Running head: Pain mitigation in 6 mo-old castrated calves

Effect of preemptive flunixin meglumine and lidocaine on behavioral and physiological indicators of pain post- band and knife castration in 6-mo-old beef calves[1]

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ABSTRACT

One hundred and seventy-four Angus bull calves (248 ± 27.1 kg of body weight (**BW**), 6-mo-old) were used in a 71 d study to assess the efficacy of the combination of flunixin meglumine and lidocaine in mitigating pain associated with band and knife castration. The experiment consisted of a 3 × 2 factorial design that included castration method -sham (**C**), band (**B**) or knife (**K)**; and medication – lidocaine (scrotal ring block 30 mL, 2% HCl lidocaine) and flunixin meglumine (single s.c. dose of 2.2 mg/kg BW) (**M**), or saline solution (**NM**). Animals were weighed on d 0 and weekly until d 71 (final BW) post-castration to obtain ADG. Physiological indicators included salivary cortisol collected on d 0 (30, 60, 120 and 240 min), d 2, 8, and weekly until d 48 post-castration; scrotal and eye temperature assessed on d 1, 2, 6, 8, and weekly until d 36 post-castration; fecal samples for *E. coli* collected on d 0, 2, 6, 8, and 22 post-castration. Behavioral measures included stride length on d 0, 8, and weekly until d 36, visual analog scale (VAS) evaluated during castration, and feeding behavior collected daily from d 0 to d 71 post-castration. Final BW and ADG were greater ($P < 0.05$) in C than B and K castrated calves. Salivary cortisol concentrations were greater ($P < 0.05$) in B and K calves than C calves up to 4-h post-castration, and remained greater in K calves up to 48-h post-castration, while concentrations were lower ($P = 0.01$) in M than NM calves. Fecal *E. coli* varied daily ($P = 0.01$) however, no obvious pattern over time. Scrotal temperature was greatest ($P < 0.05$) in K, intermediate in C and lowest in B calves, except at 30 min, and 22 and 36 d post-castration where they did not differ from C calves. Eye temperature was greater ($P < 0.05$) in B and K than C calves on d 2 and 8 post-castration. No differences ($P > 0.10$) were observed for stride length. The VAS scores were greater ($P = 0.01$) in K than C and B calves, while NM had greater scores ($P < 0.01$) than M calves. Dry matter intake and meal size were greater ($P = 0.05$) in M than NM calves. Meal duration was greater ($P = 0.01$) in B and C than K calves on d 0, while K calves had greater ($P < 0.01$) meal duration than C calves

1 and 2-wk post-castration. Overall, the combination of flunixin meglumine and lidocaine reduced physiological and behavioral indicators of pain, suggesting that their combined use was effective at mitigating pain associated with band and knife castration.

Key words: Beef cattle, Castration, Pain mitigation, Welfare

1. Introduction

Castration is a practice performed in beef cattle to reduce aggressiveness, facilitate herd management, and to improve meat quality (Stafford and Mellor, 2005; Nian et al., 2018). Among castration techniques performed in North America, band and knife castration are the most common methods used (Coetzee et al., 2010; Moggy et al., 2017), however it has been well documented that both methods cause acute and chronic pain and physiological stress in cattle (Schwartzkopf-Genswein et al., 2012; Marti et al., 2017; Meléndez et al., 2017b). Due to these concerns, current Canadian animal care guidelines recommend castration be performed in animals as young as practically possible and requires the use of pain mitigation drugs for calves that are 6-mo of age and older (CCAC, 2009; NFACC, 2013). Although the vast majority of Canadian calves are castrated earlier than 6 months of age (Moggy et al., 2017), a portion of calves, including those bull calves that do not meet the selection criteria for breeding stock or those that were not castrated properly on the ranch still need to be castrated at 6-mo of age or older, therefore identifying an effective pain mitigation strategy is highly relevant.

Several studies have shown that the combined use of an analgesic and an anesthetic can provide optimal pain control for castration related pain in cattle (Stilwell et al., 2008; González et

al., 2010; Van der Saag et al., 2018; Meléndez et al., 2018b). Nonsteroidal anti-inflammatory drugs (NSAIDs) such as flunixin meglumine have been shown to reduce behavioral and physiological indicators of pain at the time of castration (Currah et al., 2009; Webster et al., 2013; Kleinhenz et al., 2018). However, the efficacy of flunixin meglumine, alone or in combination with an anesthetic, may depend on its route of administration. Although labeled for intravenous (i.v.) administration, a study found that administration of flunixin meglumine via this route did not reduce inflammation in 25 d-old surgically castrated and medicated calves compared to non-medicated calves (Mintline et al., 2014). The extra-label administration of flunixin via intramuscular (i.m.) route has been associated with myonecrosis and damage to muscle tissue in cattle (Pyörälä et al., 1999; Smith et al., 2008). Consequently, a subcutaneous (s.c.) route of administration may be more practical than i.v. or i.m. administration, due to ease of administration, reduced needle sight lesions as well as a greater elimination half-life (5.4 ± 2.5 h) and bioavailability (104%) compared to an i.v. route (3.4 ± 1.0 h of half-life) (Kissell et al., 2012).

Although there are several studies assessing pain mitigation strategies for castration in beef calves few studies have evaluated a combination of s.c. flunixin meglumine and lidocaine to mitigate pain associated with castration. Therefore, the objectives of this study were to assess pain associated with band and knife castration, and the efficacy of using the combination of s.c. flunixin meglumine (analgesic) and lidocaine (anesthetic) at mitigating pain in 6-mo-old beef calves. We hypothesized that knife castration would have greater indicators of pain and that the combination of flunixin meglumine and lidocaine would be effective at reducing pain associated with castration.

2. Materials and methods

This protocol was approved by the Animal Care Committees of the Lethbridge Research and Development Centre (ACC # 0822). All animals were cared for according to the Canadian Council of Animal Care guidelines (CCAC, 2009).

