Aus der Tierklinik für Fortpflanzung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Optimizing calf health on German dairy farms through pain reduction after disbudding and colostrum quality control

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
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Tierärztin
aus Berlin

Berlin 2024
Journal-Nr.: 4430

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):

Calves, dairy cattle, animal health, disbudding, cow colostrum, hydrocortisone, non-steroidal antiinflammatory agents, cutaneous application, ELISA, milk testing, milk quality

Tag der Promotion:

20.02.2024

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Publication I

Publication II

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Additional unpublished work

Abbreviations

AEA	apparent efficiency of absorption
a.m.	morning
AUC	area under the curve
CI	confidence interval
°C	Celsius
d	day(s)
e.g.	exempli gratia
ELISA	enzyme-linked-immunsorbent-assay
et al.	et alii/aliae/alia
EU	European Union
FGM	fecal glucocorticoid metabolite
FPT	failure of passive transfer
g	gram(s)
g	gravity
h	hour(s)
HPA	hypothalamic–pituitary–adrenocortical axis
Ig	immunoglobulin
i.m.	intramuscular
i.v.	intravenous
kg	kilogram(s)
L	liter
m	meter(s)
mg	milligram(s)
mL	milliliter
mm	millimeter
NPV	negative predictive value
NSAID	nonsteroidal anti-inflammatory drug
P	probability
p.m.	afternoon
PPV	positive predictive value
RID	radial immunodiffusion
ROC	receiver operating characteristic
s	second(s)
SAM	sympathoadrenal medullary
SD	standard deviation
SE	standard error
Se	sensitivity
SOP	Standard operating procedure
Sp	specificity
TP	total protein
TS	total solids
US	United States
µg	microgram(s)
vs.	versus
w	half-width(s)

Introduction

Interest in animal welfare from both the general public and famers has increased in recent years in Europe. Of particular relevance are the health of the animal, the extent to which the animal suffers pain or aversive emotions, and the animal's ability to perform most normal behaviors (Fraser et al. 1997). This is also consistent with the World Organization for Animal Health (2008) definition of animal welfare. For calf welfare, Vasseur et al. (2010) summarized the most important management factors as follows: use of a calving box and calving monitoring, navel disinfection, colostrum management, milk quantity, appropriate feed conversion rate, individual housing of calves, and disbudding. The presence of horns in commercial cattle is considered as problematic, due to damaged hides and bruising of cattle, especially during transport, feeding or territory fights (Mendonça et al. 2016; Shaw et al. 1976). Furthermore, horned cattle require more space in the stable, feeding bunks or cattle truck. In contrast, disbudding cattle leads to reduced risk of injuries in staff and other cattle.

Several disbudding methods are possible depending on the legislation in each country.In general, the procedure is recommended to be performed when the calf is young (before 8 weeks of age) and the horn bud small (Winder et al. 2016). Disbudding is defined as the removal of the horn bud (Cozzi et al. 2015). The entire horn bud or at least a ring of skin 1 cm in diameter around the horn bud is removed in order to prevent horn growth. Common disbudding methods are the use of chemical paste (25.7%) or a hot iron (71.0%) in the European Union (EU) member states (Cozzi et al. 2015). Regardless of the method, disbudding is considered as a painful and stressful practice for calves (Winder et al. 2016). These negative factors include the pain from the disbudding procedure and stress from the animal being handled and restrained (Sylvester et al. 1998).

According to Selye (1976), stress describes the nonspecific response of the body to any demand made upon it. Several physiological, psychological, and behavioral changes as a response to a particular stimulus are associated with stress (Schuler 1980). Stress can be classified eustress and distress. Eustress is a form of stress that increases the animal's capacity to cope with an unknown situation, so its adaptive capacity increases (Kupriyanov and Zhdanov 2014). The negative counterpart is distress. Distress leads to the incapacity of the body to return to physiological and psychological homeostasis (Carstens and Moberg, 2000). Both forms activate a hormonal cascade in the hypothalamic–pituitary–adrenocortical axis (HPA axis) and the sympathoadrenal medullary system (SAM system). As a result, increased secretion of catecholamines and glucocorticoids, such as cortisol, occurs from the adrenal glands. Cortisol is the hormone most commonly used to assess pain and stress response and is measurable in different media i.e., blood, saliva, feces, urine, hair and milk (Palme 2019; Heimbürge et al. 2019; Möstl et al. 2002). Cortisol plays an important role in regulating the metabolism (Korte et al. 1993) and causes increased gluconeogenesis from amino acids and has protein catabolic effects (Khani and Tayek 2001). Apart from these physiological changes in the body, other detectable changes occur, i.e., in heart rate, feed rejection, and standing and lying behaviors, which can also be used as signs of pain and stress response (Nogues et al. 2021; Barragan et al. 2018; Stewart et al. 2010). Some specific avoidance behaviors and neuroendocrine changes indicative of pain and distress observed following disbudding can be dampened by administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and local anesthetics (Stock et al. 2013).

Adequate postoperative pain relief depends on properly used NSAIDs and local anesthesia as well as a combination of the two approaches. The local anesthetic, lidocaine, was reviewed as effective for 2 to 3 h by Stafford and Mellor (2005), but there is no legislation in Germany to date that allows the use of this drug for food producing animals. Furthermore, for pain control after disbudding, the combined use of a NSAID and a local anesthetic is recommended because this combination lowers plasma cortisol and drives behavioral responses close to baseline levels (Winder et al. 2018). Furthermore, calves sedated with xylazine reacted less to painful procedures like disbudding (Reedman et al. 2021). Stress caused by physical restraint due to the disbudding procedure itself or administration of local

anesthetics or NSAIDs can be alleviated by the use of a sedative, and therefore, handling can be refined due to shorter time required to administer a nerve block (Reedman et al. 2021). In conclusion, a combination of sedative, local anesthetic, and NSAID reduces the response to pain during and after disbudding, while also lowering the stress caused by restraining, as is recommended by Stock et al. (2013). Minimum standards for the protection of calves do not regulate disbudding procedures regarding the current European legislation (Directive 91/629/ECC). In the EU, hot-iron disbudding is reported to be performed mainly by farm personnel (71.7%) and the use of pre- and post-operative medications is limited (50.7%, Cozzi et al. 2015). Farmers reported relatively little use of local anesthesia (26.6%) and sedation (24.8%) when hot-iron disbudding is carried out (Cozzi et al. 2015). This is associated with the legally restricted use of anesthetics or sedatives to veterinarians in some countries (Cozzi et al. 2015). However, due to economic reasons and lack of legal requirement, veterinarians are mostly not involved in disbudding procedures (Cozzi et al. 2009). Thus, an effective and practical medication is needed in order to reduce the pain and stress caused by disbudding. The use of the NSAID meloxicam (i.m. injection) caused a significant reduction of plasma cortisol concentration 6 h after disbudding (Heinrich et al. 2009). A similar effect was described for flunixin meglumine (2.2 mg/kg, i.v. injection) at the time of disbudding (Huber et al. 2013). A new transdermal solution of flunixin meglumine allows a simple route of administration and has been licensed in the EU in 2014 (Finadyne Transdermal, MSD Animal Health; approval number 835578) for adult cows, as well as in the US (United States Department of Agriculture 2009). Minimal training is needed for the use of the transdermal solution of flunixin meglumine, due to its administration across the back line of an animal. The use of the transdermal flunixin meglumine solution as pain medication at the time of disbudding in dairy calves, however, is currently not approved in the EU.

Calf morbidity and mortality have a major impact on the profitability of cattle farms due to the direct costs of loss and veterinary treatment as well as the long-term impact on performance (Donovan et al. 1998). Due to the impermeability of cows' placentas, calves at birth are agammaglobulinemic (Mee et al. 2014). Therefore, passive immunity of neonatal calves should be provided and correlates highly with improved neonatal health including decreased mortality rates and higher daily weight gains (Robison et al. 1988).

One of the most important factors is good colostrum management (Godden et al. 2019). This includes quick and hygienic harvest of colostrum and feeding of the first postnatal meal within the first 2 h of life. Due to the closure of intestinal permeability, the process is time limited. Absorption accelerates until 12 hours after birth, and permeability ceases completely 24 hours after birth (Stott et al. 1979). Fischer et al. (2018) reported decreased transfer of IgG due to a delay in colostrum feeding past 6 h after birth. Values for apparent efficiency of absorption (AEA) for calves fed after birth within 1, 1 to 6, 6 to 12, and 12 to 18 h were 30.5, 27.4, 23.7, and 15.8%, respectively (Osaka et al. 2014). Furthermore, an appropriate volume (10% of birth weights) of colostrum that contains at least 50mg/mL IgG is required in order to enhance the successful passive transfer of IgG (Godden 2008; Weaver et al. 2000; Pritchett et al. 1991).

Colostrum contains nutrients and non-nutrient factors, such as fat, protein, vitamins, minerals, lactose, biologically active peptides, hormones, growth factors, cytokines, maternal leukocytes, and the main component, immunoglobulins (McGrath et al. 2016; Soberon et al. 2012; Kehoe et al. 2007; Faber et al. 2005). More specifically, colostrum contains milk-borne factors such as growth hormone, epidermal growth factor, insulin, insulin-like growth factor I, prolactin, leptin, and relaxin. These influence the epigenetic development and growth of specific tissues, including liver, gastrointestinal tract, spleen, kidneys, and muscle. Colostrum contains immunoglobulin G (IgG, 85-90%) (subtypes IgG1 and IgG2), immunoglobulin M (IgM; ≤7 %), and immunoglobulin A (IgA; ≤5 %, Godden 2008). Due to the impermeability of the bovine epitheliochorial placenta, the transfer of immunoglobulins in utero to the fetus is not possible for maternal antibodies. Instead, immunoglobulins and milk-borne factors are absorbed through pinocytosis across the enterocytes of the small intestine from colostrum (Bagnell et al. 2017; Bartol et al. 2008; McGuirk and Collins, 2004; Weaver et al. 2000). Failure

of passive transfer (FPT) describes inadequate passive immunity and is one of the major factors to high mortality and morbidity of neonatal calves (Boccardo et al. 2016; Beam et al. 2009; Trotz-Williams et al. 2008).

The quality of colostrum is determined by the main immunoglobulin component IgG. Since IgG is measurable, it is an optimal parameter to study in order to improve colostrum management. The Ig concentration in colostrum can be measured with direct or indirect methods. The indirect methods (i.e., specific gravity, density, viscosity) allow us to draw conclusions about the Ig concentration of colostrum based on its chemical or physical properties, used as indicators of Ig concentration. In order to integrate colostrum testing into the daily workflow, regardless of whether the test is direct or indirect, it should be accurate, easy to use, quick, and cost effective (Bartens et al. 2016; Bielmann et al. 2010).

The direct laboratory method, radial immunodiffusion (RID), is considered historically as the gold standard to measure the IgG concentration in colostrum (Gelsinger et al. 2015). However, this method requires up to 24 h to get the results, is expensive, and can only be used in a specialized laboratory (Davis and Giguère 2005). A quantitative method based on immunoprecipitation is RID. An antibody-containing agarose gel plate is used to determine the antigens in a colostrum sample. To the best of our knowledge, there is no validated RID laboratory in Europe to date.

Another direct laboratory method, enzyme-linked immunosorbent assay (ELISA), may be an economical alternative method compared to RID (Gelsinger et al. 2015). ELISA tests are also based on antigen-antibody reactions. Commercial test kits are available and have been used in colostrum analysis (e.g., Sutter et al. 2020; Gelsinger et al. 2015). Due to the specific equipment needed and reagents with limited shelf life, the user must also be trained for the procedure resulting in higher costs (Gelsinger et al. 2015; Davis and Giguère 2005). Therefore on-farm tests have been established for use in routine in dairy work.

Indirect measurements of IgG in colostrum can be obtained by the digital Brix refractometer, which measures the concentration of dissolved substances in liquids, thereby permitting a conclusion about the immunoglobulin content according to the colostrum density (Quigley et al. 2013). The Brix refractometer is easy to handle and requires minimal training but additional equipment (Bartens et al. 2016; Quigley et al. 2013). Compared to RID, correlation coefficients to identify high-quality colostrum were good (Brix refractometer: $r = 0.64$ to $r = 0.87$; (Bartier et al. 2015; Coleman et al. 2015; Vandeputte et al. 2014; Bielmann et al. 2010).

Indirect measurements of another type can be obtained by the colostrometer, which measures the specific gravity due to the liquid density and, therefore, provides information on the content of immunoglobulins in colostrum (Fleenor and Stott 1980). Compared to RID, correlation coefficients to identify high-quality colostrum were good with a colostrometer ($r = 0.77$; (Bartier et al. 2015). However, the colostrometer works at a specific temperature (20–21°C). Colostrum should be therefore evaluated at 22°C to obtain reliable results (Conneely et al. 2013). Also, due to its glass spindle, the colostrometer is more likely to break during use than other instruments for measuring immunoglobulin in colostrum (Bartens et al. 2016).

A further indirect analytical method is the outflow funnel (ColostroCheck), measuring the time in seconds a defined volume of the fluid flow through it. Based on the viscosity of the fluid and on the assumption that in colostrum, an increased viscosity is associated with greater concentrations of immunoglobulins, an estimation of the content of immunoglobulins is permitted. Like the specific gravity, the viscosity depends on the temperature of colostrum. The outflow funnel is used at 30°C to perform the measurement.

To the best of our knowledge, the only analytical method used as an on-farm test is the semi-quantitative immunochromatographic assay, SmartStrips IgG Colostrum, which directly determines the IgG concentration (mg/mL) in colostrum. Similar to other lateral flow assays, this test method is based on an antigen-antibody reaction. Bovine IgG in colostrum reacts with

anti-bovine IgG of the test strips, resulting in a visible color change due to the accumulation of the color-labeled antigen-antibody complexes. By taking a picture of the test line with the smartphone camera using the SmartStripsTM App the line intensity is interpreted and compared to a stored standard curve. The semi-quantitative immunochromatographic assay has not been evaluated according yet for estimating colostrum quality.

To provide optimal health for newborn calves, a sufficient intake of good quality colostrum (IgG > 50mg/mL) is essential. Therefore, an accurate on-farm test for assessment of colostrum quality is needed.

The overall objective of this thesis was to evaluate important management factors for calf health. In the first study, we compared the effect of pain mitigation after disbudding of two treatments of transdermal flunixin meglumine combined with local anesthesia, compared to a non-treated control group, by analyzing plasma cortisol and behavioral changes. In the second study, 2 laboratory based analytical methods to determine colostrum quality, (RID, ELISA), and 4 on farm tests (digital Brix refractometer, colostrometer, outflow funnel, semi-quantitative immunochromatographic) were evaluated. As an addition (third study) the time of disbudding was used to perform cortisol (metabolite) measurements in different media (plasma, saliva, feces).

Publications I and II were published in the Journal of Dairy Science (Impact Factor: 3.70). The additional unpublished work has been formatted according to the Journal of Dairy Science guidelines.

Publication I

The effect of transdermal flunixin meglumine on blood cortisol levels in dairy calves after cautery disbudding with local anesthesia

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Published in:

Journal of Dairy Science, April 2022, Volume 105, No. 4, Pages 3468-3476

Please find the original article via the following digital object identifier:

J. Dairy Sci. 105:3468–3476

https://doi.org/10.3168/jds.2021-21257

The effect of transdermal flunixin meglumine on blood cortisol levels in dairy calves after cautery disbudding with local anesthesia. By Röder et al., page 3468. 3476.

The objective of this study was to evaluate the effect of transdermal flunixin meglumine on plasma cortisol levels after cautery disbudding with local anesthesia in dairy calves. A sham disbudding procedure was performed first in order to determine cortisol levels related to the manipulation. Plasma samples were collected at different time points before and after sham disbudding and disbudding in order to assess the calves' stress response. Treatment with transdermal flunixin meglumine resulted in decreased plasma cortisol concentration but had no detectable effect on calves' lying behavior after cautery disbudding.

Abstract

The objective of this study was to evaluate the effect of the nonsteroidal anti-inflammatory drug (NSAID) transdermal flunixin meglumine (Finadyne Transdermal) on plasma cortisol, average daily weight gain and standing and lying behavior of calves, when given at the time of disbudding combined with local anesthesia. A sedative was not used to minimize pharmacological interactions. Seventy-one female Holstein Friesian calves aged 13 ± 2 d, with an average weight of 48.9 ± 4.26 kg were enrolled in the study. All calves were randomly assigned to one of three treatment groups: 1) control group (CON, n = 27), 2) 1-flunixin group (1-FLU, n = 26) with a single administration of transdermal flunixin meglumine at disbudding, and 3) 2-flunixin group (2-FLU, n =24) with 2 administrations of transdermal flunixin meglumine, the first treatment at disbudding and the second 6 h after disbudding. While the CON group received a placebo, 1-FLU and 2-FLU received flunixin meglumine transdermally. To account for plasma cortisol changes due to manipulation and handling of the calves, a sham disbudding procedure was performed one week before disbudding took place. Sham disbudding was conducted by using a cold cautery dehorner applied to each horn bud for 10 s. Disbudding was performed in a similar way by using a hot cautery dehorner. Plasma samples were collected to measure the stress biomarker cortisol at seven different time points. Body weights were measured four times in two weeks. Standing and lying behavior was assessed via 3D accelerometer. During sham disbudding and disbudding mean plasma cortisol concentrations were 6.09 ± 2.5 ng/mL and 5.16 ± 2.8 ng/mL, respectively. Treatment tended to have an effect on plasma cortisol concentrations during sham disbudding ($P = 0.08$) and had an effect on plasma cortisol concentrations during disbudding ($P < 0.01$). Plasma cortisol concentrations were affected by treatment two hours after disbudding (1-FLU, $P = 0.01$; 2-FLU, $P = 0.05$) in comparison to CON group. Furthermore, there was a significant effect on plasma cortisol concentrations 6 h after disbudding in contrast to CON (1-FLU, $P = 0.01$; 2-FLU, $P = 0.05$). A return to baseline plasma cortisol levels (initial concentrations) was not achieved in CON during disbudding. There was no statistical difference between average daily weight gain and the treatment procedure ($P = 0.74$). Total lying time was not affected by treatment after disbudding ($P = 0.31$). In conclusion, transdermal flunixin meglumine given at the time of disbudding combined with local anesthesia decreased concentrations of the stress biomarker cortisol, but a second dose 6 h after disbudding had no further effect on plasma cortisol levels.

Key words: disbudding, dairy calves, cortisol, flunixin meglumine, transdermal.

Introduction

Disbudding dairy calves before 8 wk of age is a common practice in the dairy industry (Winder et al., 2016). There are 2 main reasons why dairy calves are being disbudded: easier handling of cattle and reduced risk of injuries in staff and cattle. In about 81% of dairy herds in the European Union (EU; Cozzi et al., 2015) and 94% in the United States (USDA, 2009), disbudding of dairy calves is conducted. In the United States, 32% of farmers use a hot-iron for disbudding procedure (FARM v. 4.0;https://nationaldairyfarm.com/farm-animal-care-version-4-0/). Disbudding leads to pain-related distress, which can be measured in behavioral and hormonal changes (Stafford and Mellor, 2005). To control pain, medication should be used and calves should be disbudded prior 8 to wk of age (FARM v. 4.0). Stressful situations activate a hormonal cascade of the hypothalamic–pituitary–adrenocortical axis (Sylvester et al., 1998). As a result, an increased activity of the adrenal glands occurs, which leads to a higher release of glucocorticoids (i.e., cortisol) and catecholamines (Axelrod and Reisine, 1984). Cortisol can be measured in different media such as blood or its metabolites can be measured in feces (Mormède et al., 2007; Palme, 2019). After disbudding, a rapid cortisol release is observed and results in an initial peak of cortisol in plasma within 30 min after disbudding (Stafford and Mellor, 2005). A second plasma cortisol peak occurs between 3 to 8 h after disbudding if local anesthesia is used for prevention of pain (McMeekan et al., 1998; Sutherland et al., 2002; Milligan et al., 2004; Stock et al., 2013). According to Heinrich et al. (2010), pain-associated behavioral changes varied during 44 h with a peak at 6 h after disbudding, implying that calves may be discomforted during this time despite analgesia. Local anesthetic such as lidocaine decreased plasma cortisol levels after disbudding for approximately 2 h and caused a delayed rise of plasma cortisol lasting for 6 h (Sutherland et al., 2002; Stafford and Mellor, 2005). The use of the nonsteroidal anti- inflammatory drug (NSAID) meloxicam can cause a significant reduction of plasma cortisol concentration 6 h after disbudding (Heinrich et al., 2009).

In the United States and the EU, only 1.8% and 30 to 40% of farmers reported the use of an analgesic treatment before or after disbudding, respectively (Fulwider et al., 2008; Cozzi et al., 2015). This is due to economic reasons and a lack of training on how to perform disbudding properly including analgesia (Gottardo et al., 2011).

In a randomized controlled trial, a single intravenous treatment using flunixin meglumine (2.2 mg/kg) at the time of disbudding reduced cortisol concentrations in blood plasma (Huber et al., 2013). A second intravenous treatment with flunixin meglumine 3 h after disbudding led to a similar cortisol concentration up to 6 h after disbudding in comparison to the single treatment and the nondisbudded control group. Cortisol concentrations were higher in placebo-treated calves when compared with those in calves receiving 2 administrations of flunixin meglumine 3 h apart and tended to be higher compared with those with a single administration (Huber et al., 2013). Calves treated with flunixin meglumine (once or twice) showed less head shakes and head rubs compared with a placebo-treated control group (Huber et al., 2013). The use of the transdermal flunixin meglumine solution as pain medication at the time of disbudding in dairy calves is currently neither approved in the EU, nor in the United States.

