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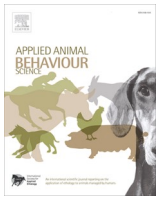

Behaviour, heart rate variability and surface temperature of calves after hot-iron disbudding or injection of clove oil or isoeugenol under the horn buds

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ABSTRACT

In recent years, injection of clove oil was investigated as potential alternative to the use of a hot iron for disbudding due to its cytotoxic and anaesthetic properties. Isoeugenol, the isomer of the main component of clove oil, is available as a pure substance and has some advantages as compared to clove oil, but its effects were studied rarely. In this paper we investigated behaviour, heart rate, heart rate variability and surface temperature around the horn bud of 40 calves in four treatments (N = 10/treatment): injection under each horn bud of 1.5 ml clove oil (CLOV), isoeugenol (ISO) or saline (CON), or hot-iron disbudding (BURN) with local anaesthesia and sedation. Behaviour, heart rate (HR) and heart rate variability (HRV) as well as surface temperature around the horn buds were measured before and at different time points after the treatment up to 7 days post treatment; behaviour was also observed during the treatment. Behavioural observations were performed real-time or per video recording. HR and HRV were analysed during undisturbed lying periods and post-treatment changes relative to baseline were calculated. The maximum surface temperature in the region around each of the two horn buds was used for further analysis. LMM, ANOVA or, for behaviour during treatment, non-parametric tests were used for statistical analysis. There was a treatment*time point interaction (LMM, $p<0.05$) for behaviours associated with pain (ear flicking, head shaking, head scratching) as well as for self-grooming. HR or HRV changes from baseline did not differ between treatments, but sample size was strongly reduced due to lack of data. The development of the maximum surface temperature around the horn buds differed clearly between treatments. Our findings suggest that the injection of clove oil and especially isoeugenol causes less pain compared to the use of a hot iron without analgesia, but more pain compared to the injection of saline solution.

1. Introduction

In Europe and the USA most dairy calves are disbudded or dehorned (Europe: 81% (Cozzi et al., 2015), USA: 94.3% (United States Department of Agriculture, 2018). While pain can be alleviated during disbudding by local anaesthesia (Graf and Senn, 1999; Stilwell et al., 2009, 2012) and post-operative by the use of anti-inflammatory drugs (Faulkner and Weary, 2000; Stafford and Mellor, 2005; 2011; Stilwell et al., 2012; for a review see: Winder et al., 2018), recent studies indicate that the massive tissue damage and associated physiological responses can lead to longer-term impairment of the animal's welfare (Adcock and Tucker, 2018; Casoni et al., 2019). Further, despite severe pain during disbudding without anaesthesia was confirmed already 25 years ago (Graf and Senn, 1999; Grøndahl-Nielsen et al., 1999) this practice is still allowed and performed in many countries including many European ones. In addition, frontal bone destruction or even brain damage can be found after thermal disbudding (Nation and Calder, 1985; Schoiswohl et al., 2022). Therefore, potential alternative methods that may exert

Abbreviations: BURN, hot-iron disbudding; CLOV, clove oil injection; CON, saline injection; HF, high frequency; HR, heart rate; HRV, heart rate variability; IRT, infrared thermography; ISO, isoeugenol; LF, low frequency; RMSSD, root mean square of successive differences; VLF, very low frequency.

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less negative impact on animal welfare compared to hot-iron disbudding are investigated in recent years. The injection of clove oil, a substance with anaesthetic and cytotoxic effects (Chaieb et al., 2007; Taher et al., 2015) under the horn bud caused less pain related behaviours as compared to hot-iron disbudding in the first hours after the procedure in calves (Sutherland et al., 2018) but a similar level in goats (Hempstead et al., 2018a). However, the nociceptive threshold, a measure of pain sensitivity, did hardly differ between the two methods after 48 h (Sutherland et al., 2018) or over the course of 3 weeks post-procedure (Juffinger et al., 2021). Similarly in goats, Hempstead et al. (2018b) conclude that clove oil induced a similar pain response than hot iron disbudding, based on several physiological indicators. Moreover clove oil injection caused severe swellings (in calves: Juffinger et al., 2021; in goats: Still Brooks et al., 2021) and, in one calf, some frontal bone damage (Schoiswohl et al., 2022) or, in a goat kid, osteomyelitis (Schoiswohl et al., 2021). In addition, effectiveness to stop horn growth was limited for clove oil (in calves: Sutherland et al., 2019; Juffinger et al., 2021, in goats: Hempstead et al., 2018c, Schoiswohl et al., 2021). However, injection of isoeugenol, a pure substance that therefore may be advantageous to clove oil (Frahm et al., 2020) increased pain sensitivity to a lower and shorter extent compared to hot-iron disbudding and to clove oil, and only few and mostly low-grade swellings occurred (Juffinger et al., 2021). Evaluation of further behavioural and physiological indicators of animal welfare could give a clearer picture of the potential of an injection of isoeugenol as alternative to hot-iron disbudding.

Ear flicking, head shaking, head scratching, tail flicking and the transitions between standing up and lying down and vice versa are behavioural indicators of pain due to hot-iron disbudding (Grøndahl--Nielsen et al., 1999; Faulkner and Weary, 2000; Sylvester et al., 2004; Stafford and Mellor, 2005). Head shakes and head rubbing differed also when comparing hot-iron disbudding and injection of clove oil (Sutherland et al., 2018); but in this study the behaviour was observed only within the first 48 h. Calves injected with clove oil showed less head shaking (within the first 2 h after the treatment) and more head rubbing (within the first day after the treatment) compared to calves disbudded with a hot iron (Sutherland et al., 2018). However, ear flicking, head shaking and tail flicking are common fly-defence behaviours (e.g. Mooring et al., 2007). Thus, the presence of flies can elicit and increase these behaviours unrelated to or in interaction with the treatment. Also, the ambient temperature, calf`s sex or age can affect pain related and other behaviours (Stilwell et al., 2009, Mang, 2012, Ugwu et al., 2021, Martin et al., 2022, Riley et al., 2023).

The use of a hot iron also leads to changes in heart rate (HR) and heart rate variability (HRV) in calves (Stewart et al., 2008, 2009). The HR increases in reaction to disbudding and stays elevated within the first hours (Grøndahl-Nielsen et al., 1999; Stewart et al., 2009); additionally a decrease in RMSSD and HFpower as well as an increase in LF/HF ration occurred (Stewart et al., 2009). To our knowledge there are no studies examining the effects of an injection with clove oil or isoeugenol on HR and HRV in calves.

Changes in surface temperature, which can be assessed by using infrared thermography (IRT), are a good indicator for illness and inflammation in humans (Lahiri et al., 2012) and animals (Rekant et al., 2015). In a recent study the surface temperature around the horn buds increased within 2 h after disbudding of calves, returning to baseline values within 8 h (Scherf et al., 2020), probably reflecting a stress response within the first hours after disbudding (e.g. Scherf et al., 2020).

In this study, we compared calves that were either disbudded with a hot iron or had received an injection of clove oil or of isoeugenol or, as control, of saline solution under the horn buds. We investigated the behaviour, HRV and surface temperature around the horn buds during seven days after the procedure. In the same animals we had also assessed further indicators of welfare that have been published already: mechanical nociceptive threshold, tissue damage and horn growth (Juffinger et al., 2021) as well as salivary cortisol and, from a subsample of the animals, histology of horn bud biopsy and computer tomographic images (Schoiswohl et al., 2022). We hypothesized that calves injected with clove oil and especially isoeugenol will show less behaviours associated with pain (ear flicking, head shaking, head scratching, tail flicking, transitions lying/standing) compared to calves disbudded with a hot iron, but show more such behaviour compared to the control injection. For play behaviour and self-grooming we expected the opposite direction, that is clove oil and especially isoeugenol showing more of these behaviours as compared to hot iron disbudded animals but lower levels as compared to control animals. Regarding HRV we expected it will be the highest in control calves, followed by calves injected with isoeugenol, then clove oil, and will be the lowest in hot-iron disbudded calves. We expected the surface temperature around the horn bud to be the highest in hot iron calves, due to stronger inflammation, followed by clove oil and isoeugenol and will stay unchanged in control calves.