2.1. Animals, experimental design and facilities

One hundred and seventy-four Angus bull calves, with an initial BW of 248 ± 27.1 kg and 6-mo of age, were used in a 71-d trial at the experimental feedlot at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (**LRDC**; Lethbridge, Alberta, Canada). Calves were randomly assigned to 1 of 4 experimental pens (42 or 44 calves/pen) where they were adapted for 21 d before the commencement of the experiment. Calves were housed in 4 outdoor pens (40.2 × 27.4 m) protected with windbreak fencing to the west and north. Each pen had a centrally located water system (Bolhmann Inc., Denison, IA) and a concrete apron (2.4 × 24.5 m) directly in front of the feeders. Straw bedding was added as needed in the corner opposite the feeders in each pen. The experiment was conducted as a 3 × 2 factorial design, where main effects included castration (**CAS**) method consisting of sham (non-castrated control - **C**), band (**B**) or knife (**K**) castration and use of medication (**MED**) consisting of either a combination of flunixin meglumine and lidocaine with epinephrine (**M**), or saline solution (**NM**) to yield the six treatments: non-castrated non-medicated (**CNM**, n = 29), non-castrated medicated (**CM**, n = 28), knife non-medicated (**KNM**, n = 29), knife medicated (**KM,** n = 29), band non-medicated (**BNM**, n = 29), and band medicated (**BM**, n = 29). All treatments were represented within each pen. Animals were assigned to two different groups of 87 calves to facilitate castration procedures, times and data collection on two consecutive days. Additionally, calves were subdivided into groups of 6 to allow

for drug onset (approximately 30 min) between the time of drug administration and the castration procedure. Within each subgroup, the medication treatments were administrated sequentially at 5 min intervals, before calves being castrated in the chute.

Non-castrated calves in the control treatment had their testicles manipulated in a similar way and for a similar amount of time as calves in the band and knife castration treatment. Knife castration was performed with the aid of a Newberry knife (Syrvet Inc., Waukee, IA) to make a vertical-lateral incision in the scrotum and each testicle was pulled out from the scrotal sac. A sterilized emasculator was applied for approximately 120 s to crush the spermatic cords. Band castration was performed by fitting a rubber band around the scrotal neck using a Callicrate bander according to the manufacturer's recommendations (Callicrate Bander, No-Bull Enterprises, St. Francis, KS). Both castration procedures were conducted by the same experienced veterinarian.

The medication protocol consisted of an intra-testicular injection of lidocaine and a lidocaine ring block (30 mL, 2% HCl lidocaine with epinephrine; Bimeda Canada, Ontario) where 10 mL of lidocaine were administrated into each testicle and another 20 mL were injected subcutaneously into the lateral walls at the base of scrotum, and a single s.c. dose of flunixin meglumine (2.2 mg/kg BW, Banamine, Schering-Plough Animal Health, Kirkland, Quebec, Canada) was administered in the neck. Both drugs were administered 30 min before castration. Non-medicated calves received a single s.c. dose of sterile Lactated Ringer's solution (10 mL, Lactated Ringer's Irrigation, Baxter Canada, Mississauga, Ontario, Canada) at the base of the scrotum, also administered 30 min before castration. The needle was not inserted into the testicles to avoid causing orchitis. Isopropyl alcohol was sprayed over the scrotum before local anesthetic was administered to all calves.

All calves were fed a total mixed back-grounding ration (57.6% DM consisting of 61.5% barley silage, 16.4% rolled barley, 17.2% rolled oats, and 5% supplement containing minerals and vitamins) *ad libitum* to meet NCR requirements (NRC, 2016). Feed was delivered twice daily at 0900 and 1300 and fresh water was available *ad libitum*.

2.2. Sampling and procedures

The experiment was conducted over a 13-wk period with data being collected 3-wk before and 10-wk after castration. A sub-sample of 48 calves (8 calves per treatment) was randomly selected to obtain more detailed physiological and behavioral measurements.

2.2.1. Performance

All animals were weighed at 0800 on d -21, -14, and for 2 consecutive days before castration (average of d -6 and -5, used as initial body weight; BW), and weekly until the end of the experiment (final BW), d 71 post-castration. Average daily gain (ADG, kg/day) was calculated by subtracting the final BW from the initial BW and dividing it by the number of days on feed.

2.2.2. Physiological parameters

2.2.2.1. Salivary cortisol and Fecal Escherichia coli

Saliva samples to determine cortisol concentration were collected on d -1, on d 0 at 30, 60, 120 and 240 min post-castration, and on d 2, 8, and weekly thereafter until d 48 after castration. Saliva was collected by swabbing the oral cavity using a cotton swab, which was then stored in a plastic tube and immediately frozen at -20° C for subsequent analysis using an enzyme

immunoassay kit (Salimetrics, State College, PA). Inter and intra-assay variability values were 14.8% and 8.9%, respectively. Fecal samples were collected on d -1, 2, 6, 8, and 22 after castration by rectal palpation for enumeration of total *E. coli* to determine pathogen shedding as described by Bach et al. (2004).

2.2.2.2. Infrared thermography

Thermographic images of the total scrotal area were obtained from all calves on d -6 before castration, 30 min, and on d 2, 6, 8 after castration, and weekly thereafter until d 36 post-castration. Eye images were obtained from all calves on d -6 and -30 min before castration, 30 min, and on d 2, 6, 8 after castration, and weekly thereafter until d 36 post-castration. Additionally, eye images were also collected at T0 (at the time castration started), T0.02 (middle of castration procedure; 1.3 min), and T0.05 (end of castration procedure; 3 min) to identify potential changes in blood flow and pain indicators during castration. The infrared pictures were obtained using a FLIR I40 infrared camera (FLIR Systems Ltd., Burlington, ON, Canada) and processed with ThermCam QuickView 1.3 (Flir Systems Inc., Burlington, Canada) by delineating the area of the eye and scrotum and recording the greater temperature within that area. An emissivity coefficient of 0.98 was used to analyze the images. Infrared images obtained from K castrated calves were taken from the area of the scrotum. Images were obtained from the scrotal area and the eye at a distance of 1 m.

2.2.3. Behavioral Observations

2.2.3.1. Stride length

Stride length was measured on d -5, 0 (immediately after castration) and on d 8, 15, 22, 29 and 36 post-castration. A digital video camera was placed 4 m in front of a 15-m-long dirt alley located directly in front of the headgate and squeeze chute where the castration procedure was performed. The camera allowed for the recording of individual animal stride length (of hind legs) as the calves exited the chute (Currah et al., 2009). Images were captured when both back hooves were placed on the ground while walking. Stride length was the distance measured from the middle of each hoof with ImageJ software (National Institutes of Health Image, Bethesda, MD).

2.2.3.2. Visual Analog Scale (VAS)

Behavioral observations during castration were recorded by one experienced observer located 2 m from the animal. The observer recorded their overall impression of the amount of pain the animal was experiencing during castration as previously described by Moya et al. (2014).

