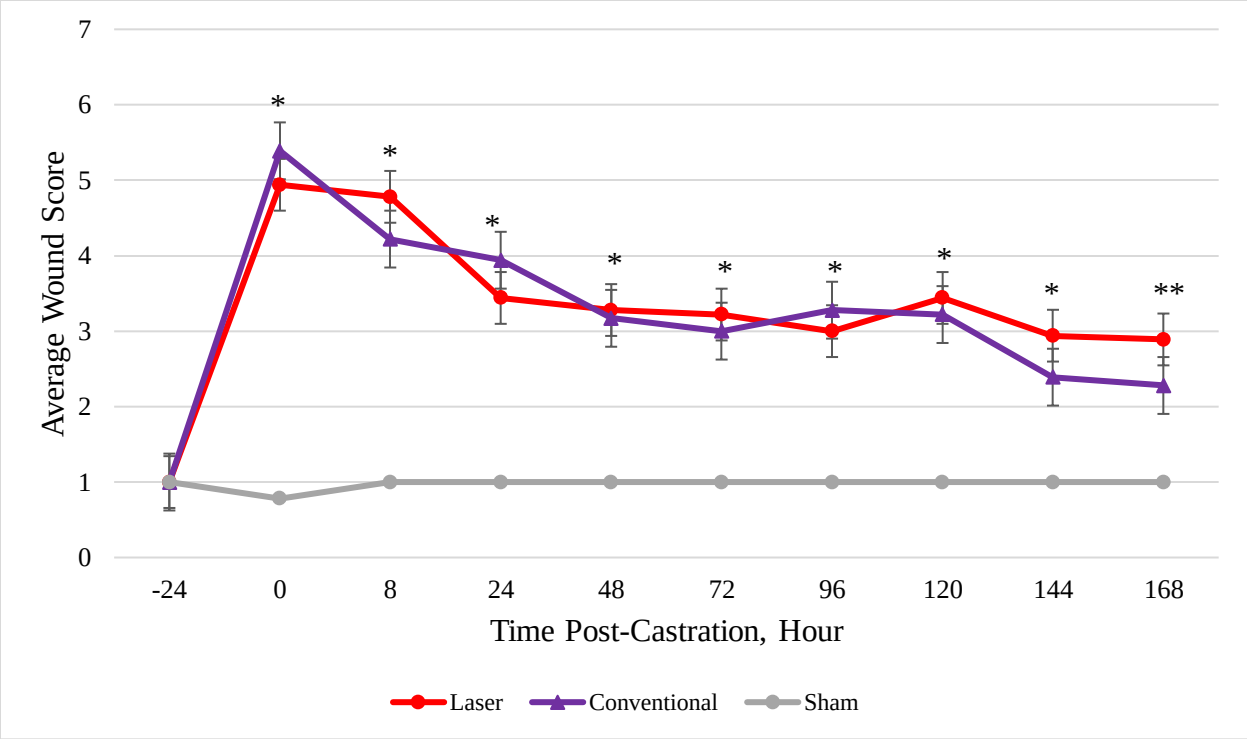


Figure 4.4. Average wound score of the castration site (mean ± SE) of piglets in each processing group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between processed and sham-handled piglets. Double asterisks represent a significant difference ($P < 0.0001$) between laser tail docked and sham-handled piglets.

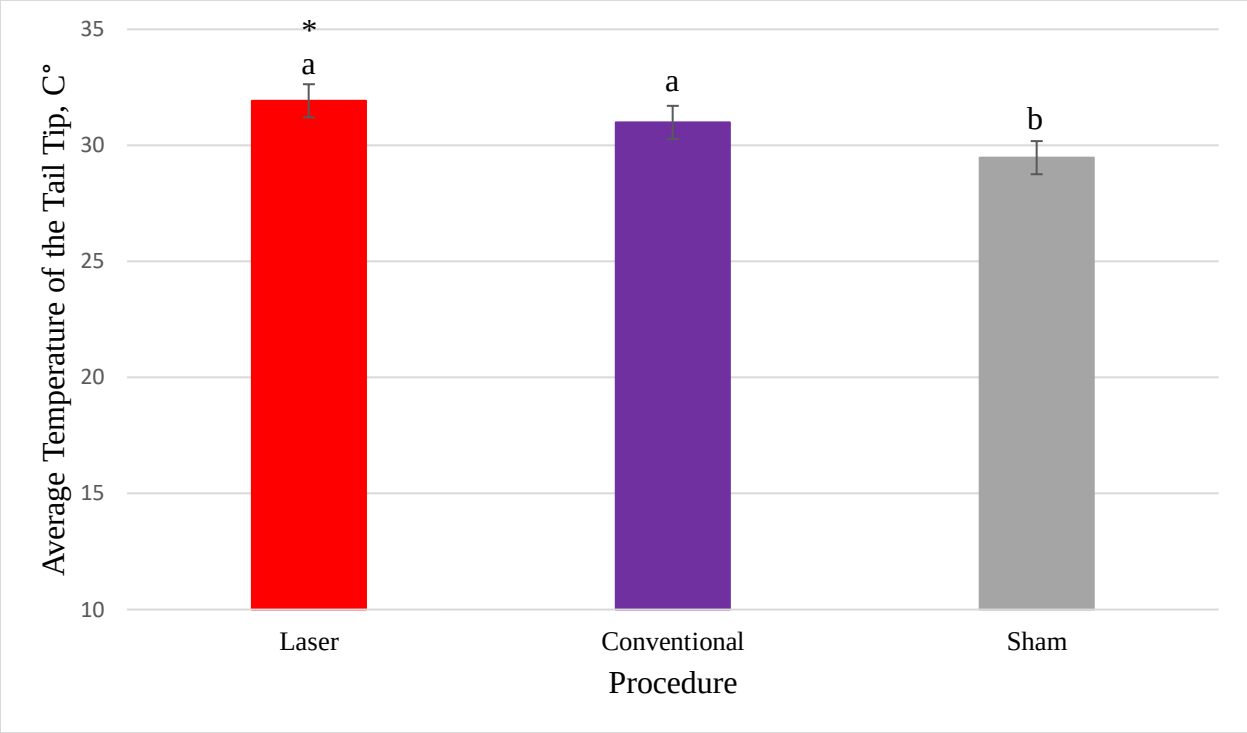


Figure 4.5. Average temperature (°C) of the tail tip (mean ± SE) of piglets in each processing group.

Different letters represent a significant difference ($P < 0.0001$) between processed and sham-handled piglets. Asterisks represents a significant difference ($P < 0.0001$) between laser and conventional tail docked piglets.

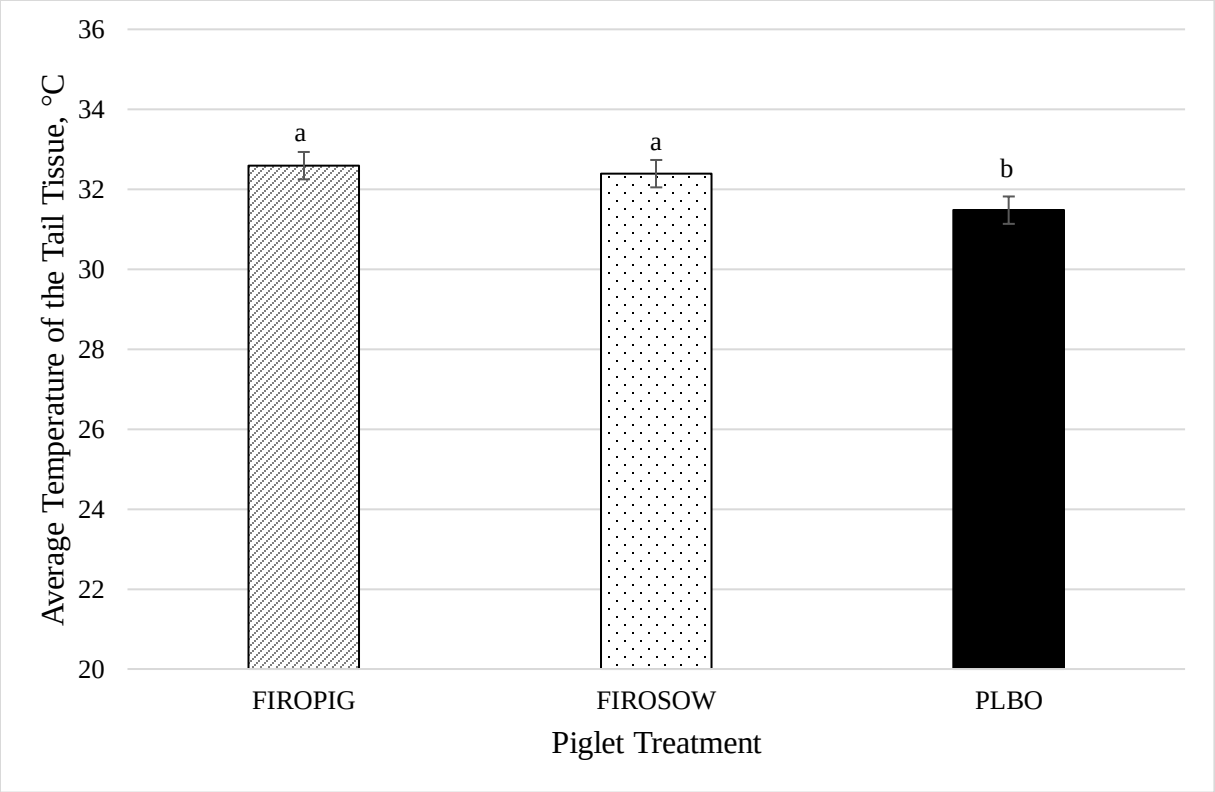


Figure 4.6. Average temperature (°C) of the tail tissue (mean ± SE) of piglets in each treatment group after tail docking.