2. Material and methods

Between August 2018 and June 2019 the study was carried out at the dairy farm of the University of Veterinary Medicine, Vienna (VetFarm) at Kremesberg in Pottenstein, Austria. All procedures were discussed and approved by the ethics and animal welfare committee of the University of Veterinary Medicine, Vienna, and by the Advisory Committee for Animal Experiments of the Federal Ministry of Science, Research and Economics (BMWFW-68.205/0049-WF/V/3b/16, date of approval: 31.03.2016) and conducted in accordance with GSP guidelines and national legislation.

2.1. Animals and housing

Forty calves, 38 pure breed Simmental (13 female and 27 male) and two crossbreed Simmental x Brown Swiss (one female and one male), that were born at the dairy facility (where about 80 Simmental dairy cows were kept) between August 2018 and April 2019, were included in this study. As a standard procedure all calves were separated from the dam within the first hours of life, bottle-fed with colostrum (at least 2 L within 6 h) and brought to outdoor single-calf hutches, as soon as their coat was dry. In addition, all calves were weighed, ear-tagged and received an injection with vitamins within the first day of life. For the first 10–14 days the calves were housed outdoors in single straw-bedded calf hutches under a roof for weather protection. The individual pens consisted of a hutch (length 147 cm x width 109 cm x height 117 cm) and an adjoined fenced area (1.68 m^2), where a water bucket and a hay rack were attached for ad libitum access. The hutches were located next to each other allowing visual, auditory and limited tactile contact with conspecifics. Calves were fed with their mothers' colostrum (2 L per meal) three times daily (around 08:00 h, 13:00 h and 18:00 h) for the first 5 days of life and afterwards twice daily (around 08:00 h and 18:00 h) with whole milk (3 L). Calves stayed in the hutches until an age of 10–14 days, when they were moved to group housing.

2.2. Study design, procedure and general data collection

At the age of 1–5 days the 40 calves were randomly allocated to one of four treatments (10 calves per treatment). Treatments were either the injection of 1.5 ml saline (Braun 0.9% - Infusionslösung, B. Braun Austria GmbH, control, **CON**), of 1.5 ml clove oil (*Syzygium aromaticum*; 80.18% eugenol, Herba Chemosan Apotheker-AG, Austria; **CLOV**) or of 1.5 ml isoeugenol (99% isoeugenol, Merck KGaA, Germany; **ISO**) under each horn bud, or hot-iron disbudding with previous sedation (0.1 mg/kg Xylazin i.m., Sedaxylan 20 mg/ml, Eurovet Animal Health B.V., AE Bladel, Netherlands) and local anaesthesia (5 ml Procainhydrochlorid s. c. each side; Procamidor 20 mg/ml, Richter Pharma AG, Wels, Austria; **BURN**). After hot-iron disbudding the cautery wounds were additionally treated with antiseptic spray ('Cyclo'-spray containing Chlorine tetracyclin hydrochloride, Eurovet Animal Health BV, Bladel, Netherlands).

A 16 G needle (BOVIVET 16 G x 1–1/2" 1.6 ×38 mm, Jørgen KRUUSE A/S, Denmark) was used for the injections of clove oil, isoeugenol or saline; it was inserted under the horn bud from rostro-medial of the bud in the direction of the base of the ear (for figure see: Juffinger et al., 2021). The treatment and all measurements were conducted while calves were in the outdoor calf-hutches. In most cases, one calf was treated per day; for 10 calves, two calves were treated at the same day; this depended on the date of birth. The area around the horn buds was clipped before the first data recording. The treatment was performed by AS or JS around 10:00 h in the morning, thus at least 2 h after the morning feeding. One or two additional persons (staff of the dairy facility) were necessary to restrain the calf during the injection. To allow an injection as precise as possible the head of the calf was pressed to the ground. Due to sedation calves in the BURN group were not restrained. The different time points of the observations and measurements are shown in Fig. 1 and explained in detail below.

All real-time behavioural observations and measurements reported in this paper were performed by one trained person (AJ). The behavioural observations using video recordings (i.e. short-period video observations at day 0 and 1 and 24 h video observations at day 1, 5 and 7) were performed by two trained observers. All persons were blind to the treatment, except for the BURN treatment during real-time observations and IRT-measurement where blinding was not possible.

2.3. Behavioural observations

Behaviour of calves was observed at different time points either real-time on farm or by using video recordings. The videos were recorded with infrared-sensitive cameras (Sanyo, 1/3", VCC-HD2300P, Full HD 1920×1080, 2.8–8 mm Optic, Sanyo Electric Co., Ltd. Osaka, Japan) and infrared spotlights (IR-LED294S-90, 880 nm, 90°, Microlight, Bad Nauheim, Germany) allowing night recordings, using the software GeoVision (Surveillance System V8.5.6, GeoVision Inc., Taipei, Taiwan). One camera captured two calf hutches at the same time. Another camera recorded the surrounding. Except for the observations during the treatment, all observations have been coded using the Behavioural Observation Research Interactive Software (BORIS, Version 7.4.7 or higher, Friard and Gamba 2016).

2.3.1. During the treatment

During injection or hot-iron disbudding, respectively, the observer was sitting outside the fenced area of the calf hutch. Every event of the following behaviours was noted down in a data sheet by hand: leg movement (movement of a leg), vocalisation (any type of vocalisation), tail flicking (rapid movement of the tail) and teeth grinding (pressing teeth on each other producing a creaky sound). If the calf showed strong movements as if trying to escape (e.g. moving several legs at once, moving the torso) accompanied by vocalisations, this was noted down as severe reaction present (yes/no). The observation for each calf started when the needle was inserted into the skin or the hot iron was touching the skin and ended when the needle or the hot iron were removed.

2.3.2. Real-time observation before and after the treatment

During the observations the observer was sitting as still as possible on an elevated chair in a distance of 1 m to the fence. She recorded the behaviours shown in the Supplementary Material Table S1 with a Netbook (Lenovo MIIX 320–10 ICR Type 80XF, Lenovo China). Behaviour was observed continuously for 20 min per time point, once before the treatment, and a total of 10 time points after the treatment, seven on day 0 (1, 2, 3, 4, 6 and 9 h after the treatment), and one in the morning of day 1, 3 and 7 each (Fig. 1). The observation started soonest 5 min after feeding or the last manipulation of the calf. Sometimes parts of the calfs body were not visible due to the calf hutch, therefore the level of visibility was recorded as well (see Supplementary Material Table S1 under "partly concealed") to be able to later correct for the actual duration of observation for specific behaviours. Seldom (five times), it happened that observations had to be stopped earlier, due to disturbances that interfered with the calfs behaviour or technical problems. In these cases and if visibility was shorter than 20 min, the behaviours were corrected to the set time of observation (20 min). That is, in case of the real observation time being shorter than 20 min, the observed frequencies or duration were multiplied with the ratio of set time to actual observed time (in total or for specific body parts and thus behaviours) to reflect a theoretical observation time of 20 min.