2.2.3.3. Feeding behavior

All calves were fitted with a radio-frequency ear tag (Allflex Canada, St-Hyacinthe, Canada) on d -21 and placed in pens equipped with an electronic feed bunk monitoring system (GrowSafe Systems Ltd., Airdrie, AB, Canada) that automatically recorded individual visits to the feeders, feed intake and feeding behavior, as previously described by González et al. (2009). All feeding behavior-related data for each calf was recorded 24 h/d from d -21 to d 71 relative to castration. An ear tag transponder was read by an antenna located in the rim of the feed bunk when calves came within 50 cm of the bunk so that feeding behavior could be documented (Schwartzkopf-Genswein et al., 1999). The system recorded daily feeding time (min/d) calculated as the time spent at the feeders within a day, feed intake (dry matter intake, DMI, kg/d), and feeding rate (g of

DM/min) calculated as the daily DMI divided by the feeding time. Feeding events were pooled by meals using a meal criterion of 300 s as previously described by Schwartzkopf-Genswein et al. (2003). The meal criterion allowed the determination of meal frequency (nº/day) calculated as the number of times per day that a non-feeding interval length exceeded the meal criterion, meal size (g DM/meal) which was the average amount of feed consumed in one meal, and meal duration (min/meal) calculated as the length of time per meal. Frequency of visits was calculated as the number of feeding visits per day (number/d) and per meal (number/meal) which were used as an indicators of the animal's activity at the feeding area. The variables were calculated from the feeding behavior data recorded per day but were summarized by week, starting in the day of castration and for each animal (Meléndez et al., 2018b).

2.3. Statistical Analysis

Statistical analyses were carried out using SAS software (SAS version 9.4, SAS Institute Inc., Cary, NC). Box plots were used to identify outliers, while PROC UNIVARIATE was used to verify the normality of error distribution. The GLM procedure was performed to confirm homoscedasticity of variance for all dependent variables using Levene's test. The experimental unit was the animal mixed within pen. Performance data, salivary cortisol concentration and feeding behavior parameters (meal frequency, meal size, feeding rate and visits frequency) were log transformed while a square root +1 transformation was used for VAS, meal duration and feeding time when data did not fit a normal distribution. All baseline data collected before castration were used as covariates for each variable analyzed during the post-castration period (salivary cortisol concentrations on average of d -1 and 0, total fecal *E. coli* on d 0, stride length

and scrotal temperatures on d -6, and eye temperatures on average of d -6 and -1 before castration). Initial BW (average of d -6 and -5) was used as a covariate for all analyzed variables. A mixed-effects model with repeated measures was performed with the PROC MIXED (SAS version 9.4, SAS Institute Inc., Cary, NC) procedure and included castration method (CAS), medication (MED), time (T), and their interactions as fixed effects, whereas group nested within pen was included as a random effect. The nesting option was included in the repeated statement of the MIXED procedure for all dependent variables with heterogeneous variance. Degrees of freedom were calculated using the Kenward-Rogers method and the covariance structure (unstructured, compound symmetry, and autoregressive order 1) was chosen to minimize Schwarz's Bayesian information criterion. A *post-hoc* (Tukey's) test was used to compare adjusted means at significance levels established at $P \leq 0.05$ and a tendency between $0.05 < P \leq 0.10$.

3. Results

During the experimental period, one KM calf was diagnosed with a clostridial infection caused by a malign edema and died 14 d after castration and another KNM calf died due to respiratory disease 37 d after castration therefore, both calves were removed from the study dataset. A total of 17 medical treatments were recorded from which 2, 3, 4 and 8 occurred in BNM, CN, KM and KNM calves, respectively. Abscesses (up to 30 cm in diameter) within the scrotal sack were observed in 8 K calves and this animals were included in the statistical analysis. The abscesses were opened with a surgical blade to allow drainage.

3.1. Performance

Final BW and ADG were greater (castration effect, $P \leq 0.02$) in C calves than B and K calves (Table 1). Final BW did not differ ($P > 0.10$) between the K and B calves. However, ADG of B calves tended ($P = 0.06$) to be greater than K calves (0.8 ± 0.10 and 0.7 ± 0.10 kg/d, respectively). No medication effects ($P > 0.10$) were observed for any performance parameters (Table 1).

3.2. Salivary cortisol

Salivary cortisol concentrations were greater (castration × time effect, $P = 0.05$; Table 1) in B and K calves compared to C calves up to 4-h post-castration, and remained greater only in K calves up to d 2 post-castration compared to C and B calves (Figure 1). Cortisol concentrations were lower (medication effect, $P = 0.01$) in M (2.7 ± 0.06 nmol/L) compared to NM calves (3.2 ± 0.06 nmol/L) after castration.

3.3. E. coli

Total fecal *E. coli* had a significant three way interaction (castration × medication × time effect, $P = 0.01$; Table 1). The BM calves shed less ($P < 0.05$) compared to CNM calves 2 d post-castration, however no differences ($P > 0.10$) were observed among BM and CNM calves and the other treatments (Figure 2). The KM calves shed less ($P < 0.05$) compared to CM, BM and KNM calves 6 d post-castration, but no differences ($P > 0.10$) were observed among these treatments and BNM and CNM calves. In addition, CNM calves shed less ($P < 0.05$) *E. coli* compared to CM,

KNM and BM calves 6 d after castration. No differences ($P > 0.10$) were observed in *E. coli* shedding among treatments on d 8 or 22 after castration.

3.4. Scrotal and eye temperature.

Scrotal temperature was greater (castration × time effect, $P < 0.01$) in K calves, intermediate in C calves and lowest in B calves at all time points with the exception of 30 min and d 22 and 36 post-castration, where K calves did not differ ($P > 0.10$) from C calves (Figure 3). No medication effects ($P > 0.10$; Table 1) were observed for scrotal temperature. Scrotal temperatures tended ($P = 0.09$) to be greater in KM and KNM calves (34.6 ± 0.29 and 34.7 ± 0.39 °C) compared to CM and CNM (33.1 ± 0.32 and 32.1 ± 0.39 °C) as well as to be lower in BM and BNM (21.5 ± 0.53 and 22.31 ± 0.55 °C) compared to others treatments (Table 1).

Eye temperature was greater (castration effect, $P < 0.05$) in B (37.3 ± 0.11 °C) and K calves (37.4 ± 0.11 °C) compared to C calves (37.1 ± 0.11 °C). Overall, eye temperature increased (time effect, $P < 0.01$) after castration, with lower temperatures recorded at the commencement of castration (T0, 36.7 ± 0.13 °C) and increasing up to 0.5 min after castration started, however no differences ($P > 0.10$) was observed among middle measures (T0.02, 36.9 ± 0.12 °C), final measures (T0.05, 37.0 ± 0.13 °C), and at 30 min post-castration (37.0 ± 0.14 °C). Eye temperature remained high on d 2 (37.8 ± 0.12 °C), 6 (38.0 ± 0.12 °C) up to d 22 (37.1 ± 0.12 °C) post-castration and then returned to similar baseline levels on d 29 (36.8 ± 0.12 °C) and increased again on d 36 (37.8 ± 0.12 °C) post-castration. No medication effects ($P > 0.10$; Table 1) were observed for eye temperature.

