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Evaluating Approaches to Measuring Ocular Pain in Bovine Calves with Corneal Scarification and Infectious Bovine Keratoconjunctivitis–Associated Corneal Ulcerations

Abstract

Infectious bovine keratoconjunctivitis (IBK) is a common ocular disease in cattle, associated with a 6.8 to 13.6 kg decrease in weaning weight. Antibiotic therapy is available but it is unclear if pain mitigation as an adjunct therapy would reduce the weight loss associated with IBK. Before assessing the impact of pain mitigation therapies, it is first necessary to validate approaches to qualifying ocular pain. The objective of this study was to evaluate approaches to qualifying ocular pain in bovine calves (*Bos taurus*) with IBK. Our a priori assumption was that scarification or corneal ulcerations consistent with IBK are painful compared to normal eyes. To quantify this difference in pain, we assessed 4 tools: pressure algometry-mechanical nociceptive threshold (PA-MNT), corneal touch thresholds (CTT) obtained with the use of a Cochet-Bonnet aesthesiometer, and assessment for the presence of blepharospasm and photophobia as metrics for pain. Using a 1-eye randomized controlled challenge trial, 31 calves with healthy eyes were randomly allocated to treatment groups, and then a left or right eye was randomly assigned for corneal scarification and inoculation with *Moraxella bovoculi* or *Moraxella bovis*. A repeated measures analysis of variance was used for PA-MNT, with significance set at $P < 0.05$. A log (base 10) transformation was used to stabilize the variance, and Tukey's *t* tests were used to test differences between assessment days for each landmark. Calves had statistically significantly lower PA-MNT scores (which indicates more pain) the day after scarification relative to baseline measurements (4 d before scarification). For example, at 1 landmark the median PA-MNT (kg/force) prescarification was 4.82 (95% confidence interval [CI]: 3.92-5.93) and 3.43 (95% CI: 2.79-4.22) postscarification. These data suggest PA-MNT may be a tool for quantifying ocular pain in calves. No differences ($P < 0.1$) in PA-MNT scores between scarified and not-scarified eyes were detected for any landmark on any day. This result suggests that the pain response occurs over the entire face, not just the affected eye. Corneal ulcerations consistent with IBK were not associated with statistically significant differences in PA-MNT or CTT at eye or calf levels. Not surprisingly, scarified eyes were more likely to exhibit blepharospasm and photophobia compared to healthy eyes. Due to blepharospasm, the use of the Cochet-Bonnet to evaluate corneal sensitivity by CTT was of limited value.

Keywords

animal welfare, calves, infectious bovine keratoconjunctivitis, nociception, ocular pain, pinkeye

Disciplines

Eye Diseases | Large or Food Animal and Equine Medicine | Veterinary Infectious Diseases

Comments

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Evaluating approaches to measuring ocular pain in bovine calves with corneal scarification and infectious bovine keratoconjunctivitis–associated corneal ulcerations¹

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ABSTRACT: Infectious bovine keratoconjunctivitis (IBK) is a common ocular disease in cattle, associated with a 6.8 to 13.6 kg decrease in weaning weight. Antibiotic therapy is available but it is unclear if pain mitigation as an adjunct therapy would reduce the weight loss associated with IBK. Before assessing the impact of pain mitigation therapies, it is first necessary to validate approaches to qualifying ocular pain. The objective of this study was to evaluate approaches to qualifying ocular pain in bovine calves (*Bos taurus*) with IBK. Our a priori assumption was that scarification or corneal ulcerations consistent with IBK are painful compared to normal eyes. To quantify this difference in pain, we assessed 4 tools: pressure algometry–mechanical nociceptive threshold (PA-MNT), corneal touch thresholds (CTT) obtained with the use of a Cochet-Bonnet aesthesiometer, and assessment for the presence of blepharospasm and photophobia as metrics for pain. Using a 1-eye randomized controlled challenge trial, 31 calves with healthy eyes were randomly allocated to treatment groups, and then a left or right eye was randomly assigned for corneal scarification and inoculation with *Moraxella bovoculi* or *Moraxella bovis*. A repeated measures analysis of vari-

ance was used for PA-MNT, with significance set at $P < 0.05$. A log (base 10) transformation was used to stabilize the variance, and Tukey's t tests were used to test differences between assessment days for each landmark. Calves had statistically significantly lower PA-MNT scores (which indicates more pain) the day after scarification relative to baseline measurements (4 d before scarification). For example, at 1 landmark the median PA-MNT (kg/force) pre-scarification was 4.82 (95% confidence interval [CI]: 3.92–5.93) and 3.43 (95% CI: 2.79–4.22) post-scarification. These data suggest PA-MNT may be a tool for quantifying ocular pain in calves. No differences ($P < 0.1$) in PA-MNT scores between scarified and not-scarified eyes were detected for any landmark on any day. This result suggests that the pain response occurs over the entire face, not just the affected eye. Corneal ulcerations consistent with IBK were not associated with statistically significant differences in PA-MNT or CTT at eye or calf levels. Not surprisingly, scarified eyes were more likely to exhibit blepharospasm and photophobia compared to healthy eyes. Due to blepharospasm, the use of the Cochet-Bonnet to evaluate corneal sensitivity by CTT was of limited value.

Key words: animal welfare, calves, infectious bovine keratoconjunctivitis, nociception, ocular pain, pinkeye

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INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK) is a common disease in cattle. Infectious bovine keratoconjunctivitis is an important ocular disease in cattle and has been associated with a 6.8 to 13.6 kg decrease in weaning weight (Funk et al., 2009). Infectious bovine keratoconjunctivitis can occur in 20 to 30% of

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calves in a single beef calf crop, with an estimated 30% of beef herds affected annually (Brown et al., 1998). Clinical signs of IBK include corneal edema, corneal ulceration, photophobia, blepharospasm, and ephiphora (Gelatt, 2008; George, 1984).

Ocular abnormalities such as corneal ulceration and perforation resulting from IBK are thought to be painful for cattle; however, the extent of pain has not been quantified. The absence of evaluated methods for quantifying ocular pain in cattle presents challenges for designing studies to assess pain mitigation strategies for IBK. Since the magnitude and variation of measurements are unknown, researchers cannot determine appropriate alternative hypotheses for required sample size determination. Validated methods for quantification of pain associated with ocular abnormalities are necessary for identification and validation of effective pain mitigation strategies.