In the EU, flunixin transdermal solution (Finadyne Transdermal, MSD Animal Health; approval number 835578) was licensed in 2014 for treatment of fever, pain, and lameness in cattle associated with respiratory diseases, acute mastitis and interdigital phlegmon. In the United States and Canada, it has been approved for the control of fever associated with bovine respiratory disease and for pain management in lame cattle (US Food and Drug Administration, 2017). This solution is administered across the back line of an animal and absorbed transdermally into the bloodstream, which allows a simple way of administration.

Flunixin meglumine is effective in the treatment of acute inflammation (Thiry et al., 2017) and chronic visceral pain (Cook and Blikslager, 2015). It also serves as a strong antiphlogistic and antipyretic drug. The transdermal solution has a longer half-life (6.42 h) compared with the injectable solution (4.99 h; Kleinhenz et al., 2016). The bioavailability is, however, reduced compared with other administration routes.

The objective of this study was to evaluate the effect of 2 treatments of transdermal flunixin meglumine combined with local anesthesia on blood cortisol concentrations after cautery disbudding in dairy calves. We hypothesize that (1) treatment with transdermal flunixin meglumine at the time of disbudding reduces plasma cortisol concentrations compared with the control group, and (2) a second treatment with transder- mal flunixin meglumine 6 h after disbudding extends the effect on plasma cortisol concentrations.

Material and Methods

The reporting guidelines for randomized controlled trials in livestock and food safety (REFLECT) by (Sargeant et al., 2010) was used as reporting guideline for the manuscript.

Dairy Farm, Animal Enrollment and Housing

The study was conducted from February to August 2020 on a commercial dairy farm in Northeast Germany. The study protocol was in accordance with the Institutional Animal Care and Use Committee of the Freie Universität Berlin (approval number: 2347-49-2019).

An a priori sample size calculation was performed using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium) considering cortisol concentrations from a previous study. According to (Kleinhenz et al., 2017) the sample size calculation was conducted assuming a decrease in cortisol concentration to 6.4 ± 7.2 ng/ml between control and treatment group. Presuming 80% power and a confidence level of 95%, resulting in a total number of 20 dairy calves per group. In order to account for follow up losses 17 additional calves were enrolled.

Calves were housed in individual calf hutches measuring 2.05 m x 1.15 m x 1.35 m and bedded with straw. Further, each calf hutch had an individual paddock with sand bedding (1.50 m x 1.10 m). All calves were separated from their dams immediately after birth and placed into an individual calf hutch. Every calf received 3. 0 L of colostrum from their dam within 4 h after parturition using a nipple bottle. Colostrum was fed during the first three d of life using a teat bucket three times a day (4.5 L/d). Feeding took place at 4:30 a.m., 12:30 p.m., and 7:30 p.m. A mixture (70:30) of milk replacer (MR; Sprayfo Vitesse 50, Trouw Nutrition Deutschland GmbH; with a concentration of 150 g of MR/L) and colostrum was fed 3 × 2.0 L/d from 4 d until 14 d. From 15 d until 35 d the calves received twice daily 3.0 L of MR (Sprayfo Vitesse 50, Trouw Nutrition Deutschland GmbH, Diepholz, Germany; 175 g MR/L). From 36 d until weaning the feed quantity was reduced to 2 x 2.3 L (4.6 L/d; 175 g of MR/L). Each calf had ad libitum access to water and starter grain from 4 d. The visual and tactile contact with adjoining calves was possible. Feeding took place at 4:30 a.m. and 7:30 p.m. p.m.

Experimental Design and Drug Administration

Holstein Friesian dairy calves (n = 77) had to meet the following inclusion criteria: minimum 265 days of gestation, female, eutocia and singleton to be enrolled. Calves were randomly assigned to one out of three groups: 1) control group (**CON**, n = 27), 2) 1-flunixin group (**1-FLU**, n = 26) with a single administration of transdermal flunixin meglumine at disbudding, and 3) 2-flunixin group (**2-FLU**, n = 24) with 2 administrations of transdermal flunixin meglumine, the first treatment at disbudding and the second 6 h afterwards. We administered a second dose at 6 h after disbudding to test the hypothesis that the analgesic effect of flunixin meglumine could be extended due to the half-life of 6.42 h to 13.2 h (Wagner et al., 2021). The CON group received a placebo treatment, a mixture of 80% lubricant (Gleitcreme Bengen, WDT e.G., Garbsen, Germany) and 20% water.

The placebo and flunixin meglumine (Finadyne Transdermal, MSD Animal Health, Unterschleißheim, Germany) were applied at the top line of the back using the dosage chamber of the bottle. The calculated doses of flunixin transdermal solution were based on actual body weight measurements. A range of 3.21 to 3.84 mg/kg (mean 3.45 mg/kg) has been reported when the dosing chamber was used (Martin et al., 2020). To ensure that the solution was not accidentally removed during the disbudding procedures, placebo or transdermal

flunixin meglumine was applied directly after disbudding. The treatments were not blinded. A random number list was generated in Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany) before the initiation of the study to allocate all calves into one of the three groups.

Sham Disbudding and Disbudding

For both disbudding procedures the area for the local anesthesia was shaved and cleaned with an alcohol-soaked gauze before administering local anesthesia. The local anesthesia was applied 10-15 min before sham disbudding or disbudding took place. The calf was restrained and a cornual nerve block was performed as described by (Reedman et al., 2021) at each horn bud. Administered with an 18-gauge, 35-mm disposable needle (B. Braun Melsungen AG, Melsungen, Germany) and disposable syringe (5 mL, Henry Schein, Langen, Germany). The puncture site was located halfway between the lateral corner of the eye and the horn bud of the corresponding site. The puncture direction was perpendicular to the skin, below the palpable external frontal crista. The *Ramus cornualis* of the trigeminal nerve was anesthetized with 5 mL of 2% procaine (Procamidor 20 mg/mL, WDT e.G., Garbsen, Germany) at a depth of approximately 1.5 cm.

A gas dehorner (Buddex, KERBL Corporation, Buchbach, Germany) was used for cautery disbudding. Before disbudding, the effect of the local anesthesia was verified by observing the reaction to 5 consecutive needle pricks into the horn bud and the surrounding skin area. Cautery disbudding was performed, if an aversive reaction was absent. Otherwise, a second administration of the local anesthetic was performed. For sham disbudding the cold gas dehorner was applied to each horn bud for 10 s. For disbudding the gas dehorner was pre-heated for 3 min to gain the recommended working temperature of 650°C and the same working procedure was used until a cautery ring around the horn tissue was achieved. After sham disbudding and disbudding, the horn bud area was treated with a topical aluminum spray (Aluminium-Spray, Albrecht GmbH, Aulendorf, Germany) and monitored for signs of infection the following 8 d. In order to determine the baseline cortisol concentration and to exclude bias due to manipulation and handling of the animal a sham disbudding procedure was performed before disbudding took place as done in previous studies (Stilwell, G., Lima, M.S. and Broom, D.M, 2007; Kleinhenz et al., 2017). Disbudding took place one week after sham disbudding. One week was considered as the washout period. Disbudding was performed in the morning hours between 6 a.m. and 9 a.m. For a given calf the time of sham disbudding matched with the time of disbudding.

Blood Sample Collection

Blood samples from the jugular vein were withdrawn via venipuncture 30 min before sham disbudding and disbudding (baseline), at sham disbudding and disbudding (0 min) and 30 min, 2 h, 6 h, 12 h, 24 h post disbudding into BD Vacutainer tubes spray-coated with lithium heparin (BD Vacutainer, Franklin Lakes, NJ). Due to a possible diurnal rhythm of cortisol concentrations (Hays et al., 1975), disbudding were always performed at the same time as sham disbudding. The blood collection times were the same for sham disbudding as for disbudding. The blood samples were inverted cautiously five times and cooled on ice until centrifugation (approximately 30 to 60 min after sampling). The centrifugation was performed for 6 min at 4,500 g. The obtained plasma was immediately pipetted into cryovials (Cryovial 2 mL, Simport Scientific Inc., Bernard-Pilon Beloeil, Canada) and stored at -18°C. One aliquot was sent on dry ice for the assessment of plasma cortisol concentration to the Unit of Physiology, Pathophysiology and Experimental Endocrinology of the University of Veterinary Medicine in Vienna. Cortisol concentrations in plasma following diethylether extraction (Schöffmann et al., 2009) were analyzed with a cortisol enzyme immunoassay (EIA) according to Palme and Möstl (Palme et al., 1997). A detailed description of the EIA, including cross-reactions, is provided in Palme and Möstl (Palme et al., 1997). The standard curve (80 to 20% relative binding) ranged from 1 to 30 pg/well, and the sensitivity of the EIA was 0.3 pg/well. Intra- (and inter-) assay coefficients of variation of high and low concentrated pooled samples were 9.2% (12.7%) and 12.8% (15.7%), respectively.

Daily Weight Gain, Lying and Standing Behavior

The animals were weighed with an electronic scale (Load bars HD5T with wooden bottom panel, Patura KG, Laudenbach, Germany) 24 h before and 24 h after both disbudding procedures. To calculate the average daily weight gain of each calf, the initial weight was subtracted from the last measured weight and divided by the difference in days.

Lying and standing behavior was recorded using a 3D accelerometer (Hobo Pendant G data logger, Onset Computer Corp., Bourne, USA) attached to the right hind leg of the calves. A randomized subset of 38 calves (CON, n = 13; 1-FLU, n = 13; 2-FLU, n = 12) was fitted with 3D accelerometers. The logger was mounted and activated 24 h before sham disbudding and left until 7 days after the disbudding procedure. It was fixed at the lateral side of the right hind leg above the metatarsophalangeal joint by using vet wrap bandage (Co-Flex, Andover Healthcare Inc., Salisbury, USA) and a velcro tape. To avoid bruises, the logger was wrapped in gauze bandage (Rolta-Soft, Paul Hartmann AG, Heidenheim, Germany) before fixation. Recording frequency was set to one reading per minute according to (Bonk et al., 2013). The data were downloaded from the accelerometers using the manufacturer's proprietary software and exported as one file per calf with comma-separated values (CSV). A custom-built script for data processing, written in the Python programming language (van Rossum et al, 1995) and utilizing the data analysis and statistics library pandas (McKinney, 2011), was developed. Output was stored as an Excel XLSX file (Office 2010, Microsoft Deutschland Ltd., Munich, Germany) containing multiple tables with the aggregated results and processing meta-data. The degree of vertical tilt (y-axis) was used to determine the lying position of the animal, such that readings ≥120° indicated the calf standing, and readings <120° indicated the calf lying down according to (Bonk et al., 2013). To verify and ensure accurate data processing of the script, PDF files with y-axis line graphs were generated per calf for visual inspection. Lying bout frequency, average lying bout length and total lying time per observation period were calculated. Data of one hour (n = 60) were summarized to a 1-hour observation period. The data was further processed in 6-hour sections (0-6 h: 0000-0600 h, 6-12 h: 0600-1200 h, 12-18 h: 1200-1800 h, 18-24 h: 1800-2400 h) until 4 days after sham disbudding and disbudding.

Statistical Analysis

To evaluate the effect of the two treatment protocols on plasma cortisol concentrations and on total lying time, number of lying bouts, and average lying bout length a generalized linear mixed model was constructed using the GENLINMIXED procedure of SPSS (version 22.0, SPSS Inc., IBM, Ehningen, Germany). The outcome variable was either concentration of plasma cortisol (ng/mL) or total lying time, number of lying bouts, or average lying bout length. Calf was the experimental unit.

The model for plasma cortisol concentration contained the following explanatory variables as fixed effects: treatment procedure (CON, 1-FLU, and 2-FLU); sampling time (hours; continuous); and the interaction of treatment protocol and sampling time. The model for total lying time, number of lying bouts, and average lying bout length contained the following explanatory variables as fixed effects: treatment procedure (CON, 1-FLU, and 2-FLU); time period (6-hour section); and the interaction of treatment protocol and time period. A negative binomial model was chosen to analyze number of lying bouts, as it provided the lowest AIC (Akaike-Information-Criterion) value in the GENLINMIXED procedure of SPSS.

Two separate models were used for sham disbudding and disbudding, respectively. To account for multiple comparisons, the *P*-value was adjusted using a Bonferroni correction. A significant statistical difference was specified for variables between the levels of a classification when $P < 0.05$; a statistical tendency was declared as differences between $P \geq 0.05$ and $P \leq 0.10$.

To evaluate significant differences between average daily weight gains of the three groups, a one-way analysis of variance (ANOVA) was performed using SPSS. The dependent

variable was average daily weight gain (kg/d; continuous), the independent variable was treatment procedure (CON, 1-FLU and 2-FLU).

Average, minima and maxima were determined using Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany).

Results

Overall, 77 calves were enrolled in the study. Six animals were excluded because an adequate local anesthesia was not provided (CON, n = 4; 1-FLU, n = 2). In the final analysis, 71 animals aged 13 ± 2 d with an average weight of 48.9 kg were considered (CON, n = 23; 1-FLU, n = 24; 2-FLU, n = 24).

Plasma Cortisol

The descriptive statistics for average plasma cortisol concentrations (ng/ml) for sham disbudding and disbudding are presented in Table 1 and 2.

Mean plasma cortisol concentrations during sham disbudding were 6.09 ± 2.5 ng/mL with a range of minimum 0.4 ng/mL up to maximum 29.6 ng/mL. Plasma cortisol was affected by time ($P < 0.01$). Treatment tended to have an effect on plasma cortisol concentration during sham disbudding ($P = 0.08$). The single treatment (1-FLU) showed a significant effect compared with CON ($P = 0.05$;−0.97 ng/mL; 95% CI: −1.99 to −0.01), whereas a second treatment (2-FLU) tended to have an effect ($P = 0.06$; −0.93 ng/mL; 95% CI: −1.88 to 0.02) compared with CON, respectively. The 2 treatment groups did not differ ($P = 0.94$; 0.04 ng/mL; 95% CI: −0.89 to 0.97). During disbudding (0 h) the mean plasma cortisol concentration was 5.16 ± 2.8 ng/mL (range: 0.2–26.4 ng/mL). Plasma cortisol concentration was affected by treatment ($P < 0.01$) and sampling time ($P < 0.01$). There was no interaction between treatment and sampling time ($P = 0.95$). The single treatment (1-FLU) showed a significant effect in comparison to CON ($P < 0.01$; −1.29 ng/mL; 95% CI −2.15 to −0.44), as well as a second treatment (2-FLU; $P < 0.01$; −1.29 ng/mL; 95% CI: −2.14 to −0.43) compared with CON, respectively. The 2 treatment groups did not differ significantly ($P = 0.99$; 0.01 ng/mL; 95% CI: −0.84 to 0.84).

Two h after disbudding the plasma cortisol concentration was reduced by a single treatment (1-FLU, $P = 0.01$) in comparison to CON group as well as 6 h post-disbudding (1-FLU, $P = 0.01$). The plasma cortisol concentration was also affected and reduced by a double treatment two h after disbudding (2-FLU, $P = 0.05$) in comparison to CON group as well as 6 h post-disbudding (2-FLU, $P = 0.05$). During disbudding the mean plasma cortisol concentration maxima from different sampling times were 8.98 ng/mL in CON (0.5 h), 8.61 ng/mL in 1-FLU (0 h) and 8.29 ng/mL in 2-FLU (0 h), respectively (Table 2).

After the initial peak during disbudding procedure (0 h) the plasma cortisol concentrations reached baseline levels two hours after sham disbudding and disbudding in all groups except for control calves after disbudding (Figure 1 and 2). After another increase of plasma cortisol concentrations 2 h post-disbudding a second peak occurred 12 h post-disbudding except for CON in disbudding. For control calves during disbudding the second peak was observed 6 h after disbudding (Fig. 1 and 2).

Daily Weight Gain, Lying and Standing Behavior

As one calf of the control group had to be excluded from the dataset of daily weight gain due to an implausible data, 70 calves were included in the statistics for ADG (CON, n = 22; 1-FLU, n = 24; 2-FLU, n = 24).

Mean (± SD) body weight was 52.7 ± 4.10 kg for CON calves, 51.1 ± 4.50 kg for 1-FLU group and 52.1 ± 3.81 kg for 2-FLU group. There was no statistical difference between average daily weight gain and treatment ($P = 0.74$; Table 3).

For the lying and standing behavior a total of 38 animals were analyzed (CON, n = 13; 1-FLU, n = 13; 2-FLU, n = 12. In total 608 time periods (CON, 208 time periods; 1-FLU, 208 time periods and 2-FLU, 192 time periods) of measurement were collected. Due to the high number of collected data regarding the lying and standing behavior per calf, there was enough power to perform the statistical analysis without the need of a Hobo Logger for each calf. Therefore, 38 calves were randomly selected by using Excel and the loggers were distributed among the three experimental groups. Therefore, 38 calves were randomly selected by using Excel and the loggers were distributed among the three experimental groups. Treatment after sham disbudding had an effect on total lying time (P = 0.01). Calves that received a double treatment with flunixin meglumine (281.8 ± 2.2 min/6 h) had increased lying time compared with CON (273.7 ± 2.1 min/6 h; P = 0.01) and 1-FLU (273.4 ± 2.1 min/6 h; P = 0.01). Treatment also had an effect on average lying bout length in calves (P = 0.01) after sham disbudding. The average lying bout length of calves that received a double treatment with flunixin meglumine (57.1 ± 2.3 min/6h) increased compared with CON (46.6 ± 2.2 min/6 h; P = 0.01) and 1-FLU (52.2 ± 1.8 min/6 h; P = 0.02). Treatment had an effect on number of lying bouts (P = 0.02). Calves that received a double treatment of flunixin meglumine (6.3 ± 0.2 observations/6 h) showed a decreased number of lying bouts compared with CON (6.8 ± 0.2 observations/6 h; P = 0.02). There was a difference between CON and 1-FLU (P = 0.02) but not for 1-FLU and 2-FLU (P = 0.85).

After disbudding, treatment had no effect on total lying time (P = 0.31). The average total lying time was 273.7 ± 2.5 min/6 h for CON, 274.1 ± 2.7 min/6 h for 1-FLU, and 278.8 ± 2.7 min/6 h for 2-FLU. Treatment also had no effect on average lying bout length in calves (P = 0.41) after disbudding. The average lying bout length was 51.7 ± 2.6 min/6 h for CON, 54.3 ± 2.3 min/6 h for 1-FLU, and 56.8 ± 2.9 min/6 h for 2-FLU. Moreover, treatment also had no effect on number of lying bouts (P = 0.28). Lying behavior was affected by time during sham disbudding (P < 0.01) and disbudding (P < 0.01) for all groups.

Discussion

The increase in plasma cortisol concentrations after sham disbudding might be related to a novel experience, unknown personnel, the restraining for drug administration, or the sham disbudding procedure (Huber et al., 2013; Heinrich et al., 2009; Vickers et al., 2005). A cortisol response in blood could be seen after several minutes after ACTH administration (Negrão et al., 2004) and after restraining and handling (Huber et al., 2013). In our study the plasma cortisol samples (0 h) were collected immediately after sham disbudding or disbudding and sampling took less than 3 min. Therefore, we conclude that the time-zero samples mainly reflected the stress associated with restraining and drug administration, rather than with the disbudding process itself. Nevertheless, the results showed that even during sham disbudding, the administration of transdermal flunixin meglumine tended to have an effect on plasma cortisol (P = 0.08). (Heinrich et al., 2009) recorded similar results with the use of meloxicam, which decreased heart rates in combination with local anesthesia during sham disbudding.After disbudding, peak plasma cortisol concentrations were measured in 1-FLU and 2-FLU at 0 h, whereas in control calves the peak plasma cortisol concentration was measured at 0.5 h after cautery disbudding. This is consistent with a previous study (Huber et al., 2013), in which cortisol concentrations (7.6 ng/mL) in a placebo-treated group peaked 0.5 h after the disbudding procedure. Thirty to 60 min after procaine injection, the anesthetic effect wears off (Skade et al., 2021) and sensitivity returns (Stafford and Mellor, 2005; Stock et al., 2013) which can explain the increase in plasma cortisol concentrations 0.5 h after disbudding in CON.

Two and 6 hours post-disbudding the plasma cortisol concentrations were affected by treatment for 1-FLU and 2-FLU calves. And a return to baseline plasma cortisol concentrations occurred in 1-FLU and 2-FLU after 2 h. According to (Wagner et al., 2021) the maximum concentration of transdermal flunixin meglumine occured 1.66 h to 2.14 h (Tmax-concentration) after administration. This timing might have influenced the return of plasma

cortisol concentrations to baseline levels in 1-FLU and 2-FLU 2 h post disbudding in our study. Due to a delay between the drug administration and the full effect of transdermal flunixin meglumine, (Kleinhenz et al., 2017) suggested an administration 2 h before disbudding.

