Blinking Patterns in Calves and Changes Following Restraint and Disbudding

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Research Article

Keywords: spontaneous eye blink rate, Blinking patterns, calves, restraint, disbudding

Posted Date: June 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-618557/v1

Abstract

The use of spontaneous eye blink rate [EBR] as a non-invasive stress measure is well documented in humans and is gleaning research attention in animal studies. Thus, this study investigated the EBR patterns and changes associated with two known stressful conditions, crush-restraint and disbudding, in dairy calves. Fifty-four female Holstein dairy calves were studied. EBR patterns were assessed in two separate studies (n = 10 in first study; n = 25 in replicate study) four times daily for 4-5 days. Additionally, the EBR of 19 calves divided into two groups (disbudded group = 13; sham group = 6) was recorded following crush-restraint and disbudding (under anaesthesia)/sham-disbudding (without anaesthesia). EBR was consistent across days and times in the first study but in the replicate study, it varied across days. The disbudded group had lower EBR following crush-restraint but was comparable with the sham group following disbudding. Findings suggest that spontaneous blinking may potentially serve as an instant measure of stress in dairy calves based on the elevations associated with crush-restraint and disbudding. However, blink rates may vary across days, and this should be considered when utilizing this measure to assess stress.

Introduction

Blinking is a normal eye activity in humans and some other animal species. It can be defined as a momentary closure and opening of the eyes which results from the coming together of the lower and upper eyelids to obliterate the palpebral fissure of the eye partially as in partial/half blinking, or completely as in complete/full blinking. Three types of eye blinking exist and these include reflex, spontaneous and voluntary types[1,2]. Generally, blinking is thought to serve notable functions including ensuring even distribution of the lacrimal gland secretion thereby preventing dry eyes; protection of the cornea and the entire eye from external insult or foreign body; and shielding the eyes from strong and dazzling light. Blink activity in animals has not had enormous research attention, however, there are some studies describing this activity in certain animal species. The first systematic animal study on eyelid movements observed 39 animal species and defined various eyelid movements possible in animals including blink, wink, half blink, pseudo blink, flicker, flink and full eye closure[3]. Thereafter, studies describing typical blinking pattern emanated such as that which reported that cattle blinked 5 times in a minute[4]. Additionally, blink activity has been described in dogs[5,6], cats[7], birds[8], guinea pigs[9], harbour seal pups[10], rabbits[11], horses[12,13], 71 primate species[14], rats[15], and red deer[16].

Of the three types of blinking, the spontaneous type is endogenous and performed on impulse without premeditation[17]. Thus, it is expected that normal, healthy, and alert humans would perform spontaneous eye blinks[17]. Consequently, this activity gleaned multidisciplinary research interest for nearly nine decades and is assessed in two dimensions; the rate at which blinks occur termed ‘eye blink rate’ and the interval between blinks termed ‘interblink interval’[17,12]. Even though this type of blinking is unpremeditated, evidence has shown that it can be influenced by both ophthalmic and non-ophthalmic factors including stress[18,19], cognitive activities[17], emotional states[9] and dopaminergic activity[20,21]. Due

to its potential to be influenced by stress, spontaneous eye blink rate is utilised as a non-invasive measure of stress. This use has been well studied in humans[18,19] and is currently gleaning research attention in animal studies[12,13]. Stress is a complex and multifactorial phenomenon[22]. In animals, stressful conditions have been shown to result in both reduction and elevation in spontaneous blinking. For instance, induced fear condition in cats caused an increase in spontaneous blinking[7] while spontaneous blinking decreased following feed restriction in horses[12]. Additionally, reports have suggested that spontaneous blink activity may be influenced by pain modulation in humans[23–26] and animals[27] via its relationship with central dopaminergic activity. It is thought that some dopaminergic neurons reside in an area of the brain, the ventrolateral periaqueductal gray (PAG), which is incorporated in the descending pain modulation process[28].

Spontaneous blink activity has not been previously described in dairy calves. Such investigation would reveal its patterns or useful range as a means of recognising changes which may potentially affect this activity. Utilising spontaneous blinking as a stress measure offers the following advantages. It is objective, easy to determine, yields instant results, non-invasive and can be obtained unobtrusively. More so, it has proven to be effective in reflecting stress based on changes in rate in other species. Thus, since evidence in the literature supports its use as a non-invasive stress marker in other species of animal, it is also worthwhile investigating its potential to vary in response to stressful and painful procedures which dairy calves are routinely subjected to. An example of such conditions is disbudding[29,30]. To be able to carry out these procedures and make handling easier, dairy calves are usually restrained in a calf crush which is recognised to be potentially stressful[31]. The influence of disbudding and physical restraint using the crush on spontaneous blinking has not been previously studied, thus, justifying the need for this investigation. Therefore, this study was designed to investigate the spontaneous eye blink rate patterns in dairy calves and ascertain the influence of restraint and disbudding on this activity.