Different letters represent a significant difference (P = 0.003) between treatments.

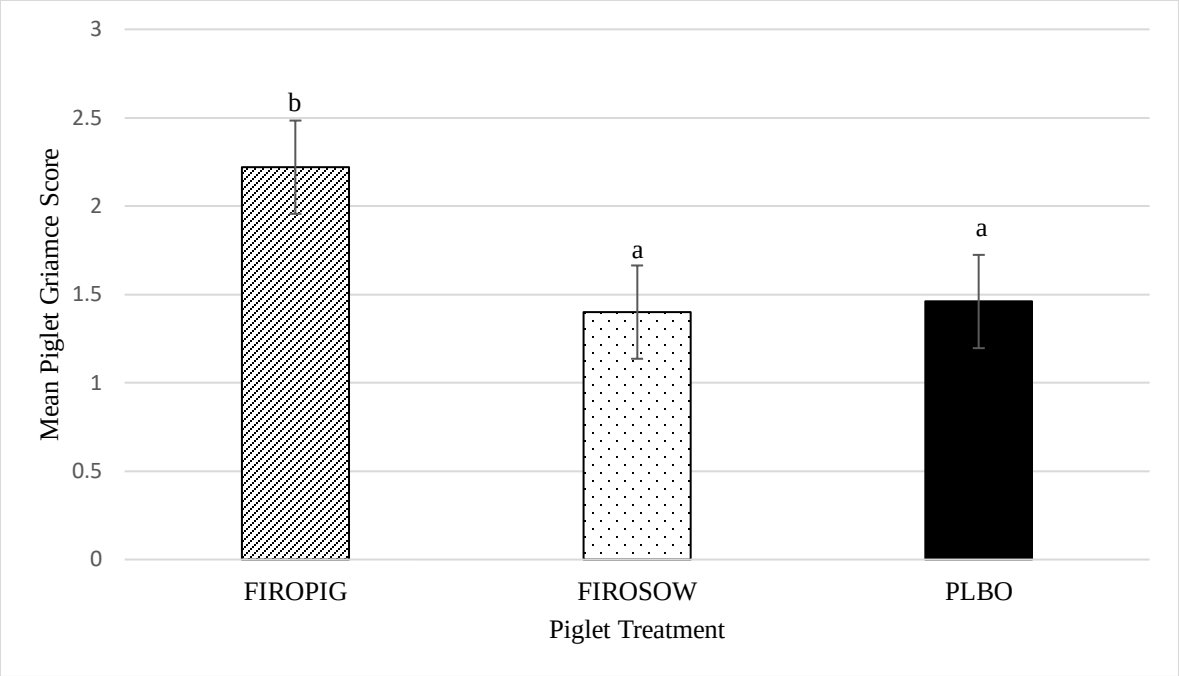


Figure 4.7. Mean Piglet Grimace Score (mean ± SE) of piglets in each treatment group. Different letters represent a significant difference ($P < 0.0001$) between treatments.

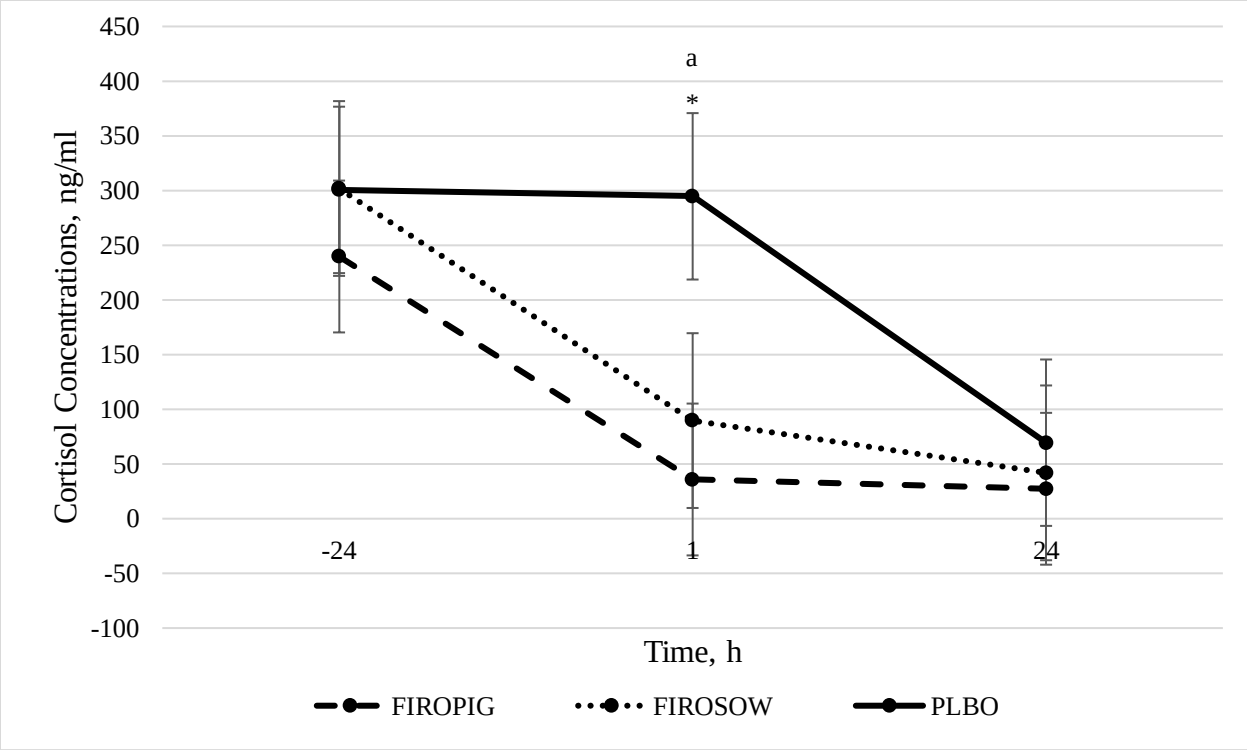


Figure 4.8. Mean cortisol concentrations (ng/mL) (mean ± SE) of piglets in each treatment group across the assessment period.

Asterisks represent a significant difference (P = 0.002) between piglets administered firocoxib (oral and transmammary) and placebo. Letter represents a significant difference (P = 0.03) between piglets administered firocoxib (oral and transmammary).

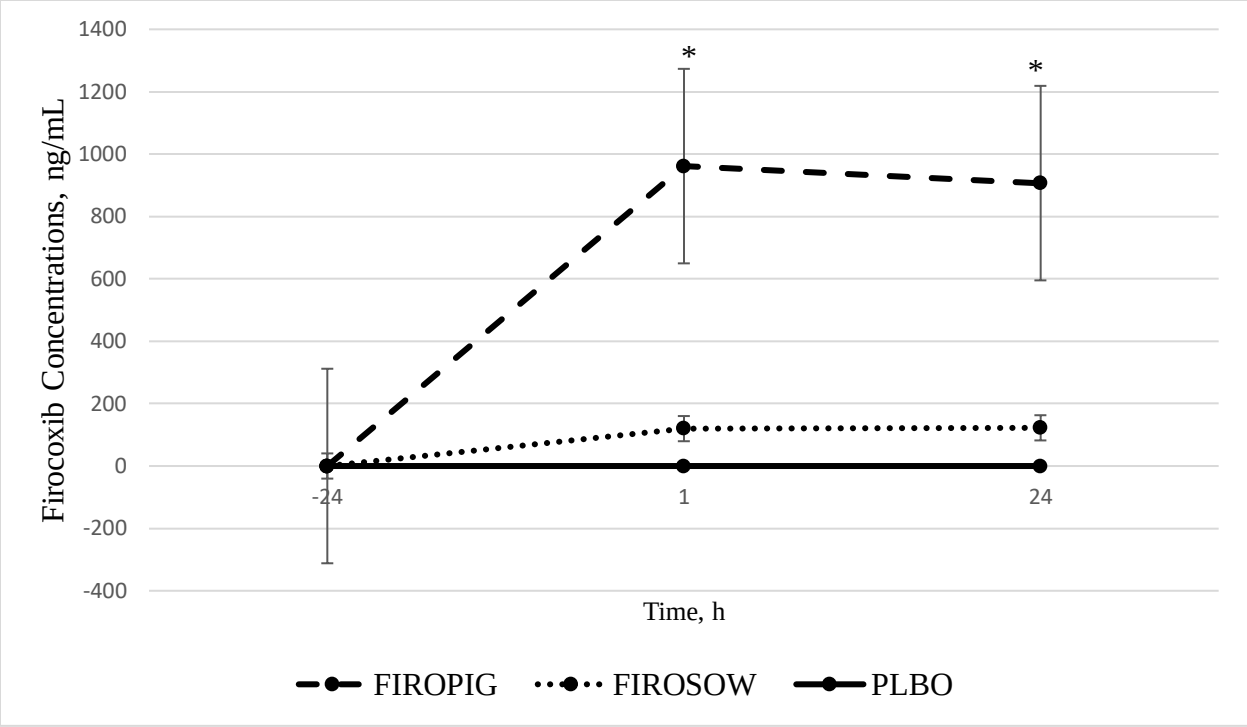


Figure 4.9. Mean firocoxib concentrations (ng/mL) (mean ± SE) of piglets in each treatment group across the assessment period.