2.3.3. Short-period video observations

In addition, behaviour of calves was observed from video recordings at four time points (Fig. 1) for 20 min each, two observations on the day

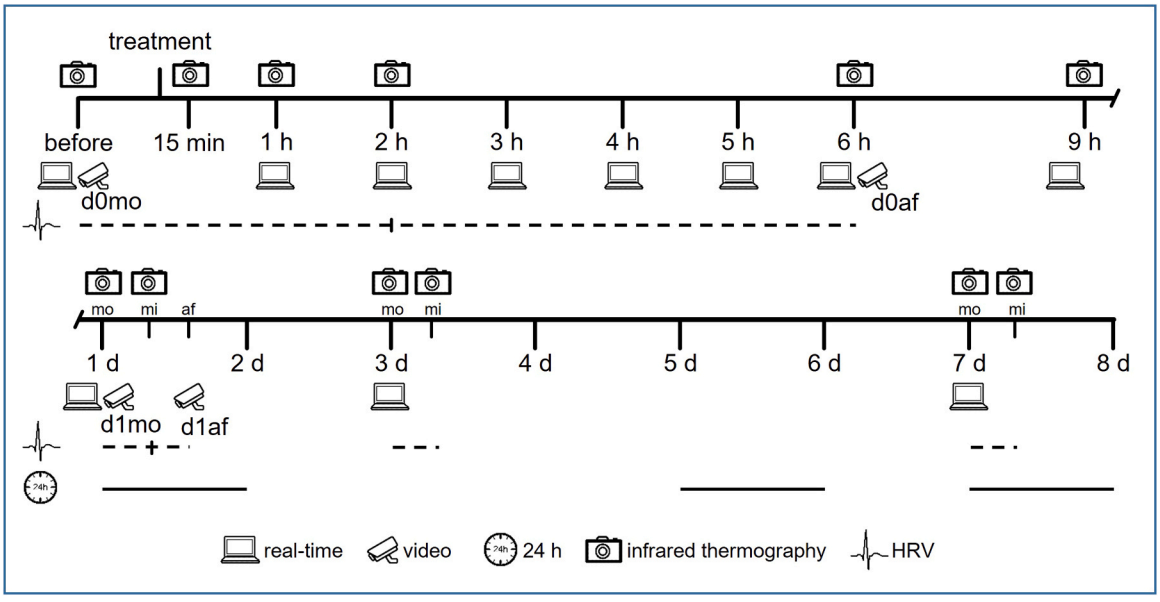

Fig. 1. Time points of the different observations and measurements reported in this paper. The top line represents all measurements on the day of the treatment (day 0). The bottom line depicts day 1 to day 8 after the treatment. Measurements/observations on day 1–7 were performed in the morning (mo), midday (mi) or afternoon (af); corresponding to the time points before, 2 h and 6 h of day 0. Images for the different measurements are aligned under the corresponding time point, except for the image of the infrared thermography, which was positioned above the corresponding time point.

of the treatment (day 0, one observation before the treatment, day 0 morning, and one observation in the afternoon, day 0 afternoon,) and two the day after (day 1, one in the morning, day 1 morning and one in the afternoon, day 1 afternoon). We had chosen time points where we expected the calf to be not or least affected by experimental manipulations or the presence of the experimenter herself. Further, time points of the two days should be comparable regarding time of the day and time relative to feeding. The morning observations started 5 min after the morning feeding on day 0 and day 1; the afternoon observations on day 0 started 5 min after the last manipulation for data recording of time point 6 h after the treatment and before the evening feeding; the afternoon observations on day 1 started at the same time of the day as on day 0. Behaviours were recorded continuously according to the ethogram in Table 1. To control for a possible effect of flies, we checked the white calf hutch for black moving spots, which we assume to be flies and coded it as 'flies present or not'. SB performed all behavioural coding. Some of the calves were not visible for a special behaviour during a large part of the observation time. Therefore, only observations where the calf or relevant calf body part was visible for at least half of the time, i.e. 10 min, were used for further analysis, reducing sample size as described in statitical analysis (see 2.6.). For the rest of the calves the behaviours were all calculated for a standardized observation time of 10 min (duration of frequency / real observation time in s * 600 s).

2.3.4. 24 h observations

For duration of lying and the frequency of transitions from standing to lying and vice versa, we analysed 24 h recordings of three different days (day 1, 5 and 7) (Fig. 1). While on day 1 and 7 some other data recording took place in the morning, there was no manipulation of the calves on day 5. At day 7 we additionally observed the duration of play behaviour (bucking, jumping, gallop, leap, turning as well as rubbing, pushing and butting of the head), because play occurs only rarely at various peaks throughout the day (Größbacher et al., 2020), 24 h observations are best suitable for reliable evaluating this behaviour. More details of the ethogram used and how potential disturbances were considered are described in Table 1. Transitions from standing to lying and vice versa where only considered if the calf was not standing up in response to a disturbance.

Table 1
Definitions of calves' behaviours, visibility and potentially disturbing events recorded during the short-term video observation (V) and the 24 h video observation (24 h). For head shaking, head scratching and tail flicking only frequency was recorded, for all other behaviour duration and frequency was recorded, but only durations were analysed.

Behaviour/ Event	definition	obs
Ear flicking	Rapid movement of one or both of the ears independent of the head movement. A separate ear flicking event is considered to occur after a clear stop of the ear-movement beforehand	V
Head shaking	Rapid movements of the head around the rostro-caudal axis. Headshakes separated by a clear stop are considered separate events. Headshakes during locomotor play are not coded as headshaking (see locomotor play)	V
Head scratching	Scratching any part of the head (excl. neck) with the rear limb. Including attempts (limb does not contact the head, but is lifted from ground and moved towards the head, at least tiptoe is in front of the caudal edge of the calfs shoulder blade). Event stops when limb touches the ground again	V
tail flicking	Rapid movement of the tail from one side to the other across the calfs rear limbs (not counted during play), one event consists of the tail moving from one side (e.g. left) to the other side (e.g. right) and back again. If the calf is lying the movement from the ground to one side and back again to the ground is counted as one event.	V
inert lying	Lying with muzzle on flank (modified after Stilwell et al. 2008)	V
locomotion	Movement of the calfs body by foot (at least one limb is in the air), including walking and running (modified by Chua et al. 2002). The calf takes at least two steps. Without behaviours described as locomotor play (see locomotor play)	V
Standing	Body position in which the limbs bear the bodyweight and the animal is stationary (for 24 h observation including locomotion without play)	V 24 h
Lying	Sternal or lateral recumbency: sternum or flank are in contact with the ground and no weight is supported by any of the limbs (including standing up for 24 h observation)	V 24 h
locomotor play	Any movement apart from basic activities, including gallop (fast four-beat gait), leap (front limbs are lifted from the ground and moved upwards and forwards, during last phase rear limbs may be lifted from the ground), jumping (similar to leap, but only a movement upwards and not forwards), bucking (body ascends sharply from front to back, maybe including a kick), turning (calf turns around while leaping) and/or a combination of some or all of these behaviours. May also include movements similar to head-shaking. For a detailed description see Jensen et al. (1998).	V 24 h
head play	Rubbing, pushing and butting the head back and forth against an object in a playful manner and during standing (including bars, hutch, floor/straw), modified after Jensen et al. (1998)	V 24 h
self grooming	Licking, scratching or rubbing its own coat with its mouth or limb but not on the head (including attempts). A separate self-grooming event is considered to occur after a clear stop. Even short contacts of the calves muzzle with its coat are sufficient and will be coded.	V
body shaking	Calf shakes his whole body from head to tail, rotating around the rostro-caudal axis, behaviour is only recorded if occurring during standing	V
exploration	Calf has muzzle (licking or nibbling) or almost muzzle contact (within 10 cm from feature, sniffing) with environmental features, except (including) feed, during standing or walking through the box or lying. The muzzle has to be at least partly visible.	V
Visibility		
Inside	At least the calfs front 1/3 (from the calfs nose to the caudal edge of its shoulder blade) or the whole body is inside of the hutch	V
both ears not visible	The calfs ears are not visible, therefore behaviours (e.g. head shaking, head rubbing, head play, ear flicking) including these parts of the calfs body cant be seen and therefore are not recorded (at least the eartags of both ears have to be visible to not code this behaviour)	V
one ear not visible	At least one ear of the calf is completely invisible (either the complete left or the complete right ear), behaviours that cant be seen clearly are not recorded	V
tail not visible	The calfs tail is not visible, thus behaviours including these parts of the calfs body cant be seen and therefore are not recorded	V
calf not visible	It is not possible to differentiate if the calf is standing or lying. If the behaviour is caused by manipulation, only manipulation is coded.	24 h
Potentially disturbing events		
humans visible	One or more humans stand or walk by close to the calf and may affect the calfs behaviour. It lasts as long as a human is visible in the camera for observing the surrounding. The experimenter is counted as a human for the short-period observation (except when she is sitting still or is located with at least one calf hutch between her and the observed calf), but not for the 24 h observation	V 24 h
vehicle visible	A vehicle passes near the calfs hutch and may affect the calfs behaviour. The behaviour starts as soon as the vehicle is visible on the video and ends when its not visible anymore.	V 24 h
experimenter visible	The experimenter is visible in the surrounding camera. It starts when experimenter is visible in the surrounding camera and last until experimenter leaves the area next to the calf hutches (not visible in the surrounding camera anymore).	24 h
manipulation	Calf is handled by a person. The behaviour starts when the door of the calf hutch is opened and ends when the person leaves the calf hutch (the door has been closed). If there are other incidents in addition to manipulation (e.g. disturbances, calf not visible) only manipulation is coded.	24 h
Feeding	The calf is being fed. The behaviour starts when the milk bucket is attached to the fence and ends when the milk bucket is removed. If the calf needs assistance during feeding, this is also counted as feeding.	24 h