297 *3.5. Stride length and VAS*

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299 No castration, medication, time or interaction effects ($P > 0.10$) were observed for stride
300 length (Table 1). VAS scores were greater (castration effect, $P < 0.05$) in K (3.1 ± 0.14 cm)
301 compared to C (1.9 ± 0.14 cm) and B calves (1.6 ± 0.14 cm) while no differences ($P > 0.10$) were
302 observed between C and B calves. VAS scores were also greater (medication effect, $P < 0.01$;
303 Table 1) in NM (3.0 ± 0.13 cm) compared to M calves (1.5 ± 0.13 cm).

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305 *3.6. Feeding behavior*

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307 A significant interaction (castration × time effect, $P = 0.04$; Table 2) for frequency of feeding
308 visits per day was observed, however, despite the observed significance the *post-hoc* test did not
309 detect treatment differences. Meal duration was greater (castration × time effect, $P = 0.01$) in B
310 (18.6 ± 0.14 min/meal) and C calves (19.4 ± 0.14 min/meal) compared to K calves (15.4 ± 0.14
311 min/meal) on the day of castration. Daily DMI and meal size were greater (medication effect, $P =$
312 0.01 and $P = 0.05$, respectively) in M (8.2 ± 0.13 kg of DM/day and 1.2 ± 0.01 kg of DM/meal,
313 respectively) compared to NM calves (7.5 ± 0.24 kg of DM/day and 1.1 ± 0.04 kg of DM/meal,
314 respectively). No castration or interactions effects ($P > 0.10$; Table 2) were observed for DMI,
315 feeding rate, feeding time, visit frequency (n/meal) meal frequency or meal size. No medication
316 effects ($P > 0.10$) were observed for feeding rate, feeding time, visit frequency, meal frequency or
317 meal duration (Table 2).

318

319 **4. Discussion**

320

321 *4.1. Performance*

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323 In the present study, band and knife castrated calves had a 29% reduction in ADG compared
324 to non-castrated calves over the 10-wk study period, which explains why the final BW of castrated
325 calves was 4.7% lower than the non-castrated calves. Similarly, González et al. (2010) reported a
326 reduction in ADG of 31% in 6-mo-old band castrated compared to intact bull calves over a 6-wk
327 period post-castration, while Marti et al. (2017) reported a 12% reduction in ADG in 1-wk-old and
328 4-mo-old beef calves over a 9-wk period post-knife castration compared to non-castrated and band
329 castrated calves. Lack of, or reduced performance has also been reported after surgical (Ting et al.,
330 2003a; Webster et al., 2013; Mintline et al., 2014) and band (Repenning et al., 2013; Moya et al.,
331 2014) castration even after the administration of NSAIDs (flunixin meglumine, ketoprofen, or
332 meloxican). The reduction of ADG and consequently final BW observed in castrated calves in this
333 study is likely related to pain and discomfort caused by tissue trauma (Weary et al., 2006; Moya
334 et al., 2014), as well as the interruption of testosterone production associated with castration (Ting
335 et al., 2003a, b; Marti et al., 2010; Nian et al., 2018). Castration triggers an immune response which
336 is associated with tissue damage (Stafford, 2007; Moya et al., 2014) and thus requires that energy
337 is allocated for tissue repair (Mintline et al., 2014) and the restoration of homeostasis after
338 castration instead of growth (Anderson and Muir, 2005).

339 The combination of flunixin meglumine and lidocaine used to control procedural and post-
340 operative pain did not improve ADG in castrated calves. However, the fact that castrated calves
341 given medication did not have lower growth rates compared to their non-castrated and non-
342 medicated counterparts indicates the pain control strategy used in this study had some benefit;

particularly when taking into account that non-castrated calves would have the advantage of continued testosterone secretion related to increase growth rates in calves (Miyamoto et al., 1989; Stafford and Mellor, 2005). Similar to the results observed in the present study, the combination of xylazine epidural and flunixin meglumine before band application (González et al., 2010), xylazine plus lidocaine epidural and ketoprofen or lidocaine alone during burdizzo castration (Ting et al., 2003b), and the administration of post-operative topical anesthetic and pre-operative buccal meloxicam alone or in combination after surgical castration (Van der Saag et al., 2018) in beef cattle, did not prevent a reduction in growth rates compared to non-castrated calves.

4.2. Physiology

Cortisol is a corticosteroid hormone released by the hypothalamic-pituitary-adrenal (HPA) axis during stress and has been widely used as an indicator of acute pain and physiological stress associated with castration (Stafford et al., 2002; Stafford and Mellor, 2005; Marti et al., 2015; Meléndez et al., 2017b; Meléndez et al., 2018a). Elevated concentrations of cortisol and catecholamine caused by castration pain have been reported to increase body temperature and heart rate frequency associated with the autonomic response (Stewart et al., 2010). In the present study, the elevated cortisol concentration evidenced after band and knife castration are in agreement with previous findings assessing band and knife castration at different ages; 1-week-old calves (Meléndez et al., 2018a), 2-mo-old (Meléndez et al., 2017b), 4-mo-old (Stewart et al., 2010), and weaned calves (Moya et al., 2014; Marti et al. 2015). The rapid rise in salivary cortisol concentration in both band and knife castration up to 4 h after castration could be explained by the acute response to castration, although the prolonged duration (2 d) of elevated salivary cortisol in

366 knife castrated calves indicates that this procedure caused greater pain and stress than band
367 castration.