Asterisks represent a significant difference ($P < 0.0001$) between piglets administered oral firocoxib and the other two treatment groups (transmammary firocoxib and placebo).

Table 4.1. Description of the study population and administration doses of firocoxib or placebo to the sows and piglets

Crate	Sow ID	Parity	Wt (kg)	Tx[a]	Dose (mg/kg)	Piglet ID	Sex	Wt(kg)	Tx[b]	Dose (10 mg/mL)
A	0527	12	254	CON	0.0	1A	M	2.56	FIROPIG	0.77
						2A	M	2.85		0.86
						3A	M	2.33		0.70
						4A	F	2.44		0.73
						5A	M	2.81		0.84
						6A	F	2.19		0.66
						7A	F	1.73		0.52
						8A	F	1.67		0.50
						9A	M	1.72		0.52
						10A	F	1.46		0.44
						11A[c]	M	---		---
						12A[c]	F	---		---
B	1314	7	259	FIRO	795	1B	M	2.25	FIROSOW	0.68
						2B	M	1.98		0.60
						3B	M	1.93		0.58
						4B	M	2.17		0.65
						5B	F	2.06		0.62
						6B	M	1.91		0.57
						7B	F	2.04		0.61
						8B	F	1.79		0.54
						9B	F	1.64		0.49
						10B	F	1.52		0.46
						11B[c]	M	---		---
						12B[c]	F	---		---
C	33918	5	229	CON	0.0	1C	F	2.67	PLBO	0.80
						2C	M	2.09		0.63
						3C	M	2.43		0.73
						4C	F	2.70		0.81
						5C	M	2.39		0.72
						6C	M	2.33		0.70
						7C	F	2.23		0.67
						8C	F	2.59		0.78
						9C	F	2.23		0.67
						10C	M	2.09		0.63
						11C[c]	F	---		---
						12C[c]	M	---		---

[a]Sow treatments - FIRO (3.0mg/kg PO firocoxib) and CON (60 mL Gatorade)
[b]Piglet treatments – FIROPIG (3.0mg/kg PO firocoxib), FIROSOW (1 mL PO Gatorade), and PLBO
[c]Piglets enrolled as "extra" in each litter

Table 4.2. Wound healing scoring system for piglet tail docking

The 6-point wound healing scale with descriptions of each score used to assess tail wounds in docked piglets (From Lout et al., 2022).

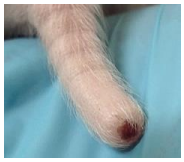
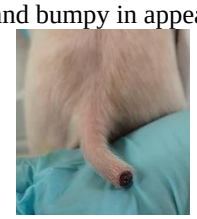
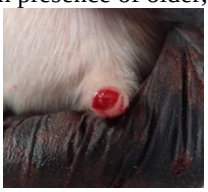
Score	Description
0	 Fully healed intact skin with no scab
1	 Intact skin with a thin, light-colored scab
2	 Fully formed scab (browner in color) with granulation (thin and bumpy in appearance)
3	 Partially opened with a thick, dark scab with presence of dried, black blood
4	 Open wound with presence of older, darker red blood
5	 Raw, open wound with presence of fresh, red blood. Bone may be visible

Table 4.3. Wound healing scoring system for piglet castration

The 6-point wound healing scale with descriptions of each score used to assess castration wounds in castrated piglets (Adapted from Sutherland et al., 2010).

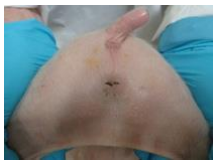
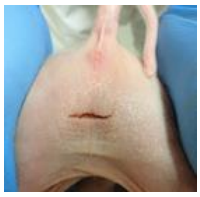
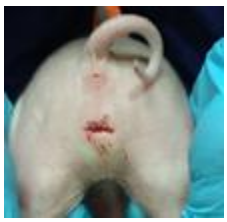
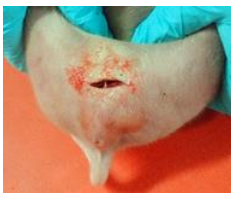
Score	Description
1	 Completely healed (no scab)
2	 Presence of minor scab
3	 Fully formed scab (thick and bumpy in appearance)
4	 Partially formed scab (thin in appearance)
5	 Open wound with fresh blood
6	 Fully open and raw wound

Table 4.4. Least square means (± SE) of outcome measures from the pressure mat gait analysis by procedure and treatment

	Treatment[1]			P-Value		
	FIROPIG	**FIROSOW**	**PLBO**	**Treatment**	**Time**	**Treatment x time**
Parameter						
Front limbs						
Stance time (s)	0.44 ± 0.02	0.42 ± 0.02	0.43 ± 0.02	0.47	0.006	0.51
Stride length (cm)	18.76 ± 0.41	18.23 ± 0.43	18.97 ± 0.46	0.47	< 0.0001	0.36
Peak vertical vorce (kg)	4.65 ± 0.15[a]	3.49 ± 0.16	3.79 ± 0.17	< 0.0001	< 0.0001	0.33
Peak vertical impulse (kg x s)	1.40 ± 0.07 [a]	1.01 ± 0.07	1.19 ± 0.08	0.0004	0.01	0.44
Peak contact pressure (kg/cm^2)	3.88 ± 0.13[a]	3.02 ± 0.13	3.36 ± 0.14	< 0.0001	< 0.0001	0.06
Hind limbs						
Stance time (s)	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.02	0.73	0.09	0.48
Stride length (cm)	18.45 ± 0.45	17.73 ± 0.46	19.11 ± 0.51	0.13	0.0003	0.74
Peak vertical force (kg)	1.87 ± 0.08[a]	1.43 ± 0.08	1.51 ± 0.10	0.0005	0.38	0.26
Peak vertical impulse (kg x s)	0.39 ± 0.03	0.29 ± 0.03	0.34 ± 0.04	0.08	0.31	0.54
Peak contact pressure (kg/cm^2)	2.66 ± 0.17	2.59 ± 0.18	2.31 ± 0.20	0.40	0.29	0.60
Gait distance (cm)	38.79 ± 1.80	41.20 ± 1.86	40.75 ± 2.04	0.63	0.05	0.17
Gait velocity (cm/sec)	26.55 ± 1.12	27.01 ± 1.16	26.41 ± 1.27	0.93	0.0001	0.47

[1]FIROPIG = 3.0 mg/kg oral firocoxib + bupivacaine liposome suspension; FIROSOW = 3.0 mg/kg transmammary firocoxib + bupivacaine liposome suspension; PLBO = bupivacaine liposome suspension only

[a,b] Values within a row with different superscripts differ significantly ($P \leq 0.05$

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Chapter 5 - Evaluating a multimodal approach to control pain associated with surgical castration of nursing male piglets: A pilot study

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Abstract

Currently, there are no available pharmaceutical products for the control of pain in swine that have been approved by the Food and Drug Administration (FDA) in the US. This has resulted in the surgical castration of commercially raised male piglets without the provision of an analgesic and/or anesthetic for pain management. Analgesic drug approval by the FDA requires evidence validating effective pain mitigation strategies through reliable animal-based outcomes. The objective of this study was to determine if 1.0 or 2.0 mg/kg firocoxib, in combination with 0.04 mg/kg buprenorphine, is effective at reducing surgical castration pain in piglets. A total of 30 male piglets were randomly allocated into 1 of 4 treatment groups: 1) castration + placebo (CON), castration + intervention 1 (CI1: 1.0 mg/kg firocoxib + 0.04 mg/kg buprenorphine), castration + intervention 2 (CI2: 2.0 mg/kg firocoxib + 0.04 mg/kg buprenorphine), and sham handling (SHAM). Firocoxib and buprenorphine were administered intramuscularly one day pre-castration and on the day of castration, respectively. Piglet body weights, cranial infrared thermography images, pressure mat gait analysis, and blood samples for cortisol and prostaglandin E_2 metabolites (PGEM) analysis were collected pre-procedure and up to 48h post-castration. Piglets with no intervention (CON and SHAM) were heavier than piglets receiving 2.0 mg/kg firocoxib ($P = 0.02$). Irrespective of treatment, cranium skin temperature ($P = 0.001$) decreased and front stride length ($P = 0.002$) increased over time from 6 to 48h post-castration, respectively. Specifically, at 6h, piglets in the CON group displayed a significantly longer front stride length than piglets in the CI1 and SHAM groups. Lastly, piglets receiving an intervention (CI1 and CI2) had lower PGEM concentrations up to 6h post-castration compared to piglets given no intervention (CON and SHAM; $P < 0.05$), with CI1 piglets having lower concentrations than CI2 piglets. These results indicate that a dose of 1.0mg/kg firocoxib (CI1) combined with 0.04 mg/kg buprenorphine may

be most effective in reducing pain associated with piglet castration as evidenced by maintenance of gait and reduction in PGEM levels.