Intra- and inter-observer reliability was tested using videos not used for further analysis by calculating Cohen's Kappa in the observational software BORIS. All behaviours and visibilities showed good reliability (Cohens's Kappa 0.75 – 0.99).

2.4. Heart rate variability

For measuring heart rate variability (HRV) we used human heart rate transmitters (T31-CODED) and monitors (S810, Polar Electro Oy, Kempele, Finland), that continuously store inter-beat intervals for about 4 h. To ensure a good transmission ample ultrasound gel was applied to the calfs coat, at the area where the transmitter was placed (left body side, behind the caudal margin of the scapula). In addition, an extra elastic girth was applied, on the one hand to keep the transmitter in place and on the other hand to store the monitor in an attached pocket. The HRV-data were downloaded from the monitor onto a computer and further analysed using the software Polar Precision Performance SW (Version 4.03.050, Polar Electro Oy, Kempele, Finland). HRV was recorded for a total of 8 h on day 0, and for 4 h on day 3 and 7, always starting at 8:00 h (Fig. 1). However for further analysis only the first 2 h were used per day, comprising the time before the treatment on day 0 as baseline and comparable time of the day at day 5 and 7. To select HRV-data where the calf was lying undisturbed (Hagen et al., 2005, von Borell et al., 2007), video recordings were analysed by coding for lying and standing as well as for potential disturbances (passing vehicles, feeding, passing or interacting humans), using the software Solomon Coder (Version beta 17.03.22, by András Péter, ELTE TTK, Budapest, Hungary). The HRV data of "undisturbed lying bouts", i.e. calves were already lying for at least 3 min and no disturbance had occurred within 1 min, were checked for the error rate per 1 min as decribed in Hagen et al. (2005) using the Polar softwareś standard settings and by checking the software's correction for plausibility visually. Minutes with an error rate higher than 5% were removed from further analysis. Initially we intended to use only sampling periods with a minimum length of 8 min, but finally had to include also sampling periods of shorter duration (minimum 5 min) to increase the data set. If usable sampling periods were longer than 20 min they were cut in shorter ones. To calculate HRV parameters of the selected sampling periods the software Kubios HRV (Version 2.1, Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland) was used. Settings for the frequency bands for VLF (0.0033 – 0.04 Hz), LF (> 0.04 – 0.20 Hz) and HF (> 0.20 – 0.58 Hz) were used according to the setting described for calves by von Borell et al. (2007).

2.5. Infrared thermography

A FLIR T650SC infrared thermography camera (FLIR Systems, Inc., Wilsonville, Oregon, United States of America) was used for the measurement of the surface temperature around the horn buds of each calf at different time points (Fig. 1). The camera settings for the distance (1 m) and emission ratio (0.95) were always kept the same, while the temperature of the surrounding and the humidity were adjusted for each picture, by using the values of a temperature/humidity logger (ECOLOG TH1, ELPRO-BUCHS AG, Buchs SG, Switzerland). The logger was placed near the calf hutches and stayed there throughout the whole experiment, except for when a calf was lying in the calf hutch; in this case the logger was carefully placed within the hutch. Images were always taken directly after the real-time observations. If a calf was too restless to get a proper image, the calf was allowed to suck on the experimenters' finger to calm down, but avoiding to touch any other part of the calf's body.

Using the software FLIR ResearchIR Max 4 (Version 4, 64 bit, FLIR Systems, Inc., Wilsonville, Oregon, United States of America), we selected the image with the best focus for each horn bud per time point. Additionally, we checked the settings and adjusted them if necessary. The maximum temperature of the area of the horn bud was analysed and used for further analysis.

2.6. Statistical analysis

Sample size of the study was calculated based on expected differences in salivary cortisol (Schoiswohl et al., 2022). Not all potentially confounding variables could be controlled for completely due to our long study period from summer to spring and the dependence on calvings and therefore they were included in statistical models where relevant (see below). Statistical analyses of behaviour and surface temperature were performed using SPSS (Version 25.0, IBM Corp., Armonk, New York, United States of America). For behaviour during the treatment we used a Fisher-Freeman-Holton exact test for the dichotomous variable severe reactions, an ANOVA for leg movement and a Kruskal-Wallis-Test (KW) for the other behaviours for comparison of three or four treatments, depending on data distribution, and Mann-Whitney-U-Test (MWU) for post-hoc pairwise comparison. Play behaviour during the short-period video observations occurred rarely. Therefore, play events were aggregated for all time points after the treatment and an ANOVA calculated. For play behaviour at day 7 during 24 h observations an ANOVA was used as well. For all other behaviours and the surface temperature linear mixed models (LMM) were used for analysis. For the LMM models with behaviours as dependent variables, we included the fixed effects treatment, time point, sex, their three-way interaction and all lower-order interactions as well as age at treatment, ambient temperature and presence of flies (only for video observation data) and, as random effect, the animal. For the model with surface temperature as dependent variable treatment, time point and their interaction, ambient temperature, head side and location of the calf (in the hutch or outdoor run) was included as fixed effects and animal as random effect.

Model assumptions (normal distribution and homogeneity of variance of residuals) were checked graphically. Models for behavioural and surface temperature data were reduced by eliminating non-significant ($p > 0.05$) factors to the model with the best (lowest) Akaike information criterion (AIC), starting with the three-way interactions, followed by the two-way interactions and finally, the main effects, except for the variables of main interest: treatment, time point and their interaction. Due to this model selection exact p-values in the behaviour models should be treated with caution; however all our main variables of interest did not change the level of significance throughout the model selection process. In addition, to correct for multiple testing of an effect of treatment on behaviour after the treatment and on surface temperature, a Benjamini-Hochberg False Discovery Rate (FDR) calculation was performed (with n=15 hypotheses, 6 behaviours of the short-period observation, 3 behaviours of the 24 h observation, 5 behaviours of the real-time observation and surface temperature) and thus to control the proportion of falsely rejected null hypothesis. Some dependent variables needed to be transformed to fulfil model assumptions; transformations are indicated in the results section and Tables. Fisherś Least Significant Difference (LSD) was used for post-hoc tests of LMM of behavioural data and Sidak for post-hoc comparisons of surface temperature data.

HRV-data were analysed in R (Version 3.6.1. or 3.6.3., R Core Team 2019). HRV data were analysed using an ANOVA based on the differences to the baseline, separately for day 1 and 7, because insufficient data for all days were available. Nevertheless, sample size was reduced to six calves each in BURN, CLOV and ISO and to four calves in CON, thus strongly limiting power. We included treatment, sex, age at treatment and body weight at day 7 as fixed effects in our ANOVA. The ANOVA was based on original data (adjusted for temperature) as well as on rank transformed data. The rank transformation was done to check if outliers affect the results. Additionally, we applied a permutation test with only treatment as an explanatory variable, to specifically check for a treatment effect.