368 Several studies have reported that the combination of an anesthetic and analgesic administered
369 at the time of castration prevented increases in cortisol concentrations after the procedure (Stafford
370 et al., 2002; Ting et al., 2003b; Stilwell et al., 2008). The present study showed that the use of a
371 combination of lidocaine and a flunixin meglumine effectively reduced salivary cortisol
372 concentrations compared to non-medicated calves. Webster et al. (2013) reported that flunixin
373 meglumine (i.v., 1.1 mg/kg BW) alone tended to shorten the duration of the cortisol response while
374 flunixin meglumine in combination with lidocaine eliminated the cortisol response in 2- to 3-mo-
375 old Holstein-Friesian bull calves after surgical castration. Additionally, González et al. (2010)
376 observed the elimination of acute cortisol response when using a xylazine epidural and flunixin
377 meglumine (i.v., 1.1 mg/kg BW) in 6-mo-old bull calves before band castration. In contrast, Marti
378 et al. (2010) found no differences in cortisol concentrations in 3-mo-old band castrated Holstein
379 bull calves compared to non-castrated calves, using the same combination of lidocaine and
380 flunixin meglumine (i.m., 3 mg/kg of BW). Meléndez et al. (2018b) did not observe a synergistic
381 effect of combined lidocaine and meloxicam (s.c., 0.5 mg/kg BW) in mitigating salivary cortisol
382 concentrations during and after knife castration in 7- to 8-mo-old Angus beef calves. However,
383 they did report a reduction in cortisol concentrations in calves that were administered lidocaine
384 and meloxicam alone, which is likely the result of each drug acting at different time points.
385 Differences among studies using the same combination of medications can be explained by
386 differences in the route and dosage of the administered drugs. For example, the pharmacokinetics
387 of flunixin meglunine differs according to the route of delivery (intramuscular, subcutaneous or
388 intravenous injection) as well as the period of action which is related to drug half-life, absorption

and availability in body tissues (Kissel et al., 2012). Currently, flunixin meglumine is only labeled for i.v. administration and therefore s.c. delivery would be considered extra-label use. However, s.c. flunixin meglumine results in a significantly longer half-life (5.4 ± 2.5 h) compared to the i.v. (3.4 ± 1.0 h) route which is associated with prolonged absorption, accumulation and slower release by the tissues (Kissel et al., 2012) and thus, effective in relieving pain in the studied cattle.

E. coli is an enteropathogen that causes diarrhea and septicemia in calves and affects the production system in general (Coura et al., 2014). The conditions in which shedding of this pathogen occurs within beef production systems is of great interest due to its link with human food safety (Stein and Katz, 2017). Previous studies have suggested a relationship between stress in livestock and increased pathogen shedding (Cray et al., 1998; Bach et al., 2004). The detrimental effects of stress on immune function are well documented and are associated with the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent production of excess glucocorticoids which results in the destabilization of innate immune barriers such that the host is more susceptible to pathogen invasion (Jeffery and Forsberg, 2007). Consequently, castrated calves may be more susceptible to *E. coli* invasion and as hypothesized in the current study, pain mitigation may play a role in reducing shedding in calves that undergo painful/stressful management procedures by mitigating the cortisol response. González et al. (2010) reported that the use of pain control (xylazine and flunixin megulime) at the time of band castration reduced *E. coli* shedding in 6- to 8-mo-old beef calves compared to non-castrated calves, which was attibuted to a reduction in glucocorticoid secretion and consequently a stronger immune system. The limited effects of castration and medication effect in this study may be due to the fact that the sampling times were not frequent enough to capture changes in *E. coli* shedding within the first hours after acute stress would have been experienced and at the time that the drugs would have had optimal

412 effect. Consequently, future studies assessing pain mitigation strategies for castration on *E. coli*
413 shedding should increase the number of sampling points within the first 2 days after castration.

414 Infrared thermography is a non-invasive technique to detect changes in surface temperature
415 through the peripheral blood flow in response to painful husbandry procedures and thus useful in
416 measuring pain/stress in animals (Stewart et al., 2010; Mintline et al., 2014; Moya et al., 2014;
417 Marti et al., 2017). In the present study, the greater maximum scrotal temperatures occurred in
418 knife castrated calves, while non-castrated and band castrated calves had intermediate and the
419 lowest scrotal temperatures, respectively for up to 15 d after castration, with an increase on scrotum
420 temperatures again on d 36. The results of the current study suggested greater scrotal inflammation
421 in knife castrated calves compared to non-castrated calves in the days after castration, related to
422 tissue damage. Similarly, recent studies assessing the effect of band and knife castration on acute
423 (up to 7 d post-castration) (Meléndez et al., 2017b) and chronic pain (between 7 d and weekly until
424 the testicles sloughed off (63, 49 and 42 d post-castration) (Marti et al., 2017) in 1-wk, 2-mo and
425 4-mo-old calves also report lower temperatures in band compared to knife castrated and non-
426 castrated calves.

427 The combination of s.c. flunixin meglumine and lidocaine used in the present study did not
428 reduce scrotal temperature (inflammation) in castrated calves. Similarly, i.v. flunixin meglumine
429 did not affect scrotal temperature in knife castrated calves (Mintline et al., 2014) while i.m.
430 ketoprofen did not affect scrotal temperatures in either knife or band castrated calves (Moya et al.,
431 2014). In contrast, some studies have reported lower scrotal temperature and reduced wound
432 inflammation in surgically castrated calves after the administration of an NSAID including buccal
433 meloxicam (Van der Saag et al., 2018), s.c. meloxicam (Meléndez et al., 2018b) or i.v. ketoprofen

(Ting et al., 2003a). Differences in scrotal temperature among studies could be due to the pharmacokinetics of each NSAID, dosage, administration time and route of administration used.

Similar to scrotal temperature, ocular temperature has been used as a practical and non-invasive indicator of body temperature to assess acute pain via the activation of autonomic nervous system (ANS) (Gloster et al., 2011; Stewart et al., 2010). In the present study, eye temperature increased in calves during band and knife castration, compared to non-castrated calves remained greater for up to 8 d post-castration. Other researchers studying the consequences of stressful procedures have also reported increases in eye temperatures up to 3 min after band and surgical castration in calves (Dockweiler et al., 2013) and immediately after events such as competitive show jumping in horses (Valera et al., 2012).

4.3. Behavior

Although as a subjective measure, the VAS has been a valuable tool to evaluate procedural pain and together with other physiological and behavioral indicators helped to assess the effects of castration (Thüer et al., 2007; Moya et al., 2014; Meléndez et al., 2018a). The findings for VAS assessed during castration were similar to previous results which reported that calves were most responsive to knife castration, indicating that knife castration results in more acute pain (Meléndez et al., 2018a). This is consistent with the greater pain related response of weaned calves to surgical castration observed by Moya et al. (2014), while in 1-wk, 2-mo and 4-mo old calves, greater VAS values were observed in both knife and band castrated calves (Meléndez et al., 2017b). The lack of difference in VAS scores between banded and non-castrated calves observed in the present study maybe due to the fact that discomfort experienced during band application was not

sufficiently different from the manipulation of the testicles in non-castrated calves to cause a behavioral change. The activation of chemical nociceptors in knife castrated calves occurs via inflammatory substances released in response to the scrotal incision, traction of testicles and cutting of the spermatic cords which is a stimulus sufficiently intense to generate immediate discomfort and also produce behavioral changes during castration (Woolf, 2010). The combination of drugs (anesthetic and analgesic) administered before to knife and band castration in this study helped to reduce the acute behavioral response (reduced VAS), but did not completely eliminate all pain related behaviors as medicated calves exhibited some indicators of pain compared to non-castrated calves. Previous studies did not observe a beneficial effect of NSAIDs including ketoprofen (Moya et al., 2014) and meloxicam (Meléndez et al., 2018a) on VAS during castration. In contrast, only lidocaine was able to minimize the immediate expression of pain during burdizzo (Thüer et al., 2007) and surgical castration (Meléndez et al., 2018b) while in non-castrated calves signs of pain were observed. The observed reduction of overt signs of pain during castration in the present study is likely related to the effects of lidocaine on reducing acute nociception.