Because of the half-life of 6.42 h to 13.2 h (Wagner et al., 2021), we administered a second dose at 6 h post-disbudding to test the hypothesis that the analgesic effect of flunixin meglumine could be extended. Inflammation induced prostaglandin E2 production peaks 24 h as well as 48 h post-disbudding (Allen et al., 2013; Stock et al., 2015). According to (Thiry et al., 2017) the inflammation-inhibiting effect of transdermal flunixin meglumine persisted for up to 48 h. However, the second treatment 6 h post- disbudding did not have an additional effect on the cortisol concentrations compared with a single treatment.

The treatment with transdermal flunixin meglumine in combination with local anesthesia reduced plasma cortisol after cautery disbudding in 1-FLU and 2-FLU group compared to the placebo-treated CON group. Our results showed that a local anesthetic in combination with a NSAID was more effective in reducing the stress response after cautery disbudding in calves compared to a local anesthetic alone.

Standing and lying behavior in the present study was monitored using a 3D accelerometer according to (Bonk et al., 2013). Due to individual variation in lying and standing time, (Coetzee et al., 2012) compared the behavior of each calf before and after the disbudding procedure. Discomfort after disbudding or castration was associated with decreased lying time (Heinrich et al., 2010; Theurer et al., 2012). After sham disbudding treatment increased total lying time by 10 min in 6 h. This effect was rather small and can be considered as irrelevant. After disbudding, treatment had no effect on total lying time ($P = 0.31$), average lying bout length in calves ($P = 0.60$) and number of lying bouts ($P = 0.49$). Total lying time was similar for all presented groups and was not affected by treatment. This is not in accordance to a previous study (Sutherland et al., 2018) in which meloxicam affected lying behavior 2 h post-disbudding. Furthermore, calves treated with meloxicam had up to 10% greater lying time on the first 4 d post-disbudding compared to a non-treated control group (Theurer et al., 2012). Therefore, we presume that flunixin meglumine is less effective in reducing discomfort after disbudding than meloxicam as measured by lying behavior. Further research is warranted.

Study limitations

In the present study, there was no difference in average daily weight gain among the treatment groups. Most probably this result was confounded by the restrictive feeding program (max. 6.0 L/d) of the dairy farm and the short interval (2 wk) between the two weight assessments. The milk amount of the feeding protocol and average daily weight gain of our study calves was lower than recommended (Khan et al., 2011).

Conclusion

This study demonstrates that transdermal flunixin meglumine decreased plasma cortisol concentrations following cautery disbudding of calves. Further research is warranted in order to evaluate analgesic effects compared to other NSAID medications. To ensure adequate pain relief more research is warranted in order to explore the effect of a second dose of flunixin meglumine administered at different times post-disbudding. However, a second dose of transdermal flunixin meglumine 6 h post-disbudding had no additional effect on plasma cortisol concentrations. Average daily weight gain and total lying time were not affected.

Acknowledgements

We gratefully thank the participating dairy farm for their collaboration, and Edith Kobletz-Rassam (Unit of Physiology, Pathophysiology, and Experimental Endocrinology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria) for cortisol analysis. Mareike Röder was funded in part by Tiergyn e. V. (Berlin, Germany).

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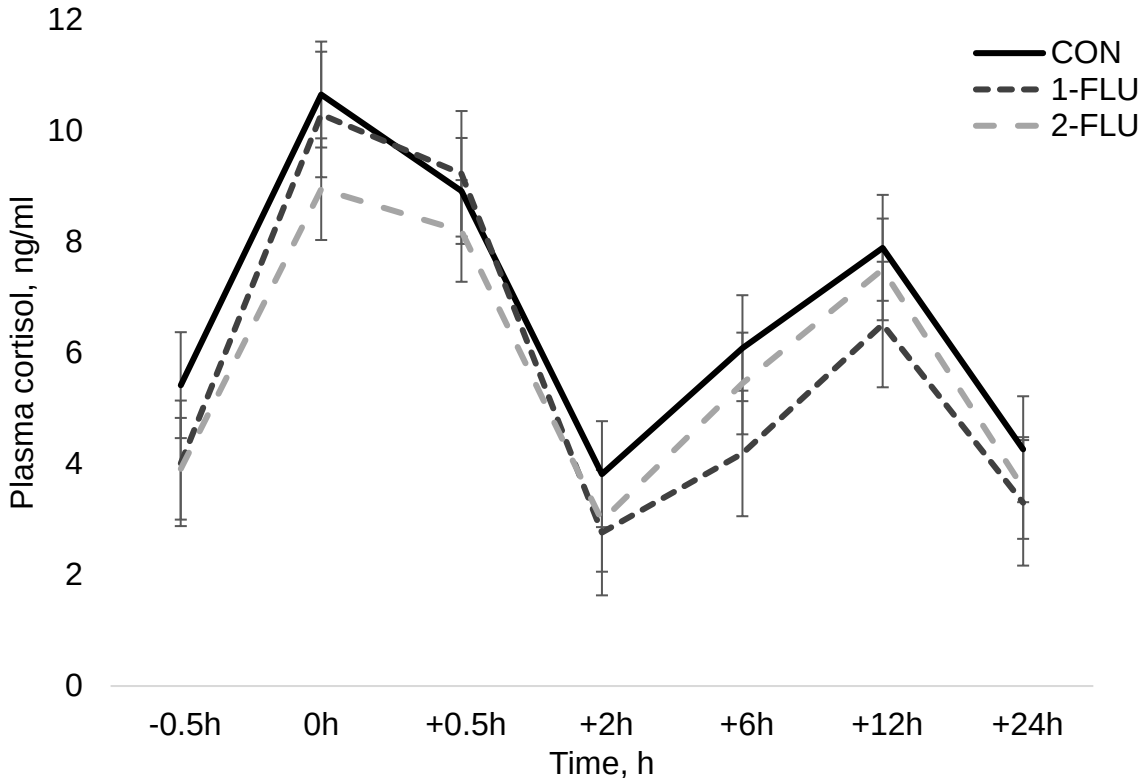

Figure 1. Plasma cortisol concentrations (mean ± SE) after sham disbudding for control group (**CON**, n = 23**)**, 1-flunixin group (**1-FLU**, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at sham disbudding, and 2-flunixin group (**2-FLU**, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at sham disbudding and the second 6 h after sham disbudding. The treatment procedures tended to have an effect on plasma cortisol concentrations ($P = 0.08$). Plasma cortisol concentrations were affected by sampling time ($P < 0.01$). There was no interaction between treatment and sampling time ($P = 0.99$).

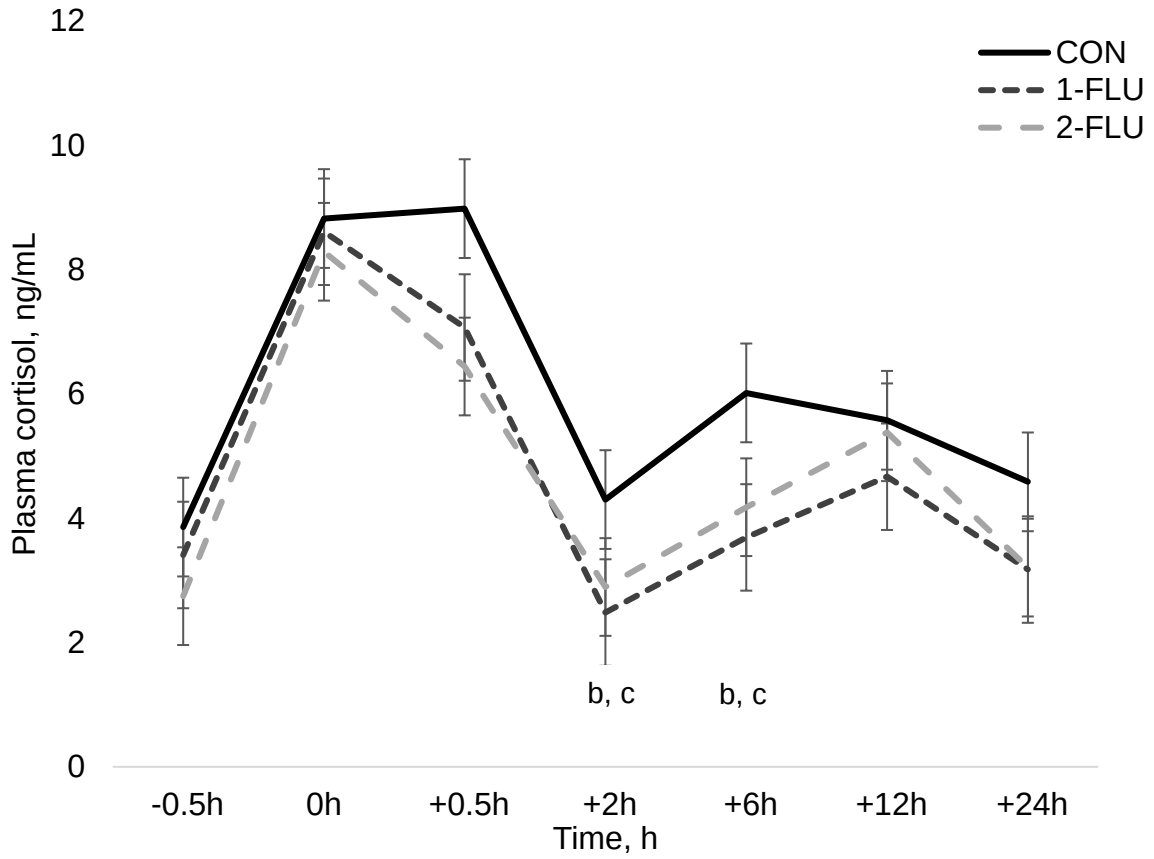

Figure 2. Plasma cortisol concentrations (mean ± SE) after disbudding for control group (a, **CON**, n = 23**)**, 1-flunixin group (b, **1-FLU**, n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at disbudding, and 2-flunixin group (c, **2-FLU**, n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after disbudding. Plasma cortisol concentrations were affected by treatment procedures ($P < 0.01$) (b, c) and sampling time ($P < 0.01$) (b, c). There was no interaction between both ($P = 0.95$).

Table 1. Descriptive statistics for average plasma cortisol concentrations (ng/ml) during sham disbudding of calves (n = 71) at the different sampling times.

Group[2]	Plasma cortisol concentrations (ng/ml) x ± SE[1] (95% CI)						
	Time						
	-0.5 h	0 h	0.5 h	2 h	6 h	12 h	24 h
CON	5.42 ± 0.81 (3.83-7.01)	10.65 ± 1.23 (8.23-13.07)	8.92 ± 1.02 (6.91-10.91)	3.82 ± 0.69 (2.47-5.16)	6.08 ± 0.91 (4.30-7.87)	7.89 ± 0.99 (5.94-9.83)	4.23 ± 0.70 (2.99-5.64)
1-FLU	4.01 ± 0.79 (2.45-5.57)	10.29 ± 1.18 (7.97-12.60)	9.22 ± 0.95 (7.31-11.13)	2.76 ± 0.66 (1.47-4.05)	4.19 ± 0.87 (2.48-5.90)	6.51 ± 0.95 (4.65-8.37)	3.30 ± 0.67 (1.98-4.61)
2-FLU	3.91 ± 0.78 (2.39-5.44)	8.94 ± 1.18 (6.63-11.26)	8.19 ± 0.95 (6.28-10.11)	2.97 ± 0.66 (1.68-4.26)	5.45 ± 0.87 (3.74-7.15)	7.50 ± 0.95 (5.64-9.36)	3.57 ± 0.67 (2.25-4.88)

[1]Standard error.

[2]Control group (**CON,** n = 23**)**, 1-flunixin group (**1-FLU,** n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at sham disbudding, and 2-flunixin group (**2-FLU,** n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after sham disbudding.

Table 2. Descriptive statistics for average plasma cortisol concentration (ng/ml) during disbudding of calves (n = 71) at the different sampling time points.

Group[2]	Average plasma cortisol (ng/ml) ± SE[1] (95% CI)						
	Time						
	-0.5 h	0 h	0.5 h	2 h	6 h	12 h	24 h
CON	3.86 ± 0.65 (2.59-5.13)	8.82 ± 1.20 (6.47-11.17)	8.98 ± 1.01 (6.99-10.97)	4.30 ± 0.50 (3.32-5.83)	6.02 ± 0.67 (4.71-7.32)	5.58 ± 0.74 (4.11-7.04)	4.59 ± 0.80 (3.01-6.15)
1-FLU	3.41 ± 0.63 (2.17-4.65)	8.61 ± 1.17 (6.31-10.91)	7.07 ± 0.99 (5.12-9.02)	2.48 ± 0.49 (1.52-3.44)	3.69 ± 0.65 (2.42-4.97)	4.67 ± 0.71 (3.27-6.07)	3.17 ± 0.76 (1.67-4.68)
2-FLU	2.75 ± 0.63 (1.50-4.00)	8.29 ± 1.17 (5.99-10.59)	6.44 ± 0.99 (4.49-8.39)	2.89 ± 0.49 (1.93-3.90)	4.18 ± 0.65 (2.90-5.45)	5.38 ± 0.71 (3.98-6.78)	3.21 ± 0.76 (1.70-4.70)

[1]Standard error.

[2]Control group (**CON,** n = 23**)**, 1-flunixin group (**1-FLU,** n = 24) with a single administration of transdermal flunixin meglumine (3.33 mg/kg) at disbudding, and 2-flunixin group (**2-FLU,** n = 24) with 2 administrations of transdermal flunixin meglumine (3.33 mg/kg), the first treatment at disbudding and the second 6 h after disbudding.

Table 3. Descriptive statistics for average body weight (kg) and average daily weight gain (kg) of 70 female Holstein dairy calves during sham disbudding and disbudding distributed on 3 groups. The weight was recorded 24 h before sham disbudding and 24 h after disbudding.

Group [1]	Parameter	n	Mean	SD[2]	Minimum	Maximum
CON	Weight (1) (before sham disbudding)	22	49.5 kg	4.97 kg	39.0 kg	57.0 kg
	Weight (2) (after disbudding)	22	55.3 kg	3.90 kg	48.0 kg	64.0 kg
	Average daily weight gain	22	0.57 kg	0.30 kg	-0.05 kg	1.11 kg
1-FLU	Weight (1) (before sham disbudding)	24	48.0 kg	3.80 kg	39.6 kg	56.5 kg
	Weight (2) (after disbudding)	24	53.8 kg	4.89 kg	46.5 kg	68.0 kg
	Average daily weight gain	24	0.64 kg	0.31 kg	0.36 kg	1.28 kg
2-FLU	Weight (1) (before sham disbudding)	24	49.1 kg	3.80 kg	41.5 kg	54.5 kg
	Weight (2) (after disbudding)	24	54.7 kg	3.67 kg	47.5 kg	61.0 kg
	Average daily weight gain	24	0.62 kg	0.27 kg	0.61 kg	1.06 kg

[1]Control group (**CON**; n = 22), 1 treatment with transdermal flunixin meglumine at disbudding (**1-FLU**; n = 24), and 2 treatments with transdermal flunixin meglumine at disbudding and 6 h after disbudding (**2-FLU**; n = 24).

[2]Standard deviation

Publication II

Evaluation of an ELISA and four on-farm tests to estimate colostrum quality for dairy cows.

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Published in:

Journal of Dairy Science, August 2023, Volume 106, Issue 12, Pages 9164–9173

Please find the original article via the following digital object identifier:

J. Dairy Sci. 106:9164–9173

https://doi.org/10.3168/jds.2023-23467

Evaluation of an ELISA and four on-farm tests to estimate colostrum quality for dairy cows.
By Röder et al.
The objective of this study was to evaluate a laboratory-based method (ELISA) and different on-farm tests to determine colostrum quality of dairy cows in comparison to the IgG concentration measured by the accepted reference method (radial immunodiffusion). By definition high-quality colostrum has an IgG concentration of ≥50 mg/mL. One ELISA and four on-farm tests (i.e., a digital Brix refractometer, a colostrometer, an outflow funnel, and a lateral flow assay), were employed to assess colostrum quality compared to radial immunodiffusion. The ELISA provided accurate results in comparison to radial immunodiffusion. The Brix refractometry and the colostrometer were adequate to determine colostrum quality, while the lateral flow assay and the outflow funnel did not provide accurate results compared to radial immunodiffusion.

Abstract

The objectives of this study were to evaluate different analytical methods to determine colostrum quality in dairy cattle, including one laboratory-based method (ELISA) and four on-farm tests. We hypothesized that the colostral immunoglobulin G (IgG) concentration using different analytical methods, i.e., ELISA (mg/mL), digital Brix refractometer (% Brix), colostrometer (specific gravity and mg/mL), an outflow funnel (seconds), and a lateral flow assay (mg/mL) were highly correlated with the reference method, radial immunodiffusion (RID; mg/mL) and would generate comparable results. Colostrum samples were collected from 209 Holstein Friesian cows on two commercial dairy farms in Germany. Colostrum weight and colostrum temperature were measured. Test characteristics, such as optimum thresholds, sensitivity (Se), specificity (Sp), and area under the curve (AUC) were determined using a receiver operating characteristic (ROC) curve analyses for each test.

Out of 209 colostrum samples assessed by RID, 186 (89%) samples had high quality (≥50 mg IgG/mL), while 23 colostrum samples (11%) showed poor quality with IgG concentrations less than 50 mg/mL. The mean IgG concentration (±SD) was 101.3 ± 45.9 mg/mL and the range was 6.0 to 244.3 mg IgG/mL. The Pearson correlation coefficient between RID and ELISA was r = 0.78. In comparison to RID, Pearson correlation coefficients for the on-farm tests were: r = 0.79 (digital Brix refractometry), r = 0.58 (colostrometer: specific gravity), r = 0.61 (colostrometer: temperature corrected), r = 0.26 (outflow funnel) and r = 0.43 (lateral flow assay), respectively. The optimal threshold to identify high-quality colostrum using ELISA was at 50.8 mg/mL with sensitivity 91.3%, specificity 92.3%, and AUC of 0.94. For the on-farm tests sensitivity ranged from 95.7% (Brix refractometry) to 60.9% (lateral flow assay). Specificity ranged from 88.6% (lateral flow assay) to 75.9% (colostrometer after temperature correction). The AUC ranged from 0.93 (Brix refractometry) to 0.73 (outflow funnel). Based on the AUC, ELISA (0.94) and Brix refractometry (0.93) can be considered highly accurate.

In conclusion, the ELISA is accurate to assess colostrum quality. Regarding the on-farm tests only the digital Brix refractometer and the colostrometer were adequate to determine colostrum quality.

Introduction

Due to the impermeability of the bovine placenta for maternal antibodies calves are born immunonaive (Weaver et al., 2000; Barrington and Parish, 2001). To acquire passive immunity it is essential for neonatal calves to ingest adequate volumes of high-quality colostrum during their first hours of life (Weaver et al., 2000; Baumrucker et al., 2010; Fischer-Tlustos et al., 2021; Godden et al., 2019). For the utmost transfer of passive immunity (TPI), colostrum should be ingested within the first 2 h post natum. The absorption of maternal IgG from colostrum across the small intestinal epithelial cells is greatest in the first hours of life and progressively decreases after the first day of life (Weaver et al., 2000; Barrington and Parish, 2001). The concentration of antibodies in calf serum allows health monitoring of the calf population. Failed transfer of passive immunity (FTPI) was defined as serum immunoglobulin G (IgG) concentrations <10 mg/mL in 24 to 48 h old calves (Weaver et al., 2000; McGuirk and Collins, 2004; Godden et al., 2019) and is associated with an increased risk for mortality and morbidity. Lombard et al. (2020) recently proposed new standards including 4 serum IgG categories (excellent, good, fair, and poor) with serum IgG levels of >25.0, 18.0-24.9, 10.0-17.9, and <10 mg/mL, in order to reduce the risk of mortality and morbidity in dairy calves. Raboisson et al. (2016) detected greater hazard ratios for bovine respiratory disease (1.75), diarrhea (1.51), overall morbidity (1.91) and mortality (2.12) for calves suffering from FTPI. Therefore, it is essential to provide high-quality colostrum for the first feeding to assure a sufficient maternal IgG supply. In bovine colostrum 85% to 90% of the total immunoglobulins are represented by IgG (Larson et al., 1980). The concentration of colostral IgG is considered the reference method to assess colostrum quality (Godden et al., 2019). Bovine colostrum of high-quality is defined as colostrum with an IgG concentration ≥50 mg/mL (McGuirk and Collins, 2004).

There are different on-farm tests commercially available to estimate the colostrum quality. Nevertheless, the measurement of colostrum quality with on-farm tests should be easy to perform

and it has to be accurate (Bartier et al., 2015). The measurement of dissolved solids in colostrum determined by refractometry is a user-friendly way to indirectly assess the colostral IgG concentration (Bartier et al., 2015). Digital Brix refractometry uses the refraction of a light beam to assess how much dissolved solids are present in a fluid, detecting its relative density in % Brix. It has been shown that it is a reliable tool for determining colostral IgG concentration (Chigerwe et al., 2008; Bielmann et al., 2010; Morrill et al., 2012; Quigley et al., 2013). Correlation coefficients to identify high-quality colostrum range from r = 0.64 to r = 0.87 comparing Brix refractometry and radial immunodiffusion (RID; Bielmann et al., 2010; Vandeputte et al., 2014; Bartier et al., 2015; Coleman et al., 2015). According to Bielmann et al. (2010) high-quality colostrum in Holstein Friesian cows can be identified using a threshold of ≥22% Brix.