Pressure algometry is a noninvasive technique to quantify changes in pain sensitivity by gradually increasing force applied to a specific area until a withdrawal response is observed at the mechanical nociceptive threshold (MNT). Similarly, Cochet-Bonnet aesthesiometers determine pain sensitivity associated with the corneal touch threshold (CTT) and have been used to assess corneal sensitivity in mammals (humans [Beuerman and McCulley, 1978], equine [Brooks et al., 2000; Kaps et al., 2003], feline [Blocker and van der Woerd, 2001] canine [Good et al., 2003], guinea pig [Trost et al., 2007], and camelid [Welihoziy et al., 2011; Rankin et al., 2012]). The association of photophobia and pain is suggested by the reported demonstration in humans and rats of a neurologic association between pain through noxious neural stimulation in response to a bright light (Moulton et al., 2009; Okamoto et al., 2010). In cattle, blepharospasm is observed in cattle experiencing IBK-associated corneal ulceration or corneal scarification and assumed to be a manifestation of pain; however, association between blepharospasm and ocular pain has not been empirically validated (Williams, 2010).

The objective of this study was to address this absence of information by investigating 4 potential techniques for measuring ocular pain in cattle: MNT as measured by pressure algometry, CTT as measured by Cochet-Bonnet aesthesiometer, and presence of blepharospasm and photophobia.

MATERIALS AND METHODS

The study reported herein is a hypothesis generating study conducted using animals enrolled in a randomized and blinded disease challenge study. The original study tested the hypothesis that *Moraxella bovoculi* was associated with IBK incidence (Gould et al., 2013). Here, we describe aspects of the design relevant to the question about ocular pain. The rationale for the secondary use of the animals was to maximize the information obtained from the animals consistent with the 3R principles (Russell and Burch, 2005). Approval for the study was obtained from the Iowa State University (ISU) Institutional Biosafety Committee (IBC number 11-D-0017-A) and the Institutional Animal Care and Use Committee (IACUC 8-11-7187-B).

Study Location and Animal Sourcing

The study was conducted with 3 replicates in January 2011 (**Trial 1**), May 2012 (**Trial 2**), and August 2012 (**Trial 3**). A timeline is presented in Fig. 1. Bovine calves were sourced from the ISU dairy farm (Trial 1 and Trial 2) and a private Iowa-based owner (Trial 3). Calves were predominantly Holstein genetics with some Jersey influence and were 8 to 12 wks of age. Calves were housed in a Biosecurity Level 3 facility at ISU Livestock Infectious Disease Isolation Facility in Ames, IA. For each replicate, all enrolled calves were housed in a single room maintained at 20 to 21°C (68–70°F). Each calf was housed in raised 0.9- by-1.8 m (3- by 6-foot) pens that provided no opportunity for calf-

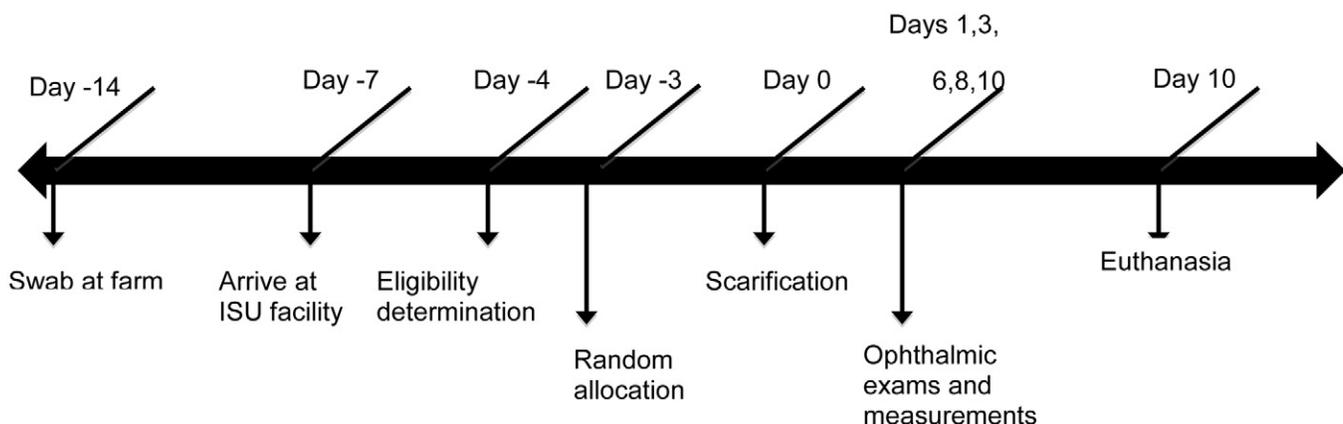


Figure 1. Study timeline. ISU = Iowa State University.

to-calf contact and separate water drinkers and feeders. Calves were provided free choice water and were fed twice daily. Total daily ration included approximately 2.272 kg mixed grass hay and a 1.36 to 2.28 kg of pre-mixed medicated calf starter (Heartland Co-op, Des Moines, IA). Caretakers and research personnel wore protective gloves and clothing when working with the calves, which were changed between calves during animal husbandry and study related activities.

Enrollment of Animals

For each of the 3 replicates, a baseline exam was performed by a team composed of a bovine veterinarian (a veterinary ophthalmologist), and calves without identified ocular disease were enrolled in the study on d -4 relative to scarification and inoculation. A 3.5-V Finoff transilluminator (Welch-Allyn Inc., Skaneateles Falls, NY) and slit-lamp biomicroscope (Welch-Allyn Inc., Kowa Optimed, Inc. Torrance, CA) were used for examination of the eyelids, conjunctiva, cornea, anterior chamber, iris, lens, and anterior vitreous. Additionally, the cornea was assessed for epithelial defects by fluorescein staining (Fluor-I-Strip; Ayerst Laboratories Inc., Philadelphia, PA). Intraocular pressure was measured using a TonoVet (Tiolat Oy, Helsinki, Finland) and tear production was assessed with the Schirmer tear test I. The ocular fundus was evaluated via indirect ophthalmoscopy with a 2.2 Panretinal lens (Volk Optical Inc., Mentor, OH) following pupil dilation with topical tropicamide 1% ophthalmic solution. To prevent allocation bias, the researcher responsible for treatment group allocation did not participate in pre-enrollment ophthalmic exams.