For all statistical tests, an alpha level of 0.05 was set for significance, p-values of $0.05 < p \leq 0.1$ are reported as trend or tendency; after FDR correction the adjusted p-value was 0.017 for significance and 0.053 for a trend (see Supplementary Material Table S4 for calculation of FDR

correction and adjusted p-values). Sample sizes were reduced not only for HRV but also for behavioural observations due to technical problems with videos (loss of videos due to power blackout) or exclusion of animals due to too long periods of invisibility (see 2.3.3). Therefore, sample size for short-period video-analysis was as follows, order of time points: for ear flicking 7, 7, 7, 9 CON, 5, 6, 7, 8 BURN, 7, 6, 5, 8 CLOV and 9, 6, 9, 9 ISO; for head shaking, head scratching, self grooming: 7, 8, 8, 9 CON, 7, 6, 7, 9 BURN, 8, 7, 6, 8 CLOV, 9, 7, 9, 9 ISO and for tail flicking 7, 6, 6, 10 CON, 7, 6, 6, 8 BURN, 8, 8, 6, 6 CLOV and 9, 8, 7, 6 ISO. For 24 h observations 6 BURN calves and 7 calves each in CON, CLOV and ISO could be analysed. We will not report all details of results regarding confounding factors in the text but concentrate mainly on our variables of interest.

3. Results

3.1. Behavioural observations

3.1.1. During the treatment

The duration of the procedure (injection or disbudding) differed between the treatments (KW: $p = 0.022$), with CON lasting the shortest (median: 42.0; range: 29 – 103 s) followed by CLOV (52.5; 27 – 113 s) and ISO (60.0; 50 – 163 s); BURN lasted the longest (103.0; 35 – 285 s), differing significantly from CON and CLOV (MWU: each $p \leq 0.05$).

There was a significant effect of treatment on the number of movements (ANOVA: $p = 0.008$), and by trend on tail flicking (KW: $p = 0.059$) and severe reactions (Fisher Exact Test: $p = 0.082$) but none on vocalisations (KW: $p = 0.257$). However, the three injection treatments did not differ in any of the behaviours observed (all MWU or T-Test or Fisher Exact Test: $p > 0.1$), but BURN, being sedated, showed less of these behaviours compared to the other treatments (MWU: $p < 0.05$, Fig. 2; only one BURN calf showed severe reactions, standardized residual −1.6 indicated difference to all other treatments).

3.1.2. Real-time observation before and after the treatment

For the **frequency of ear flicking** a main effect of treatment ($p < 0.001$), time point ($p < 0.001$), temperature ($p = 0.046$), as well as an effect of the interactions treatment*time point ($p < 0.001$) and sex*age ($p = 0.033$) occurred (details see Table 2). In BURN the frequency of ear flicking increased after the treatment, with highest values at 2 h post treatment (while there was no change over time points in CON, and no statistically confirmed difference to baseline in CLOV and ISO; Table 2, Fig. 3). Accordingly, BURN calves showed more ear flicking than all other treatments at the time points 1 h, 2 h, 3 h and 6 h ($p \leq 0.001$ to $p = 0.045$, Table 2), CLOV and ISO did not differ from each other and showed either less ear flicking, by trend, compared to CON at the time point 3 d (CLOV, $p = 0.063$) or more ear flicking at 7 d (ISO, $p = 0.026$, Table 2, Fig. 3).

For no other behaviour of the real-time observation an effect of treatment was confirmed (i.e. no effect of the interaction treatment*time point); for details on model results for these behaviours see Supplementary Material Table S2.

3.1.3. Short-period video observations

The detailed results including estimated means and effects of confounding variables are depicted in Table 2. Animals differed between treatments depending on time point (interaction treatment*time point) with respect to duration of ear flicking ($p=0.009$), head shaking ($p=0.013$), head scratching ($p=0.023$), self grooming ($p=0.016$) and, over all time points after the procedure, play behaviour (treatment, $p=0.003$), but not in tail flicking ($p=0.173$). While in BURN calves the **duration of ear flicking** was longest in the afternoon of day 0 (d0af), there was a clear decrease in CLOV for two time points after the treatment (d0af, d1af), and it stayed the same for ISO, while in CON there was a lower duration in the morning of day 1 (d1mo; Table 2). Accordingly, BURN calves showed longer ear flicking in one or two of the time points following the treatment (d0af, d1mo) compared to CLOV or CON, but did not differ to ISO (Table 2, Fig. 4). CLOV showed lower ear flicking duration compared to ISO in the afternoon of day0 (d0af) and of day 1 (d1af) after the treatment (Fig. 4). CON showed lower ear flicking duration as compared to the other three treatments in the morning of day 1 (d1mo). **Frequency of head shaking** increased in BURN calves for two time points after the treatment, while in CON and ISO they were lower after 24 h and did not change in CLOV.

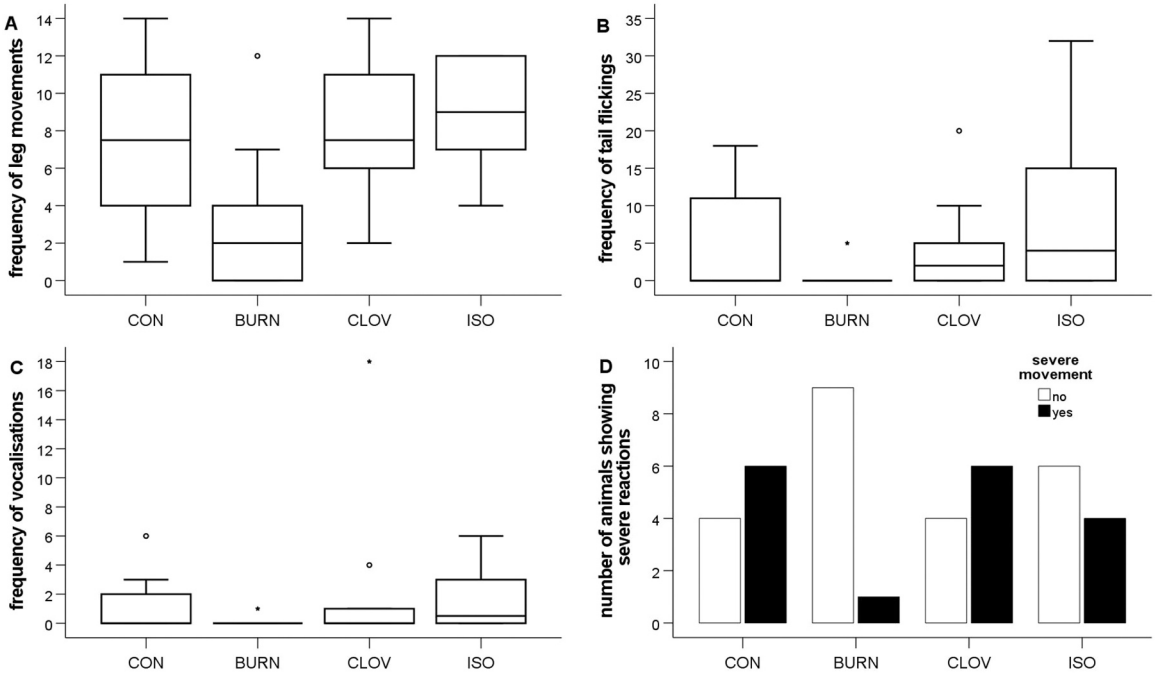

Fig. 2. Boxplots of frequency of leg movements (**A**). tail flickings (**B**). vocalisations (**C**) and frequency charts of the number of animals that showed severe reactions (**D**). observed during either injection of clove oil (CLOV). isoeugenol (ISO) or saline (CON). or during disbudding with a hot iron (BURN). BURN calves were sedated with xylazine and had received local anaesthesia 10 min before the treatment.