The determination of specific gravity of colostrum with a colostrometer is a conventional method that has been established a long time ago (Fleenor and Stott, 1980). It measures the specific gravity and determines colostral IgG concentration indirectly. It has been shown to have good correlation with the IgG concentration assessed by RID (r = 0.77; Bartier et al., 2015) or measured by ELISA (r = 0.79; Lemberskiy-Kuzin et al., 2019). The specific gravity is influenced by the dissolved solids in colostrum. The greater the IgG concentration of colostrum, the higher its specific gravity and the greater the uplift of the colostrometer. In a less dense colostrum sample, the colostrometer sinks deeper. Colostrum of good quality shows a specific gravity ≥1,047 in Holstein Friesian cows (Fleenor and Stott, 1980). Though, this method has some limitations as the specific gravity depends, for instance, on breed of the dam (Morin et al., 2001) and the temperature of the colostrum (Mechor et al., 1991, 1992). Colostrum should be evaluated at 22°C to obtain reliable results (Conneely et al., 2013). Furthermore, the colostrometer is made of glass and therefore fragile.

Another indirect analytical method is the use of an outflow funnel. The outflow funnel measures the time in seconds a defined volume of the fluid flow through it. This type of measurement is based on the viscosity of a fluid and on the assumption that an increased viscosity is associated with greater concentrations of immunoglobulins in colostrum. Like the specific gravity, the viscosity depends on the temperature of colostrum. The outflow funnel requires a temperature of 30°C to perform the measurement.

A direct analytical method is the lateral flow assay which determines the IgG concentration (mg/mL) in colostrum based on an antigen-antibody reaction. Bovine IgG in colostrum reacts with anti-bovine IgG of the test strips. To perform the analysis, the test strip is immersed in a diluted colostrum sample. It is a semiquantitative immunochromatographic test, which uses color-labeled antibodies that bind colostral IgG. The resulting antigen-antibody complexes migrate through the test strip until they encounter the reading area (antibodies for fixation of the antigen-antibody complexes). The fixation results in a visible color change due to the accumulation of the color-labeled antigen-antibody complexes. In addition, the test contains a control line to ensure that the sample migrated completely through the test strip. The concentration of IgG in the colostrum sample is proportional to the intensity of the test line. By taking a picture of the test line with the smartphone camera using the SmartStripsTM App the line intensity is interpreted and compared to a stored standard curve. The measurement range of the test is from between 2 and 120 mg/mL. Values lower than 2 mg/mL are displayed as <2 and values higher than 120 are displayed as >120 mg/mL.

A laboratory-based direct analytical method is the ELISA which is based on an antigen-antibody reaction. Immune complex enzyme reactions result in a color change that can be measured photometrically to determine the colostral IgG concentration.

Up to now, RID is still considered the reference method for measuring IgG concentration in colostrum (Ahmann et al., 2021). Disadvantages of RID are the limited test range, low reproducibility, and long incubation times. Furthermore, this method is time-consuming and expensive and therefore not feasible for calf health monitoring and management (Fleenor and Stott, 1980). Therefore, ELISA could be a useful alternative analytical method.

The objectives of this study were to evaluate 4 on-farm tests (digital Brix refractometer, colostrometer, outflow funnel, lateral flow assay) and an ELISA with the reference method in order to determine colostrum quality. We hypothesized that the colostral IgG concentration using ELISA, digital Brix refractometer, colostrometer, outflow funnel, and lateral flow assay were highly correlated with the reference method RID.

Material and Methods

Colostrum was collected on two dairy farms in northern Germany from February 2020 to August 2020. Since all colostrum samples were obtained during routine farm management practices, the study was in accordance with the Institutional Animal Care and Use Committee of the Freie Universität Berlin. A sample size calculation was performed as described by Moinester and Gottfried (2014) for different half-widths (w) and Pearson correlation coefficients (r), expecting the desired 95% CI. For r = 0.80 and w = 0.05 a colostrum sample size requirement of n = 205 was needed.

A total of 213 colostrum samples were collected from clinically healthy primiparous (n = 86) and multiparous cows (n = 127). Colostrum samples were excluded if they were considered bloody or mastitic. The farms had a separate side-by-side milking parlor for 6 fresh cows near the calving area. Cows were milked three times daily at regular milking times (4:30 a.m., 12:30 p.m., and 7:30 p.m.) regardless of the individual calving time. Therefore, colostrum was tested mostly at the regular milking times. Otherwise, colostrum was stored in the milking bucket with a lid in the refrigerator at 8°C. Colostrum was milked into a separate milking bucket after the teats were pre-dipped, forestripped and dry wiped using a clean paper towel. Forestripping involved the manual removal of two streams of colostrum from each teat after thoroughly milking out the teat sealant. This manual stimulation lasted 30 s. The time interval between manual stimulation and attachment of the milk unit clusters was 60 s. The vacuum of the milking equipment (Flo-Star MAX, Boumatic Robotics GmbH, Kempten, Germany) was 45 kPa and the milk-to-rest ratio was at 60:40. After milking, the teats of the cows were dipped with iodine (Jod 5000, CID Lines N.V., Ieper, Belgium).

Relevant information such as cow identification, parity, date, and time of parturition were obtained from the on-farm documentation.

Colostrum Sample Analysis

The assessment of colostrum quantity, temperature and quality was carried out in the milking parlor, immediately after milking until approximately 3 h after colostrum harvest. After each milking, the bucket with the colostrum was weighed with a digital hanging scale (LS 06 luggage scale, Beurer GmbH, Ulm, Germany) and the weight of the empty bucket subtracted. The temperature was measured by immersing a digital thermometer (Digital probe thermometer 30.1018, TFA Dostmann GmbH & Co. KG, Wertheim-Reicholzheim, Germany) approximately 20 cm into the colostrum at the center of the bucket.

The quality of all colostrum samples was evaluated with 4 on-farm tests. Sampling was done directly from the milking bucket after mixing thoroughly the colostrum. 1) Digital Brix refractometer (Misco PA201, Misco, Solon, Ohio) with an automatic temperature calibration. Accurate measurements were possible between 0 and 50°C. Before each batch of samples, the device was calibrated with distilled water at room temperature (20°C). Two drops of colostrum were applied to the prism of the digital Brix refractometer using a disposable syringe (2 mL, Henry Schein, Langen, Germany) and the quality was measured in % Brix.

2) The specific gravity was assessed with a colostrometer (Colostrometer, Albert Kerbl GmbH, Buchbach, Germany) in a cylinder (diameter 3.5 cm, height 25 cm) filled with 500 mL colostrum. The device was immersed in the colostrum filled cylinder and the specific gravity determined by reading the scale. To control the influence of different colostrum temperatures on colostral IgG concentration, the equation according to Mechor et al. (1992) was used:

IgG concentration (mg/mL) = 853 x (specific gravity) + 0.4 x (Celsius degrees) – 866.

3) The viscosity of colostrum was assessed using an outflow funnel (ColostroCheck, QUIDEE GmbH, Homberg, Germany) with a volume of 100 mL. It was immersed into the milking bucket and raised. Thereafter, the time was stopped until the colostrum passed through the funnel. Colostrum with an outflow velocity of >24 s was classified as good quality colostrum according to the specifications of the manufacturer.

4) A lateral flow assay (SmartStrips IgG Colostrum, Bio-X Diagnostics, Rochefort, Belgium) was carried out according to the manufacturer's instructions. To obtain a first dilution, 20 µl of colostrum was transferred into the first dilution vial by using the pipette from the test kit. The vial was closed and swiveled. From this first vial, another 20 µl of the dilution was pipetted into a second vial in the same manner, using a new pipette. The test strip was placed into the second vial for 10 min and the test line photographed with a smartphone by using the SmartStripsTM App.

Aliquots of colostrum were collected and transferred into sterile vials (Cryovial 2 mL, Simport, Bernard-Pilon, Canada). For further analysis, one aliquot each was shipped on dry ice to the Veterinary Science Department, Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich for ELISA analysis and to The Saskatoon Colostrum Co. Ltd. (SCCL) in Canada for IgG analysis via RID.

At the Department of Veterinary Science in Munich the colostral IgG testing was performed via sandwich ELISA according to Erhard et al. (1999). With PBS-Tween the colostrum samples were diluted in a ratio of 1:50,000. The assessment of the IgG concentration via ELISA was performed as described in Sutter et al. (2019). The ELISA was based on coating and conjugating the IgG with anti-bovine IgG coupled to a peroxidase enzyme. The catalyzed color change was measured photometrically. The mean value of the IgG concentration in each well of one column resulted in the final colostrum concentration (mg IgG/mL). Colostrum containing ≥50 mg/mL IgG was regarded as high quality colostrum (McGuirk and Collins, 2004).

The assessment via RID (reference method) was performed in SCCL (Saskatoon, SK, Canada) as described in Shivley et al. (2018). The RID was based on the measurement of antibodies and antigens by their precipitation which involves diffusion through an in-house prepared 24 mL agarose plate, using commercially available ingredients and reagents. The diffusion denotes precipitation in gel. A plate reader (digital RID reader AD400, The Binding Site Inc., San Diego, CA) was used to measure the diameters of the precipitin rings surrounding the wells. A regression line was generated for each plate for the variable R (ring diameter) versus log 10 (concentration) by using the results (ring diameters) obtained for each of the 2-fold dilutions and a spreadsheet (Excel, Microsoft Corp., Redmond, WA). By using the regression line of the bovine IgG standard obtained for each plate the IgG concentration for the test sample was determined. The diameters were entered into a template where IgG concentration (mg/mL) and the regression line was calculated.

Statistical Analysis

Pearson correlation coefficient (r) were determined using distribution plots. The results of ELISA and the on-farm tests were plotted against the reference method obtained by RID. Correlation coefficients and Bland-Altman plots were generated using Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). Bland-Altman plots were used to quantify the agreement between two quantitative measurements by using statistical limits of agreement, which were calculated by using the mean and the standard deviation (SD) of the differences between these two measurements (Bland and Altman, 1999, 2003). The quantitative difference of the two measurements were plotted against the average of the two measurements (RID and ELISA). The limits of agreement were expressed as the mean difference ±1.96 SD (Bland and Altman, 1999, 2003). By definition 95% of the data points lie within ±1.96 SD of the mean difference.

For all test methods, the mean IgG concentration (means ± SD) and sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) and area under the curve (AUC) were calculated using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium) with RID as reference method. Sensitivity was defined as the probability of a test result correctly indicating poor colostrum quality (i.e., IgG <50 mg/mL). Specificity was defined as the probability of a test result correctly indicating good colostrum quality with IgG >50 mg/mL. The PPV was defined as a predictive probability of a test result correctly indicating poor colostrum (IgG < 50 mg/mL). The NPV was defined as a predictive probability of a test result correctly indicating good colostrum quality (IgG >50 mg/mL).

By plotting the true positive rate against the false positive rate, a receiver operating characteristic (ROC) curve was generated, and the optimal thresholds were assessed. The optimal threshold was defined as the point on the curve with the highest combined sensitivity and specificity and its deduction was based on the AUC according to Swets (1988) as perfect (AUC = 1), highly accurate (0.9 < AUC < 1), very accurate (0.7 < AUC < 0.9), accurate (0.5 < AUC < 0.7), and as non-informative (AUC = 0.5). Accuracy describes the variance of a measurement from its true value while precision refers to the dispersion of the measurements (Ranstam, 2008). A significant statistical difference was defined for variables when $P < 0.05$; a statistical tendency was specified as differences between $P \geq 0.05$ and $P \leq 0.10$. The ROC curve analyses using RID as the reference method generated the test characteristics (optimal thresholds, Se, Sp, PPV, NPV and AUC) for the on-farm tests and ELISA to identify high-quality colostrum (≥50 mg/mL).

Results

Four samples could not be analyzed by RID due to high viscosity (out of 213). Due to missing data some colostrum samples were excluded from the analysis with the lateral flow assay (n = 1), the outflow funnel (n = 2), ELISA (n = 5) and colostrometer (n = 13). For the final analyses, 209 colostrum samples were considered.

Descriptive Statistics

The RID analysis identified 186 (89%) high quality colostrum samples (>50 mg IgG/mL) and 23 poor quality colostrum samples (<50 mg IgG/mL). Furthermore, 50.2% contained ≥100 mg IgG/mL, respectively. The mean IgG concentration (±SD) was 101.3 ± 45.9 mg/mL, and the range was 6.0 to 244.3 mg IgG/mL (Table 1). The mean temperature of colostrum (±SD) was 27.3°C ± 9.4 with a range from 2.6 to 38.2°C. The mean weight of colostrum (±SD) was 6.4 kg ± 3.8 with a range from 0.7 to 25.3 kg. The descriptive statistics for all tests are listed in Table 1.

Correlation Coefficients and Bland-Altman plots

The Pearson correlation coefficients for the on-farm test compared to the reference method (RID) were between r = 0.71 (Brix refractometer) and r = 0.26 (outflow funnel; Figure 1A-1E). The ELISA showed a moderate correlation to RID (r = 0.78; $P < 0.01$; n = 204; Figure 1F).

On average, the IgG concentration measured by ELISA was 21.6 mg/mL lower compared to RID (Figure 2). The limits of agreement were -37.9 mg/mL and 81.1 mg/mL (±1.96 SD; 95% CI).

Test Characteristics

Test characteristics to identify high-quality colostrum determined by the different on-farm tests, as well as by the laboratory method ELISA are summarized in Table 2. The analysis for ELISA identified an optimal threshold at 50.8 mg/mL with sensitivity 91.3%, specificity 92.3%, and AUC of 0.94. Sensitivity ranged from 95.7% (Brix refratometry) to 60.9% (lateral flow assay). Specificity ranged from 88.6% (lateral flow assay) to 75.9% (colostrometer after temperature correction). The AUC ranged from 0.93 (Brix refractometry) to 0.73 (outflow funnel).

Discussion

As on-farm test, the digital Brix refractometer and colostrometer were adequate to determine colostrum quality but not the lateral flow assay and the outflow funnel. As laboratory method ELISA was accurate to assess colostrum quality compared to RID.

Colostrum Quality

In the present study, the mean and range of IgG concentration measured with RID was slightly higher than in previous studies (Kehoe et al., 2007; Chigerwe et al., 2008; Bielmann et al., 2010; Shivley et al., 2018), but in agreement with Morrill et al. (2012). For a comparison of IgG concentration across studies, it is important to consider the analytical details of the implementation of RID (Kehoe et al., 2011; Rivero et al., 2012) and ELISA (Baumrucker et al., 2010; Nowak et al., 2012) as removal of fat or heat treatment. The RID analysis detected 89% of the samples as high-quality colostrum, whereas 11% of the samples contained less than 50 mg IgG/mL. Chigerwe et al. (2008) and Bartier et al. (2015) reported 32% and 29.1% of poor-quality colostrum samples, respectively. One possible explanation is that the current study was conducted on 2 commercial farms. A wider range of environmental conditions and farm management practices is likely to be represented by a larger number of farms. Factors affecting colostrum quality can be divided into animal-related and environmental-related factors. It is known that dietary practices and trace mineral supplementation are associated with colostrum quality (Godden et al., 2019; Kincaid and Socha, 2004), as well as colostrum harvesting times more than 2 h after parturition negatively affect the IgG concentration in colostrum (Moore et al., 2005; Chigerwe et al., 2008). Higher colostrum yield can have a dilution effect on colostrum as well as dry period length (Cabral et al., 2016). According to Conneely et al., (2013) a genetic standard deviation for IgG concentration is given (16.0 g/l) as well as a positive influence of parity on colostrum quality.

Correlation Coefficients and Bland-Altman Plot

The results of the established methods including Brix refractometer ($r = 0.71$; $P < 0.01$) and colostometer (specific gravity: $r = 0.58$; $P < 0.01$; after temperature correction: $r = 0.61$; $P < 0.01$), showed similar results to previously published studies. Correlation coefficients between Brix refractometry and RID ranged from 0.64 to 0.87 (Vandeputte et al., 2014; Bartier et al., 2015; Coleman et al., 2015; Morrill et al., 2015). Considering the correlation coefficients of the colostrometer and RID the published range of previous studies was 0.53 to 0.84 (Fleenor and Stott, 1980; Morin et al., 2001; Bartier et al., 2015; Løkke et al., 2016). This wide range of reported correlation coefficients might be caused due to variation of non-IgG protein content in the colostrum (Elsohaby et al., 2017). Morin et al. (2001) detected that specific gravity had higher correlation with colostral total protein (TP; $r = 0.76$) than with IgG_1 ($r = 0.53$). Also Fleenor and Stott (1980) reported a correlation of $r = 0.84$ ($R^2 = 0.699$) between specific gravity and the entire ɣ-globulin content. Furthermore, breed and species specific colostrum compositions affect the dissolved solids in colostrum (Kessler et al., 2021). Løkke et al. (2016) detected that fat content had a significant negative effect on specific gravity and a positive effect on % Brix results.

The outflow funnel and the lateral flow test showed poor correlations to the reference method RID. The Pearson correlation coefficient of the outflow funnel compared to RID was $r = 0.26$ ($P < 0.01$) clearly indicating no correlation between viscosity and IgG concentration. There is only limited research regarding the association between viscosity and IgG concentration of colostrum. Hallberg et al. (1995) and Maunsell et al. (1999) approached this topic in their study. Nevertheless, the viscosity determination was carried out by visual assessment and therefore it was rather subjective. Maunsell et al. (1999) concluded that there was no correlation between viscosity and IgG content, whereas Hassan et al. (2020) detected that the viscosity of colostrum from cows suffering from mastitis was lower than from healthy cows. Hassan et al. (2020) compared visually assessed colostral viscosity and dynamic colostral viscosity using a viscometer of 40 Holstein dairy cattle to colostral IgG concentration measured by colostrometer and % Brix refractometry. The correlation between the viscometer and colostrometer was moderate ($r = 0.58$; $P < 0.01$). To the best of our knowledge the outflow funnel has not been validated yet.

The lateral flow assay used in our study correlated weak with the reference method RID ($r = 0.43$; $P < 0.01$). Besides the main components represented by immunoglobulins colostrum contains different components with immune enhancing properties which can influence the quality of colostrum as well as the test results (Puppel et al., 2019). Furthermore, the test performance may be susceptible to variations due to the individual performance steps (first and second dilution). On-farm tests that directly measure the IgG concentration in colostrum have only been evaluated in one study using an IgG assay measuring the optical density (Drikic et al., 2018). The correlation with RID for dairy and beef colostrum were $r = 0.72$ and $r = 0.73$, respectively. The weak correlation of the current lateral flow assay for colostrum contrasts a previous study in which the lateral flow assay for serum of the same manufacturer was evaluated in beef and dairy calves (Delhez et al., 2021). The correlation between lateral flow assay for serum and ELISA was $r = 0.86$ ($P < 0.01$), which indicates that it is an appropriate on-farm test for the assessment of TPI in calf serum. The results for the lateral flow assay obtained with colostrum do not provide accurate results in comparison to the reference method. To the best of our knowledge the lateral flow assay for colostrum has not been validated yet.

The highest correlation in the present study was assessed with the laboratory method ELISA ($r = 0.78$; $P < 0.01$) which is in accordance with Dunn et al. (2018), who observed a strong correlation ($r = 0.91$; $R^2 = 0.83$; $P < 0.01$). However, Gelsinger et al. (2015) reported a weaker correlation ($r = 0.60$; $R^2 = 0.36$; $P = 0.01$). The discrepancy is probably caused by different test kits both for ELISA and RID used. The Bland-Altman plot showed lower IgG concentrations measured by ELISA (21.6 mg/mL) compared to RID. The limits of agreement were wide (-37.9 to 81.1 mg/mL) because of a high mean difference and SD in both methods. The discrepancy between RID and ELISA increased with IgG concentration. Rising concentrations can cause increased variability of the measured values (Grouven et al., 2007). These results underline the findings of Gelsinger et al. (2015) and Dunn et al. (2018) that the additional validation of specific assay kits is needed to determine thresholds appropriate for application to ELISA, so that ELISA and RID values can be accurately compared. Further research is warranted to validate the established threshold with relevant clinical outcomes such as disease incidence rates and mortality.

Test Characteristics

The laboratory method ELISA, and the on-farm test digital Brix refractometer were highly accurate ($0.9 < AUC < 1$) using the AUC as an indicator of overall test characteristics by Swets (1988). The 95% CI did overlap for the ELISA and the Brix refractometer and high Se and Sp was detected (ELISA: Se: 91.3%, Sp: 92.3%; Brix refractometer: Se: 95.7%, Sp: 82.8%). The colostrometer (specific gravity and temperature corrected) was very accurate, though, 95% CI of the AUC did not overlap with the CI of ELISA and Brix refractometry, indicating that test accuracy of the colostrometer was slightly less accurate than the test accuracy of ELISA and Brix refractometry. The 95% CI did not overlap for the outflow funnel and the lateral flow assay as well. Further, Se and Sp were moderate for the outflow funnel (Se: 65.2%, Sp: 76.1%) and the lateral flow assay Se: 60.9%, Sp: 88.6%, lateral flow assay), indicating that these tests cannot distinguish between high- and poor-quality colostrum without considerable numbers of false negatives and positives.

Based on our data the threshold for the digital Brix refractometer to identify high-quality colostrum is 21.3% Brix. This is consistent with previous studies in which varied between 20 and 23% Brix (Chigerwe et al., 2008; Bielmann et al., 2010; Quigley et al., 2013). Implementing an optimal threshold ensures that high- and poor-quality colostrum is correctly identified and not discarded or fed to calves, respectively.