Sample Size

It was not possible to calculate the sample size necessary to detect meaningful differences in MNT due to the absence of estimates of normal thresholds or variation of calves with or without ocular disease, which would be needed to develop an alternative hypothesis. The sample size for the original study was based on estimated IBK risk between groups, such that the aim was to enroll 12 animals in each treatment (i.e., a total of 36 calves) to obtain 80% power to detect an estimated 60% difference in IBK incidence with significance level 0.05

Treatment Group Description and Allocation

On d -4, within each replicate, enrolled calves were allocated to treatment groups using a random number function (Microsoft Excel; Microsoft Corp., Redmond, WA), and then the left or right eye was randomly assigned for corneal scarification. The treatment groups were:

Treatment 1) corneal scarification only,

Treatment 2) corneal scarification and inoculation with *M. bovoculi* (ATCC strain: BAA-1259; origin: California), and

Treatment 3) corneal scarification and inoculation with *Moraxella bovis* (strain Epp63-300; Dr. Rosenbusch lab; origin: National Animal Disease Center).

Corneal scarification was accomplished by a researcher trained in the procedure and according to a published protocol (Rosenbusch and Ostle, 1986). Calves were restrained using a portable modified head restraint placed on the front of each pen. Topical anesthesia (0.5% proparacaine hydrochloride) was applied 3 to 5 min before scarification. A sterilized wire brush approximately 5 mm in length was used to create 3 to 4 horizontal and vertical superficial scratches. For all calves, a swab was rolled across the cornea as well as introduced into the medial conjunctival sac of each scarified eye (type of swab). To inoculate eyes with *M. bovoculi* or *M. bovis*, the sterilized swab had previously been rolled across a blood agar plate containing the organism. For noninoculated calves, the swab was rolled across a sterile blood agar plate. To ensure equal application of scarification, the researcher preparing the swabs concealed the allocation status from the researcher conducting the scarification procedure.

After scarification, calves were evaluated for MNT and CTT on d +1, +3, +6, +8, and +10 to 1) identify centrally located corneal ulcerations consistent with IBK (hereafter termed “IBK-associated corneal ulcerations”) and 2) to assess pain in the ocular region. If the IBK-associated corneal ulceration was 15 mm or greater in diameter, the calf was euthanized. To further prevent information bias, the researcher involved with treatment allocation was not present when outcomes were measured. Likewise, personnel involved with outcome measurement did not participate in allocation procedures. Only 2 researchers assessed corneal lesions in each of the 2 replicates.

Outcomes: Measures of Pain

For this study, animal pain may be defined as “an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal’s physiology and behavior to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery” (Molony and Kent, 1997, p. 266). Pressure algometry was used to measure changes in pain sensitivity, with MNT determined when sufficient force was applied to elicit head withdrawal response, recorded in kilograms of force

(kgf). A hand-held pressure algometer (Wagner Force Ten FDX 50 Compact Digital Force Gage; Wagner Instruments, Greenwich, CT) with a 1-cm² flat rubber tip was used. Calves were restrained using a portable modified head restraint, and the pressure algometer was applied in triplicate at 7 landmarks: 3 landmarks surrounding each eye and a control landmark in the middle of the calf's face (Fig. 2). The pressure algometer was applied perpendicular to the landmark at a rate of approximately 1 kgf/s.

The rationale for the landmarks was:

Landmark 1: the juncture of the dorsal bony orbit and the projection of the calvarium. This landmark was selected to stimulate the supraorbital nerve, which is a terminal branch of the ophthalmic nerve (Budras et al., 2003).

Landmark 2: the notch formed by the frontal and temporal processes of the zygomatic bone. This landmark was selected to stimulate sensory branches (ophthalmic and maxillary divisions) of the trigeminal nerve (Budras et al., 2003).

Landmark 3: 1 cm rostral to the medial canthus of the eye. This landmark was selected to stimulate the infratrochlear nerve, a sensory branch of the ophthalmic nerve (Budras et al., 2003).

Landmark 4 (control): on the face midway between the eyes. This point was included as a control point when testing scarified and nonscarified eyes, because pain sensitivity at this landmark was not expected to change over time.



Figure 2. Algometry landmarks. Landmark 1: the juncture of the dorsal bony orbit and the projection of the calvarium. Landmark 2: the notch formed by the frontal and temporal processes of the zygomatic bone. Landmark 3: 1 cm rostral to the medial canthus of the eye. Landmark 4 (control): on the face midway between the eyes. See online version for figure in color.

The sequence of testing the 4 landmarks per eye was allocated for each calf using a random number generator function (Microsoft Excel; Microsoft Corp., Redmond, WA). Once the testing sequence was generated for the calf, it was consistently used on each evaluation day. The sequence of testing eyes on d -4 was randomly assigned, so half of the calves were tested on the right eye first and half were tested on the left eye first. The subsequent order of testing (right versus left) was alternated daily (i.e., if the right eye was tested first on d -4, then the left eye was tested first on d 1). To reduce potential effects of ophthalmic exams interfering with calf responses, all pressure algometry measurements were collected 1 to 2 h before or after scheduled ophthalmic exams. The same researcher applied the pressure algometry throughout the study and was blinded to the treatment groups. To facilitate blinding to the numeric MNT output and potential effects on application rate, a second researcher recorded the output data from the device.

Corneal touch threshold was measured for each eye using a Cochet-Bonnet aesthesiometer (Luneau; Western Ophthalmics, Lynnwood, WA), which included a nylon filament 0.12 mm in diameter with length ranging from 5 to 60 mm. The force required to elicit the blink reflex and reach the CTT is inversely proportional to the length of the filament. Because a longer length of filament produces less force, blink reflex responses at longer lengths are associated with increased corneal sensitivity. The Cochet-Bonnet was oriented perpendicular to the central cornea, and the eye was touched until an approximately 4 to 5% bend in the filament was produced. Beginning with a 60 mm filament length, the eye was touched up to 5 times and the filament length was subsequently shortened by 5 mm increments until a blink reflex was elicited in 3 out of 5 applications. If 3 blink responses were attained before the fifth application, the CCT testing ceased for that eye on that assessment day. If an eye could not be opened with minimal resistance due to blepharospasm and required topical anesthesia (0.5% proparacaine hydrochloride) to complete other components of the ophthalmic assessment, CTT was not assessed. Evaluations were performed by 2 veterinary ophthalmologists (KLT and RDW) on d -4, and all postscarification evaluations were performed by the same investigator (KLT). To prevent transfer of pathogens between calves, the Cochet-Bonnet filament was disinfected after every use. A sample of the PBS fluid used to rinse the Cochet-Bonnet after disinfection was saved from each calf for every assessment and tested for the presence of *M. bovoculi* and *M. bovis*. These bacteria were not recovered from any of the submitted microtubules.