Table 2
Results of the models for ear flicking observed during the real-time observation and the behaviours observed during the short-period video observation or the 24 h video observation (24 h) comparing the four treatments injection of clove oil (CLOV), isoeugenol (ISO) or saline (CON) or hot-iron disbudding (BURN). Estimated means or, if transformation as indicated in brackets was necessary, backtransformed estimated means are presented for the main variable of interest (treatment for play and 24 h observations; interaction treatment*time point for all other short period observations and ear flicking). For the main hypothesis of interest a FDR calculation confirmed the P-values in bold for rejection of the null-hypothesis with an alpha-level of < 0.05, while P-values in bold & italics correspond to a trend (alpha-level of < 0.1).

Behaviour (transformation)	**F**	**Df**	**P**	**CON**	**BURN**	**CLOV**	**ISO**
Real-time observation							
F ear flicking (log10), events/20 min							
Treatment	10.058	3/44	< 0.001				
time point	4.193	10/61	< 0.001				
treatment*time point	3.679	30/57	< **0.001**				
Before				0.14	0.12^{w}	0.25^{xy}	0.37^{xy}
1 h				0.21^{a}	$4.12^{b\ yz}$	$0.45^{a\ xy}$	$0.66^{a\ xy}$
2 h				0.19^{a}	$7.28^{b\ y}$	$0.20^{a\ xy}$	$0.35^{a\ xy}$
3 h				0.05^{a}	$2.56^{b\ z}$	$0.30^{a\ xy}$	$0.10^{a\ x}$
4 h				0.27	0.58^{wx}	0.19^{xy}	0.07^{x}
5 h				0.05	0.29^{w}	0.13^{xy}	0.05^{x}
6 h				0.25^{a}	$0.91^{b\ x}$	$0.06^{a\ xy}$	$0.10^{a\ x}$
9 h				0.15	0.26^{w}	0.05^{xy}	0.05^{x}
1 d				0.06	0.22^{w}	0.28^{x}	0.00^{x}
3 d				0.27^{ab}	$0.34^{a\ wx}$	$-0.14^{b\ y}$	$0.13^{ab\ x}$
7 d				$0,07^{a}$	$0,13^{a\ w}$	$0,28^{ab\ xy}$	$0,77^{b\ y}$
Sex	3.287	1/30	0.080				
age (at treatment)	1.436	1/30	0.240				
Temperature	4.126	1/71	0.046				
time point*temperature	1.807	10/56	0.080				
sex*age	4.978	1/30	0.033				
Short-period video observations							
D play (log10), s / 30 min							
Treatment	5.585	3/32	**0.003**	10.02^{a}	6.64^{a}	0.07^{b}	5.82^{a}
Sex	2.410	1/32	0.130				
age (at treatment)	4.140	1/32	0.050				
temperature	0.221	1/32	0.641				
sex*temperature	6.210	1/32	0.018				
D ear flicking (log10), s/10 min							
Treatment	2.176	3/65	0.099				
time point	1.946	3/35	0.140				
treatment*time point	3.013	9/35	**0.009**				
day0morning (before treatment)				2.20^{x}	1.89^{x}	2.22^{x}	3.89
day0afternoon				$2.58^{ab\ x}$	$3.92^{a\ y}$	$0.82^{b\ y}$	3.37^{a}
day1morning				$0.25^{a\ y}$	$2.79^{b\ xy}$	$0.92^{b\ xy}$	1.97^{b}
day1afternoon				$3.54^{a\ x}$	$1.89^{ab\ x}$	$0.99^{b\ y}$	3.35^{a}
Temperature	24.618	1/65	< 0.001				
flies present	59.401	1/44	< 0.001				
treatment*flies	1.367	3/47	0.264				
treatment*temperature	3.458	3/56	0.022				
F head shaking, events/10 min							
Treatment	1.992	3/54	0.126				
time point	2.698	3/46	0.057				
treatment*time point	2.694	9/47	**0.013**				
day0morning (before treatment)				$1.32^{ab\ x}$	$1.03^{a\ x}$	0.91^{ab}	$2.05^{b\ x}$
day0afternoon				1.26^{xy}	2.36^{xy}	0.65	1.30^{xy}
day1morning				$0.33^{a\ y}$	$1.79^{c\ y}$	0.49^{ab}	$0.96^{b\ y}$
day1afternoon				0.62^{xy}	0.79^{x}	0.73	$1.29^{\ y}$
age (at treatment)	7.951	1/25	0.009				
Temperature	6.044	1/36	0.019				
flies present	7.329	1/65	0.009				
treatment*flies	2.015	3/53	0.123				
F head scratching (log10), events/10 min							
Treatment	0.322	3/33	0.810				
time point	6.159	3/46	0.001				
treatment*time point	2.493	9/40	**0.023**				
day0morning (before treatment)				0.01^{x}	-0.05^{x}	-0.10^{x}	0.43^{x}
day0afternoon				0.85^{xy}	0.84^{xy}	0.82^{xy}	0.73^{xy}
day1morning				$0.03^{a\ x}$	$1.09^{b\ y}$	$0.07^{a\ xy}$	$-0.07^{a\ x}$
day1afternoon				$1.40^{ab\ y}$	$0.63^{b\ xy}$	$1.03^{ab\ y}$	$2.41^{a\ y}$
age (at treatment)	4.936	1/32	0.033				
flies present	1.441	1/107	0.233				
time point*flies	4.920	3/52	0.004				
D self grooming (log10), s/10 min							
Treatment	5.476	3/21	0.006				
time point	0.282	3/32	0.838				
treatment*time point	2.791	9/32	**0.016**				
day0morning (before treatment)				11.50	3.58^{xy}	2.27	6.55
day0afternoon				18.63^{a}	$0.00^{b\ x}$	-0.02^{b}	30.12^{a}

(*continued on next page*)

Table 2 (*continued*)

Behaviour (transformation)	**F**	**Df**	**P**	**CON**	**BURN**	**CLOV**	**ISO**
day1morning				7.00^{ab}	9.05$^{b\ y}$	-0.03^{a}	8.77^{b}
day1afternoon				14.38	6.46^{y}	0.55	13.55
Sex	0.000	1/23	0.996				
age (at treatment)	28.892	1/16	< 0.001				
flies present	1.231	1/23	0.278				
Temperature	4.979	1/14	0.042				
treatment*sex	0.743	3/23	0.537				
treatment*age	2.036	3/16	0.149				
treatment*temperature	4.515	3/15	0.019				
treatment*flies present	0.326	3/25	0.806				
treatment*time*sex	1.454	12/32	0.193				
F tail flicking (log10), events/10 min							
Treatment	5.393	3/22	0.006				
time point	0.590	3/37	0.652				
treatment*time point	1.530	9/38	0.173				
day0morning (before treatment)				4.55	3.56	13.93	4.96
day0afternoon				7.69	5.47	6.94	5.30
day1morning				1.24	9.57	10.43	4.89
day1afternoon				7.89	9.19	11.94	3.56
Sex	3.590	1/16	0.076				
age (at treatment)	31.589	1/22	< 0.001				
flies present	53.407	1/50	< 0.001				
treatment*age	6.827	3/21	0.002				
treatment*flies present	3.571	3/40	0.022				
24 h observations							
D lying, h / 24 h							
treatment	3.629	3/15	***0.039***	17.82$^{a.b}$	17.54^{a}	19.08^{b}	18.71^{b}
time point	31.833	2/21	< 0.001				
treatment*time point	1.887	6/21	0.131				
day 1 after treatment				18.20	18.67	20.36	19.39
day 5 after treatment				17.65	16.92	18.49	18.40
day 7 after treatment				17.62	17.04	18.40	18.34
age (at treatment)	15.795	1/14	0.001				
treatment*age	4.862	3/14	0.016				
Transitions (log10), events / 24 h							
Treatment	0.584	3/23	0.632	30	30	27	31
time point	3.415	2/34	0.045				
treatment*time point	0.899	6/34	0.507				
day 1 after treatment				31$^{\cdot}$	30	23	33
day 5 after treatment				30	30	29	29
day 7 after treatment				29	30	31	31
Sex	7.204	1/21	0.014				
age (at treatment)	0.513	1/23	0.481				
Temperature	11.381	1/36	0.002				
time point*age	3.152	2/34	0.056				
D play (sqrt), s / 24 h							
Treatment	7.065	3	**0.002**	140.2^{a}	588.4^{b}	260.0^{a}	288.4^{ab}
Temperature	1.241	1	0.279				
treatment*temperature	4.990	3	0.010				