Results

On average, dairy calves blinked 8 times per minute [40 blinks /5 minutes], though EBR ranged from 1 to 21 blinks per minute.

Determination of EBR patterns in dairy calves.

Results from the first study for the determination of EBR patterns revealed that the mean EBR of dairy calves (n = 4; six calves were excluded because of poor visibility of the calves' eye from video recording) did not vary significantly across days ($P = 0.76$) or times ($P = 0.84$) during the observation period. Also, no day*time interaction effect ($P = 0.53$) was observed. In the replication study, the mean EBR of dairy calves in day one was significantly higher [$F_{4, 384} = 20.96$; $P \leq 0.001$; small effect size: Cohen's d = 0.1; power = 100%] than the other four days as shown in Fig. 1 below. The mean EBR of dairy calves did not vary significantly across time ($P = 0.07$) and there was no day*time effect ($P = 0.09$).

EBR did not vary between disbudded vs non-disbudded calves ($P = 0.70$); weaned vs un-weaned ($P = 0.67$); calves in standing vs lying posture ($P = 0.61$) or between calves performing oral behaviour vs those not performing any oral activity ($P = 0.66$).

EBR changes following crush restraint and disbudding.

Between group analysis: There were no significant differences in mean EBR ($P = 0.37$) between the disbudded (Group A) and sham-disbudded group (Group B) on baseline days − 5 to -1. On the day of disbudding also, the baseline mean EBR (day $_0$) did not vary significantly ($P = 0.47$) between groups. EBR assessment following crush restraint revealed that the mean EBR of group A was significantly lower [t (15) = -4.49; $P \leq 0.001$] than that of group B (Fig. 2). There was no significant difference between the mean EBR of the two groups following disbudding ($P = 0.59$) and on post-disbudding days ($P = 0.44$).

Within group analysis: Time comparison within the disbudded group revealed that the mean EBR following disbudding was significantly higher than baseline mean [t (11) = -5.63; $P \leq 0.001$], baseline (day $_0$) mean [t (11) = -2.96; $P \leq 0.013$], crush-restraint mean [t (11) = -4.26; $P \leq 0.001$] and post-disbudding mean [t (11) = -5.28; $P \leq 0.001$] as shown in Fig. 2 below. In the disbudded group, mean EBR following crush-restraint did not vary significantly with the baseline mean ($P = 0.21$), baseline (day $_0$) mean ($P = 0.93$) and post-disbudding mean ($P = 0.28$) as shown in Fig. 2 below. In the sham-disbudded group, mean EBR following sham-disbudding (though higher) did not differ significantly from all the other time points [baseline mean ($P = 0.11$); baseline (day $_0$) mean ($P = 0.06$); crush-restraint mean ($P = 0.09$); post-disbudding mean ($P = 0.053$)]. However, following crush restraint, the mean EBR was significantly higher than baseline mean [t (4) = -4.28; $P = 0.013$], baseline (day $_0$) mean [t (4) = -5.42; $P = 0.006$] and post-disbudding mean [t (4) = -6.52; $P = 0.003$] as shown in Fig. 2 below.

Discussion

The results of this study which investigated spontaneous eye blink rate (EBR) patterns and changes associated with stressful husbandry procedures in dairy calves is the first to provide an insight into their blink activity and the potential of this activity in stress measurement. The results from the first study objective suggest that spontaneous blinking may vary across factors such as day but not across time of day. In line with this finding, time of day also had no significant effect on spontaneous blink rate in humans[32]. It is noteworthy that the day variation only occurred between day one vs other assessment days in the replicate study. In this replicate study, acclimatization was not carried out due to time constraints even though it was initially planned for. Thus, results suggest that the lack of calf-familiarity with the observer (and video recorder) may have significantly influenced spontaneous EBR on day one. The presence of an observer can be stressful to animals and consequently influence behaviour[33], more so in prey animals like calves[34]. Cattle maybe fearful in the presence of unfamiliar persons and thus, exhibit measurable stress-induced changes in behavioural and physiology[35] such as in elevated spontaneous EBR observed in this study. Repeated exposure to the observer during the four data

collection times on day one may have played an acclimatory role that resulted in a non-significant spontaneous EBR observed between the other assessment days. Thus, the findings of this study support acclimatization prior to data collection and this was practiced in the second study.