Photophobia was defined as intolerance to a bright light stimulus and was demonstrated by a head withdrawal response or by closing the eye (Williams, 2010). Pho-

tophobia was assessed using a 3.5-V Finoff transilluminator in a darkened room. The trained evaluator assessed the presence/absence of photophobia as distinct from the dazzle reflex (blinking in response to a bright light). An eye was considered to exhibit blepharospasm when spastic reflex contractions of the eye lids (orbicularis oculi muscles) were observed (Gelatt et al., 2013). Blepharospasm was assessed at normal room lighting. Both photophobia and blepharospasm were measured in a consistent sequence during ophthalmic assessments on d -4, +1, +3, +6, +8, and +10. Evaluations on d -4 were performed by 2 investigators (KLT and DW). After d -4, only 1 researcher assessed blepharospasm and photophobia (KLT).

Statistical Analysis

The aim of our study was to describe the magnitude of the measurements and variation and assess the association of measurement with pain. The a priori assumption was that scarification would be associated with increased pain sensitivity as determined by the 4 measures of pain reported. For all categorical variables the coding used was present (1) or absent (0).

Pressure Algometry–Mechanical Nociceptive Threshold Analysis. Descriptive data including means, medians, minimum and maximum values, and standard deviations of the unadjusted pressure algometry–mechanical nociceptive threshold (PA-MNT) scores were calculated for scarified and nonscarified eyes by assessment day.

Fixed explanatory variables considered for inclusion in the PA-MNT model were assessment day (d -4, +1, +3, +6, +8, and +10), eye-level IBK-associated corneal ulceration status (present or absent), calf-level IBK-associated corneal ulceration status (present or absent), and landmark (7 levels). The 7 landmarks included 3 landmarks surrounding the scarified eye, 3 landmarks surrounding the not scarified eye, and 1 shared control landmark. The designator “scarified” refers to the group allocation of the eye but not necessarily the presence of corneal lesion associated with scarification. As such, the scarification classification of the landmark did not change over time even though the eye may have healed. Infectious bovine keratoconjunctivitis–associated corneal ulceration status was a time-dependent variable because IBK-associated corneal ulceration may have been positive on one assessment day and negative on another. Explanatory variables evaluated as random effects were calf and the interaction between calf and the 7 landmarks.

Mechanical nociceptive thresholds obtained through pressure algometry data were continuous (kgf). A repeated measures ANOVA (PROC GLIMMIX; SAS Inst. Inc., Cary, NC) was used. The approach to model building was to include all available fixed effects for consideration and biological sensible interaction terms. Type III tests of fixed

effects were used to test significance of the fixed variables. Fixed effect variables with P -values > 0.05 were removed from the final model. Random effects were included to account for expected random variation and their significance in model was not tested. As the study design was balanced, no linear relationship assumptions were made and explanatory variables were not checked for collinearity. All models were checked to ensure they meet model assumptions and transformed reported where preformed. Missing data were assumed missing at random.

After evaluating residual plots using nontransformed data, a log (base 10) transformation was used to stabilize the variance of the PA-MNT measurements. Fixed effects included in the final model were landmark and assessment day and the interaction between landmark and assessment day. Random effects included in the final model were calf and calf \times landmark interaction.

This model provided estimates of the mean log (PA-MNT) for each landmark for each eye each day. To test if scarification was associated with differences in log (PA-MNT) between scarified and nonscarified eyes at each landmark position each day, Tukey’s t test was used. To test the assessment day was associated with differences between the mean log (PA-MNT) between assessment days for each landmark Tukey’s t test was used. The results are reported as model fitted median values for PA-MNT.

Corneal Touch Threshold Analysis. Corneal touch threshold data were continuous (mm of filament length). As with the PA-MNT analysis, a repeated measures ANOVA (PROC GLIMMIX; SAS Inst. Inc.) was used. Fixed variables included in the final model were assessment day, eye-level scarification status (present or absent), and the interaction between eye-level scarification status and assessment day. Random effects included in the final model were calf and eye-level scarification status (present or absent).

To test the null hypothesis that the CTT measurement of scarified eyes and eyes with IBK-associated corneal ulcerations would not differ compared to CTT measurements of nonscarified eyes, the difference in least squares means between CTT for each eye was calculated on each day and tested using a t test. If the mean difference was close to zero, this suggested that there were no differences in CTT between eyes that had been scarified and those that were not. If the mean differences were negative, CTT was higher in nonscarified eyes, contrary to the working hypothesis that CTT was associated with increased pain sensitivity. If least squares mean difference was positive, scarified eyes had lower CTT values consistent with the working hypothesis.

Blepharospasm and Photophobia Analysis. We also attempted an analysis of the blepharospasm and photophobia data using a mixed effect logistic regression analy-

sis (PROC GLIMMIX; SAS Inst. Inc.) using a binomial outcome distribution. However, the strong correlation between eye-level treatment (scarification present or absent) and IBK-associated corneal ulceration status confounded the model. Therefore, instead we present the cross-tabulation of the incidence of blepharospasm and photophobia in calves scarified and IBK-associated corneal ulcers. Because this cross tabulation does not account for within calf correlation, no hypothesis tests were performed.

RESULTS

Study Population and Animal Flow

Thirty-one enrolled calves were randomly allocated in 3 replicates. Of the 36 calves eligible for enrollment, 5 were excluded due to preexisting ophthalmic abnormalities identified on d -4 that did not include diagnosis of IBK. Hence, the final number of calves allocated to 3 treatments included scarification only ($n = 11$), scarification and inoculation with *M. bovis* ($n = 10$), and scarification and inoculation with *M. bovoculi* ($n = 10$). The discussion pertaining to causality of *M. bovoculi* and *M. bovis* with IBK is presented elsewhere (Gould et al., 2013).

Nineteen calves from Trial 2 and Trial 3 with records for all PA-MNT categories (landmark, completion of at least 2 repetitions) were included in the PA-MNT analyses. Nine calves (9/19) in Trial 2 had missing data on d +10 because practical constraints prevented collection of PA-MNT data around scheduled ophthalmic exams and euthanasia. One calf in Trial 1 developed severe respiratory disease and was euthanized on d +7. Before euthanizing, the calf had not developed an IBK-associated corneal ulceration. The CTT measurements were limited to Trial 1 ($n = 12$). Blepharospasm and photophobia information were captured from all calves in all trials ($n = 31$).

Onset and Duration of Infectious Bovine Keratoconjunctivitis-Associated Corneal Ulcerations

Ten calves developed IBK-associated corneal ulcerations (4 in Trial 1, 3 in Trial 2, and 3 in Trial 3). Nine of the ten IBK-associated corneal ulcerations were identified on d +1; all had been scarified and inoculated with *M. bovis*. One scarified eye in the control group developed an IBK-associated corneal ulceration on d +3 in Trial 1. In Trial 2, the 3 calves with IBK-associated corneal ulcerations were euthanized after assessment on d +1 because the IBK-associated corneal ulceration had met or exceeded 15 mm in diameter. Only 1 eye remained "IBK-associated corneal ulceration positive" from d +1 through d +10. All other IBK-associated corneal ulcerations were resolved at the following assessment day.