The optimal threshold to identify high-quality colostrum with ELISA was 50.8 mg/mL. However, a laboratory specific threshold should be established based on relevant outcomes.

Specific gravity without and with temperature correction barely varied [specific gravity: Se: 81.8, Sp: 78.2, and AUC: 0.83; IgG (mg/mL): Se: 86.4, Sp: 75.9, and AUC: 0.84, mg IgG/mL]. The optimal threshold for specific gravity and IgG after temperature correction were 1,047 and

46.0 mg/mL, respectively. These findings are similar to previous research (Fleenor and Stott, 1980; Pritchett et al., 1994). More recent publications recommended higher thresholds (1,050 to 1,055) for specific gravity (Bartens et al., 2016; Løkke et al., 2016) and 60 to 90 mg/mL for IgG concentration (Chigerwe et al., 2008; Bartier et al., 2015). By increasing the thresholds, the risk of identifying poor colostrum (<50 mg/mL) falsely as good is decreased which ensures that more calves will be fed with colostrum of good quality (≥50 mg/mL). Alternatively, some high-quality colostrum would be falsely classified as not acceptable.

Conclusion

The laboratory method ELISA and the on-farm test digital Brix refractometer and colostrometer were suitable to assess colostrum quality. The predictive value of the colostrometer for colostral IgG concentration was lower than digital Brix refractometer and ELISA. Different threshold values must be considered. The outflow funnel and the lateral flow assay cannot be recommended as on-farm tests to determine colostrum quality.

Acknowledgments

We gratefully thank the participating dairy farm for their collaboration and the farm personnel for their kind support, and Chantelle Gosslin (Saskatoon Colostrum Company Ltd., 30 Molaro Pl, Saskatoon, Saskatchewan, S7K 6A2, Canada) for colostrum analysis. Mareike Röder was funded in part by Tiergyn e. V. (Berlin, Germany). The authors have not stated any other conflicts of interest.

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Table 1. Descriptive statistics of four on-farm and two laboratory tests to determine colostrum quality.

Test	Unit	n	Mean ± SD	Minimum	Maximum
Brix refractometry[1]	% Brix	213	24.3 ± 4.7	11.9	38.0
Colostrometer[2]	Specific gravity	200	1054.6 ± 12.9	1025.0	1077.0
Colostrometer	mg/mL	200	49.7 ± 11.2	23.2	70.0
Outflow funnel[3]	Seconds	211	32.1 ± 36.9	15.6	240.8
Lateral flow assay[4]	mg/mL	212	96.4 ± 33.5	20.0	120.0
ELISA	mg/mL	205	78.9 ± 29.2	3.5	179.5
Radial immunodiffusion	mg/mL	209	101.3 ± 45.9	6.0	244.3

[1]Misco PA201 Brix refractometer; [2]Colostrometer (Albert Kerbl GmbH); [3]ColostroCheck (QUIDEE GmbH); [4]SmartStrips IgG Colostrum (Bio-X Diagnostics)
SD = Standard deviation of the estimate

Table 2. Test characteristics for four on-farm tests and one laboratory method for determining high quality colostrum using radial immunodiffusion (RID) as reference method (≥50 mg/mL).

Test method	n	Threshold[5]	AUC[6] (95% CI)	SE[6]	*P*-value	Sensitivity	Specificity	PPV[6] (95% CI)	NPV[6] (95% CI)
Brix refractometry[1]	209	21.3% Brix	0.93 (0.89 – 0.96)	0.04	<0.001	95.7	82.8	40.7 (27.4 – 55.1)	99.4 (96.5 – 100.0)
Colostrometer[2] (specific gravity)	196	1,047	0.83 (0.77 – 0.88)	0.04	<0.001	81.8	78.2	32.1 (20.2 – 46.1)	97.1 (92.8 – 99.2)
Colostrometer (IgG mg/mL)	196	46.0 mg/mL	0.84 (0.78 – 0.88)	0.03	<0.001	86.4	75.9	31.1 (19.9 – 44.3)	97.8 (93.6 – 99.5)
Outflow funnel[3]	207	20.8 s	0.73 (0.67 – 0.79)	0.06	<0.001	65.2	76.1	25.4 (14.9 – 38.6)	94.6 (89.6 – 97.6)
Lateral flow assay[4]	208	55.7 mg/mL	0.74 (0.67 – 0.79)	0.06	<0.001	60.9	88.6	40.0 (23.6 – 58.2)	94.8 (90.4 – 97.6)
ELISA	204	50.8 mg/mL	0.94 (0.90 – 0.97)	0.03	<0.001	91.3	92.3	60.0 (42.1 – 76.1)	98.8 (95.8 – 99.9)

[1]Misco PA201 Brix refractometer; [2]Colostrometer (Albert Kerbl GmbH); [3]ColostroCheck (QUIDEE GmbH); [4]SmartStrips IgG Colostrum (Bio-X Diagnostics)

[5]Optimal threshold was determined by receiver operating characteristic curve analysis using the threshold with the highest sum of sensitivity and specificity to identify colostrum of good quality

[6]AUC = area under the curve; SE = Standard error; PPV = Positive predictive value; NPV = Negative predictive value

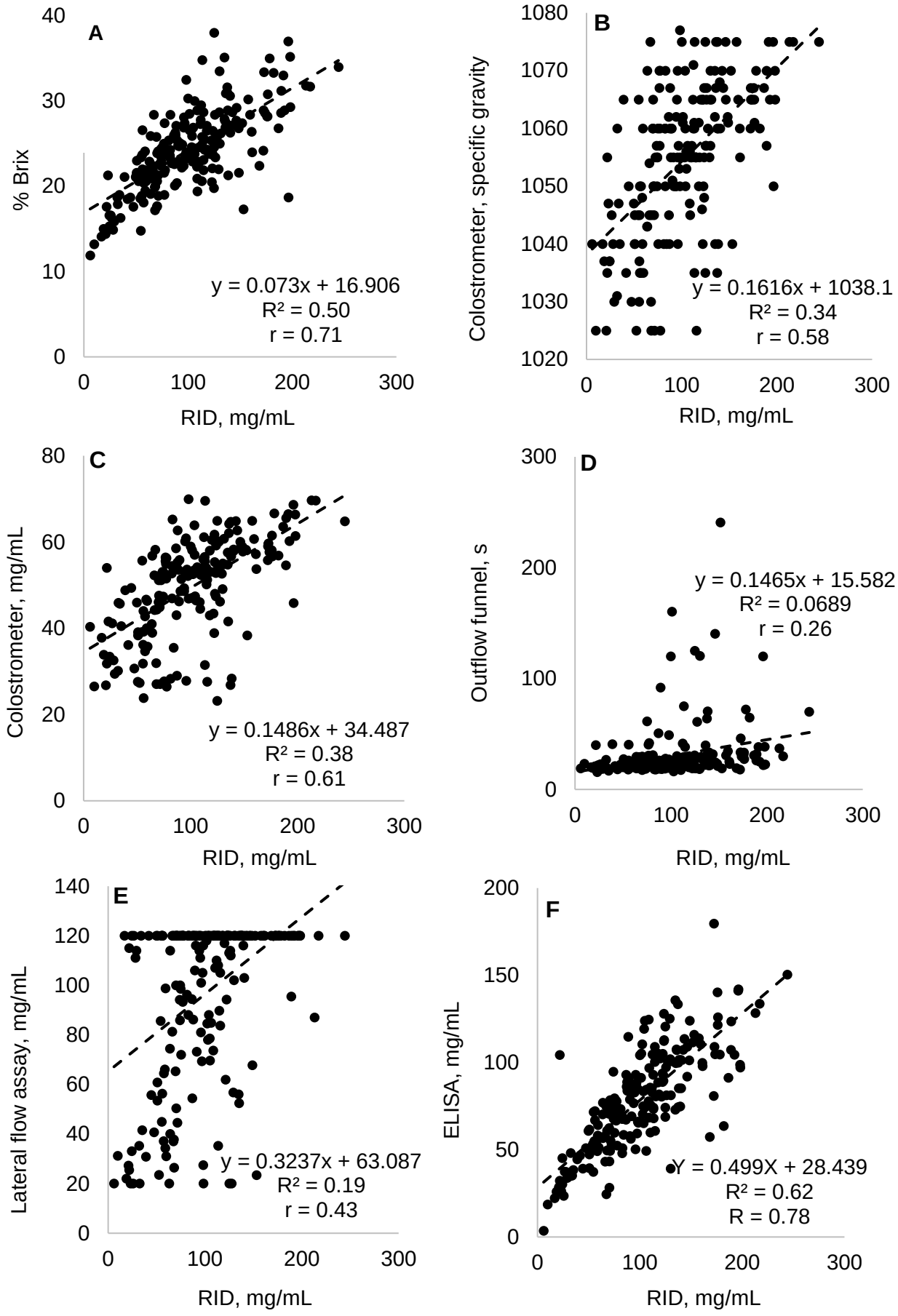
A
% Brix
RID, mg/mL
y = 0.073x + 16.906
R² = 0.50
r = 0.71
B
Colostrometer, specific gravity
RID, mg/mL
y = 0.1616x + 1038.1
R² = 0.34
r = 0.58
C
Colostrometer, mg/mL
RID, mg/mL
y = 0.1486x + 34.487
R² = 0.38
r = 0.61
D
Outflow funnel, s
RID, mg/mL
y = 0.1465x + 15.582
R² = 0.0689
r = 0.26
E
Lateral flow assay, mg/mL
RID, mg/mL
y = 0.3237x + 63.087
R² = 0.19
r = 0.43
F
ELISA, mg/mL
RID, mg/mL
Y = 0.499X + 28.439
R² = 0.62
R = 0.78

Figure 1. Comparison of four on-farm and one laboratory tests to determine colostrum quality using the colostral IgG concentration (mg/mL) determined by radial immunodiffusion (RID) as reference method. **Panel A**: Colostral IgG concentration assessed by RID compared with % Brix (n = 209; R^2 = 0.50; r =0.71); **Panel B**: Colostral IgG concentration assessed by RID compared with specific gravity using the colostrometer (n = 196; R^2 = 0.34; r =0.58); **Panel C**: Colostral IgG concentration assessed by RID compared with the IgG concentration (mg/mL) measured by colostrometer after correction analysis (Mechor et al., 1992); n = 196; R^2 = 0.38; r =0.61); **Panel D**: Colostral IgG concentration assessed by RID compared with the outflow velocity using the outflow funnel (n = 207; R^2 = 0.07; r =0.26); **Panel E**: Colostral IgG concentration assessed by RID compared with the IgG concentration (mg/mL) measured by the lateral flow assay (n = 208; R^2 = 0.19; r =0.43); **Panel F**: Colostral IgG concentration assessed by RID compared with the IgG concentration (mg/mL) determined by ELISA (n = 204; R^2 = 0.62; r = 0.78).

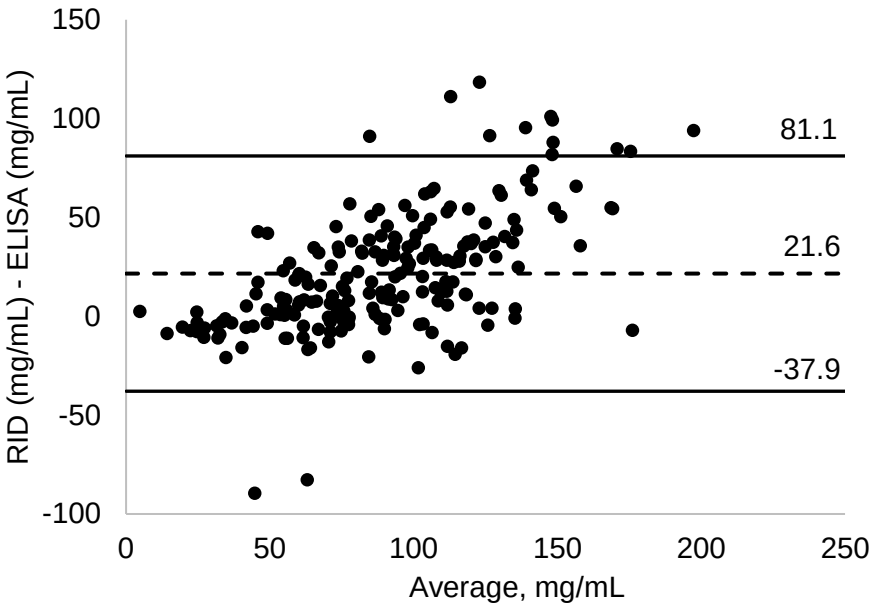

Figure 2. Bland-Altman plot comparing the difference between total IgG concentration measured by radial immunodiffusion (RID; mg/mL) and ELISA (mg/mL; n = 204). On average, the IgG concentration measured by ELISA was 21.6 mg/mL lower compared to RID. The limits of agreement were 81.1 mg/mL and -37.9 mg/mL (±1.96 SD; 95% CI).

Additional unpublished work

Saliva cortisol and fecal glucocorticoid metabolites suitable for acute stress assessment in preweaned dairy calves?

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Instructions to Authors.

Saliva cortisol and fecal glucocorticoid metabolites suitable for acute stress assessment in preweaned dairy calves? By Röder et al.

The objective of this study was to measure cortisol concentrations in saliva, plasma and its metabolites in the feces following disbudding. Saliva cortisol had a good correlation with plasma cortisol, reflected the acute stress response well and thus proved as a suited parameter. Fecal glucocorticoid metabolites showed a weak correlation with plasma cortisol and were less suitable for the assessment of an acute stress response in dairy calves.

Abstract

The objective of the study was to measure cortisol in saliva and glucocorticoid metabolites in feces of calves after cautery disbudding and compare them with plasma cortisol levels. Seventy-one female Holstein Friesian calves aged 13 + 2 days, with an average weight of 48.9 + 4.26 kg were enrolled in the study. One week before disbudding took place, a sham disbudding procedure was performed. Sham disbudding was conducted by using a cold cautery dehorner applied to each horn bud for 10 s. Disbudding was performed identically but using a hot cautery dehorner. Both procedures were conducted after local anesthesia. In total 987 plasma samples (Sham: 492; Disbudding: 495), 969 saliva samples (Sham: 486; Disbudding: 483) and 1,950 fecal samples (Sham: 825; Disbudding: 1165) were collected. Cortisol was measured in plasma and saliva and its metabolites in the feces (with an 11-oxo-etiocholanolone enzyme immunoassay) at seven time points (-0.5 h, 0 h, 0.5 h, 2 h, 6 h, 12 h and 24 h) after disbudding. The sampling order of the 3 media at each time point was randomized for every calf. Sampling started between 6:00 a.m. and 9:00 a.m. and the individual sampling time for all calves was taken into account to minimize the influence by circadian rhythm of cortisol. Additionally, further fecal samples were collected 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h and 240 h post disbudding. Mean (± SD) plasma cortisol concentration was 6.0 ± 0.5 ng/mL for sham disbudding and 5.1 ± 0.5 ng/mL for disbudding. The mean salivary cortisol concentration was 2.5 ± 0.2 ng/mL for sham disbudding and 1.9 ± 0.2 ng/mL for disbudding, respectively. The mean fecal glucocorticoid metabolite (FGM) concentration was 260.5 ± 27.5 ng/g for sham disbudding. For disbudding the mean FGM concentration was 248.2 ± 23.2 ng/g. Saliva cortisol showed a good correlation with plasma cortisol ($r = 0.73$, $P < 0.01$). The correlation was greater when the plasma sample was withdrawn before the saliva sample ($r = 0.76$; $P < 0.01$). In contrast, there was a weak correlation between plasma cortisol and glucocorticoid metabolites in feces considering a 6 h and 12 h delay after the cortisol-releasing stimulus (6 h: $r = 0.24$; $P < 0.01$; 12 h: $r = 0.21$; $P = 0.02$). Saliva can be used as a sampling method for acute stress response assessment after cautery disbudding, if the lower cortisol concentration in saliva is taken into account. Due to the variable delay times in fecal excretion and a lack of samples between 6 and 12 h post disbudding, FGMs were not suited to determine the acute stress response in dairy calves.

Key words: disbudding, cortisol, saliva, glucocorticoid metabolites, minimal-invasive sampling

Introduction

Rearing calves on a dairy farm can be associated with events that can cause stress such as separation, re-grouping, disbudding, or castration. Stress activates a hormonal cascade in the hypothalamic–pituitary–adrenocortical axis (HPA axis) and the sympathoadrenal medullary system (SAM system). As a result, an increased secretion of glucocorticoids, such as cortisol, and catecholamines occur from the adrenal glands.

Cortisol is the hormone most commonly used to assess pain and stress (Merl et al., 2000; Palme, 2012; Sadoul and Geffroy, 2019). Cortisol can be measured in several media, i.e. blood, saliva, feces, urine, hair and milk (Möstl et al., 2002; Cook, 2012; Heimbürge et al., 2019; Palme, 2019). While serum or plasma are the media most commonly used for measuring cortisol, the blood sampling procedure itself can cause stress and lead to elevated cortisol concentrations because of handling and restraining the animal (Stilwell and Broom, 2008). In dairy cows the measurement of fecal glucocorticoid metabolites (FGMs) has already been evaluated as an indirect stress parameter after experiencing transport or veterinary education courses (Möstl et al., 2002) or high udder pressure after sudden dry-off (Bertulat et al., 2013). This method has the potential to improve animal welfare due to easy and smooth stress monitoring (Palme, 2012). Feces can be collected after spontaneous defecation as a non-invasive sampling method. However, FGMs reflect the situation a certain, species-specific time before (~12 h in cattle) due to the delay in fecal excretion (Palme et al., 1999; Palme, 2019) .

Saliva has been shown to be a suitable media for measurements of cortisol. Correlations between saliva and plasma vary between 0.75 and 0.83 after ACTH injections or

udder inflammation (r = 0.75; Schwinn et al., 2016) as well as social isolation in cows and calves (r = 0.83; Hernandez et al., 2014). However, the number of animals in those studies has been low (n = 5 to 23).

Overall, minimal-invasive to non-invasive analysis methods such as saliva cortisol or FGM have been barely evaluated in calves (Cook, 2012). Therefore, the objective of this study was to compare two minimal-invasive sampling methods with venipuncture in dairy calves to measure cortisol concentration in plasma, saliva and glucocorticoid metabolites in feces, respectively. Sample collection was timed around cautery disbudding to include a distinct cortisol releasing stimulus. We hypothesized that cortisol concentrations in plasma, saliva and FGMs will increase following the acute stressor, and are suited to assess stress in dairy calves.

Material and Methods

Animal Enrollment and Experimental Design

This experiment was carried out as part of a larger study, on a commercial dairy farm in northeastern Germany from February to August 2020 (Röder et al., 2022). The study protocol was in accordance with the Institutional Animal Care and Use Committee of the Freie Universität Berlin (approval number: 2347-49-2019). The sample size was based on assumptions of the first study using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium) considering a difference of cortisol concentrations (6.4 ± 7.2 ng/mL) between a control and two treatment groups (Kleinhenz et al., 2017).

Calves were housed in individual calf hutches (2.05 x 1.15 x 1.35 m) bedded with straw. Each calf hutch had an individual paddock with sand bedding measuring 1.50 x 1.10 m. The study design and the disbudding procedures (sham disbudding and disbudding) are described in detail in Röder et al. (2022). In total, 77 dairy heifer calves (13 + 2 d) were enrolled in the study and sham disbudded at d 14 and disbudded at 21 d of age. In brief disbudding was conducted between 6:00 a.m. and 9:00 a.m. after local anesthesia of the Ramus cornualis with 5 mL of procaine 2% (Procamidor 20 mg/mL, WDT e.G., Garbsen, Germany) at each horn bud, as described by Reedman et al. (2021). Afterwards, sham disbudding took place by using a cold cautery dehorner (Buddex, KERBL Corporation, Buchbach, Germany), whereas a preheated gas dehorner (Buddex, KERBL Corporation) was used for disbudding one week later.

Sampling of Blood, Saliva and Feces

Samples of blood, saliva and feces were collected 0.5 h before, at (0 h), and 0.5 h, 2 h, 6 h, 12 h, 24 h after sham disbudding and disbudding, respectively. The sampling order of the 3 media at each time point was randomized for every calf and the individual sampling time for all calves was taken into account to minimize the influence by circadian rhythm of cortisol. Fecal sample collection started 48 h prior sham disbudding and ended 8 d after disbudding. Additional fecal samples were collected once daily in the morning hours between 7:00 a.m. and 8:00 a.m.

Blood samples were taken from the jugular vein with lithium heparin vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) and inverted cautiously five times. Blood samples were stored on ice until centrifugation at room temperature (6 min at 4,500 g) approximately 45 ± 15 min after withdrawal. Plasma was transferred into cryovials (Cryovial 2 mL, Simport, Bernard-Pilon, Canada) and stored at -18°C. Saliva sample collection was performed with cotton swaps (Salivette Cortisol, Sarstedt AG & Co, Nürnbrecht, Germany). The swaps were inserted it into the oral cavity for approximately 3 min. The samples were stored on ice until centrifugation for 6 min at 4,500 g at room temperature took place. The saliva was pipetted into cryovials (Cryovial 2 mL, Simport, Bernard-Pilon, Canada) and stored at -18°C. To ensure fecal collection at the specified sampling times, a digital transrectal stimulation was performed and the obtained feces was transferred into fecal sample tubes (HS-fecal sample tubes 18 mL, Henry Schein Medical GmbH, Melville, US). If defecation occurred spontaneously, the sample was collected without stimulation. The samples were stored at -18°C.