The results from the second study objective which assessed spontaneous EBR changes associated with crush-restraint and disbudding/sham-disbudding revealed that crush restraint (in non-anaesthetized calves) and disbudding was associated with hyper blinking in calves. It is important to reiterate that this study did not assess stress using conventional methods which are invasive but relied on literature confirmation of crush-restraint and disbudding as known stressors[30,31]. It was expected that the exposure of disbudded calves to aversive conditions such as local anaesthetic administration[36], clipping[37] in addition to crush-restraint[31] would have resulted in a rise in the EBR. Rather than increase, the EBR remained near baseline level. Even though appropriate comparative literature is lacking, some studies have reported that topical anaesthesia (ocular) caused a reduction in spontaneous blink rate[38,39]. The mechanism by which local anaesthetic mitigated an increase in spontaneous EBR response in the anaesthetized group following crush-restraint is not clear but it might relate to its ability to mitigate stress response in dairy calves[40] and not necessarily because it has a direct effect on blink activity. Additionally, it should be noted that calves in this group entered the crush twice and EBR was assessed following their second exposure to the crush as against first exposure assessment in the sham group. This design was chosen to ensure EBR assessment was done just before disbudding/sham-disbudding in both groups. The elevation in EBR seen in the sham group may also have been recorded in the disbudded calves if it was assessed following initial exposure to crush restraint prior to anaesthesia. Based on the increase in spontaneous EBR following crush-restraint and disbudding/sham-disbudding and the literature evidence associating stressful and painful stimuli with spontaneous hyper blink activity, it can be inferred that these procedures were stressful and perhaps painful (disbudding) to dairy calves.

Stress, a complex multifactorial phenomenon, represents the body's response to inward and/or outward stimulus and demand[18]. Thus, this complexity may account for the variable effect of stressful stimuli on spontaneous EBR. Similar with the findings of this present study, increase in spontaneous EBR followed exposure to stressful stimuli (emotional and social recollection tests) in humans[9], handling in emotionally aroused guinea pigs[9], induced fear condition in domestic cats[7], clipping in reactive horses[13], fear[41] and frustration[42] in dogs. Increase in EBR also occurred following painful laser stimulation in healthy human subjects[26]. In contrast, feed restriction in horses resulted in a significant decrease in spontaneous blink rate[12]. From the above cited studies, it may be inferred that the EBR response following exposure to stress is largely dependent on the type of stressor, thus, emphasizing the need to validate this measure against any potential stressful condition. The mechanism by which stress and pain result in exaggerated spontaneous blink activity is via the action of dopamine neurotransmitter, whose neurons are activated following aversive or acute nociception stimulus[26,43–45] and possibly, chronic pain[46]. Supporting this phenomenon is the fact that drugs mimicking the action of dopamine had an analgesic influence in tonic-induced pain[47,48]. Central dopamine actions on brainstem structures which

control facial reflexes including the superior colliculus and nucleus raphe magnus[47] may also account for the stress-induced increase in spontaneous EBR.

It is important to note that the elevation in spontaneous EBR following crush restraint and disbudding/sham-disbudding was not sustained as the spontaneous EBR of dairy calves returned to baseline values within 24 hours post-procedure. This may signify quick adaptation of dairy calves to stress as was reported in cattle[31]. Another possible explanation to this finding would be that the spontaneous EBR is only a transient stress or pain marker and thus, may be dependent on time interval between stress exposure and assessment[13] amidst other factors. This suggests that validity of spontaneous EBR may be limited to acute measure of stress and pain and thus questions its applicability as a long-term measurement of pain in dairy calves. The finding from the post-disbudding EBR assessment is also corroborated by the absence of significant variation between previously disbudded and non-disbudded calves. In the replicate study discussed above, it was mentioned that calves previously disbudded (13 days) prior to assessment had comparable EBR values with their non-disbudded counterparts.

The study is not without limitations. Firstly, the lack of acclimatization in the replicate study may have influenced all the results obtained, even though this constraint demonstrated the importance of acclimatization prior to data collection. Secondly, assessing the EBR for only two minutes following crush-restraint may have been subject to natural fluctuations which occur when measurement is obtained for < 3 minutes notwithstanding that this was done to ensure measurements were obtained within the first ten minutes following anaesthesia. Regardless of these limitations, this present study is the first to assess EBR patterns in healthy calves within their home environment normal and changes associated with physical restraint and disbudding.