Descriptive Information and Hypothesis Testing Results for Pain Metrics

Untransformed PA-MNT results by scarification status and landmark for each day are presented in Table 1. Model derived estimates of median PA-MNT and 95% confidence intervals are presented in Table 2. No statistically significant ($P < 0.1$) differences in PA-MNT scores between scarified and not scarified eyes were detected for any landmark on any day. For example, the comparison of PA-MNT in scarified and nonscarified eyes on d +1 at landmark 1 (4.82 compared to 4.79 kgf) was not statistically significant (Table 2). However, assessment day was associated with PA-MNT. For example, the difference between PA-MNT at landmark 1 on d -4 (prescarification) compared to d +1 (postsscarification) was statistically significant: 4.82 kgf on d -4 compared to 3.43 kgf on d +1, which was significant at the $P < 0.05$ level as indicated by the superscripts in Table 2. On d -4, PA-MNT levels were lower at all landmarks when compared to d +1, +3, +6, and +8 (Table 2). The estimated variance components of the random effects on the base 10-log scale were among calf (0.013) and interaction between calf and landmark (0.051), and the residual was 0.065.

Corneal touch threshold measurements (mm of filament length) were obtained for 138 observations among 12 calves and ranged from 1 to 4.75 mm. During 8 of the 138 observations, 2 calves presented with an eye that was deemed too painful for CTT to be assessed; the eye could not be opened with minimal resistance or without using a topical anesthetic for completion of the ophthalmic exam. Data for CTT were also not obtained after d +6 for 1 calf that was euthanized due to respiratory disease. Least squared means for CTT by day in scarified and nonscarified eyes are shown in Table 3. The estimated variance components of the random effects included in the final model were calf ID (0.005) and eye (0.011), and the estimated residual variance was 0.489. When CTT measurements from scarified and nonscarified eyes obtained on d +1, +3, +6, +8, and +10 were compared by day, there were no statistically significant differences (Table 3). When the association between IBK-associated corneal ulceration status (yes/no) and CTT was assessed, the presence of an IBK-associated corneal ulceration was not associated with CTT (Type III test of fixed effects; $P = 0.2981$).

Information about the occurrence of blepharospasm or photophobia in eyes that were scarified or that developed IBK-associated corneal ulcers is presented in Table 4. Blepharospasm was documented at 21 of 342 time points (6.14%) and in 10 of the 31 (32.26%) calves enrolled in the study. Of the 21 blepharospasm observations among the 10 calves, blepharospasm occurred in 10 calves on d +1, 3 calves on d +3, 4 calves on d +6, and 2 calves on d +8 and +10. Blepharospasm was

Table 1. Mean (standard deviation, median, minimum, and maximum) for pressure algometry–mechanical nociceptive threshold (PA-MNT) on d 0 (n = number of eyes)

Day (n)	Landmark 1	Landmark 2	Landmark 3	Landmark 4 (control)
Scarified eyes				
–4 (19)	5.2 (1.9, 4.8, 1.4, 10.1)	2.0 (1.2, 1.8, 0.2, 6.5)	4.0 (2.4, 3.7, 0.5, 10.3)	5.5 (2.2, 5.2, 1.1, 11.1)
1 (19)	3.9 (1.9, 3.7, 0.7, 10.0)	1.7 (1.1, 1.4, 0.1, 5.1)	3.1 (2.1, 2.5, 0.1, 8.3)	3.9 (1.7, 3.9, 0.7, 11.2)
3 (16)	3.1 (1.2, 3.0, 0.9, 6.9)	1.3 (0.8, 1.2, 0.2, 3.3)	2.6 (2.5, 1.6, 0.2, 11.2)	3.2 (1.1, 3.2, 0.4, 6.6)
6 (16)	3.4 (1.3, 3.2, 0.5, 6.2)	1.6 (1.1, 1.3, 0.2, 4.0)	3.1 (2.3, 2.6, 0.2, 9.9)	3.6 (1.8, 3.6, 0.4, 11.4)
8 (16)	3.7 (1.7, 3.2, 0.8, 9.4)	1.5 (1.0, 1.3, 0.2, 3.2)	2.8 (1.7, 2.6, 0.3, 7.2)	3.7 (1.7, 3.3, 0.7, 9.6)
10 (7)	3.7 (0.8, 2.5, 1.2, 3.7)	1.0 (0.5, 0.8, 0.4, 2.0)	2.1 (1.3, 2.0, 0.2, 4.8)	3.4 (2.0, 2.3, 0.7, 11.1)
Not-scarified eyes				
–4 (19)	5.5 (2.1, 4.9, 1.4, 10.5)	2.3 (1.1, 2.3, 0.2, 5.1)	4.2 (3.0, 3.8, 0.3, 13.6)	5.5 (2.2, 5.2, 1.1, 11.1)
1 (19)	4.0 (2.1, 3.7, 0.6, 10.0)	1.6 (0.9, 1.5, 0.1, 3.8)	3.4 (2.2, 2.5, 0.1, 8.4)	3.9 (1.7, 3.9, 0.7, 11.2)
3 (16)	3.2 (1.2, 3.0, 1.3, 6.4)	1.3 (0.7, 1.2, 0.1, 2.8)	3.1 (2.6, 2.4, 0.2, 12.8)	3.2 (1.1, 3.2, 0.4, 6.6)
6 (16)	3.5 (1.4, 3.2, 0.9, 9.4)	1.4 (0.7, 1.4, 0.2, 3.9)	2.5 (1.6, 2.0, 0.4, 6.4)	3.6 (1.8, 3.6, 0.4, 11.4)
8 (16)	3.1 (1.5, 3.0, 0.2, 8.4)	1.7 (1.0, 1.6, 0.1, 4.8)	3.0 (2.5, 2.1, 0.3, 10.6)	3.7 (1.7, 3.3, 0.7, 9.6)
10 (7)	3.1 (1.7, 2.8, 0.6, 6.9)	1.2 (0.8, 0.8, 0.2, 2.7)	2.3 (1.6, 1.5, 0.4, 5.6)	3.4 (2.0, 2.3, 0.7, 11.1)