Introduction

The United States raises over 113 million pigs each year for food production. Male piglets are surgically castrated to enhance meat quality through the elimination of boar taint, and to reduce aggression among conspecifics (Sutherland, 2015). This procedure is routinely performed on commercial swine farms when piglets are ≤ 7 days of age without the administration of an analgesic or anesthetic for pain management (Rault et al., 2011). Currently, the provision of pain management for piglets undergoing surgical castration is limited due to the lack of drugs approved by the U.S. Food and Drug Administration (FDA) for this specific indication (Kleinhenz et al., 2021). Extensive literature demonstrates the need for analgesic drug approval to control pain associated with surgical castration, given the short and long-term adverse physiological and behavioral effects castration can have on piglet welfare (Ison et al., 2016).

Drugs, such as firocoxib and buprenorphine, are approved by the FDA for the control of pain in non-food producing animals, and both have previously been investigated for their ability of to manage pain associated with piglet castration (Coetzee et al., 2019 ;Viscardi and Turner, 2018). Firocoxib is specifically approved for the control of pain associated with osteoarthritis in horses and dogs (US Food and Drug Administration, 2004). Firocoxib is a non-steroidal anti-inflammatory drug (NSAID) producing analgesia and anti-inflammatory effects through the highly-selective inhibition of cyclooxygenase 2 (COX-2) (Papich, 2021). The COX-2 enzyme primarily synthesizes prostaglandins (e.g., prostaglandin E_2 metabolites), which mediate pain and inflammation. Buprenorphine is an opioid with a high affinity towards the μ-, κ-, and δ-opioid receptors (Foster et al., 2013). As a partial μ-opioid agonist, buprenorphine provides analgesia

binding to receptors in the brain, spine, and periphery to inhibit the transmission of pain signals (Rockwell, 2019).

The objective of this study was to determine if 1.0 or 2.0 mg/kg firocoxib, in combination with 0.04 mg/kg buprenorphine, is effective at reducing surgical castration pain in piglets. We hypothesized that a dose 2.0mg/kg firocoxib in combination with 0.04mg/kg buprenorphine would be most effective in reducing pain associated with piglet castration.

Materials and Methods

This experiment was conducted at Midwest Veterinary Services. All animal use and procedures were approved by the Midwest Veterinary Services Institutional Animal Care and Use Committee prior to study commencement (Protocol #MCL-20042).

Animals

Five Large White Crossbred sows sourced from Midwest Veterinary Services (MVS) Klitz Farm (Oakland, NE) were enrolled onto this study (**Table 1**). Sows were loaded onto a livestock trailer and transported from MVS Klitz Farm to the Central States Research Center (CSRC; MVS facility, Oakland, NE). The distance of travel was approximately 17 mi and took less than 30 min. Sows were randomly assigned to a farrowing crate using the random number function in Excel (Excel, Microsoft, Redmond, WA). This enabled sows to farrow in their farrowing crate within the study room to eliminate sow and piglet transportation to the study room after farrowing.

Prior to enrollment, each sow received a full comprehensive physical examination by a veterinarian to ensure they were healthy, pregnant, and of a parity between 1-3. Sows were identified by use of an ear tag with a unique number. Approximately 3 days post-farrowing, a total of 30 male piglets (mean BW: 1.89 ± 0.37 kg) were enrolled in this study, distributed across five sows. Piglets underwent a physical examination by a veterinarian had to meet the following

inclusion criteria to be enrolled: physically healthy, 2-4 days of age, undocked tails, two descended testicles, and a minimum body weight of 1.1 kg. Cross-fostering was performed for only one litter (Sow 19152) within 12h prior to study enrollment to limit effects on baseline behavior and activity.

Sows and their piglets were housed in a standard (82" x 54") farrowing crate equipped with a heat mat and a water nipple. Water nipples provided sows and piglets ab libitum access to water. Sows were fed a diet through individual feeders that met or exceeded National Research Council nutrient requirements for lactating sows (National Research Council, 2012). All animals were exposed to a 12 h light:12 h dark cycle, respectively. The temperature (Max; 89.6% and Min; 22.1°C) and humidity (Max; 87% and Min; 2.2°C) of the study room were recorded daily.

Experimental design

This study was performed using a randomized and masked 2 x 2 factorial design with the piglet as the experimental unit. The day before piglets were processed (Study Day -1), piglets were given a unique identifier consisting of a number and capital letter. The number portion of the piglet identifier was composed of 4 digits. The first 2 digits were the same last 2 digits of the sow ID and the last 2 digits was a random number (1-10). A capital letter was used within litters, such that each piglet was assigned a different letter from the following list: A, E, I, K, O, S, T, or V. A black permanent marker was used to write the number on both hind legs and the letter on the forehead and back of each piglet.

Piglets were randomly allocated into 1 of 4 treatment groups: surgical castration + placebo (CON: 0.9% sterile saline), surgical castration + intervention 1 (CI1: 1.0 mg/kg firocoxib + 0.04 mg/kg buprenorphine), surgical castration + intervention 2 (CI2: 2.0 mg/kg firocoxib + 0.04 mg/kg buprenorphine), and sham handling (SHAM). Each treatment was represented at least once in every litter.

On Study Day -1 (approximately 24 h pre-castration), piglet body weights were recorded to calculate the appropriate dosage of firocoxib (1.0 or 2.0 mg/kg; Equioxx, Merial, Inc. Duluth, GA) and buprenorphine (0.04 mg/kg; Buprenorphine Hydrochloride, Par Pharmaceutical, Chestnut Ridge, NY). Body weights for piglets in the C group were used to calculate the corresponding volumes for appropriate dosages of sterile saline (Sterile Saline Solution, USP, Nova-Tech, Inc., Grand Island, NE) for placebo. Within litter and treatment, piglet body weight was used as blocking factor when randomizing treatments. Firocoxib or placebo was administered approximately 16h prior to castration between 14:00-15:00, after all baseline outcomes were collected. Buprenorphine or placebo was administered on Study Day 0 from 06:00 and 07:00, immediately before piglets were castrated.

To facilitate treatment administration, piglets were first removed from their farrowing crate and placed into a cart. Piglets in the sham treatment group were immediately returned back to their farrowing crate. Drug administration was performed by the same two trained individuals: one person held each piglet as the other administered their assigned treatment. Syringes (1.0 mL) were pre-labeled with each piglet's unique identifier and pre-filled with their appropriate calculated drug dosage; therefore, the individuals administering the injections were blinded to piglet treatment. Prior to administration, both individuals verified that the label on the syringe matched the identification number of the piglet. Treatments were then administered intramuscularly in the piglet's neck muscle using a 21-guage, 0.5-inch needle. Specifically, the left and right neck muscle were injected on Day -1 (firocoxib and placebo) and Day 0 (buprenorphine or placebo), respectively.

Piglet castration and sham-castration were performed by the same trained individual. A scalpel was used to make two vertical incisions (one over each testicle) into the scrotum. Manual

pressure was applied to excise the testicles through the incisions, which were then pulled until the spermatic cord was torn. A new scalpel was used for each litter and was disinfected with chlorhexidine between each piglet. The same handling procedures used on surgically castrated piglets were used on piglets in the sham treatment group, except no incisions were made. The dull handle of the scalpel was used to simulate the making of the two incisions over each testicle and manipulation of the scrotum mimicked the removal of the testicles and spermatic cord. Once castrated, piglets were returned to their farrowing crate.

Data collection

The following outcomes in the following order were evaluated from each piglet: body weight, infrared thermography, pressure mat gait analysis, blood cortisol and prostaglandin E_2 metabolites and salivary cortisol.

Body weight

A plastic tote (50.8 cm x 43.2 cm) was placed onto a digital scale. Prior to each use, the accuracy of the scale was verified using known masses (i.e., certified weights), with an acceptable margin of error of 2%. Each piglet was then individually placed into the tote to record their body weight. To ensure proper piglet footing, the tote was equipped with a towel. Piglet body weights were collected at baseline (-24 h pre-castration) and at 24 and 48h post-castration.

Infrared thermography

Images of the cranium, right and left ear, and snout of each piglet were taken at baseline and at 6, 12, 24, and 48 h post-castration using a research-grade infrared thermography (IRT) camera (FLUKE TiX580: FLUKE Corporation, Everett, WA) to assess changes in piglet skin temperature induced by stress. For approximately 10 sec, each piglet was individually placed inside a plastic tote (50.8 cm x 43.2 cm) to capture the IRT images. In instances where there was

excessive piglet movement resulting in a blurry or pixelated image, the piglet was gently restrained by an individual to obtain an image of acceptable quality.

A research-grade software (SmartView 4.3; FLUKE Corporation, Everett, WA) designed specifically to interface with the IRT camera was used to analyze and record the average skin temperature (°C) of the specific anatomical locations. To obtain the average temperature of the cranium, 2 lines were drawn forming an X over the forehead. The lines connected the base of the right ear to the medical canthus of the left eye and connected the base of the left ear to the medial canthus of the right eye. A line over both the right and left ear connecting the base of the ear with the tip of the ear were drawn to obtain average ear temperature. Lastly, a circle was drawn around the snout to collect average snout temperature.