Conclusion

In conclusion, the elevation in spontaneous eye blink rate following physical restraint in a crush and disbudding in dairy calves supports its use as a stress measure. However, to ensure that reliable results are obtained, the recommended three-minute observation time should be adhered to. In addition, factors which may influence its consistency should be assessed and controlled for. Against conventional stress indicators, spontaneous EBR benefits from the fact that it is non-invasive, yields instant result, can be obtained unobtrusively and costless since it may not require any special equipment.

Methods

Ethical permit. The study was assessed and approved by the Bristol Veterinary School's Animal Welfare and Ethical Review Body [AWERB] and registered under the protocol numbers, UOB/19/00 (20th February 2019) and UIN 19 000 (1st April 2020). The methods used in this study were performed in accordance with the relevant guidelines and regulations of the University of Bristol. Methods also conformed with ARRIVE guidelines.

Study site. The study was conducted on a college farm in the southwest of the United Kingdom. The farm has a capacity of 220 dairy cows, over 50 heifers and young calves, and 50 sheep. The farm also produces beef calves which are sold within 10 days of birth. Even though it runs a commercial dairy unit with average milk production of 30 litres per day per cow, it fundamentally serves teaching purposes for college students and occasionally, undergraduate veterinary students from nearby universities. Artificial insemination using selected semen for females is practised in the farm to ensure that only female dairy calves are produced.

Dairy calves' management: Feeding. Diary calves were fed a combination of calf milk powder (Blossom Easymix®, Volac, Hertfordshire, UK) and water via an automatic calf feeder, concentrate and barley straw. During each visit to the automatic calf feeder, dairy calves were identified by their sensor-based identification collar and received some quantity of milk depending on their daily requirement. Concentrates (Deccox®, Mole Valley Feed Solutions, Devon, UK) were delivered twice daily at the hours of 08:00 [between 06:00 and 08:00] and 16:00 [16:00 and 17:00] in a plastic feeder attached to the metal gate. Drinking water was also made available in a plastic drinking trough anchored to the right side in the front part of each pen.

Housing. Calves were housed in group pens with dimensions of 3 x 10m [7.5 m^2/calf in Pen 1; 5.0 m^2 /calf in Pen 2] with a 1 x 3 m [length x width] metal gate at the entrance. The floor of each pen was made of concrete, the first half bedded with sawdust which was changed daily during feeding while the last half was covered with barley straw bedded up daily and cleaned out at the end of each batch of calves. In some pens, it was possible for dairy calves in one pen to have tactile, visual, auditory, and olfactory contact with calves in the other pen via an adjoining metal gate situated in the front part of the demarcating pen wall. One heat lamp was provided at the back portion of each pen. The pens were aligned in a row facing a large pen of heifers.

Study period. The first round of data collection was carried out between 19th to 23rd October 2019 including a one-day acclimatization period. The rest of the data collection exercise commenced on 13th October 2020 and ended on 28th October.

Spontaneous eye blink rate ethogram. Pilot observations of dairy calves in their environment revealed that they displayed both partial [half] and complete [full] blinks. Thus, the eye blink rate [EBR] was documented when at least 80% of the eye under observation was closed for one second or less. It was determined from either eye depending on which eye was clearly visible. Irrespective of which eye was chosen, the EBR was consistently obtained from only one eye during each observation period, and not both. Even though the EBR was destined to be expressed as events per minute, a five-minute recording time per calf was preferred to control for variations observed at one-minute recording time during pilot observations. This is also in line with a literature review of human studies which stated that a less than three-minute observation time for the analysis of spontaneous eye blink rate may be markedly subjected to natural fluctuations[17]. Other eyelid movements such as eye twitch and pseudo blinks [closing of the eye using the third eyelid or nictating membrane following local anaesthetic induction] were also

observed in dairy calves during the study period, although, their assessment is beyond the focus of this study.

Determination of EBR patterns in dairy calves. Initially, the EBR of 10 six weeks old calves was obtained for a period of four days using videography and counting method[17] following a one-day acclimatisation period. These calves were housed in 2 group pens (n = 4 in pen 1; n = 6 in pen 2) which were interconnected. To acclimatise the calves, the observer stood in-front of each pen with a video camera, Canon [Legria HF R206] mounted on a tripod and positioned directly in front of each calf for five minutes (or the entire pen if dairy calves were in proximity with each other) in four sessions at the hours of 10:00, 12:00, 14:00 and 16:00 to simulate the data collection periods. This was then followed by data collection and each calf's face was video-recorded for five minutes at the hours of 10:00; 12:00; 14:00 and 16:00 on days 1 – 4 as shown in Figure 3 below. The EBR of each calf was subsequently counted from the video recordings using a tally counter and transferred onto a Microsoft excel sheet. This aspect of the study took place between 19th to 23rd October 2019.