recorded for 1 calf on d +1 in an eye that had not been scarified and did not have any identified ocular abnormalities. Blepharospasm was not recorded for this eye for any other assessment days and no ocular abnormalities were recorded during the study. Photophobia was documented in 11/336 time points (3.3%) and was noted at least once in an eye for 8/31 enrolled calves (25.8%). Of the 11 photophobia observations among the 8 calves, photophobia was noted in 5 of the affected calves on d +1, 2 calves on d +3 and +6, and 1 calf on d +8 and +10. Photophobia was recorded for 1 of the 5 calves on d +1 in an eye that had not been scarified and did not have an IBK-associated corneal ulceration or any other detected ocular abnormalities. Photophobia was not

identified for this eye on any other assessment days and no other ocular abnormalities were recorded for that eye. When photophobia was documented during an ophthalmic examination, blepharospasm was also documented in 8/11 (62.5%) observations. For 4 of the 21 calves in which blepharospasm was recorded for an eye in normal lighting, the veterinary ophthalmologist did not further assess for photophobia using the transilluminator in a darkened room because he deemed the eye too painful to properly examine. Although the eye was almost certainly photophobic, the presence of photophobia could not be absolutely verified according to the study protocol and therefore could not be identified as such. Blepharospasm and photophobia occurred consistently in eyes

Table 2. Estimated median and 95% confidence intervals for pressure algometry–mechanical nociceptive threshold (PA-MNT; kg of force) scores associated with ocular pain for calves scarified on d 0 (n = number of eyes)

Day (n)	Landmark 1	Landmark 2	Landmark 3	Landmark 4 (control)
Scarified eyes				
–4 (19)	4.82 ^a (3.92–5.93)	1.70 ^a (1.38–2.09)	3.15 ^a (2.56–3.88)	5.05 ^a (4.24–6.03)
1 (19)	3.43 ^b (2.79–4.22)	1.28 ^{ab} (1.04–1.57)	2.34 ^{ab} (1.91–2.88)	3.54 ^b (2.97–4.23)
3 (16)	2.80 ^b (2.25–3.48)	1.03 ^b (0.83–1.28)	1.80 ^b (1.45–2.24)	2.96 ^b (2.46–3.56)
6 (16)	3.02 ^b (2.43–3.76)	1.14 ^b (0.91–1.42)	2.20 ^b (1.77–2.74)	3.19 ^b (2.65–3.83)
8 (16)	3.25 ^b (2.61–4.05)	1.18 ^b (0.95–1.47)	2.21 ^b (1.78–2.75)	3.31 ^b (2.76–3.98)
10 (7)	2.78 ^b (2.07–3.74)	1.17 ^{ab} (0.87–1.58)	2.11 ^{ab} (1.57–2.84)	3.28 ^b (2.60–4.14)
Nonscarified eyes				
–4 (19)	4.79 ^a (3.88–5.90)	2.0 ^a (1.63–2.45)	3.24 ^a (2.64–3.99)	5.05 ^a (4.24–6.03)
1 (19)	3.40 ^b (2.77–4.18)	1.23 ^b (1.00–1.51)	2.50 ^{ab} (2.03–3.07)	3.54 ^b (2.97–4.23)
3 (16)	2.97 ^b (2.39–3.69)	1.01 ^b (0.81–1.26)	2.25 ^b (1.81–2.80)	2.96 ^b (2.46–3.56)
6 (16)	3.18 ^b (2.55–3.95)	1.18 ^b (0.95–1.47)	2.04 ^b (1.64–2.54)	3.19 ^b (2.65–3.83)
8 (16)	2.66 ^b (2.14–3.31)	1.21 ^b (0.97–1.51)	2.03 ^b (1.63–2.52)	3.31 ^b (2.76–3.98)
10 (7)	3.11 ^{ab} (2.31–4.18)	1.37 ^b (1.02–1.84)	2.25 ^{ab} (1.67–3.03)	3.28 ^b (2.60–4.14)

^{a-c}Different superscripts within a column indicate statistical differences at the $P = 0.05$ level. For example when comparing the PMT-MT response of scarified eyes, the PMT-MT kilograms of force (kgf) recorded at landmark 1 on d –4 differed from the PMT-MT kgf recorded at landmark 1 on d 1, 3, 6, 8, and 10 whereas d 1, 3, 6, 8, and 10 did not differ from each other (i.e., d –4 has superscript ^a while d 1, 3, 6, 8, and 10 has superscript ^b). Fixed effects are assessment day, landmark, and the interaction between assessment day and landmark. Random explanatory variables are calf and the interaction between calf and the 7 landmarks. The estimated variance components of the random effects on the base 10-log scale were among calves (0.013) and the interaction between calf and landmark (0.051), and the residual was 0.065.

Table 3. Least squares means for scarified and nonscarified eyes by assessment day for corneal touch threshold (CTT; mm of filament length; *n*; 95% confidence intervals [CI]) and *P*-value for calves scarified on d 0

Day	Scarified	Not scarified	Difference estimate (CI)	<i>P</i> -value
-4	3.40 ^a (12; 3.01, 3.80)	3.50 ^a (12; 3.11, 3.910)	-0.10 (-0.67, 0.46)	0.7141
1	3.25 ^a (10; 2.81, 3.68)	2.88 ^a (12; 2.48, 3.28)	0.37 (-0.22, 0.96)	0.2168
3	3.26 ^a (10; 2.83, 3.70)	3.15 ^a (12; 2.75, 3.54)	0.11 (0.47, 0.71)	0.6911
6	3.34 ^a (10; 2.90, 3.77)	3.48 ^a (12; 3.08, 3.88)	-0.14 (-0.73, 0.45)	0.6389
8	2.81 ^a (9; 2.37, 3.25)	2.82 ^a (10; 2.40, 3.24)	-0.01 (-0.62, 0.59)	0.9700
10	2.99 ^a (9; 2.53, 3.45)	2.81 ^a (10; 2.37, 3.24)	0.18 (-0.45, 0.81)	0.5694

^aSuperscripts indicate comparisons within a column. All days share the same letters and are not significantly different ($P < 0.05$). Explanatory variables evaluated as random effects are calf and eye. Fixed variables included in the model are assessment day, eye-level scarification status, and the interaction between eye-level scarification status and assessment day. The estimated variance components of the random effects included in the final model were calf ID (0.005) and eye (0.011), and the estimated residual variance was 0.489.

with IBK-associated corneal lesions. However, this was not the case for scarification; an eye that was scarified did not necessarily display blepharospasm.