Samples were transported on dry ice to the laboratory of the Unit of Physiology, Pathophysiology and Experimental Endocrinology at the University of Veterinary Medicine in Vienna for analyses.

Plasma, Saliva and Fecal Sample Analyses

Cortisol concentration in plasma and saliva was measured following diethylether extraction (Schöffmann et al. 2009) using a cortisol enzyme immunoassay (EIA; Palme and Möstl, 1997). Its application in calves was performed according to Wagner et al. (2013). Fecal samples (0.5 g) were extracted with 5 mL 80% methanol (Palme et al., 2013) and FGMs were measured in an aliquot of the supernatant using an 11-oxoetiocholanolone EIA (Möstl et al., 2002). The intra- (and inter-) assay coefficients of variation (CV) of low and high concentration plasma pool samples were 9.2% (12.7%) and 12.8% (15.7%), respectively. The intraassay CV for saliva and fecal samples were below 10%. Interassay CVs for low (high) concentration pool samples were 14.1 (17.1%) and 12.2% (14.6%) for saliva and fecal samples, respectively. Sensitivity of the cortisol and 11-oxoetiocholanolone EIA were 0.2 ng/mL saliva and 2 ng/g feces, respectively.

Statistical Analysis

The saliva cortisol concentrations (ng/mL) were plotted against the plasma cortisol concentrations (ng/mL). Pearson correlation coefficients (r) and Bland-Altman plots were generated using Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). In addition, Spearman's rank correlation coefficients of plasma cortisol, saliva cortisol and FGMs were generated using SPSS (version 22.0, SPSS Inc., IBM, Ehningen, Germany) comparing each sampling time. Statistical significance was reached at $P < 0.05$ and a statistical tendency was declared as differences between $P \geq 0.05$ and $P \leq 0.10$. Furthermore, to determine the average, minima, maxima and regression equations Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany) was used. The data was expressed as mean ± SEM.

To evaluate the dynamics of the different cortisol concentrations (i.e., plasma or saliva) or FGMs during the disbudding procedure, repeated measures ANOVA with first-order autoregressive covariance structure were performed using the GENLINMIXED procedure of SPSS. The outcome variable was either plasma cortisol or saliva cortisol (ng/mL) or FGMs (ng/g). The initial model contained sampling time relative to disbudding (-0.5 h, 0 h, 0.5 h, 2 h, 6 h, 12 h, 24 h) as fixed effects. For FGM analyses further sampling times were considered (-48 h, -24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h, 240 h). A Bonferroni adjustment was used to account for multiple comparisons. Variables were declared to be significant when $P < 0.05$. A statistical tendency was declared when $P \geq 0.05$ and $P \leq 0.10$.

Results

In total 77 dairy calves were enrolled in this study. Due to inadequate local anesthesia six animals were excluded and 71 animals with an average body weight of 48.9 + 4.26 kg (mean ± SD) were considered in the final analyses. During sham disbudding 492 plasma samples, 486 saliva samples and 825 fecal samples were collected. During disbudding 495 plasma samples, 483 saliva samples and 1165 fecal samples were collected.

Correlation Coefficients

In total 969 saliva samples with corresponding plasma samples were considered for the Pearson correlation coefficients. Overall, saliva cortisol had a good correlation with plasma cortisol ($r = 0.73$; $P < 0.01$; Figure 1A). Considering the sampling order, saliva cortisol had a greater correlation if the blood was withdrawn first ($n = 333$; $r = 0.76$; $P < 0.01$; Figure 1B), whereas if the saliva sample was collected first, the correlation was $r = 0.69$ ($n = 309$; $P < 0.01$; Figure 1C).

The Spearman's rank correlation coefficients comparing plasma and saliva cortisol (ng/mL) sampling times are shown in Table 1. The highest correlations were obtained when samples taken at the same time were compared. The highest Spearman's rank correlation coefficient ($r = 0.85$) was measured between plasma and saliva cortisol. The Spearman's rank correlation coefficients comparing plasma cortisol (ng/mL) and FGMs (ng/g) at the different

sampling times are shown in Table 2. Correlation coefficients for plasma and fecal samples collected at the same time were low and not significant, except for the time sampling time 24 h (Table 2). Considering a time lag, the highest correlations were found at these four sampling times: FGM concentration (72 h) compared to plasma cortisol at -0.5 h ($r = 0.28$; $P = 0.02$); FGM concentration at 6 h compared to 0 h plasma cortisol ($r = 0.24$; $P < 0.01$), as well as FGM concentration at 72 h and 96 h compared to plasma cortisol at 6 h ($r = 0.28$; $P = 0.02$) and 24 h ($r = 0.25$; $P < 0.01$), respectively. The correlation between plasma cortisol and FGM considering a 6 h and 12 h delay after the cortisol-releasing stimulus (0 h) was $r = 0.24$ ($P < 0.01$) and $r = 0.21$ ($P = 0.02$), respectively.

Time Course of Plasma and Saliva Cortisol and Fecal Glucocorticoid Metabolites

The mean plasma cortisol concentration was 6.0 ± 0.5 ng/mL (means + SEM) with a range from 0.4 ng/mL to 29.6 ng/mL for sham disbudding and 5.1 ± 0.5 ng/mL (range: 0.2 ng/mL to 26.4 ng/mL) for disbudding. The mean salivary cortisol concentration was 2.5 ± 0.2 ng/mL (range: 0.06 ng/mL up to 12.8 ng/mL) for sham disbudding and 1.9 ± 0.2 ng/mL (range: 0.05 ng/mL to 13.3 ng/mL) for disbudding, respectively. The mean FGM concentration was 260.5 ± 27.5 ng/g with a range from minimum 11.0 ng/g to maximum 2090.3 ng/g for sham disbudding. For disbudding the mean FGM concentration was 248.2 ± 23.2 ng/g (range: 12.5 ng/g to 1972.0 ng/g). Descriptive statistics for plasma cortisol, salivary cortisol and FGM for sham disbudding and disbudding are given in Table 3.

After sham disbudding the first peaks in cortisol or FGM concentrations occurred at 0 h, 0.5 h and 6 h in plasma, saliva and feces, respectively. After disbudding the first peaks in cortisol or FGM concentrations occurred at 0 h, 0.5 h and 48 h in plasma, saliva and feces, respectively. The changes of concentration for plasma and saliva cortisol over 24 h after sham disbudding and disbudding are shown in Figure 2. The changes in FGM concentration 48 h prior until 226 h after sham disbudding and disbudding are shown in Figure 3.

Plasma cortisol concentration was affected by time ($P < 0.05$). Compared to baseline (-0.5 h) plasma cortisol was significantly higher 0 h, 0.5 h and 6 h after sham disbudding as well as 0 h, 0.5 h, 6 h and 12 h after disbudding (Table 3). Saliva cortisol concentration was affected by time ($P < 0.05$). Compared to baseline (-0.5 h) saliva cortisol was significantly higher 0 h, 0.5 h, 6 h and 12 h after sham disbudding as well as 0 h, 0.5 h, 2 h, 6 h and 12 h after disbudding (Table 3). FGM concentration was affected by time ($P < 0.05$). Compared to baseline (-0.5 h) FGM concentration was significant higher 12 h, 76 h and 92 h after sham disbudding as well as 48 h, 144 h, 192 h, 216 h and 240 h after disbudding (Table 4).

Discussion

The main findings of this study were: 1) Correlation of plasma and saliva cortisol in dairy calves after sham disbudding and disbudding was good while the correlation of plasma cortisol and FGM concentration was weak; 2) the correlation was higher, when the plasma sample was withdrawn before the saliva sample.

Plasma and Saliva Cortisol

The coefficient of correlation between plasma and saliva cortisol ($r = 0.73$) was in line with previous studies reporting correlations between these two mediums in calves ($r = 0.71$, $P < 0.01$; Negrão et al., 2004) and adult dairy cows ($r = 0.75$; Schwinn et al., 2016) using a ACTH administration as stimulus for cortisol secretion. Our calves were sampled around disbudding which is a stressor typically occurring between 2 and 6 weeks of age. Considering these findings, we conclude that saliva is a suitable medium for the measurement of cortisol in calves. Compared to adult dairy cows, less manipulation and handling is required during saliva sampling but sampling took more time than blood sampling (Negrão et al., 2004).

In our study, the correlation was higher, when blood samples were withdrawn before the saliva samples ($r = 0.76$). The timely collection of blood and saliva samples allowed us to obtain the variations of cortisol concentrations between the two media considering sampling time. The highest correlation was achieved when plasma and saliva samples were collected

at the same time or saliva samples were collected until 0.5 h post-disbudding. This is consistent with a 10 min time lag between peak cortisol concentrations in plasma and saliva (Hernandez et al., 2014). This is due to the disproportionate increase in free cortisol under stress conditions (Cook et al., 1997).

Plasma Cortisol and Fecal Glucocorticoid Metabolites

The correlation between plasma cortisol (0 h) and FGM concentration was weak. Correlations between plasma cortisol and FGM varied in different studies and species. Only the free, non-bound cortisol is metabolized by the liver and excreted via feces (Palme et al., 2005) which could explain the weak correlation. In our study we found a weak correlation between plasma cortisol (0 h) and FGM concentration after the cortisol releasing stimulus at time point 6 h and as well as 12 h. Palme et al. (1999) described a species-specific time lag of 10 to 14 h in cattle due to gastrointestinal passage. In our study number of feedings were reduced on day 14 from three to two. This could have affected metabolism of glucocorticoids due to changes in gastrointestinal passage and digestion. As gastrointestinal passage time was not measured in our study, we cannot determine what time lag should be considered when using fecal samples to evaluate acute stress in calves. In a recent, preliminary study, Vogt et al. (2022) described peak FGM concentrations in preweaned calves after 9.8 h and 8.3 h in calves after weaning exposed to transport-induced stress, respectively. This indicates that peak FGM samples may have been missed by our sampling regime (6 h and 12 h post disbudding).

Time Course of Cortisol and Fecal Glucocorticoid Metabolite Concentration

Disbudding induced an increase in plasma cortisol concentration (Röder et al., 2022), which was also obvious in saliva and feces. The peak cortisol concentration in plasma was observed at sham disbudding and disbudding (0 h), whereas saliva cortisol peaked 0.5 h after sham disbudding and disbudding and the maximum FGM concentration could be detected 6 h after sham disbudding and 48 h after disbudding.

Time affected cortisol concentration ($P < 0.05$) in plasma, saliva cortisol and as well as in FGM compared to baseline (-0.5 h). Therefore, we conclude that the cortisol concentrations in plasma harvested from blood samples collected at disbudding reflected the associated stress with the procedure, while in saliva and feces the increase was delayed. Hernandez et al. (2014) reported a time lag of 10 min between plasma and saliva cortisol peak after social separation of dairy calves.

In our study, elevated FGM concentrations could be detected until 240 h after disbudding. After exposure to transport stress or stimulation of cortisol secretion through ACTH injection, FGMs were elevated 8 h to 16 h in adult dairy cows (Palme et al., 2000; Morrow et al., 2002). After disbudding, FGM concentration reached its peak at 48 h and 144 h. Furthermore, the FGM concentration remained higher than the baseline during the entire observation period after disbudding. The measurement of FGM has been evaluated for the assessment of cortisol in cows, especially for the assessment of chronic stress (Möstl et al., 2002; Morrow et al., 2002), this could be an explanation for the elevated FGM levels.

Diet had a non-significant influence on peak FGM concentration following transport-induced stress in preweaned and weaned calves (Vogt et al., 2023). Therefore, the restrictive milk feeding program during our study might have affected the gastrointestinal passage time. Given our sampling points (6 h and 12 h) and above recently published study (Vogt et al., 2023). peak FGM concentrations might have been missed.

Conclusion

Results from this study support the suitability of saliva samples to estimate cortisol concentrations in plasma after cautery disbudding as an acute stressor in preweaned dairy calves. We do not recommend fecal samples for evaluating an acute stress response, because disbudding was not reflected well and more frequent sampling would be needed. However, for assessing longer lasting stressors fecal samples may be suited, but further research is needed.

Even though sampling saliva has been considered as minimal-invasive, further research is warranted to determine if calves experience less stress when saliva samples are collected compared to blood samples.

Acknowledgements

We gratefully thank the participating dairy farm for the collaboration and Edith Kobletz-Rassam (Unit of Physiology, Pathophysiology, and Experimental Endocrinology, University of Veterinary Medicine, Vienna, Austria) for EIA analyses. Mareike Röder was funded in part by Tiergyn e. V. (Berlin, Germany). The authors have not stated any other conflicts of interest.

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Table 1. Spearman's rank correlation coefficients comparing plasma and saliva cortisol concentrations (ng/mL; n = 969) of 71 dairy calves during disbudding at 7 different times. Only the significant results ($P < 0.05$) were included in the table.

			Plasma cortisol						
			Time relative to disbudding, h						
	Time, h		-0.5 h	0 h	0.5 h	2 h	6 h	12 h	24 h
Saliva cortisol relative to disbudding	-0.5 h	r	0.79**	0.34**	0.30*	-	0.32**	0.18*	0.33**
		P-value	<0.01	<0.01	0.01	-	<0.01	0.03	<0.01
	0 h	r	0.46**	0.69**	0.41*	-	0.27*	-	0.36**
		P-value	<0.01	<0.01	0.02	-	0.01	-	<0.01
	0.5 h	r	0.35**	0.69**	0.85**	0.30**	0.21*	-	0.36**
		P-value	<0.01	<0.01	<0.01	<0.01	0.01	-	<0.01
	2 h	r	0.20*	0.37**	0.37**	0.54**	0.30*	-	0.29*
		P-value	0.01	<0.01	<0.01	<0.01	0.01	-	0.03
	6 h	r	0.38**	0.25**	0.25**	-	0.63**	0.18*	0.19*
		P-value	<0.01	<0.01	<0.01	-	<0.01	0.03	0.03
	12 h	r	-	-	-	-	-	0.65**	-
		P-value	-	-	-	-	-	<0.01	-
	24 h	r	0.26**	0.35**	0.30*	-	-	-	0.67**
		P-value	<0.01	<0.01	0.02	-	-	-	<0.01

**Correlation is significant at $P < 0.01$
*Correlation is significant at $P < 0.05$

Table 2. Spearman's rank correlation coefficients comparing plasma cortisol (ng/mL) and FGM concentration (ng/g; n = 987) of 71 dairy calves during disbudding at 7 different times. Only the significant results ($P < 0.05$) were included in the table.

			Plasma cortisol, ng/mL Time relative to disbudding, h						
	Time, h		-0.5 h	0 h	0.5 h	2 h	6 h	12 h	24 h
FGM, ng/g	-0.5 h	r	-	-	-	-	-	-	-
		P-value	-	-	-	-	-	-	-
	0 h	r	-	-	-	-	-	-	-
		P-value	-	-	-	-	-	-	-
	0.5 h	r	-	-	0.22*	-	-	-	-
		P-value	-	-	0.01	-	-	-	-
	2 h	r	-	-	0.23**	-	-	-	-
		P-value	-	-	0.007	-	-	-	-
	6 h	r	-	0.24**	0.18*	-	-	-	-
		P-value	-	0.005	0.04	-	-	-	-
	12 h	r	-	0.21*	0.20*	-	-	-	-
		P-value	-	0.02	0.02	-	-	-	-
	24 h	r	-	-	-	-	-	-	-0.26**
		P-value	-	-	-	-	-	-	0.003
	48 h	r	-	-	-	-	-	-	-
		P-value	-	-	-	-	-	-	-
	72 h	r	0.28*	-	-	-	0.28*	-	-
		P-value	0.02	-	-	-	0.02	-	-
	96 h	r	-	-	-	-	-	-	0.25*
		P-value	-	-	-	-	-	-	0.05

**Correlation is significant at $P < 0.01$
*Correlation is significant at $P < 0.05$

Table 3. Descriptive statistics of cortisol concentrations (ng/mL) in plasma (sham: n = 492; disbudding: n = 495) and saliva (sham: n = 486; disbudding: n = 483) and fecal glucocorticoid metabolites (FGMs, ng/g; sham: n = 483; disbudding: n = 480) during sham disbudding and disbudding of 71 dairy calves. Average (± SEM) were listed for each sampling time point and the relative saliva cortisol concentration (%) was calculated referring to plasma cortisol concentration.

		Time relative to disbudding, h						
		-0.5 h	0 h	0.5 h	2 h	6 h	12 h	24 h
Sham disbudding	Plasma, ng/mL	4.4 (± 0.4) [a]	9.8 (± 0.7) [b]	8.7 (± 0.6) [b]	3.2 (± 0.4)	5.4 (± 0.5) [b]	7.0 (± 0.5)	3.6 (± 0.4)
	Saliva, ng/mL	1.7 (± 0.2) [a]	3.3 (± 0.2) [b]	4.3 (± 0.3) [b]	1.4 (± 0.1)	2.5 (± 0.2) [b]	2.9 (± 0.2) [b]	1.3 (± 0.1)
	Relative saliva, %	38.4	33.4	49.4	43.8	46.3	41.4	36.1
	FGMs, ng/g	228.6 (± 20.7) [a]	300.9 (± 36.5)	250.4 (± 21.4)	284.5 (± 33.7)	321.2 (± 32.9)	244.7 (± 32.3) [b]	193.1 (± 15.4)
Disbudding	Plasma, ng/mL	3.4 (± 0.4) [a]	8.5 (± 0.7) [b]	7.5 (± 0.6) [b]	3.3 (± 0.3)	4.6 (± 0.4) [b]	5.2 (± 0.4) [b]	3.6 (± 0.5)
	Saliva, ng/mL	1.1 (± 0.1) [a]	2.5 (± 0.2) [b]	3.2 (± 0.3) [b]	1.4 (± 0.1) [b]	1.6 (± 0.1) [b]	2.0 (± 0.2) [b]	1.3 (± 0.2)
	Relative saliva, %	32.4	29.4	42.7	42.4	34.8	38.5	36.1
	FGMs, ng/g	237.7 (± 18.4) [a]	247.7 (± 20.8)	269.3 (± 25.8)	284.6 (± 28.8)	218.5 (± 24.1)	191.0 (± 21.4)	289.1 (± 37.8)

[a] - [b] Columns with different superscripts differ significantly ($P < 0.05$)

Table 4. Descriptive statistics of fecal glucocorticoid metabolites (ng/g) during sham disbudding (n = 825) and disbudding (n = 1165) of 71 dairy calves. Average (± SEM) were listed for each time point.

[a] - [b] Rows with different superscripts differ significantly ($P < 0.05$)

	FGM concentration[1]			
Time, h	Sham disbudding	SEM	Disbudding	SEM
-48	295.9	23.5	267.9	24.4
-24	269.3	24.7	252.8	24.0
-0.5 [a]	228.6 [a]	20.7	237.7 [a]	18.4
0	300.9	36.5	247.1	20.8
0.5	250.4	21.4	269.3	25.8
2	284.5	33.7	284.6	28.8
6	321.2	32.9	218.5	24.1
12	244.7[b]	32.3	191.0	21.4
24	193.1	15.4	289.1	37.8
48	252.4	20.4	346.5 [b]	34.1
72	305.5 [b]	23.8	302.5	31.0
96	218.1 [b]	17.2	325.1	52.8
120			319.3	28.9
144			395.3 [b]	37.7
192			383.3[b]	44.1
216			429.7 [b]	47.6
240			446.2 [b]	59.8

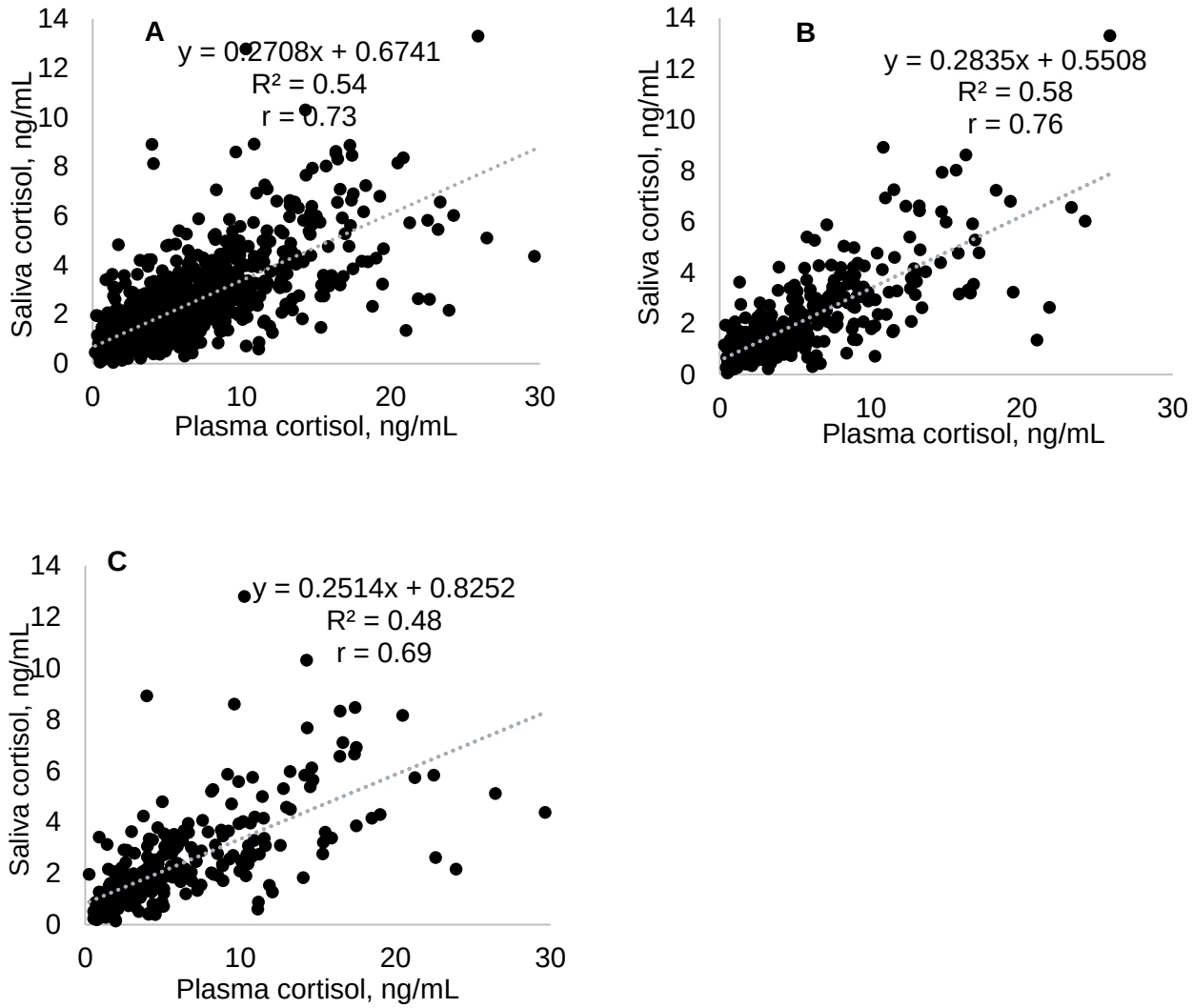

Figure 1. Panel A Comparison of plasma and saliva cortisol concentration (ng/mL) of female dairy calves (n = 71; number of samples = 969; r = 0.73); **Panel B**: subgroup in which blood samples were collected before saliva samples (number of samples = 333; r = 0.76); **Panel C**: subgroup in which saliva samples were collected before blood samples (number of samples = 309; r = 0.69).