Pressure mat gait analysis

A commercially available high-sensitivity pressure mat (Strideway, Tekscan, Inc. South Boston, MA, USA) was used to collect the following gait-associated outcomes from each piglet: contact pressure, contact area, stride length, and stance phase duration. Piglets were walked across the pressure mat at baseline and at 6, 12, 24, and 48 h post-castration. The pressure mat was connected to a laptop computer (ThinkPad W541, Lenovo, China) with the research grade software (HUGEMAT Research 5.83, Tekscan. Inc., South Boston, MA) programed to interface with the pressure mat and used to analyze each gait-associated outcome. The pressure mat was also equipped with a digital video camera (Logitech HD 720P, Suzhou, China) that recorded the piglets walking. The software synchronized the piglet walk and video to ensure consistent piglet gait at each time point.

On Study Day 0 and 1, the pressure mat was calibrated using a known mass (i.e., certified weights). Piglets were allowed to walk across the length (91.4 cm) of the pressure mat and given

3 attempts at completing an acceptable walk. A walk was accepted if a pig executed 3 full strides (i.e., feet fully striking the contact sensing elements inside the pressure mat) within the activated margin of the pressure mat tiles providing data of good quality. A piglet running, stopping, slipping, falling, walking over a non-activated pressure mat margin, or being maneuvered by an individual would be categorized as an unacceptable walk.

Blood sampling and processing

Blood was collected from each piglet at baseline and at 1, 6, and 24 h post-castration for cortisol, and prostaglandin E_2 metabolites (PGEM) analysis. Whole blood (3.0 mL) was collected through venipuncture of the vena cava as piglets were held in the supine position using a 20-gauge x 1-inch multiple use drawing needle (VACUETTE, Greiner Bio-One, Monroe, NC). The blood was then transferred into 4.0 mL vacutainer serum separator tubes (BD Vacutainer Lithium Heparin, Becton, Dickinson, and Company, Franklin Lakes, NJ). Blood sample tubes were placed immediately on ice after collection. The samples were then centrifuged at 3000 x g for 10 min and serum was pipetted into cryovials. Blood samples were stored at -80℃ prior to analysis. All blood samples were analyzed by laboratory personnel at Kansas State University blinded to treatment, procedure, and time point.

Blood serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals cat. no. 07-221106-R) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. A 500 ng/mL standard was created by diluting the supplied 1,000 ng/mL kit standard. The standard curve ranged from 1 to 1,000 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were run at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm

polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a PerkinElmer Wizard2 gamma counter for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation over 18% were re-analyzed.

Prostaglandin E_2 metabolite concentrations were analyzed using a commercially available ELISA kit (cat. no. 514531, Cayman Chemical, Ann Arbor, MI) following the manufacturer's specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were incubated at −20 °C for 30 min and then centrifuged at 3,000 × g for 5 min. The supernatant was transferred to clean 13 × 100-mm glass tubes and evaporated using a CentriVap Concentrator (cat. no. 7810014, Labconco, Kansas City, MO) overnight (approx. 18 h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300-µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted at 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest accept- able standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well (%B/B_0) of ≥ 80% or ≤ 20%. Any individual sample outside the standard curve, with a %B/B_0

outside the 20% to 80% range, or a CV > 15% was re-analyzed. The projected average for PGEM intra-assay CV was 15.08% and inter-assay CV was 11.40%.

Salivary sampling and processing

Saliva was also collected from each piglet at baseline and at 1, 6, and 24 h post-castration for cortisol analysis using Salivette® Cortisol tubes (SARSTEDT Ag & Co., Germany). In order to promote salivation, 2-3 drops of 5% citric acid (Citric acid anhydrous crystalline; Fisher Bioreagents, Ottawa, ON) were administered into the piglet mouth. Piglets were placed into a plastic tote (50.8 cm x 43.2 cm) for approximately 5 min to provide the citric acid time to stimulation salivation. Piglets were then individually handled to place a cotton roll into the piglet's mouth which was gently held closed. After approximately 2 min, the cotton roll was removed and placed in a specialized Salivette tube to be centrifuged at 1500 x g for 15 min and pipetted into wells. Saliva samples were stored at -20℃ prior to analysis. All salivary samples were analyzed by laboratory personnel at Kansas State University blinded to treatment and time point. Saliva cortisol concentrations were determined using a commercially available enzyme immunoassay (EIA) kit (Salimetrics, State College, PA) following manufacturer specifications. Cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals cat. no. 07-221106-R) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. A 500 ng/mL standard was created by diluting the supplied 1,000 ng/mL kit standard. The standard curve ranged from 1 to 1,000 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples

was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a PerkinElmer Wizard2 gamma counter for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation over 18% were re-analyzed.

Statistical analyses

All data were analyzed using the GenStat 23rd Edition software with piglet as the experimental unit. Baseline (24 h pre-castration) measurements were also included as a covariate. Data (body weight, infrared thermography, pressure mat gait analysis, and blood cortisol and PGEM) were analyzed using a repeated measures linear mixed model (REML). Treatment, time, and time by treatment were included as fixed effects. Litter, weight block within litter, animal within weight block, and their interactions with time were random effects. Statistical significance was set at $P \leq 0.05$ and trends towards significance were set at $0.05 < P \leq 0.01$.

Results

Body weight

There were significant treatment ($P = 0.02$) and time ($P < 0.001$) differences for piglet weight. Piglets in the CON (2.41 ± 0.07 kg) and SHAM (2.38 ± 0.07 kg) groups were heavier than piglets in the CI2 group (2.24 ± 0.07 kg) ($P < 0.05$). There were no weight gain differences between piglets in the CI1 (2.34 ± 0.07 kg) and CI2 group. Irrespective of treatment, piglets were heavier at 48h post-castration (2.43 ± 0.06 kg) compared to 24h post-castration (2.26 ± 0.06 kg) ($P < 0.001$)

Infrared thermography: Cranium

Ear and snout images were not included in the final analysis given poor image quality. For the average temperature of the cranium, there were no significant treatment ($P = 0.20$) or treatment x time ($P = 0.28$) interactions. However, there were significant differences regarding time alone ($P = 0.001$) (**Figure 5.1**). Average cranium temperatures were higher at 6 (38.27 ± 0.3°C) and 12h (38.17 ± 0.4°C) post-castration compared to 24 and 48h ($P < 0.05$). Average cranium temperatures were also higher at 48h post-castration (37.41 ± 0.4°C) compared to 24h (36.83 ± 0.4°C) ($P = 0.28$).

Pressure mat gait analysis: Front stride length

The following pressure mat gait analysis outcomes were not included in the final analysis due to a low level of sensitivity: contact pressure, contact area, and stance phase duration.

For front stride length, there were no significant differences based on treatment ($P = 0.06$). However, there were significant differences with respect to time ($P = 0.002$) (**Figure 5.2**). Stride length was shorter at 6h (18.85 ± 1.01 cm) and 12h (20.45 ± 1.05cm) post-castration compared to 48h (22.54 ± 0.96 cm; $P < 0.05$). Stride length was also shorter at 24h post-castration compared to 48h ($P = 0.002$). Additionally, there was a significant treatment x time interaction ($P = 0.02$) (**Figure 5.3**). At 6h, piglets in the CON group (22.7 ± 1.2cm) had a significantly longer front stride length than piglets in the CI1 (17.9 ± 1.2cm) and SHAM (16.8 ± 1.2cm) groups ($P = 0.013$).

Blood sampling: cortisol and prostaglandin E2 metabolites

For blood cortisol concentrations, there were no significant treatment ($P = 0.99$), time ($P = 0.19$), or time x treatment interactions ($P = 0.37$).

For prostaglandin E_2 metabolites (PGEM), there were significant treatment ($P < 0.001$) and time x treatment ($P = 0.006$) interactions. Piglets in the CI1 (81.2 ± 9.8 pg/ml) and CI2 (82.4 ±

10.4 pg/ml) group had significantly lower PGEM concentrations than piglets in the CON (124.5 ± 15.7 pg/ml) and SHAM (140.1 ± 17.5 pg/ml) groups ($P < 0.05$)

At 1 and 6h post-castration, piglets in the CI1 (86.1 ± 9.4 and 61.9 ± 10.9 pg/ml, respectively) and CI2 (113.0 ± 12.9 and 63.9 ± 11.8 pg/ml, respectively) groups had significantly lower PGEM levels than piglets in the CON (156.2 ± 17.8 and 114.3 ± 21.2 pg/ml, respectively) and SHAM (157.4 ± 18.0 and 138.9 ± 25.9 pg/ml, respectively) groups ($P < 0.05$) (**Figure 5.4**). At 1h post-castration, piglets in group CI1 have significantly lower PGEM levels than piglets in group CI2 ($P = 0.006$) (**Figure 5.5**).

Salivary sampling

Results are not presented for salivary cortisol as an insufficient amount of piglet saliva was collected.