Currah et al. (2009) was the first to use stride length measurements in combination with the number of steps calves took to assess castration related pain. The same authors reported that bull calves receiving lidocaine and flunixin meglumine had decreased stride lengths at 4 and 8 h post-surgical castration compared to calves without analgesia (Currah et al., 2009). Based on these findings, our hypothesis was that band and knife castrated calves would show some variation in stride length associated with pain and the use of medication could eliminate changes in stride length, however the hypothesis was not substantiated as no castration or medication effects were observed in the current study. This finding is in agreement with Marti et al. (2017) who found no differences in the stride length of band or knife castrated calves compared to controls at either 1-

wk, 2 or 4-mo-old calves, and Meléndez et al. (2018b) who assessed the effects of meloxicam and lidocaine alone or in combination on indicators of pain associated with castration in 7-8-mo-old beef calves.

The positive impact (absence of weight loss compared to non-castrated) of pain medication on growth performance previously mentioned was also supported by the differences observed in calf feeding behavior. For example, medicated calves had greater daily DMI, and meal size compared to non-medicated calves, regardless of the method of castration which may help explain why no growth rate differences between band and knife castration were observed. Similarly, no differences in DMI or feeding behavior were observed after burdizzo castration using either a combination of xylazine and lidocaine epidural, or ketoprofen and lidocaine administered alone (Ting et al., 2003b), or after band or surgical castration using only ketoprofen (Moya et al., 2014). In contrast, some studies have shown DMI reduction and reduced feeding activities after the administration of a combination of flunixin meglumine and xylazine epidural before band castration which may be a consequence of a reduced calf mobility after epidural anesthesia (González et al., 2010). Knife castrated calves on the day of castration in the current study had fewer daily visits to the feed bunk and the meal duration was shorter than B and C calves, possibly due to reluctance to walk to the feeder as a result of castration associated pain. However, in the first and second week, the K castrated calves had compensatory feeding behaviour that resulted in increased meal duration compared to C calves. The noxious stimulus during surgical castration was sufficiently intense to activate nociceptive neurons related to pain which decreased food intake (Malick et al., 2001; Weary et al., 2006) similar to the results observed in knife castrated calves in this study.

5. Conclusions

On the basis of our results, there is evidence that knife castrated calves experienced more acute pain than band castrated calves as demonstrated by greater VAS scores during castration, increased salivary cortisol concentrations up to 2 d post-castration, and greater scrotal temperatures associated with greater inflammation for up to 15 d post-castration. In addition, band and knife castrated calves had greater cortisol concentrations for up to 4-h post-castration and increased eye temperatures after castration, indicating elevation of body temperature. The combination of flunixin meglumine and lidocaine was effective at reducing behavioral (reduced VAS scores and increased feeding activities) and physiological (reduced salivary cortisol concentrations) indicators of pain in knife and band castrated calves. The combination of medication administered in this study mitigated both procedural and post-operative pain in castrated calves and therefore is a viable strategy for pain control during and after castration in 6-mo-old beef calves. Further studies assessing the effects of lidocaine in combination with a NSAID that have a longer half-life could provide pain control over an extended period of time.

Conflict of interest statement

The authors declare that have no financial or personal relationship with people or organizations that would create a conflict of interest.

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686 **Figure captions**

687

688 **Fig 1.** Least square means and SEM for salivary cortisol concentrations (nmol/L) in non-castrated
689 (C), knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves. Least square means
690 with differing superscripts within sampling time (and days) differ ($P \leq 0.05$).

691

692 **Fig 2.** Least square means and SEM for fecal *Escherichia coli* (log CFU) in non-castrated (C),
693 knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves with (M) or without (NM)
694 a combination of single s.c. administration of flunixin meglumine and lidocaine. Least square
695 means with differing superscripts within sampling day differ ($P \leq 0.05$).

696

697 **Fig 3.** Least square means and SEM for maximum scrotal temperature (°C) in non-castrated (C),
698 knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves. Least square means with
699 differing superscripts within sampling time (and days) differ ($P \leq 0.05$).

Table 1

Least square means (± SEM) of performance (initial BW, final BW and average daily gain - ADG), salivary cortisol concentrations, fecal *Escherichia coli* count, total scrotal temperature, eye temperature, and behavioral observations (stride length and VAS) in non-castrated (C), knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves with (M) or without (NM) a combination of single s.c. administration of flunixin meglumine and lidocaine.

	Treatment[1]													
	C		K		B			*P*-value[2]						
Item	NM	M	NM	M	NM	M	*SEM*	CAS	MED	CAS × MED	T	CAS × T	MED ×T	CAS × MED x T
Performance[3]														
Initial BW, kg	279.8	280.6	283.5	287.5	282.6	280.2	5.76	0.53	0.84	0.83	-	-	-	-
Final BW (d 71), kg	348.3	354.7	331.8	336.8	336.1	335.7	6.63	0.02	0.49	0.86	-	-	-	-
ADG, kg/d	0.98	1.15	0.67	0.75	0.81	0.80	0.14	<0.01	0.12	0.80	-	-	-	-
Salivary cortisol[3], nmol/L	2.6	2.4	3.4	2.9	3.6	2.8	0.09	0.01	0.01	0.85	<0.01	0.05	0.63	0.58
Fecal *E. coli*, log CFU	6.0	6.3	6.3	5.9	5.9	6.1	0.24	0.86	0.75	0.16	0.20	0.73	0.09	0.01
Eye temperature, ºC	37.2	37.1	37.2	37.5	37.4	37.3	0.14	0.04	0.67	0.14	<0.01	0.29	0.14	0.79
Scrotal temperature, ºC	32.1	33.1	34.7	34.6	22.3	21.5	0.42	<0.01	0.92	0.09	<0.01	<0.01	0.88	0.85
Behavioral observations														
Stride length, cm	53.7	51.1	53.0	55.3	52.8	52.9	1.77	0.53	0.97	0.26	0.31	0.88	0.72	0.48
VAS[4], cm	2.8	1.0	3.9	2.4	2.1	1.0	0.17	0.02	<0.01	0.85	-	-	-	-

[1]C: Non-castrated calves submitted to the same handling procedure as castrated ones; K: Calves castrated using a Newberry knife; B: Calves castrated using rubber band; NM: single s.c. injection of lactated ringer's, 30 min before castration; M: combination of a single intra-testicular injection of lidocaine and a lidocaine ring block (2% HCl lidocaine with epinephrine), and single s.c. dose of flunixin meglumine in the neck (2.2 mg/kg), 30 min before castration.