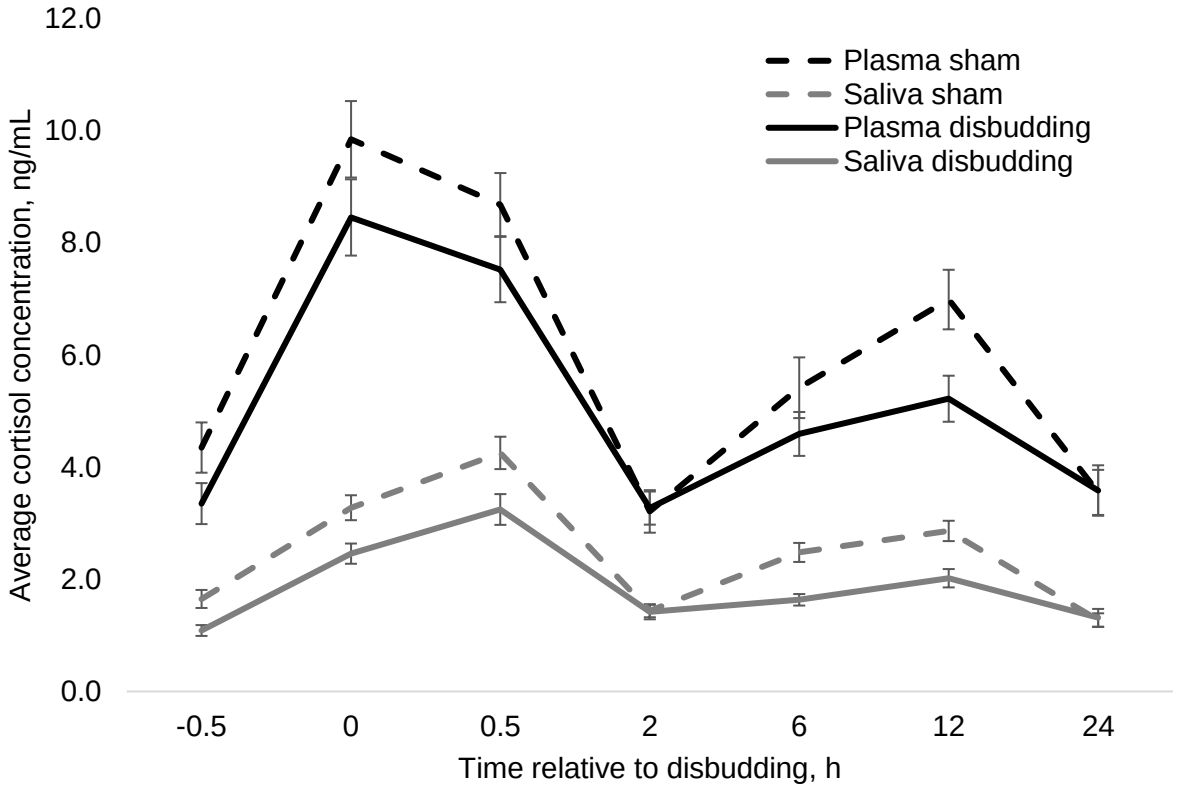

Figure 2. Plasma and saliva cortisol concentrations (ng/mL; mean ± SE) plotted relative to the time of sham disbudding (plasma samples: n = 492; saliva samples: n = 486) and disbudding (plasma samples: n = 495; saliva samples: n = 483).

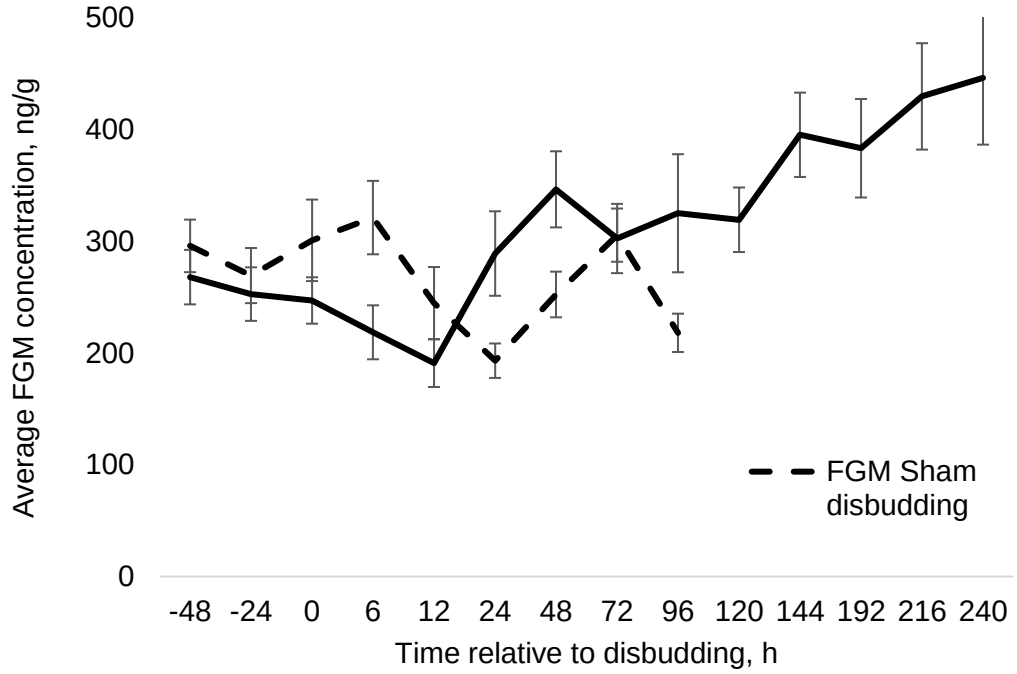

Figure 3. Average (±SE) fecal glucocorticoid metabolite (FGM) concentrations (ng/g) were plotted relative to the time of sham disbudding (n = 825) and disbudding (n = 1165).

Discussion

The overall objective of this thesis was to evaluate important management factors for calf health. In order to evaluate the effect of two treatments of transdermal flunixin meglumine combined with local anesthesia compared to a non-treated control group during disbudding 1) plasma cortisol 2) and behavioral changes were analyzed. Concerning the estimation of colostrum quality, different analytical methods, including laboratory methods (RID, ELISA), as well as on-farm tests (digital Brix refractometer, colostrometer, outflow funnel, semi-quantitative immunochromatographic) were evaluated. Additionally, disbudding procedure was used to evaluate cortisol measurements in different media (plasma, saliva, feces).

The main findings of the first study indicate that a disbudding procedure conducted with a cold dehorner (sham disbudding) must be considered as stressful for dairy calves, since their cortisol increases after they are restrained for drug administration or for the disbudding procedure. Our results showed that a NSAID in combination with a local anesthetic was more effective in reducing the stress response after cautery disbudding in calves compared to a local anesthetic alone. The use of transdermal flunixin meglumine decreased plasma cortisol concentrations following cautery disbudding of calves. A second dose of transdermal flunixin meglumine 6 h post-disbudding, however, had no additional effect on plasma cortisol concentrations. Average daily weight gain and total lying time were also not affected. This is in contrast to a previous study from Bates et al. (2015) in which growth rate was higher (0.10 kg/ day) for meloxicam treated calves in comparison to non-treated calves in the period from day 0 to day 15 after disbudding. Further research is warranted to evaluate the effect of flunixin meglumine transdermal on growth rate after procedures such as disbudding and also in comparison to other NSAIDs.

Aversion studies evaluate how negatively the experience of hot-iron disbudding is perceived (Ede et al. 2019). A combination of meloxicam and local anesthetic made calves (n = 16) less aversive during 6 h after hot-iron disbudding (Ede et al. 2019). In contrast, calves (n = 15) treated with Ketoprofen alone showed more aversion behavior than the untreated calves (Ede et al. 2019). Furthermore, the aversion to approach following different injection routes has been evaluated by Ede et al. (2018). Calves (n = 24) receiving saline injection (0.5 mL) via subcutaneous or intranasal routes showed less aversion to approach than calves receiving intramuscular injections. As an administration route, transdermal administration has not yet been compared to aversion to approach. Needle free administration of medication such as transdermal application can also have advantages in order to regulate transmission of diseases. Darpel et al. (2016) demonstrated that sharing needles can result in Bluetongue virus transmission in sheep and cattle. However transdermal application of NSAID has disadvantages in its use due to the dependency on weather conditions like rain. Altenbrunner-Martinek et al. (2020) reported that absorption and bioavailability of transdermal flunixin meglumine was impacted under rainy conditions. An assessment on the usability of transdermal application remains to be evaluated.

In the present study, standing and lying behavior was monitored using a 3D accelerometer according to Bonk et al. (2013). After sham disbudding, a small effect of treatment (considered as irrelevant) increased total lying time by 10 min within a 6 h period. After disbudding, total lying time was similar for all groups and not affected by treatment. Treatment had no effect on total lying time ($P = 0.31$), average lying bout length in calves ($P = 0.60$), or number of lying bouts ($P = 0.49$). Monitoring of procedure-induced pain behavior can help to improve animal welfare and to evaluate the efficiency of treatment protocols. In contrast, meloxicam affected lying behavior 2 h post-disbudding (Sutherland et al., 2019). Moreover, in the first 3 h after disbudding, calves showed more playing and bucking behaviors when treated with local anesthesia and meloxicam, compared with calves disbudded without analgesia or with local anesthesia alone (Mintline et al. 2013). However, over a period of 24 to 27 h after disbudding, no difference in play behavior or lying behavior could be found in dairy calves (Sutherland et al. 2019; Mintline et al. 2013). Due to wound healing, discomfort and behavioral changes can occur later in the healing process after disbudding. Lidocaine

treatment 11 d post-disbudding leads to fewer head shakes and ear flicks in comparison with untreated calves (Adcock et al. 2020). Furthermore, wound protective behavior was suppressed, indicating that pain persists for longer after the disbudding procedure when lidocaine is used (Adcock et al. 2020).

Behavioral observations are commonly used in order to rate discomfort and defined as non- invasive methods. Non-invasive methods are designed to gather information or perform interventions without causing significant discomfort, pain, or harm to the subject due to as little interference as possible (Schilling et al. 2022). In contrast, while serum or plasma are the media most commonly used for measuring cortisol, this procedure may require the use of needles or catheters, it leads to elevated cortisol concentrations and is therefore rated as an invasive sampling method because of handling and restraining the animal (Stilwell and Broom 2008). A non-invasive method refers to a procedure that does not involve the insertion of instruments, devices, or substances into the body or the disruption of the body's natural barriers (such as the skin or mucous membranes) to obtain measurements or samples rs, minimizes disturbance to animals (Pauli et al. 2010). Possible media for measuring cortisol concentration in calves with a non-invasive method could be hair, feces, urine or saliva (Möstl et al. 2002; Cook 2012; Heimbürge et al. 2019; Palme 2019). Feces and urine can be collected spontaneously or even without contact with the animal (Auer et al. 2020). Due to the metabolization by the liver only the free, non-bound cortisol is excreted via feces (Palme et al. 2005). Therefore, fecal glucocorticoid metabolites (FGM) reflect the cumulated cortisol output after a species-specific time lag of cortisol release and excretion (Di Francesco et al. 2021). Saliva cortisol reflects the unbound or free cortisol levels (Vining et al., 1983). In contrast plasma cortisol reflects the total cortisol concentration in the bloodstream, including both bound and unbound cortisol (Lewis et al. 2005). Analysis methods such as saliva cortisol or FGM have been barely evaluated in calves (Cook 2012). Therefore, the objective of the additional study was to compare two minimal-invasive sampling methods with venipuncture in dairy calves to measure cortisol concentration in plasma, saliva and glucocorticoid metabolites in feces, respectively. The main findings of the additional study were: 1) Correlation of plasma and saliva cortisol in dairy calves after sham disbudding and disbudding was good ($r = 0.73$, $P < 0.01$). while the correlation of plasma cortisol and FGM concentration was weak considering a 6 h and 12 h delay after the cortisol-releasing stimulus (6 h: $r = 0.24$; $P < 0.01$; 12 h: $r = 0.21$; $P = 0.02$); 2) the correlation was higher, when the plasma sample was withdrawn before the saliva sample. Further the comparability of different media with bound and unbound cortisol with each other as well as the comparison of the invasiveness of sampling methods should be further investigated.

With regard to the to the duration of disbudding's effects, a follow up study could look as follows: Calves are sham and disbudded at the age of 3 weeks and randomly assigned to one group each. Group assignment includes administration of local anesthesia in combination with a) meloxicam, b) flunixin meglumine transdermal or c) placebo. The choice of NSAIDs in Germany is limited due to their authorization. Cortisol (blood or saliva) can be used as measurement parameter. The measurement and observation of daily weight gain, milk intake, standing and lying behaviors, play behavior or pain-induced behavior (head shaking, ear flicks) should be considered over a longer period of time of 11 d due to pain in the course of wound healing. Behavioral observation should start 24 h prior the sham disbudding procedure and end 3 weeks after disbudding procedure. Standing and lying behavior should be monitored using a 3D accelerometer according to Bonk et al. (2013). Re-administration of local anesthesia 11 d after disbudding in combination with NSAIDs or NSAIDs alone during the wound healing period could complete the study according to Adcock et al. (2020).

The main findings of the second study indicate that the digital Brix refractometer and colostrometer were accurate to determine colostrum quality on-farm. The lateral flow assay and the outflow funnel, however, were not accurate enough to determine colostrum quality. Compared to RID as a laboratory method, ELISA was suitable to assess colostrum quality. The predictive value of the colostrometer for colostral IgG concentration was lower than the

digital Brix refractometer and ELISA. Different thresholds should be considered. If this is not the case, high quality colostrum could be falsely classified as not acceptable.

In this study, colostrum samples were considered as high quality with >50 mg IgG/mL and poor quality with <50 mg IgG/mL based on RID analysis. Considering this threshold, the RID analysis identified 186 (89%) high quality colostrum samples and 23 (11%) poor quality ones. Compared to the reference method (RID), the Pearson correlation coefficients of the on-farm test were between r = 0.71 (Brix refractometer) and r = 0.26 (outflow funnel). The ELISA showed a moderate correlation to RID (r = 0.78; P < 0.01).

As a summary measure of the ability of a diagnostic test, we used the area under the Receiver Operating Characteristic curve (AUC), in this study. The diagnostic test should result in an accurate diagnosis. Accuracy describes the variance of a measurement from its true value while precision refers to the dispersion of the measurements (Ranstam 2008). The AUC was the highest for the Brix refractometer (AUC: 0.93) and the lowest for the outflow funnel (AUC: 0.73). The 95% CI overlapped for the Brix refractometer (0.89 – 0.96) and ELISA (0.90 – 0.97). High Se and Sp were detected for ELISA and Brix refractometry (ELISA: Se: 91.3%, Sp: 92.3%; Brix refractometer: Se: 95.7%, Sp: 82.8%), indicating that these tests can distinguish between high- and poor-quality colostrum without considerable numbers of false negatives or false positives. The colostrometer (specific gravity and temperature corrected) was less accurate than the ELISA and Brix refractometry, as the 95% CI of the AUC did not overlap with the CI for the ELISA and Brix refractometry.

Based on the AUC results, 95% CI, Se, and Sp, we concluded that the lateral flow assay and outflow funnel were not capable of reliably identifying colostrum quality.

The results for the threshold to identify high-quality colostrum in our study (digital Brix refractometer: 21.3% Brix, ELISA: 50.8 mg/mL) were consistent with thresholds identified in previous studies (Quigley et al. 2013; Bielmann et al. 2010; Chigerwe et al. 2008). By implementing an optimal threshold, high- and poor-quality colostrum can be identified correctly and not discarded or falsely fed to calves, respectively. However, a laboratory-specific threshold should be established based on failure of passive transfer (FPT).

Colostrum management can be considered as one of a few processes in the dairy farm routine that can be monitored and evaluated as well as preferably regularly reviewed by veterinarians (Godden et al. 2019). Stillbirth and diseases can cause high economic losses on dairy farms (Rocha Valdez et al. 2019; Mahnani et al. 2018). Diarrhea is considered as the most common disease in calves aged under 30 d (Svensson et al. 2006) and can be mitigated by optimizing colostrum management (Cho and Yoon 2014). In order to improve calf health, it is recommended that calves are provided with a sufficient amount of clean high-quality colostrum, within the first few hours of life, i.e. 2 h (Godden et al. 2019). Pletts et al. (2018) describes the additional benefits of providing further feedings as well as extended feedings of colostrum even after gut closure-time (past 12 h). Colostrum replacers are useful if clean, high-quality maternal colostrum is not available (Lago et al. 2018). Routinely monitoring of colostrum management and calf health can help to analyze and remedy problems within the colostrum management program (Lopez and Heinrichs 2022; Godden 2008). Monitoring of colostrum management should include the farmer’s or employee’s capability. Standard operating procedures (SOPs) can be a useful tool to improve routine practice and the confidence of farmers (Hesse et al. 2019).

The appropriate method for assessing colostrum quality remains farm-specific and depends on factors such as the individually perceived user-friendliness, compliance, and profitability. Nevertheless, every colostrum should be tested before feeding or stored with a Brix refractometer or colostrometer. The control of colostrum quality should always be considered in combination with the monitoring of the animal health status via measurement of failure of passive transfer in order to ensure optimal calf health management.

Summary

Optimizing calf health on German dairy farms through pain reduction after disbudding and colostrum quality control

The overall objective of this thesis was to evaluate different important management factors for calf health. A) The effects of transdermal flunixin meglumine in combination with local anesthesia during dehorning on 1) plasma cortisol and 2) behavioral changes were assessed. B) Different analytical methods (laboratory-based methods such as RID, ELISA, as well as on-farm tests like digital Brix refractometer, colostrometer, outflow funnel, semi-quantitative immunochromatographic) were analyzed to determine the quality of colostrum. Additionally, the time of disbudding was used to evaluate cortisol measurements in various media (plasma, saliva, feces).

The objective of the first study (A) was to evaluate the effect of the nonsteroidal anti-inflammatory drug (NSAID) flunixin meglumine applied transdermally (Finadyne Transdermal) on plasma cortisol, average daily weight gain and standing and lying behavior of Holstein dairy calves, given at the time of disbudding (thermos cautery) in combination with local anesthesia. Before and after disbudding, blood samples were taken to obtain plasma, and body weight was measured. Standing and lying behavior were measured by using a 3D accelerometer.

The calves were randomly assigned as follows: 1) Control group (CON, n = 27), 2) 1-Flunixin group (1-FLU, n = 26) with a single administration of transdermal flunixin meglumine during disbudding, and 3) 2-Flunixin group (2-FLU, n = 24) with 2 administrations of transdermal flunixin meglumine (during disbudding and a second dose 6 hours after disbudding). A sham disbudding was conducted one week prior to the actual disbudding using a cold thermocauter.

During sham disbudding, the average plasma cortisol concentrations were 6.09 ± 2.5 ng/mL. Plasma cortisol concentration was influenced by time ($P < 0.01$) and tended to be influenced by treatment ($P = 0.08$). More precisely, compared to CON group, the single treatment (1-FLU) showed a significant effect ($P = 0.05$; -0.97 ng/mL; 95% CI: -1.99 to -0.01), and the second treatment (2-FLU) tended to have an effect ($P = 0.06$; -0.93 ng/mL; 95% CI: -1.88 to 0.02).