DISCUSSION

The objectives of this study were to 1) describe the magnitude and variation of measures of ocular pain in cattle with corneal scarification and IBK-associated corneal ulcerations and to 2) evaluate the associations of ocular pain measures with corneal scarification and IBK-associated corneal ulcerations in calves. Such information will enable appropriate design of studies for assessing the extent of ocular pain associated with ophthalmic abnormalities in cattle and efficacy of pain mitigation strategies. Our conclusions are based on the a priori assumption that scarification and IBK-associated corneal ulcerations are painful. Of the metrics we assessed, PA-MNT is a candidate metric for assessing pain and approaches to pain mitigation in cattle with corneal lesions. This conclusion is based on the observation that PA-MNT measurements significantly decreased postscarification (Table 3). However, PA-MNT did not differ between scarified and nonscarified eyes within a calf, and decrease in MNT from prescarification to postscarification was a calf-level response, occurring at all landmarks and

both eyes. Blepharospasm and photophobia were less sensitive to scarification, predominately occurring with IBK-associated corneal ulcerations. The Cochet-Bonnet aesthesiometer could not be reliably used on animals with IBK-associated corneal ulcerations due to blepharospasm and was not sufficiently robust to detect changes in pain sensitivity associated with scarification.

We predicted that scarified eyes would be more sensitive to pressure algometry than nonscarified eyes, but this was not observed. It is possible that scarification causes generalized hyperalgesia because changes in pain sensitivity were observed at all landmarks, including the control point at the center of the face. It is also possible the general response postscarification resulted from "head shyness" and/or aversion to handlers, resulting from motivation to protect the abnormal eye or learned aversion to the handlers following the scarification surgery. It is not possible given the data collected and described in Tables 2 and 3 to differentiate between these possibilities. Contrary to our working hypothesis, ocular IBK-associated corneal ulcerations were not associated with decreased PA-MNT scores. When analyzed at the eye level or calf level, we could not detect differences between the PA-MNT response for eyes that were scarified with IBK-associated corneal ulcerations, scarified-only eyes, and normal eyes. We postulate 3 explanations. Because only 6 of 19 calves (6/38 eyes) evaluated for PA-MNT developed IBK-associated corneal ulcerations and only 1 of those eyes was evaluated as an affected eye after d +1, our ability to detect statistically meaningful differences was limited.

Increased response to pressure algometry postscarification could simply result from hypersensitization to the procedure due to the repeated measurements. The decreasing PA-MNT scores over time could have occurred as an artifact of repeated handling (conditioning) and subsequent calf aversion and sensitization to the procedures. This may have occurred in spite of efforts to offset behaviors associated with increased handling, such as completing pressure algometry assessments on the same

Table 4. Frequency distribution of blepharospasm and photophobia in calves stratified by infectious bovine keratoconjunctivitis-associated corneal ulceration (IBK-ACU) status and scarification status

	Scarified		Not scarified		Total number of eyes
	No IBK-ACU	IBK-ACU	No IBK-ACU	IBK-ACU	
Blepharospasm/photophobia present	9	0	0	1	10
Blepharospasm absent	0	22	0	30	52
Total number of eyes	9	22	0	31	62
Photophobia present	7	0	0	1	8
Photophobia absent	2	22	0	30	54
Total number of eyes	9	22	0	31	62

day as other ophthalmic assessments and handling calves for pressure algometry collections at least 2 h before or after ophthalmic assessments were conducted. The ophthalmic assessments were conducted in a consistent manner and the same information was collected for each assessment day. Healthy humans have been found to consistently report decreased MNT measurements (greater pain sensitivity) over time when compared to baseline values and no differences in pain sensitivity between landmarks (Jones et al., 2007). Conversely, changes in MNT of livestock have been observed in association with disbudding in cattle (Heinrich et al., 2010), cattle lameness (Whay et al., 1997; Higginson-Cutler et al., 2012), and sow lameness (Tapper et al., 2013). Disbudding results in increased pain sensitivity around the horn buds in calves, but this effect is not observed until approximately 2 h postsurgery when effects of the local anesthetic (lidocaine) wear off. This increased pain sensitivity during the postsurgical period is mitigated when calves are provided with a nonsteroidal anti-inflammatory drug (Heinrich et al., 2010). Furthermore, when calves receive a long-acting local anesthetic (ethanol), MNT values do not differ from baseline values despite repeated measures over several hours and days (Tapper et al., 2011). Together, these results suggest that calves do not likely become hypersensitized or desensitized by repeated pressure algometry measurements in the absence of pain. Hence the lower MNT values we observed are more likely to be associated with changes in pain sensitivity rather than a byproduct of repeated measures, and further research is needed to confirm this interpretation.

It is feasible that the increased responsiveness to pressure algometry in the postscarification period was associated with hyperalgesia (Millman, 2013). Humans with chronic back pain report increased pain sensitivity as measured using pressure algometry at all landmarks compared to humans not suffering from chronic back pain (Giesbrecht and Battie, 2005). Proinflammatory cytokines are associated with increased nociception (Watkins et al., 1995; Driessen and Zarucco, 2007), and one explanation is that inflammatory mediators were produced from the scarification procedure, producing generalized sensitization of nociceptors, involved not only with the scarified eye but also with more peripheral innervation to the nonscarified eye (Anderson and Muir, 2005; Bussieres et al., 2008).

Our data reveal multiple important pieces of information for future design of pain mitigation strategies using PA-MNT. One implication of this finding is that paired eye designs are not likely to be useful for pain mitigation strategies that could have been assigned at the eye level. As most pain mitigation strategies in cattle are likely to be applied at the animal level, this is likely not of major importance. However, information about variation

at each landmark and among landmarks will be useful. The estimate of the intraclass correlation for the calf is low at 10% $[0.013/(0.013 + 0.051 + 0.065)]$. The intraclass correlation for landmark within the calf was higher at 50% $[(0.013 + 0.051)/(0.013 + 0.051 + 0.065)]$. This information can be used to design studies. For example, a researcher may desire to design a study with a 100% increase in the median PA-MNT. This translates to a mean difference in median PA-MNT of 0.30 ($\log(2)$). Using data collected from a single measurement at a single landmark for each calf and using α of 5% and β of 20%, the sample size calculations for this mean difference and the calf level variance estimate of 0.131 $(0.0127 + 0.051 + 0.067)$ would suggest 22 calves per group would be required. However, if instead 3 measurements were collected at 3 landmarks per calf the variation in the estimate of the calf level measurement would be decreased (Step 1: $[0.06/3$ (number of measurements)] = 0.02, Step 2: $(0.013 + [(0.051 + 0.02)/3$ (number of landmarks)], and Step 3 = $(0.013 + 0.071) = 0.084$), and the number of calves required would decrease to approximately 15 calves per group. Alternatively, if the aim was to detect a 50% increase in median MNT ($\log(1.5) = 0.17$, variance = 0.084), the number of animals needed would be approximately 46 per group. Given the pain associated with the scarification model, reducing the number of animals enrolled may be more important than decreasing the number of measurements per animal.