[2]CAS: Castration treatment effect; MED: Medication treatment effect; T: Sampling time effect.

[3]The values presented correspond to non-transformed means; SEM and *P* - values correspond to ANOVA analysis using the base-*e* log transformed data.

[4]The values presented correspond to non-transformed means; SEM and *P* - values correspond to ANOVA analysis using the square-root+1 transformed data.

Table 2

Least square means (± SEM) of feeding behavior in non-castrated (C), knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves with (M) or without (NM) a combination of single s.c. administration of flunixin meglumine and lidocaine.

	Treatment[1]													
	C		K		B			*P*-value[2]						
Item	NM	M	NM	M	NM	M	*SEM*	CAS	MED	CAS × MED	T	CAS × T	MED × T	CAS × MED × T
DM intake, kg/d	7.5	8.5	7.4	8.2	7.8	7.9	0.29	0.68	0.01	0.29	<0.01	0.90	0.96	0.68
Feeding rate[3], g of DM/min	98.2	80.7	80.2	89.8	92.1	78.2	0.03	0.96	0.30	0.30	<0.01	0.93	0.21	0.77
Feeding time[4], min/d	96.6	111.1	99.4	99.4	96.4	104.1	0.41	0.78	0.24	0.69	<0.01	0.89	0.93	0.72
Visit frequency[3], n/d	75.6	69.8	67.0	76.5	66.7	78.5	0.05	0.90	0.42	0.43	<0.01	0.04	0.34	0.82
Visit frequency, n/meal	13.75	11.37	10.75	12.78	11.49	13.16	0.059	0.71	0.56	0.15	<0.01	0.13	0.72	0.91
Meal frequency[3], n/d	6.09	6.45	6.85	6.46	6.38	6.40	0.017	0.34	0.84	0.39	<0.01	0.09	0.46	0.26
Meal size[3], kg of DM/meal	1.23	1.28	1.07	1.24	1.18	1.22	0.021	0.15	0.05	0.56	<0.01	0.16	0.95	0.88
Meal duration[4], min/meal	16.51	18.57	15.65	16.91	15.37	17.37	0.180	0.67	0.20	0.90	<0.01	0.01	0.48	0.63

[1]C: Non-castrated calves submitted to the same handling procedure as castrated ones; K: Calves castrated using a Newberry knife; B: Calves castrated using rubber band; NM: single s.c. injection of lactated ringer's, 30 min before castration; M: combination of a single intra-testicular injection of lidocaine and a lidocaine ring block (2% HCl lidocaine with epinephrine), and single s.c. dose of flunixin meglumine in the neck (2.2 mg/kg), 30 min before castration.

[2]CAS: Castration treatment effect; MED: Medication treatment effect; T: Sampling time effect.

[3]The values presented correspond to non-transformed means; SEM and *P* - values correspond to ANOVA analysis using the base-*e* log transformed data.

[4]The values presented correspond to non-transformed means; SEM and *P* - values correspond to ANOVA analysis using the square-root+1 transformed data.

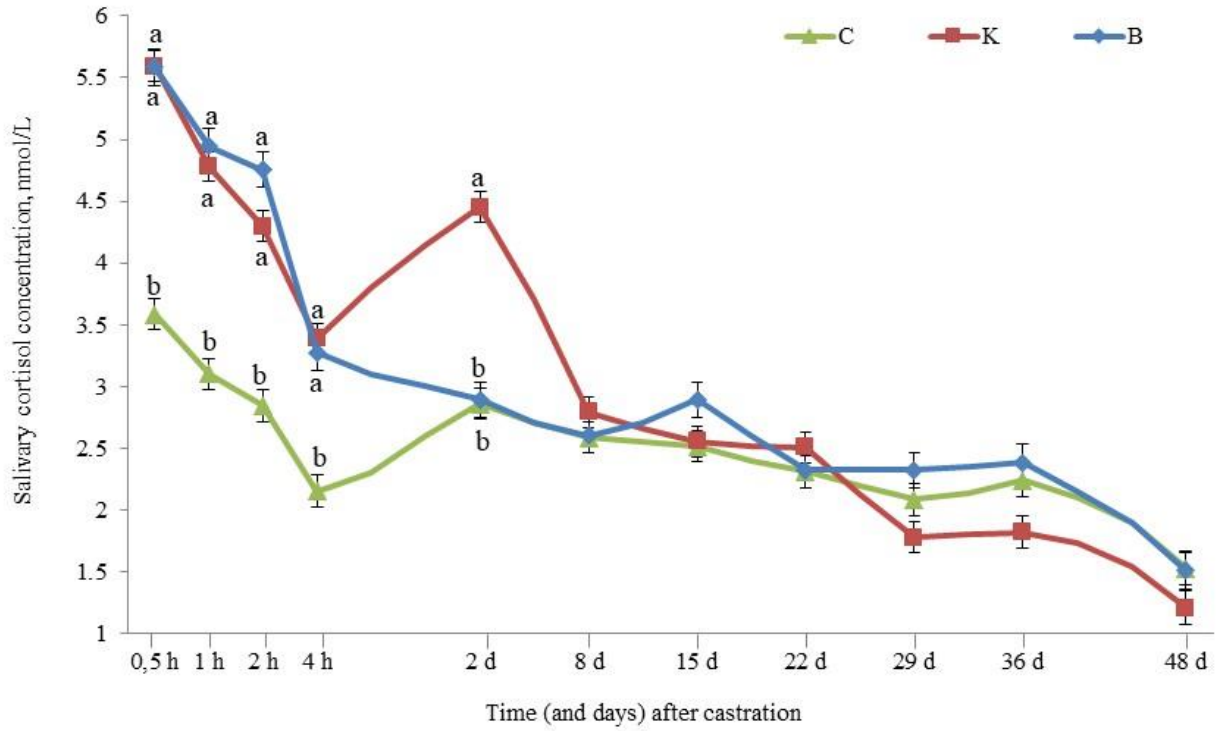
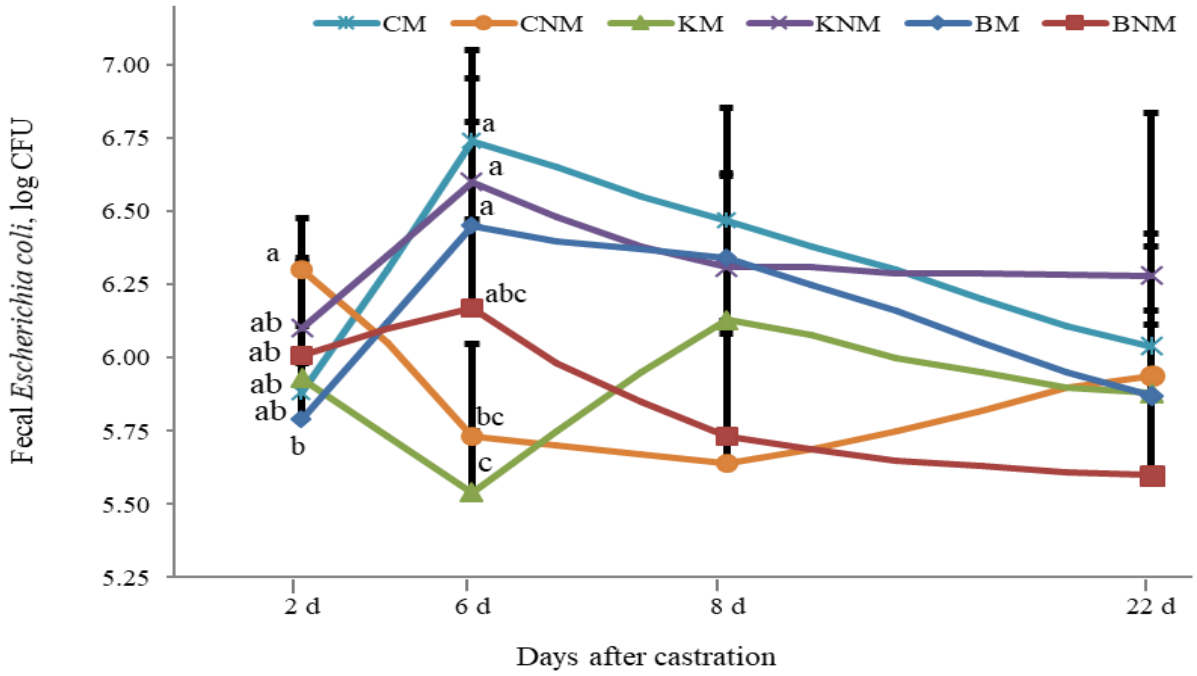