Discussion

The aim of this study was to evaluate the multimodal approach of firocoxib and buprenorphine, both of which have shown to be effective in reducing stress and pain responses in pigs. Firocoxib was evaluated for its effects in reducing pain in piglets after surgical castration and tail docking. The transmammary delivery of firocoxib at a dose of 2.0 mg/kg approximately 7h prior to processing showed reduced cortisol concentrations and enhanced piglet performance compared lower doses (0.5-1.5 mg/kg) (Coetzee et al., 2019). This same study performed a pharmacokinetic analysis and determined that firocoxib has a long plasma elimination half-life (30-48 h) in piglets, which is associated with long-lasting analgesic effects (Coetzee et al., 2019). The half-life of firocoxib in piglets is longer than in other NSAIDs including meloxicam, ketoprofen, and flunixin (Coetzee et al., 2019).

Buprenorphine (0.04 mg/kg) is considered the "gold standard intervention" for managing pain in piglets after surgical castration (Baysinger et al., 2021). When buprenorphine was administered 20 min before castration, piglets grimaced less and spent less time exhibiting pain-specific behaviors compared to piglets administered placebo for a period beyond 24h post-procedure (Viscardi and Turner, 2018). Similarly, buprenorphine has shown to have analgesic effects lasting between 7-24 h in pigs post-procedure compared to other opioids (etorphine and pethidine), where analgesic effects were observed for less than 6 h (Hermansen et al., 1986). Furthermore, activity and gait in pigs experiencing lameness was improved after administration of buprenorphine (Meijer et al., 2015). Thus, the positive outcomes from earlier work indicating the long and effective analgesic effects of firocoxib and buprenorphine in reducing pain in pigs provided the rationale this study.

Animals in a state of discomfort or pain reduce their feed intake, resulting in decreased weight gain compared to animals not in pain as energy reserves are directed towards the healing process (Anil et al., 2005). Results of studies investigating the impact of castration on piglet growth performance are mixed (Sheil and Polkinghorne, 2020). The age of piglets at the time of castration appears to have some influence, where piglets castrated at 3 days of age gained less weight than piglets castrated at 10 days of age (Kielly et al., 1999). Similarly, piglets castrated at 14 days of age had higher weaning weights than piglets castrated on day 1 of age (McGlone et al., 1993). In contrast, no effect on piglet growth was reported between castrates and non-castrates (Hay et al., 2003). It has been suggested that castration may have less of an impact on piglet growth if performed after piglet teat order has been established (Kluivers-Poodt et al., 2012).

Previous work has demonstrated that the administration of an analgesic or anesthetic may have minimal impact on piglet growth after castration. Meloxicam alone (Tenbergen et al., 2014;

Morales et al., 2017) or in combination with lidocaine (Hansson et al., 2011; Kluivers-Poodt et al., 2012; Bonastre et al., 2016) had no significant effect on piglet weight gain after castration. Ketoprofen also had no effect on piglet growth rate when administered before castration (Cassar et al., 2014). The results of this pilot study align well with previous studies, as there were no differences in weight among both treatment groups administered firocoxib and buprenorphine (CI1 and CI2). It is worth noting, that piglet growth in this pilot study was recorded until 48h post-castration, while others may determine weight differences by calculating average daily gain across a 21-day period.

In this present study, procedures for IRT imaging were adapted from (Hansson et al., 2011; Bates et al., 2014; Sutherland et al., 2015), where images from the following three anatomical locations were taken: the head, ears, and snout, by individually placing piglets in a non-restrictive plastic tote to reduce effects influenced by handling. Similar to Bates et al. (2014) ear and snout images were also not analyzed in our study given their poor quality as consistent angles could not be captured with mobile piglets in different positions. However, suitable cranium images were included in the final analysis.

Under circumstances of stress or pain, skin temperature may decrease as a result of the sympathetic nervous system (SNS) being activated and blood shifting from the periphery of the skin to vital organs aiding in survival (Stewart et al., 2005). Therefore, it is theorized that piglets in pain would present with reduced skin temperatures compared to piglets not in pain or given an effective analgesic drug (Sheil and Polkinghorne, 2020). Changes in skin temperature can be non-invasively measured using infrared thermography (IRT). Anatomical locations chosen for IRT assessment should ideally produce a high heat signature indicating a physiological response (e.g., the head, as a major source of heat), but also allow for a full range of visible access during imaging

(McCafferty, 2007). Once a practical location has been determined, researchers commonly assess skin temperature before castration ("no pain" state) and after castration (known painful state) (Hansson et al., 2011; Bates et al., 2014; Lonardi et al., 2015; Bonastre et al., 2016; Coetzee, 2019).

Bates et al. (2014) determined that measuring the skin temperature of the cranium may be a reliable location for pain assessment in castrated pigs. That same study found that trans-mammary delivery of meloxicam to piglets undergoing surgical castration and tail docking did have a reduction in cranial skin temperature compared to piglets receiving a placebo. These results indicate that control piglets were experiencing vasoconstriction from SNS activation (i.e., pain), while this same response was attenuated in piglets treated with meloxicam.

In contrast, our study found no differences in cranial temperature among the treatment groups. Our results are consistent with others assessing different anatomical locations. In comparing non-treated castrated piglets to castrated piglets receiving lidocaine alone and with meloxicam, there were no skin temperature differences at the castration site (Hansson et al., 2011). This was also observed at the castration site between non-treated castrated piglets and treated castrated piglets administered meloxicam, lidocaine, bupivacaine, or a multimodal combination (Bonastre et al., 2016). However, unlike cranium temperature, temperatures of the castration site aim to assess inflammation.

We did, however, observe differences in cranial temperature based on time. The highest temperatures were recorded on the day of castration (6 and 12h) with lower temperatures at 24 and 48h after castration. Factors that may influence body temperature, such as seasons (e.g., winter vs. summer) and circadian rhythms, have been considered (Sheil and Polkinghorne, 2020; Coetzee, 2019). Our observations across the study period followed a similar circadian pattern as reported by (Bates et al., 2014), with peaks and troughs in the evenings and morning, respectively.

Floor-based pressure mats have provided the ability to objectively evaluate the gait of an animal using parameters such as contact pressure (kg/cm^2), stance phase duration (s), contact area (cm^2), and stride length (cm) (Baysinger et al., 2021). Gait analysis led to the approval of the first drug to manage pain in cattle with foot rot, after being incorporated as a primary outcome in an FDA pivotal study (Kleinhenz et al., 2021). As a result, the FDA recognizes gait analysis as a reliable parameter in animal pain assessment studies. To obtain consistent and accurate data, the pressure mat must be of proper length to allow several continuous footfalls and be assembled on a flat, stable surface to enable a steady paced walk (not run), where the feet can fully strike the titles containing the contact sensors. Front stride length was the only gait parameter that could be analyzed in this current study, as the pressure mat lacked a high level of sensitivity needed to capture footfalls of piglets weighing less than 3.0 kg.

Stride length is the distance (cm) between the front (or back) heel after two consecutive foot falls (Reppert et al., 2020). It is assumed that animals in pain will exhibit a deviation in stride length from baseline as an indication they are reluctant to walk and are unable to maintain a normal pace, as instincts may reduce activity to promote healing. Current literature on pressure mat outcomes in relation to piglet castration pain is limited, as the initial focus were pain models focused on lameness in cattle, swine, and goats, cattle parturition, and cattle castration (Reppert et al., 2020; Kleinhenz et al., 2021; Weeder et al., 2023). Post-partum cows administered placebo displayed a longer stride length than post-partum cows given meloxicam (Kleinhenz et al., 2019). Regarding castration, castrated calves treated with lidocaine and flunixin meglumine had a longer stride length compared to castrated calves given only lidocaine with placebo (Currah et al., 2009). These results suggest that the administration of an NSAID reduced pain associated with calving and castration, enabling the cattle to travel a longer distance with each step.

While Coetzee (2019), did not encounter any differences in stride length, the authors did observe an increase of force on the front limbs of control piglets in comparison to piglets administered transmammary firocoxib. The same shift in weight distribution from the hind to the front limbs was observed in all castrated cattle, but with no differences based on treatment (placebo vs. flunixin meglumine) (Kleinhenz et al., 2018). The application of a stronger force on the front limbs over the hind limbs after castration may imply a purposeful shift in weight distribution away from a painful location on the body. In our study, front stride length increased over time from 6 (shortest distance) to 48h (longest distance) post-castration, regardless of treatment. Contrary to earlier reports of medicated animals having a longer stride length, CI1 piglets in our study had a much shorter front stride length than control piglets at 6h post-castration. This may imply that a dose of 1.0 mg/kg firocoxib may have been more effective at reducing pain than 2.0 mg/kg firocoxib.

The hypothalamic-pituitary- adrenal (HPA) axis releases cortisol which initiates the fight-or-flight response to help regulate the body while under stress or pain (Ison et al., 2016). Cortisol is a biomarker researchers have measured in piglet blood and saliva to assess stress associated with castration; however, handling or time of day alone can produce fluctuating cortisol levels (Herskin and Di Giminiani, 2018; Lou et al., 2022). Within 15-90 min after castration, blood cortisol levels were elevated in comparison to sham-castration (Prunier et al.,2005; Moya et al., 2008; Marchant-Forde et al., 2009; Lonardi et al., 2015; Maršálek et al., 2015; Merenda et al., 2022). While others have found no differences in blood cortisol concentrations between castrated and sham-handled piglets (Viscardi et al., 2020).