This protocol was repeated in between 13th October 2020 and 17th October 2020 using a new set of calves (n = 25) but calves were not acclimatized prior to data collection due to time constraints. The calf population comprised of disbudded calves aged over 7 weeks (n = 12) and non-disbudded calves aged 7 weeks and younger (n = 13). Ten out of 12 disbudded calves were already weaned while the remaining 2 in addition to 13 non-disbudded calves were un-weaned. Calves were housed as follows; 7 and 5 of disbudded calves in pens 2 and 4 while 8 and 5 of calves in the non-disbudded category resided in pens 1 and 3. Calves in one pen had no contact with calves in neighbouring pen. Using the same design as above, the EBR of each dairy calf was recorded four times daily for five days [08:00; 12:00; 14:00 and 16:00]. Each calf was allotted five minutes recording time per time point. Additionally, the postural [standing or lying] behaviour of each calf was noted. Furthermore, if the dairy calf was engaged in any oral behaviour such as chewing, eating, drinking, licking an object or grooming, it was also noted. The experimental design is summarized in Figure 3 below.

Determination of EBR changes following restraint and disbudding in dairy calves. Nineteen calves [mean age: 5 weeks] were recruited for this study. Calves were divided into two groups. Group A comprised of 13 calves to be disbudded while group B (n = 6) served as the sham control. Group A was further divided into two unequal subgroups based on time of analgesic intervention during disbudding for the purpose of a separate study (behavioural assessment). Five of 13 calves (A1) received meloxicam injection (2% Metacam®; Boehringer Ingelheim, Ingelheim am Rhein, Germany) prior to disbudding while the remaining eight (A2) received post-disbudding analgesia. These 13 calves were the non-disbudded calves studied in the replicate study described above and thus, did not need acclimatization. The rest of the study population were acclimatized to the observer for three days before data collection commenced. Groups A1, A2 and B calves were housed in three separate group pens and calves in one group pen had no contact with other pens. The experimental design is summarized in Figure 4 below. Prior to disbudding, baseline EBR was obtained for each calf once daily (between 08:00 to 10:00 hours; 5 mins per calf) for five consecutive days (Baseline days -1 to -5). On the day of disbudding and prior to calf restraint and

disbudding, baseline EBR of each calf was determined for five minutes (between 08:00 to 10:00 hours) using the same protocol described above (Baseline day 0).

Restraint and pre-disbudding protocol. Calves were restrained in a calf crush with a head bail (Short Mobile Cattle Crush®, Glendale Engineering, UK). Dairy calves in group A entered the calf crush [Short Mobile Cattle Crush®, Glendale Engineering, UK] twice; first to receive cornual nerve block (and analgesic treatment in Group A1) and secondly for other stages of disbudding (clipping, disbudding, analgesic treatment in Group A2) to be performed. Local anaesthesia was achieved using procaine hydrochloride plus epinephrine tartrate [2.5 ml per horn; 4% Pronestetic®, FATRO S.p.A, Italy] on the cornual nerve located midway between the base of the horn and the lateral cantus of the eye[51]. Following loss of pain sensation, the hairs around each horn were clipped using a cordless clipper and this lasted approximately 1 minute in Group A calves. Data collection for the determination of EBR changes during crush restraint was done immediately calves were restrained in Group B and after pre-operative protocols in group A. The EBR was assessed for only two minutes per calf in the two groups. The assessment duration was shorter to ensure that calves were disbudded within 10 minutes following anaesthetic induction as recommended[52].

Disbudding procedure. Disbudding was performed using the hot iron method by trained personnel assisted by college students. A pre-heated gas dehorner was placed over each horn bud for approximately 1 minute per horn to ensure the destruction of the horn bud. The horn buds were detached, and cauterization was done using the gas dehorner to prevent bleeding. Following disbudding, chlortetracycline hydrochloride (2.45% w/v Cyclo spray, Dechra®, North Yorkshire) was sprayed on each surgical site and meloxicam at 0.5mg/kg was also administered subcutaneously in Group A2 on the neck region. Following the completion of the disbudding procedure in Group A calves, the EBR was assessed for three minutes and calves were then returned to their group pens.

Sham-disbudding procedure. Sham disbudding was performed in similar fashion as in disbudding, but a cold gas dehorner was used to manipulate each horn bud of group B calves for approximately 1 minute. Sham disbudding was performed by the same trained personnel assisted by college students on a different day but at similar times. Immediately after sham-disbudding, the eye blink rate of each dairy calf was assessed for three minutes as was done for the disbudded group and calves were returned to their corresponding pre-disbudding pen. No sham-disbudded dairy calf received anaesthetic or analgesic treatment.