During disbudding, the average plasma cortisol concentration was 5.16 ± 2.8 ng/mL (range: 0.2–26.4 ng/mL). Plasma cortisol concentration was influenced by treatment ($P < 0.01$) and the time of sample collection ($P < 0.01$). No interaction between treatment and time of sample collection was observed ($P = 0.95$). Compared to CON group, the single treatment (1-FLU) showed a significant effect ($P < 0.01$; -1.29 ng/mL; 95% CI: -2.15 to -0.44), as did the second treatment (2-FLU; $P < 0.01$; -1.29 ng/mL; 95% CI: -2.14 to -0.43). The two treatment groups did not differ significantly from each other ($P = 0.99$; 0.01 ng/mL; 95% CI: -0.84 to 0.84).

The treatment had an effect on the total lying time as well as the lying duration per lying period during sham disbudding ($P = 0.01$) but had no effect during disbudding ($P = 0.31$).

The results showed that transdermal flunixin meglumine in combination with a local anesthetic was more effective in reducing the stress response following cautery disbudding in calves compared to the use of a local anesthetic alone. This led to lower plasma cortisol levels, although it had no detectable effect on the lying behavior of the calves.

As an addition to the initial study, time of disbudding was used to compare cortisol measurements in different media (plasma, saliva, feces). Cortisol and glucocorticoid metabolites were compared to assess the utility of different media for cortisol measurement in dairy calves. Saliva and fecal samples were collected at the same time as blood samples. Due to intestinal passage, fecal sampling began 48 hours before sham disbudding and ended 8 days after disbudding. The key findings of the additional study were as follows: 1)The correlation between plasma and saliva cortisol in dairy calves was good ($r = 0.73$; $P < 0.01$),

while the correlation between plasma cortisol and fecal glucocorticoid metabolite (FGM) concentration was weak (6 hours: r = 0.24; P < 0.01; 12 hours: r = 0.21; P = 0.02). 2) The correlation was even higher when the plasma sample was collected before the saliva sample collection.

The suitability of saliva samples for estimating plasma cortisol concentration after disbudding as an acute stressor in dairy calves is supported by the results of this study. However, we do not recommend fecal sampling for assessing an acute stress response. For evaluating longer-lasting stressors, fecal samples might be suitable, but further research is needed. Although saliva sample collection is considered as minimall-invasive, additional investigations are required to determine whether calves experience less stress during saliva sampling compared to blood sampling.

The objectives of the second study (B) were to evaluate different analytical methods for determine colostrum quality, including a laboratory-based method (ELISA) and four on-farm tests: digital Brix refractometer (% Brix), colostrometer (specific gravity; mg/mL), outflow funnel (seconds), and lateral flow assay (mg/mL). Colostrum samples were collected from 209 Holstein-Friesian cows on two commercial dairy farms in Germany. Assessment of colostrum volume, temperature, and quality was performed at the milking parlor immediately up to approximately 3 hours post-milking.

Using Receiver Operating Characteristic (ROC) curve analysis, test characteristics for each method were determined, including optimal threshold values, sensitivity (Se), specificity (Sp), and the Area Under the Curve (AUC). Radial Immunodiffusion (RID) was considered the reference method. Colostrum was defined as high-quality when the IgG content from the RID measurement was >50 mg IgG/mL.

After RID analysis, 186 (89%) samples showed high quality ($\geq$50 mg IgG/mL) colostrum, while 23 colostrum samples (11%) exhibited low quality (<50 mg/mL). The average IgG concentration (±SD) was 101.3 ± 45.9 mg/mL (range: 6.0 to 244.3 mg IgG/mL). The average colostrum temperature (±SD) was 27.3°C ± 9.4 (range: 2.6 - 38.2°C). On average, colostrum weighed (±SD) 6.4 kg ± 3.8 (range: 0.7 - 25.3 kg). The Pearson correlation coefficients between RID were: r = 0.78 (ELISA), r = 0.79 (digital Brix refractometry), r = 0.58 (colostrometer: specific gravity), r = 0.61 (colostrometer: temperature-corrected), r = 0.26 (outflow funnel), and r = 0.43 (lateral flow assay). The optimal threshold for identifying high-quality colostrum with ELISA was 50.8 mg/mL, with a sensitivity of 91.3%, specificity of 92.3%, and an AUC of 0.94. Sensitivity of on-site tests ranged from 95.7% (Brix refractometry) to 60.9% (lateral flow assay), while specificity ranged from 88.6% (lateral flow assay) to 75.9% (colostrometer after temperature correction). AUC varied from 0.93 (Brix refractometry) to 0.73 (outflow funnel). Based on the AUC, both ELISA (0.94) and Brix refractometry (0.93) can be considered highly accurate.

In summary, the ELISA is accurate for assessing colostrum quality. Among the on-farm tests, only the digital Brix refractometer and the colostrometer were suitable for determining colostrum quality. The outflow funnel and lateral flow assay cannot be recommended.

Overall, this thesis shows that: 1) transdermal flunixin meglumine can lower plasma cortisol after disbudding; 2) but does not affect average daily weight gain or total lying time. 3) ELISA is accurate in assessing colostrum quality; 4) as on-farm tests only the digital Brix refractometer and the colostrometer assess colostrum quality accurately.

Zusammenfassung

Optimierung von Kälbergesundheit auf deutschen Milchviehbetrieben durch Schmerzreduktion nach Enthornung und Kontrolle von Kolostrum Qualität

Das übergeordnete Ziel dieser Arbeit bestand darin, verschiedene wichtige Managementfaktoren für gesunde Kälber zu bewerten. Es wurden A) die Wirkung transdermalem Flunixin Meglumin in Kombination mit lokaler Anästhesie während der Enthornung auf 1) Kortisol im Plasma und 2) Verhaltensänderungen bewertet und B) verschiedene analytische Methoden (laborbasierte Methoden (RID, ELISA) sowie vor-Ort Messmethoden (digitales Brix-Refraktometer, Kolostrometer, Ausfluss-Trichter, semiquantitative Immunochromatographie)) untersucht, um die Qualität des Kolostrums zu bestimmen. Zusätzlich wurde der Zeitpunkt der Enthornung genutzt, um Kortisol-Messungen in verschiedenen Medien (Plasma, Speichel, Kot) durchzuführen.

Das Ziel der ersten Studie (A) bestand darin, die Wirkung des nichtsteroidalen Antirheumatikums (NSAID) transdermales Flunixin Meglumin (Finadyne Transdermal) auf Kortisol im Plasma, die durchschnittliche tägliche Gewichtszunahme sowie Steh- und Liegeverhalten von Holstein-Milchkälbern zu bewerten, zum Zeitpunkt der Enthornung (Thermokauter) in Kombination mit lokaler Anästhesie. Vor und nach der Enthornung wurden jeweils sowohl Blutproben entnommen, um Plasma zu gewinnen, als auch das Körpergewicht gemessen. Steh- und Liegeverhalten wurden mithilfe eines 3D-Beschleunigungssensors gemessen.

Die Kälber wurden zufällig wie folgt aufgeteilt: 1) Kontrollgruppe (CON, n = 27), 2) 1-Flunixin-Gruppe (1-FLU, n = 26) mit einer einzigen Verabreichung von transdermalem Flunixin Meglumin beim Enthornen und 3) 2-Flunixin-Gruppe (2-FLU, n = 24) mit 2 Verabreichungen von transdermalem Flunixin Meglumin (beim Enthornen und zweite Gabe 6 Stunden nach Enthornung). Eine Schein-Enthornung wurde eine Woche vor dem eigentlichen Enthornen mit einem kalten Thermokauter durchgeführt.

Während der Schein-Enthornung betrugen die durchschnittliche Konzentration vom Kortisol im Plasma 6,09 ± 2,5 ng/mL. Die Konzentration von Kortisol im Plasma wurde durch die Zeit ($P < 0,01$) und in Tendenz durch die Behandlung ($P = 0,08$) beeinflusst. Genauer gesagt: im Vergleich zu CON zeigte die Einzelbehandlung (1-FLU) eine signifikante Wirkung ($P = 0,05$; -0,97 ng/mL; 95% CI: -1,99 bis -0,01), und eine zweite Behandlung (2-FLU) eine tendenzielle Wirkung ($P = 0,06$; -0,93 ng/mL; 95% CI: -1,88 bis 0,02).

Während der Enthornung betrug die durchschnittliche Konzentration vom Kortisol im Plasma 5,16 + 2,8 ng/mL (Spanne: 0,2–26,4 ng/mL). Die Konzentration vom Kortisol im Plasma wurde durch die Behandlung ($P < 0,01$) und die Zeit der Probenentnahme ($P < 0,01$) beeinflusst. Es wurde keine Wechselwirkung zwischen Behandlung und Zeit der Probenentnahme festgestellt ($P = 0,95$). Im Vergleich zu CON zeigte die Einzelbehandlung (1-FLU) eine signifikante Wirkung ($P < 0,01$; -1,29 ng/mL; 95% CI -2,15 bis -0,44), ebenso wie die zweite Behandlung (2-FLU; $P < 0,01$; -1,29 ng/mL; 95% CI: -2,14 bis -0,43). Die beiden Behandlungsgruppen unterschieden sich nicht signifikant voneinander ($P = 0,99$; 0,01 ng/mL; 95% CI: -0,84 bis 0,84).

Die Behandlung hatte einen Einfluss auf die Gesamtliegezeit sowie die Liegedauer pro Liegeperiode bei der Schein- Enthornung ($P = 0,01$) jedoch keinen Einfluss bei der Enthornung ($P = 0,31$).

Die Ergebnisse zeigten, dass transdermales Flunixin Meglumin in Kombination mit einem Lokalanästhetikum im Vergleich zu alleinigem Einsatz eines Lokalanästhetikums wirksamer war, um die Stressreaktion nach der Kauter-Enthornung bei Kälbern zu reduzieren. Dies führte zu geringerem Plasma-Kortisol, hatte jedoch keinen nachweisbaren Effekt auf das Liegeverhalten der Kälber.

Als Ergänzung zur ersten Studie wurde der Zeitpunkt der Enthornung genutzt, um Messungen von Kortisol in verschiedenen Medien (Plasma, Speichel, Kot) durchzuführen. Kortisol und Kortisol-Metabolite wurden miteinander verglichen, um die Nutzbarkeit verschiedener Medien für die Messung von Kortisol bei Milchkälbern zu bewerten. Speichel- und Kotproben wurden zu denselben Zeitpunkten wie Blutproben gesammelt. Unter Einbezug der Darmpassage begann die Kotprobenahme 48 Stunden vor der Schein-Enthornung und endete 8 Tage nach der Enthornung. Die wichtigsten Ergebnisse der zusätzlichen Studie waren: 1) Die Korrelation von Kortisol im Plasma- und Speichel bei Milchkälbern war gut ($r = 0,73$; $P < 0,01$), während die Korrelation von Kortisol im Plasma und FGM-Konzentration schwach war (6 Stunden: $r = 0,24$; $P < 0,01$; 12 Stunden: $r = 0,21$; $P = 0,02$). 2) Die Korrelation war höher, wenn die Plasmaprobe vor der Speichelprobe entnommen wurde.

Die Eignung von Speichelproben zur Abschätzung der Konzentration von Kortisol im Plasma nach Enthornung als akuten Stressor bei Milchkälbern wird durch die Ergebnisse dieser Studie belegt. Hingegen empfehlen wir die Entnahme von Kotproben nicht zur Bewertung einer akuten Stressreaktion. Für die Beurteilung länger anhaltender Stressoren könnten jedoch Kotproben geeignet sein, jedoch bedarf es weiterer Forschung. Obwohl die Entnahme von Speichel als minimalinvasiv betrachtet wird, sind weitere Untersuchungen erforderlich, um festzustellen, ob Kälber bei der Entnahme von Speichel im Vergleich zu Blut weniger Stress erfahren.

Die Ziele der zweiten Studie (B) war die Bewertung verschiedener analytischer Methoden zur Bestimmung der Qualität im Kolostrum, einschließlich einer laborbasierten Methode (ELISA) und vier Vor-Ort-Tests: digitales Brix-Refraktometer (% Brix), Kolostrometer (spezifisches Gewicht und mg/mL), ein Auslauftrichter (Sekunden) und ein Lateraler-Flow-Assay (mg/mL). Auf zwei kommerziellen Milchviehbetrieben in Deutschland wurden Kolostrumproben von 209 Holstein-Friesian-Kühen gesammelt. Unmittelbar bis etwa 3 Stunden nach dem Melken wurde die Beurteilung von Menge, Temperatur und Qualität vom Kolostrum im Melkstand durchgeführt.

Unter Verwendung einer Receiver Operating Characteristic (ROC)-Kurvenanalyse wurden für jeden Test die Testcharakteristika wie optimale Schwellenwerte, Sensitivität (Se), Spezifität (Sp) und die Fläche unter der Kurve (AUC) bestimmt. Die radiale Immunodiffusion (RID) wurde als Referenzmethode betrachtet. Das Kolostrum wurde als hochwertig definiert, wenn der IgG-Gehalt der RID Messung $\geq$50 mg IgG/mL betrug.

Nach der RID-Analyse wiesen 186 (89 %) Proben eine hohe Qualität ($\geq$50 mg IgG/mL) auf, während 23 Kolostrumproben (11 %) eine schlechte Qualität (<50 mg/mL) zeigten. Die durchschnittliche IgG-Konzentration (±SD) betrug 101,3 ± 45,9 mg/mL (Spanne: 6,0 bis 244,3 mg IgG/mL). Die durchschnittliche Temperatur des Kolostrums (±SD) betrug 27,3°C ± 9,4 (Spanne: 2,6 - 38,2°C). Durchschnittlich wog das Kolostrum (±SD) 6,4 kg ± 3,8 (Spanne: 0,7 - 25,3 kg). Die Pearson-Korrelationskoeffizienten zwischen RID waren: $r = 0,78$ (ELISA), $r = 0,79$ (digitale Brix-Refraktometrie), $r = 0,58$ (Kolostrometer: spezifisches Gewicht), $r = 0,61$ (Kolostrometer: temperaturkorrigiert), $r = 0,26$ (Auslauftrichter) und $r = 0,43$ (Lateral-Flow-Assay). Der optimale Schwellenwert zur Identifizierung von hochwertigem Kolostrum mit ELISA lag bei 50,8 mg/mL, mit einer Sensitivität von 91,3 %, Spezifität von 92,3 % und einer AUC von 0,94. Die Sensitivität der Tests vor Ort variierte von 95,7 % (Brix-Refraktometrie) bis 60,9 % (Lateral-Flow-Assay). Die Spezifität reichte von 88,6 % (Lateral-Flow-Assay) bis 75,9 % (Kolostrometer nach Temperaturkorrektur). Die AUC variierte von 0,93 (Brix-Refraktometrie) bis 0,73 (Auslauftrichter). Basierend auf der AUC können ELISA (0,94) und Brix-Refraktometrie (0,93) als sehr präzise angesehen werden.

Zusammenfassend ist der ELISA zur Bewertung der Qualität des Kolostrums genau. In Bezug auf die Tests vor Ort waren nur das digitale Brix-Refraktometer und das Kolostrometer geeignet, um die Qualität des Kolostrums zu bestimmen. Der Auslauftrichter und der Lateral-Flow-Assay können nicht als Tests für die vor Ort Bestimmung der Qualität des Kolostrums empfohlen werden.

Insgesamt zeigt diese Arbeit, dass 1) transdermales Flunixin Meglumin die Konzentration von Kortisol im Plasma nach der Enthornung senken kann, aber 2) keinen Einfluss auf die durchschnittliche tägliche Gewichtszunahme und die Gesamtliegezeit hat. 3)

Der ELISA ist genau und 4) das digitale Brix-Refraktometer und das Kolostrometer waren geeignet, um die Qualität des Kolostrums zu bewerten.

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Publications

Research articles

Röder, M., W. Heuwieser, S. Borchardt, J. L. Plenio, R. Palme, and F. Sutter. 2022. The effect of transdermal flunixin meglumine on blood cortisol levels in dairy calves after cautery disbudding with local anesthesia. Int. J. Dairy Sci. https://doi.org/10.3168/jds.2021-21257. © Elsevier Inc. (www.elsevier.com)

Röder, M., S. Borchardt, W. Heuwieser, E. Rauch, R. Sargent, and F. Sutter. 2023

Evaluation of an ELISA and four on-farm tests to estimate colostrum quality for dairy cows. Accepted in: Journal of Dairy Science, Mai 2023 © Elsevier Inc. (www.elsevier.com)

Oral presentation

Röder, M.; F. Sutter, S. Borchardt, E. Rauch, R. Sargent, C. Gosslin, W. Heuwieser

Evaluierung verschiedener analytischer Methoden zur Bestimmung der IgG-Konzentration von Kolostrum bei Milchkühen.

45. Internationale Leipziger Laborfortbildung - Tradition und Zukunft in der Veterinärmedizin

Leipzig: 8. Oktober 2021

Röder, M.; F. Sutter, S. Borchardt, E. Rauch, R. Sargent, C. Gosslin, W. Heuwieser

Evaluierung analytischer Methoden zur Bestimmung der IgG-Konzentration im Kolostrum bei Milchkühen

14. Mitteldeutscher Rinder-Workshop - Ansätze zur Verbesserung von Gesundheit, Fruchtbarkeit und Nutzungsdauer

Bernburg (Saale): 06.05.2022

Acknowledgements

Als ich nach dem Studium von der ausgeschriebenen Promotion an der Tierklinik für Fortpflanzung im Bereich der Neonatologie erfuhr, war ich direkt hellauf begeistert! An dieser Stelle möchte ich mich herzlich bei **Herrn Prof. Wolfgang Heuwieser** für die Möglichkeit bedanken, an der Tierklinik für Fortpflanzung promovieren zu können. Vielen Dank für die stets zügige Zuarbeit und das Vertrauen in mich und meine Arbeit.

Vielen Dank an **Herrn Prof. Dr. Rupert Palme** und sein gesamtes Team für die zügige Proben-Bearbeitung und Mitarbeit an den Papern. Danke auch für die vielen persönlichen Tipps zur Erkundung Wiens.

Mein größter Dank geht an meine ganz besondere Betreuerin und – noch viel wichtiger – Freundin **Dr. Franziska Sutter**. Ich bin dir unendlich dankbar, dass du mich mit so viel Durchhaltevermögen durch die Promotion begleitet, meine Marotten hingenommen und mich nie aufgegeben hast! Du kannst sehr stolz auf dich sein. Denn als Betreuerin und auch als Freundin hast du ganze Arbeit geleistet und mir oft genug den nötigen Motivationsschub gegeben! Danke für deine freundliche und hilfsbereite Art, dass du mich an deinem Wissen hast teilhaben lassen und vor allem, dass du dir immer Zeit für mich und meine Fragen genommen hast.

Ebenfalls möchte ich mich bei **Dr. Stefan Borchardt** bedanken, mit dem stets zu rechnen war. Danke, dass du mir bei den statistischen Berechnungen und noch viel mehr zur Seite gestanden hast!

Ein weiterer Dank gilt allen Mitarbeitenden der an der Studie teilgenommenen Milchviehbetriebe.

Ich danke allen **Mitarbeiter*innen und Doktorand*innen** der Tierklinik für Fortpflanzung, denn durch euch habe ich die fettesten Bauchmuskeln vom Lachen bekommen und bin ausnahmslos jeden Tag gerne zur Arbeit gekommen! Besonders hervorheben möchte ich **Sophia und Ole**. Danke, dass ihr so bärenstark an meiner Seite gestanden seid!

Meiner **Familie und meinen Freunden** danke ich für offene Ohren, Tränen-trocknende Taschentücher, Arschtritte, finanzielle Unterstützung, Ablenkung und die stetige Nachfrage, wie es mit meiner Promotion so vorangeht. Ohne euch, wäre ich vermutlich nie fertig geworden!

Conflict of interest

Im Rahmen dieser Arbeit bestehen keine Interessenskonflikte durch Zuwendungen Dritter.

In the context of this work, there are no conflicts of interest due to donations from third parties.

Funding Sources

Die Studie wurde finanziell durch Tiergyn e.V. Berlin unterstützt.

Es besteht kein Interessenkonflikt durch die finanzielle Unterstützung der Studie.

The study was financially supported by Tiergyn e.V. Berlin.

There is no conflict of interest due to the financial support of the study.

Declaration of Independence

Hiermit erkläre ich, Mareike Röder, dass ich alle Studien selbstständig durchgeführt und die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil1 an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1	Studie 2	Studie 3
Studienplanung	++	++	++
Datenerhebung	+++	+++	+++
Datenanalyse	++	+++	++
Verfassen des Manuskripts	++	+++	+++
Editieren des Manuskripts	++	++	++

Legende: +++: > 70%, ++: 50-70%, +: < 50%

[1]The effect of transdermal flunixin meglumine on blood cortisol levels in dairy calves after cautery disbudding with local anesthesia.

[2]Evaluation of an ELISA and four on-farm tests for the determination of IgG concentration in colostrum of dairy cows

[3]Saliva cortisol and fecal glucocorticoid metabolites suitable for acute stress assessment in preweaned dairy calves?

Berlin, 20.02.2024 Mareike Röder