Regarding the ability of analgesic or anesthetic drugs to reduce cortisol levels in castrated piglets, previous finding have been varied. Castrated piglets treated with meloxicam had lower

cortisol concentrations than placebo-treated piglets (Tenbergen et al., 2014). Bonastre et al. (2016) found that a multimodal approach of meloxicam and lidocaine decreased cortisol concentrations in castrated piglets more than meloxicam only. Moreover, transmammary delivery of both meloxicam and firocoxib (2.0 m/kg) reduced cortisol concentrations after piglet castration (Bates et al., 2014; Coetzee et al., 2019). Yet, the opposite was reported with cortisol levels not differing between castrated piglets administered flunixin or placebo (Merenda et al., 2022). Cortisol concentrations were also not different between castrates with or without lidocaine (Maršálek et al., 2015). Our study found no differences in cortisol among any of the treatment groups, which are in agreement with others who also found no differences after analgesia provision.

It has been suggested that the effects of piglet age at processing on the development of their circadian rhythm and nociceptive response may be factors contributing to conflicting cortisol results (Ison et al., 2016). Piglets in our study were 3-4 days of age at the time of castration to reflect common protocols recognized on commercial swine farms in the U.S. (Baysinger et al., 2021). The adoption of standard pain management protocols on commercial production facilities will require a level of practicality for ease of use. Additionally, we selected sampling time points that track basal and peak cortisol levels in piglets as indicated by earlier studies, with a low sampling frequency to reduce the likelihood of an adverse event (e.g., death) from blind venipuncture blood collection.

An integral part of the pain pathway is the production of inflammatory mediators called prostaglandins that are released at sites of tissue damage (Mathews et al., 2014). Specifically, prostaglandin E2 (PGE_2) reduces the threshold of action potentials resulting from painful stimuli and heightens the sensation of pain (Meintjes, 2012). A function of firocoxib is to downgrade the synthesis of prostaglandins by inhibiting the production of the cyclooxygenase 2 (COX-2) enzyme

to help mitigate pain and inflammation. Unlike cortisol, PGE_2 and its metabolites are biomarkers that are understudied for piglet castration pain. As expected, castrated piglets had higher PGE_2 concentrations compared to piglets undergoing sham-handling (Prunier et al., 2005; Maršálek et al., 2015; Viscardi et al., 2020; Merenda et al., 2022).

The efficacy of NSAIDS in reducing PGE_2 production has been demonstrated for piglet castration and tail docking. A consistent reduction of PGE_2 concentrations was attained in castrated and tail docked piglets after transmammay delivery of 1.5 mg/kg firocoxib compared to 0.5, 1.0, and 2.0 mg/kg firocoxib (Coetzee, 2019). Furthermore, the transmammary delivery of meloxicam produced a greater inhibition of PGE_2 concentrations than placebo in castrated and tail docked piglets (Bates et al., 2014). Interestingly, Merenda et al. (2022) found that piglets sham-castrated with transdermal flunixin leaned towards having lower PGE_2 concentrations than sham-castration without transdermal flunixin indicating that handling alone induces a PGE_2 response that can be mitigated by an NSAID. Consistent with previous studies, piglets that received firocoxib (CI1 and CI2) had lower PGEM concentrations than the non-treated piglets (CON and SHAM). This positive effect was observed up to 6h post-castration, with the CI1 group having lower concentrations than the CI2 piglets, which followed a similar pattern described by (Bates et al., 2014).

Conclusion

In conclusion, the results of this pilot study support that surgical castration is painful procedure for piglets as evidenced by changes in weight distribution across limbs and elevated levels of PGEM. A dose of 1.0mg/kg firocoxib in combination with 0.04mg/kg buprenorphine may be most effective at alleviating pain associated with piglet castration between 3-4 days of age producing lower PGEM concentrations and reducing gait alterations. Following this pilot study, a

larger cohort of animals will be enrolled into a pivotal study to further investigate the efficacy firocoxib and confirm these findings.

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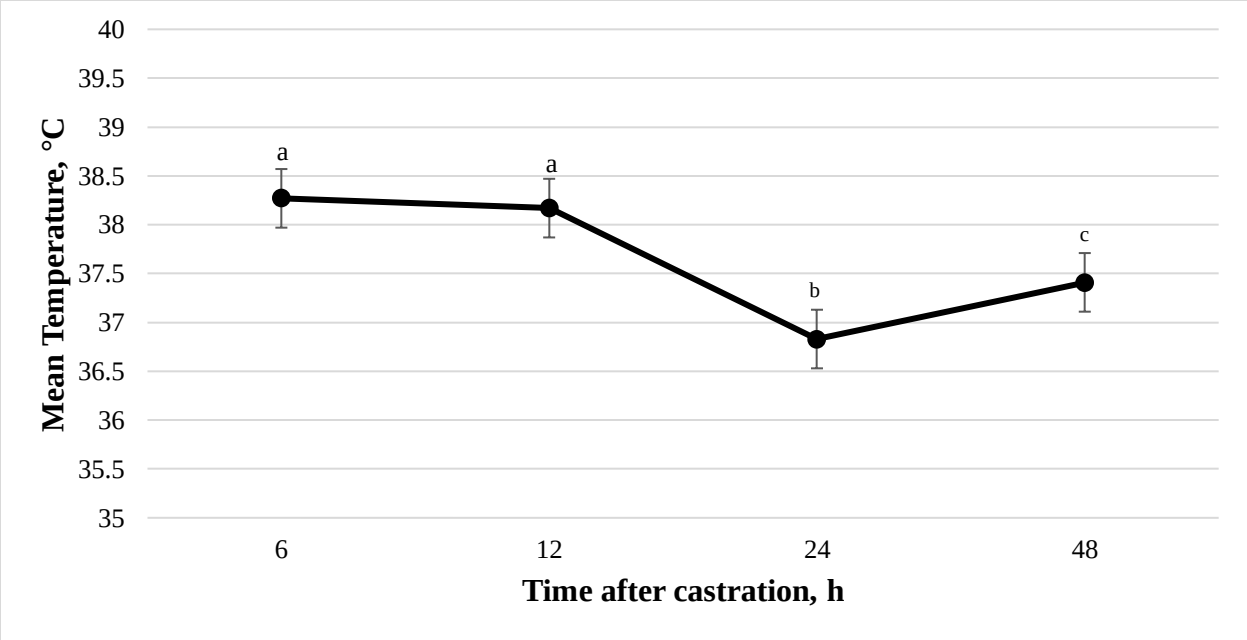

Figure 5.1. Mean (± SE) cranium skin temperature (°C) from all piglets (n=30) across the study period.

Means with different letters (a-c) indicate a significant difference between time points (P = 0.001).

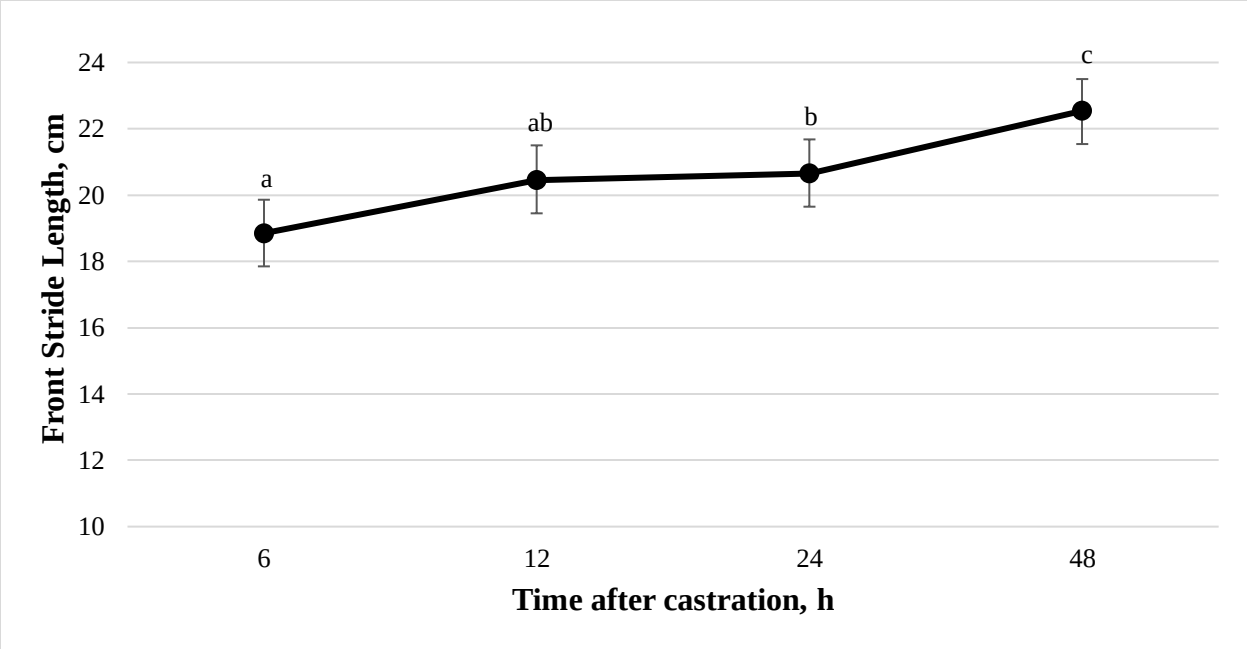

Figure 5.2. Mean (± SE) front stride length (cm) from all piglets (n=30) across the study period.