Fig 1. Least square means and SEM for salivary cortisol concentrations (nmol/L) in non-castrated (C), knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves. Least square means with differing superscripts within sampling time (and days) differ ($P \leq 0.05$).



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16 **Fig 2.** Least square means and SEM for fecal *Escherichia coli* (log CFU) in non-castrated (C),
17 knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves with (M) or without (NM)
18 a combination of single s.c. administration of flunixin meglumine and lidocaine. Least square
19 means with differing superscripts within sampling day differ ($P \leq 0.05$).
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21

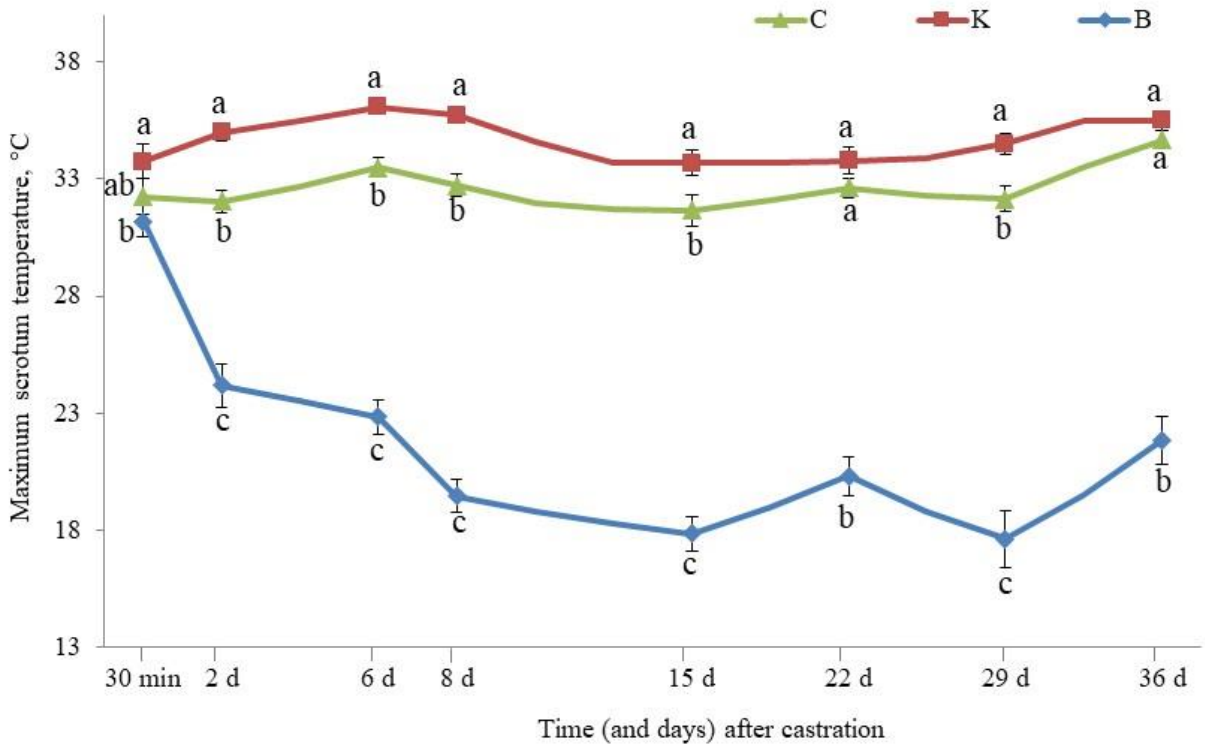

Fig 3. Least square means and SEM for maximum scrotal temperature (°C) in non-castrated (C), knife castrated (K) and band castrated (B) 6-mo-old Angus bull calves. Least square means with differing superscripts within sampling time (and days) differ ($P \leq 0.05$).