Post-disbudding and post-sham-disbudding, the EBR of each calf was assessed daily for 5 days between the hours of 08:00 and 10:00 using similar method as described above (post-disbudding days +1 to +5).

Statistical analysis. All statistical analyses were performed using IBM SPSS version 25.0 for Windows[53]. The EBR values were transformed into an average blink rate per minute. Data for the determination of EBR patterns were subjected to One-way Repeated measures ANOVA (Confidence interval adjustment: Bonferroni test) since they were normally distributed, and variances were homogenous. Day and time

served as the ‘within subject’ and ‘between subject’ variables, respectively. The assumption of sphericity ascertained by Mauchly’s test was not violated. Furthermore, due to the varying calf population in the replication study, the EBR of dairy calves (blocked for day and time effects) was compared across variables including disbudding [previously disbudded/ not disbudded], weaning [weaned/ un-weaned], posture [standing vs lying] and oral behaviour [oral vs no oral].

Data for the determination of EBR changes following restraint and disbudding met the assumptions of normality and homogeneity of variance and were subjected to One-way repeated measures ANOVA. Where significant differences were observed, independent sample t-tests were run to detect differences between groups while paired sample t-tests were run to detect within group variations. For all statistical analysis, significance was accepted at $P \leq 0.05$ and results are presented as mean [±SEM].

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Declarations

Acknowledgements

Authors thank the staff of Rodway Farm, Bridgwater and Taunton College, Cannington, for their support during the data collection period. N.U. is funded by Tertiary Education Trust Fund (TETFUND) and the University of Bristol.

Author contributions

The study was conceptualized by N.U., JH and H.R.W.; designed and edited by N.U., J.H., J.M. and E.L.; data collection, analysis, and original manuscript draft by N.U. while T.G.K reviewed the data analysis. All the authors (N.U., E.L., J.M., H.R.W., T.G.K. and J.H.) discussed the findings, reviewed the manuscript, and approved the final version to be submitted.

Competing interests

No competing interest is declared.