$^{a.b.c}$ estimated means followed by a different letter differ significantly ($p \leq 0.05$. after least significant difference test (LSD) in the row
$^{x.y.z}$ values in a column with different superscripts differ significantly ($p \leq 0.05$ after LSD)

Accordingly, the highest value in ISO before the treatment switched to a highest value of BURN the morning after the treatment (d1mo), differing from the three other treatments with CON showing lowest values (Fig. 4). Similarly BURN calves showed an increased **frequency of head scratching** the morning after the treatment, being higher than for CON, ISO and CLOV calves (d1mo, Table 2, Fig. 4). There was no difference between the three injection treatments. BURN calves as well as CLOV calves also showed the lowest **duration of self-grooming** in the afternoon of the day of the treatment (d0af), being lower than for CON and ISO (Fig. 4, Table 2).

The presence of flies had a highly significant effect on the duration of ear flicking and frequencies of head shaking and tail flicking (Table 2). The effect of flies was especially high on ear flicking duration and frequency of tail flicking: the back-transformed estimated means (EMbt) of ear flicking with flies present were 5,81 s / 10 min and thus 15-fold higher compared to without flies with 0.38 s / 10 min. For tail flicking the EMbt were 1.22 tail flicks / 10 min without and 22.39 /10 min with flies, thus 18-fold higher with flies. Calves also more than doubled their head shaking behaviour with flies (1.52 events / 10 min) compared to without (0.72 events/10 min).

The **duration of play** summarized over all post-treatment time points was affected by treatment: CLOV calves played for the shortest duration, differing from all other treatments. Numerically, CON calves played for the longest; BURN and ISO were intermediate (Fig. 4, Table 2).

3.1.4. 24 h observations

Play behaviour at day 7 after the treatment differed between treatments: BURN calves played longest and CON shortest with CLOV and ISO inbetween, so that CON and CLOV calves played less than BURN (Table 2, Fig. 3). **Duration of lying** tended to differ with shortest duration over all three days in BURN calves (17.5 h/24 h), differing from CLOV with the longest duration of lying (19.1 h) and ISO (18.7 h), but not from CON (17.8 h; Table 2, Fig. 3). No effect of treatment, nor an interaction with time point, was confirmed for **transitions** between standing and lying, although numerically CLOV showed very low transitions on day 1 (Table 2, Fig. 3).

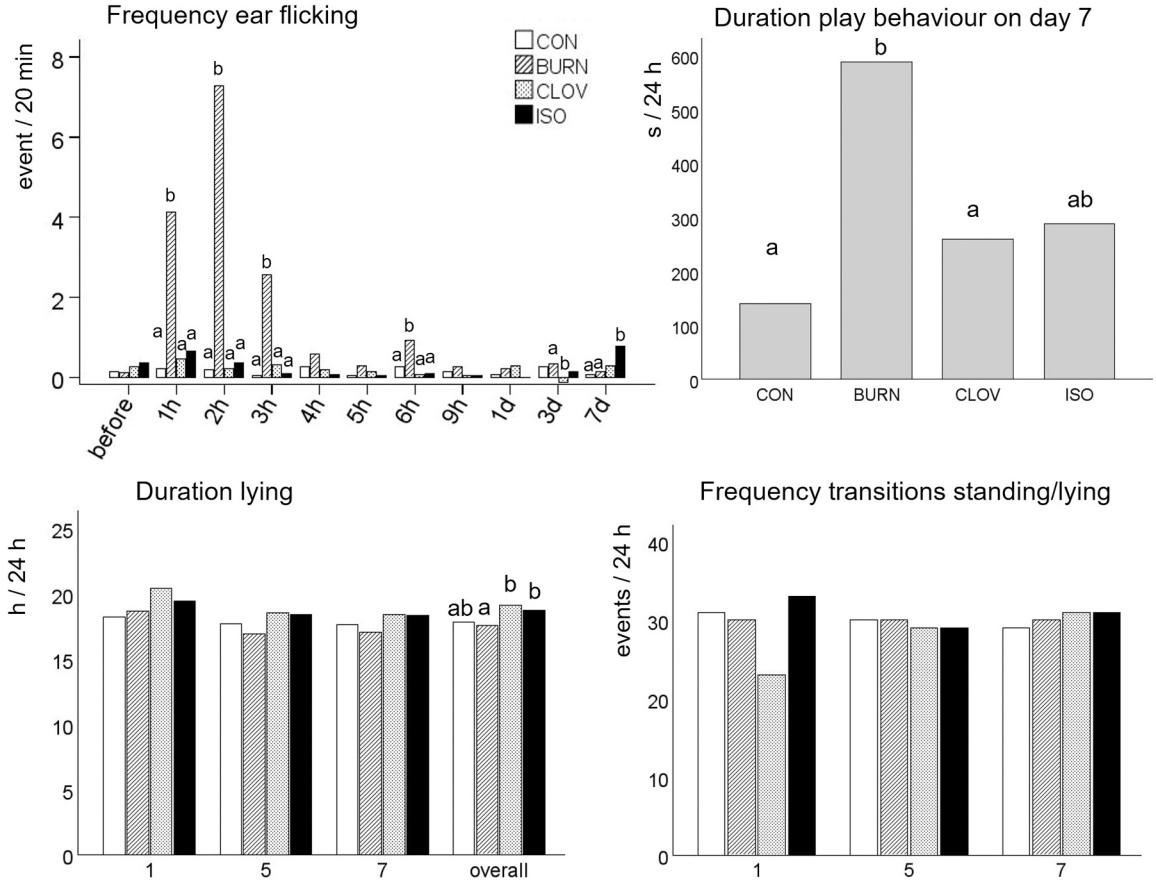

Fig. 3. Model results for frequency of ear flicking recorded during real-time observations at 11 time points and behaviour recorded during 24 h video observations on day 1, 3 and 7 for lying duration and transitions between lying and standing, or on day 7 for play behaviour. The four treatments injection of saline (CON), clove oil (CLOV), isoeugenol (ISO) or hot-iron disbudding (BURN) are compared. Bars show estimated means for duration of lying and back-transformed estimated means for the other behaviours. Treatments with different letters differ within one time point. There was a main effect of treatment (and no interaction with time point).for lying, so that overall means are shown as well.

3.2. Heart rate variability

There was no effect of treatment on the change of heart rate variability from baseline to day 1 or 7 for any of the heart rate variability variables. i.e. $p > 0.1$ for all (see Supplementary Material Figure S2 for boxplots). Weight had an effect on the change in RMSSD on day 7 compared to baseline ($F_1 = 4.849$; $p = 0.045$). with RMSSD increasing more in heavier calves. Accordingly. weight had a comparable effect on the RMSSD/SDNN difference on day 7 ($F_1 = 6.494$; $p = 0.023$).