Means with different letters (a-c) indicate a significant difference between time points (P = 0.002).

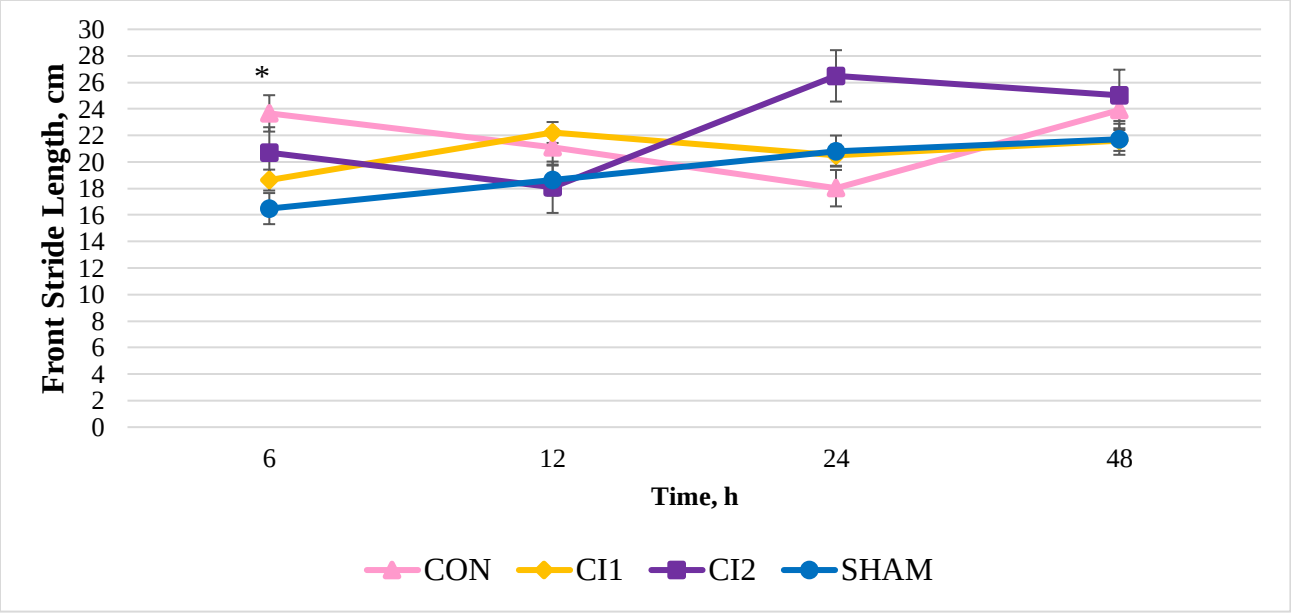

Figure 5.3. Mean (± SE) front stride length (cm) from piglets across the study period for each of the 4 treatment groups.

An asterisk denotes a time point where a significant treatment difference was observed between at least 2 treatment groups ($P < 0.05$).

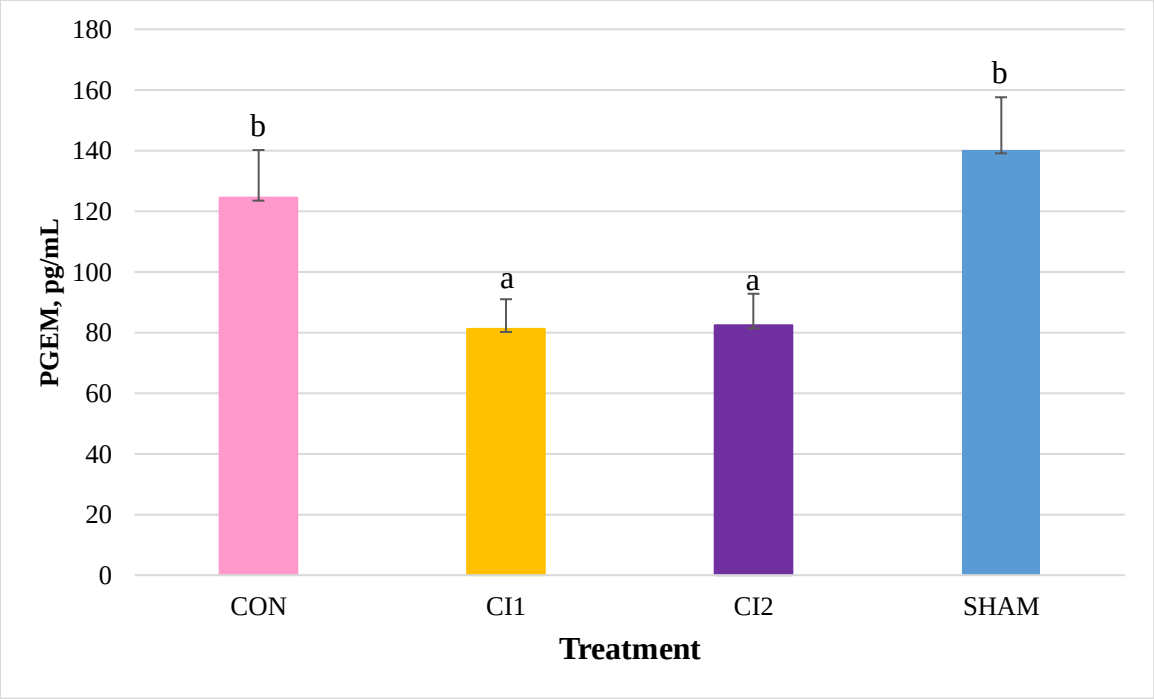

Figure 5.4. Mean (± SE) prostaglandin E_2 metabolite concentrations from piglets in each of the 4 treatment groups.

Different letters represent a significant difference ($P < 0.05$) between treatments.

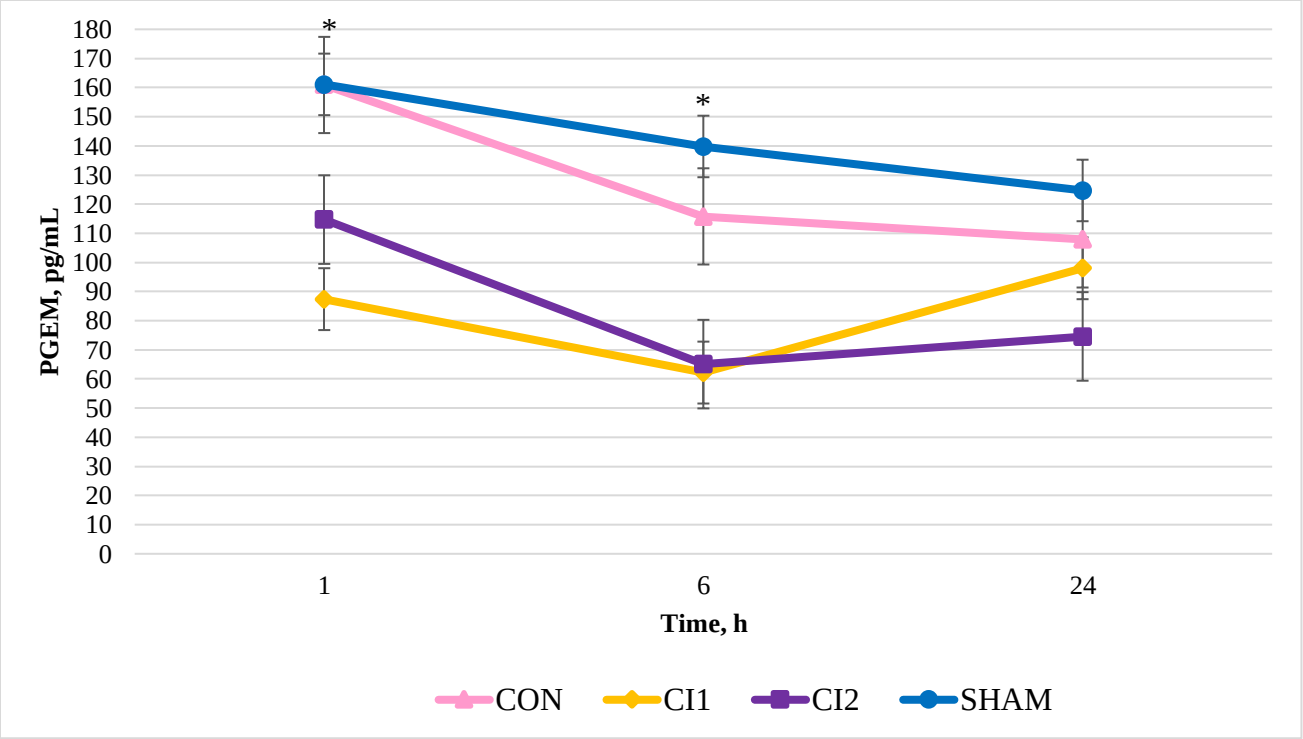

Figure 5.5. Mean (± SE) prostaglandin E_2 metabolite concentrations from piglets across the study period for each of the 4 treatment groups.

An asterisk denotes a time point where a significant treatment difference was observed between at least 2 treatment groups ($P < 0.05$).

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