Refinement and reduction of landmarks would allow also for more time-efficient collection of data and reduced handling of calves. Because this is the first study to assess ocular pain using pressure algometry, landmarks were selected based on anatomical considerations. Landmark 4 was selected as a nonpainful control, based on previous research in our laboratory for assessing disbudding pain (Tapper et al., 2011). Landmarks 1 and 3 were selected to assess if IBK-associated corneal ulcerations and scarification were associated with increased sensitivity to areas extending beyond the eye. In contrast, it is possible that pressure algometry testing at Landmark 2 placed indirect pressure on the eye and associated adnexa, potentially stimulating the 2 sensory branches of the trigeminal nerve that innervate the cornea (Gelatt, 2008). This could result in a withdrawal response regardless of whether the eye was scarified or had an IBK-associated corneal ulceration, but hyperalgesia would likely exacerbate this response. If the general decreases in PA-MNT values compared to baseline are attributed to using numerous landmarks, then focusing on Landmark 4 and Landmark 2 may result in a more accurate and time-efficient assessment of changes in MNT with ocular pain. However, despite this rationale, there did not appear to be 1 ideal landmark because the ratio of the medians from Baseline and d +1 was 1.3 to 1.7 for all landmarks.

Given that PA-MNT appears to measure changes in pain sensitivity at the calf level, it is not surprising that the theoretical control landmark did not act as such, decreasing in a similar manner to other landmarks.

The use of the Cochet-Bonnet to evaluate corneal sensitivity by CTT was of limited value for comparison of scarified and nonscarified eyes. In severe IBK cases, which we expect are most painful, it was not possible or ethical to complete CTT. Therefore, the use of the Cochet-Bonnet to measure CTT is unlikely to be useful when evaluating eyes with IBK-associated corneal ulceration. For example, if a researcher aimed to conduct a trial to determine if a pharmaceutical product reduced pain in calves with naturally occurring IBK, it would not be possible to obtain baseline CTT measurements at the time of treatment if the eye showed blepharospasm. In the present study, only the central cornea was assessed with the Cochet-Bonnet. It is possible that another area of the bovine cornea is more sensitive than the central corneal area and that additional testing of those corneal regions could have produced different results. However, other reports in the literature have used multiple regions to measure CTT and have demonstrated that the central corneal region was the most sensitive (Blocker and van der Woerd 2001; Brooks et al., 2000; Kaps et al., 2003; Trost et al., 2007; Welihozkiy et al., 2011).

Corneal scarification was associated with an increased prevalence of demonstrating photophobia or blepharospasm. However, the occurrence of photophobia and blepharospasm was greatly increased in eyes that had IBK-associated corneal ulcerations. In humans, both blepharospasm and photophobia have been associated with corneal pain (Borsook and Rosenthal, 2011; Martino et al., 2005; Peckham et al., 2011). It is postulated that both blepharospasm and photophobia result from stimulation of trigeminal nociceptors (Borsook and Rosenthal, 2011; Muller et al., 2003). In our study, calves with IBK-associated corneal ulcerations were likely to exhibit blepharospasm and photophobia, yet the frequency of an IBK-associated corneal ulceration was much less than the frequency of corneal scarification in our data set, so it is unlikely that lack of statistical power affected the outcome. Corneal scarification is likely to be initially painful, but because of the rapid ability of the corneal surface to heal, the pain associated with scarification may be transient compared to an ongoing IBK-associated corneal ulceration.

Conclusion

Our study results suggest that scarification results in generalized facial hyperalgesia as measured by pressure algometry. Studies incorporating a nonscarified treatment group would clarify if differences in MNT values resulted

from increased pain sensitivity, learned aversion to handlers associated with the scarification procedure, or reactivity due to repeated pressure algometry measurements over time. The results of this study suggest that blepharospasm and photophobia, both associated with ocular pain in humans, are likely to be observed in calf eyes affected with an IBK-associated corneal ulceration but not scarification. The use of the Cochet-Bonnet to evaluate corneal sensitivity by CTT was of limited value in this study.

This study describes important first steps in identifying and quantifying ocular pain in calves and results can be used in properly calculating sample size for additional studies. To further validate and improve the potential practicality of objective measurements for ocular pain in calves, further research and refinement is required. Development and validation of objective methods to detect and assess pain in cattle will advance science-based health and husbandry protocols. An objective method to assess pain in bovids will allow more focused research to be conducted on intervention and prevention and may lead to approval and subsequent labeling of pharmaceuticals to alleviate pain and improve cattle welfare.

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APPENDIX

SAS Codes

SAS Code for Analysis for Pressure Algometry–Mechanical Nociceptive Threshold

```
proc glimmix data = WORK. Eyes order = data;
class Calf_ID Landmark IBK_Lesion_Day_eye IBK_
Lesion_Day_Calf Assessment_Day;
model logForce = Assessment_Day | Landmark;
random Calf_ID Calf_ID *Landmark;
lsmeans Assessment_Day * Landmark/slice = Assess-
ment_Day
slicediff = Assessment_Day;
lsmeans Assessment_Day * Landmark/slice = Landmark
slicediff = Landmark adjust = tukey;
run;
```

SAS Code for Analysis for Corneal Touch Threshold

```
proc glimmix data = IBK plots = all;
class ID scar_y_n Day IBK_Lesion;
```

```
model C_B = Eye_level_scarification_y_n | Assess-
ment_Day;
random Calf_ID Calf_ID*Eye_level_scarification_y_n;
lsmeans Eye_level_scarification_y_n*Assessment_Day/
plot = meanplot(sliceby = Eye_Level_scarification_y_n
join) slicediff = Assessment_Day slicediff = Eye_Level_
scarification_y_n join) cl;
run;
```

SAS Code for Analysis of Blepharospasm

```
proc glimmix data = IBK plots = all;
class Calf ID Eye_level_scarification_y_n Assesment_
Day IBK_Lesion_y_n;
model Blepharospasm = Eye_level_scarification_y_n |
Assessment Day IBK_Lesion_y_n/s cl;
run;
```

SAS Code for Analysis of Photophobia

```
proc glimmix data = IBK plots = all;
class Calf_ID scar_y_n Day IBK_Lesion;
model photophobia = scar_y_n | Assessment_Day IBK_
Lesion/s cl;
run;
```