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Figures

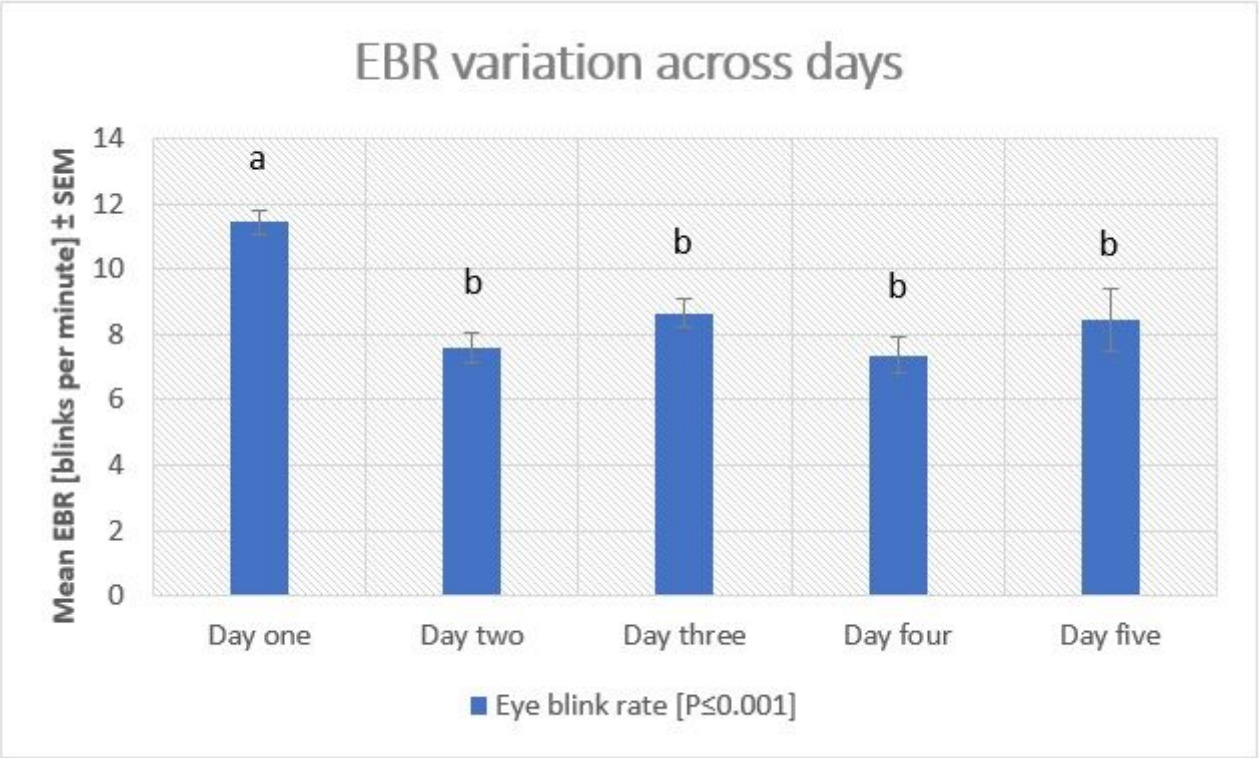

Figure 1

The mean EBR [±SEM] comparison across days of dairy calves (n = 25). Different superscripts a, b represent significance at P ≤ 0.001. Mean EBR on day one was significantly higher the mean on days two to five.

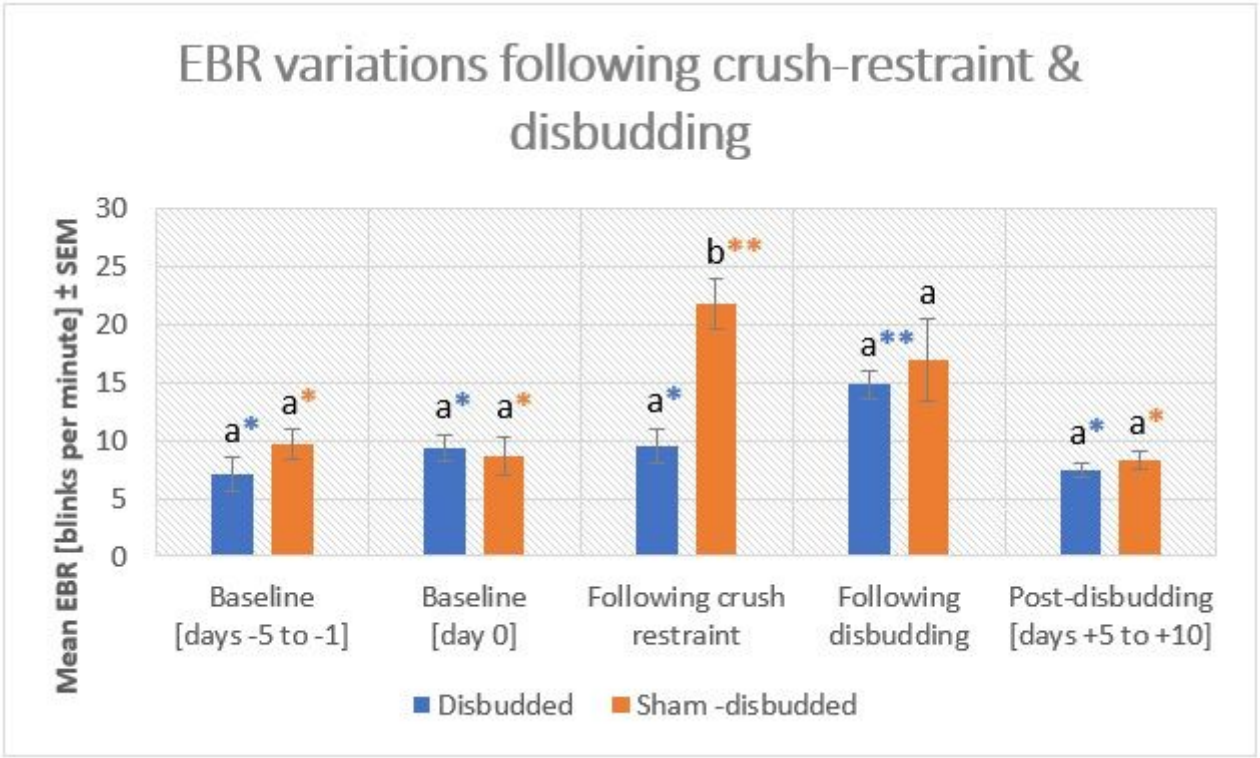

Figure 2

EBR variations following crush-restraint and disbudding. Different superscripts a, b represent significant differences between the disbudded and sham group. Following crush restraint, mean EBR was significantly higher in the sham group. Mean EBR following disbudding was significantly higher than other time points as shown by the blue* and while mean EBR following crush restraint and sham-disbudding was significantly higher than other time points represented by the orange*.

Days	EBR Assessment times 5 minutes observation/ calf at each time point	
	First study N = 10 calves	Replicate study N = 25 calves
0 [Acclimatization]	✓	✖
One	10:00 12:00 14:00 16:00	08:00 12:00 14:00 16:00
Two	10:00 12:00 14:00 16:00	08:00 12:00 14:00 16:00
Three	10:00 12:00 14:00 16:00	08:00 12:00 14:00 16:00
Four	10:00 12:00 14:00 16:00	08:00 12:00 14:00 16:00
Five	-------	08:00 12:00 14:00 16:00
Total	800 minutes for 10 calves	2500 minutes for 25 calves

Figure 3

Data collection design for the determination of eye blink rate of dairy calves (n = 35)

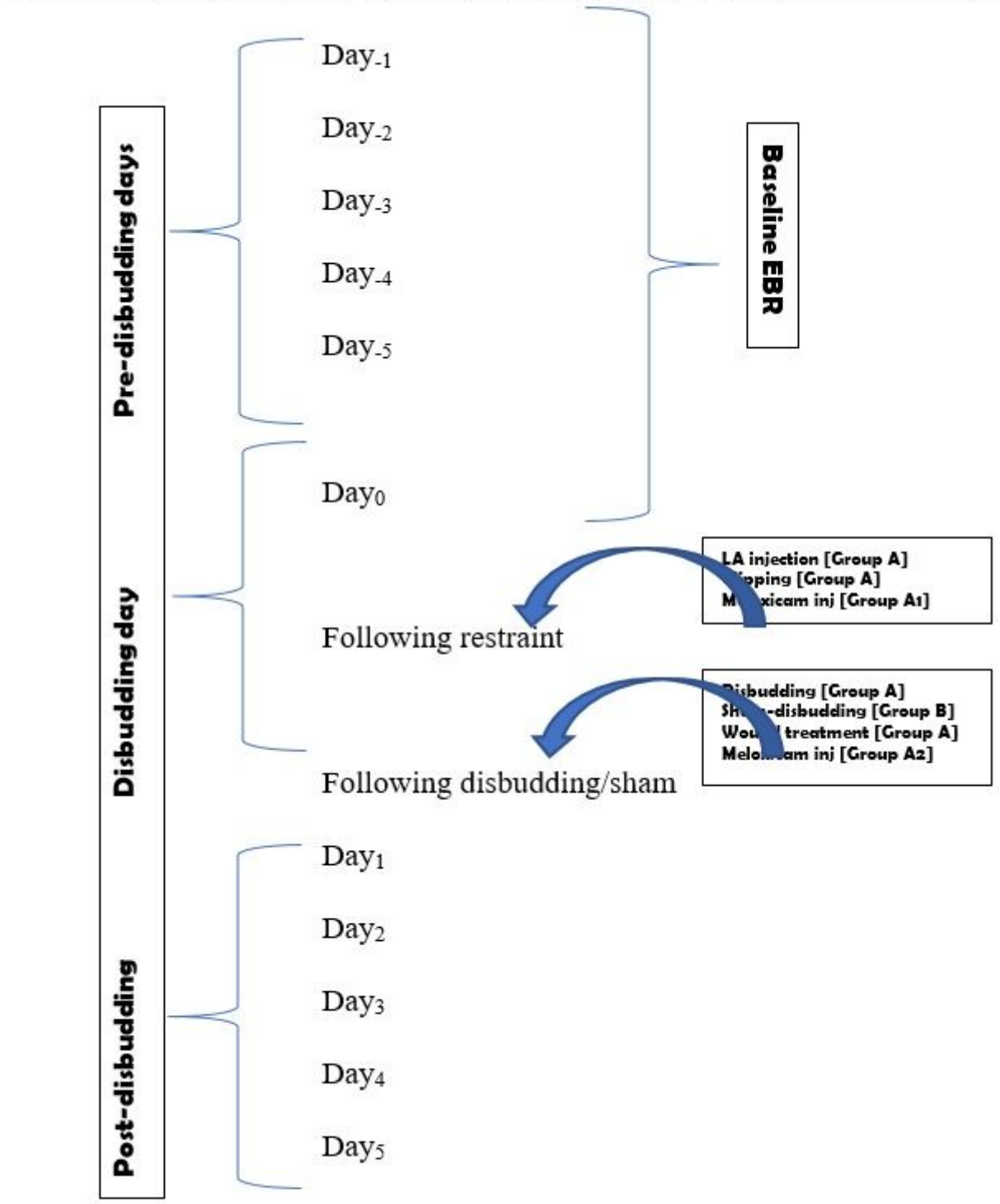

Figure 4

Data collection design for the determination of EBR changes following restraint and disbudding