3.3. Infrared thermography

The development of the **surface temperature** (ST) along time points differed clearly between treatments (Fig. 5) as reflected in a highly significant treatment*time point interaction ($F_{33.456} = 2.109$. $p < 0.001$, for further details of the model and estimated means see Supplementary Material Table S3), although post-hoc tests rarely revealed significant differences. In all injection treatments ST increased significantly (ISO ≈ 1.5°C higher) or numerically (CON ≈ 1°C, CLOV ≈ 0.4°C higher) while in BURN surface temperature decreased numerically immediately after the procedure so that BURN differed from the three other treatments at this time point. Thereafter CON calves' values were back to baseline. Also BURN calves' surface temperature came back to baseline level until day 7 morning where it had the highest value, being numerically higher than CON (Fig. 5, Supplementary material Table S3). Surface temperature of ISO calves was back to baseline 1 h post-treatment and above baseline on day 3 and 7, being about 1–1.5°C higher than in CON at the morning time points. Surface temperature of CLOV calves was up to 1.5°C higher compared to CON already on day 1. but only numerically thereafter. Regarding the comparison of the three disbudding treatments apart from time point 15 min, ISO calves had lower surface temperature than CLOV in the first measurement before the treatment but did not differ thereafter; higher values compared to BURN were found in CLOV at day 1 and ISO at day 3 and 7 in morning. There was also an effect of the surrounding temperature ($F_{1.95} = 57.482$. $p < 0.001$). head side ($F_{1.465} = 6.943$. $p = 0.009$) and the location of the calf during measurement ($F_{1.482} = 11.092$. $p = 0.001$): higher surface temperatures occurred on the right head side compared to the left ($p = 0.009$) and when the calf was in the calf hutch compared to being in the outdoor run ($p = 0.001$).

4. Discussion

In sum the results support our hypothesis of less pain and lower impairment of welfare after injection of isoeugenol and clove oil as compared to hot-iron disbudding but still higher impairment of welfare compared to control, a saline injection, although not all differences were statistically confirmed and results were not always in line with predictions for single behaviours or at specific time points. Results regarding the pain-related behaviours in general confirm our predictions: in hot-iron disbudded calves durations/frequencies of the behaviours were highest and in control calves lowest with CLOV and ISO inbetween up to the morning of day 1 after the treatment, i.e. about 22 h post-treatment: Control calves showed lowest duration of ear flicking

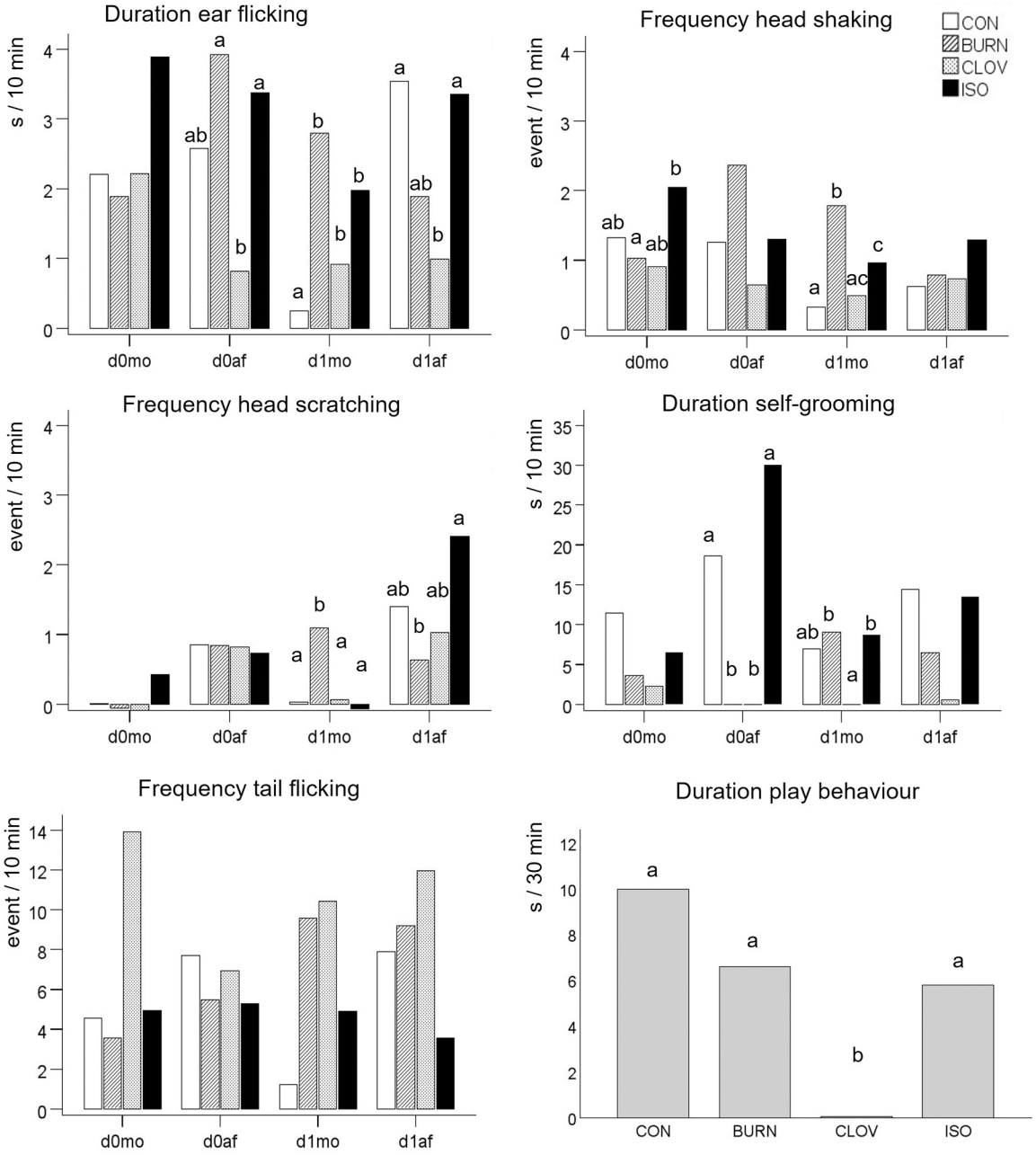

Fig. 4. Model results for behaviours recorded during short-term video observations comparing the four treatments injection of saline (CON), clove oil (CLOV), isoeugenol (ISO) or hot-iron disbudding (BURN). Bars show estimated means, all except for frequency of head shaking were back-transformed due to necessary transformation for model calculation. Behaviour was observed at four time-points for 20 min, in the morning before the treatment on the day of treatment, day 0 (d0mo), in the afternoon of day 0 (d0af), day 1 after treatment in the morning (d1mo) and in the afternoon (d1af). Behaviours were calculated per 10 min observation duration. Play behaviour was summed for all three timepoints after the treatment. Treatments with different letters differ within one time point.

and frequency of head shaking in the morning of day 1 post-treatment, differing significantly from all other treatments in ear flicking and from BURN and ISO in head shaking, with numerically highest values in BURN for both behaviours. BURN also showed highest frequency of ear flicking during live observations one to six hours post-treatment and highest frequency of head scratching at day1morning post-treatment, differing from all three injection treatments which showed quite low values. Similarly, BURN, but also CLOV, showed very low amount of self-grooming behaviour in the afternoon after the treatment (day0-afternoon) compared to both CON and ISO, thus partly confirming our predictions; in the morning of day 1 CLOV calves still self-groomed less, differing from ISO and, contrary to expectations, from BURN but not CON. CLOV calves also played least during the first two days after treatment, differing from the three other treatments; numerically CON showed highest level of play (in line with expectations). Seven days after the treatment ear flicking was still lowest for CON, this time differing from ISO with highest values, with CLOV and BURN inbetween. Further, while values of CON and BURN again showed the largest difference (statistically confirmed) in play at day 7, the direction was in the opposite than expected direction with BURN playing longer than CON but also than CLOV, with only a numerical difference between CON and the other injection treatments. The development of surface temperature points at inflammatory processes in ISO and in CLOV calves as predicted, but could not be confirmed in BURN. Further, behaviour during and surface temperature immediately after the treatment point at stress and potentially pain of calves when being injected with ISO, CLOV as well as

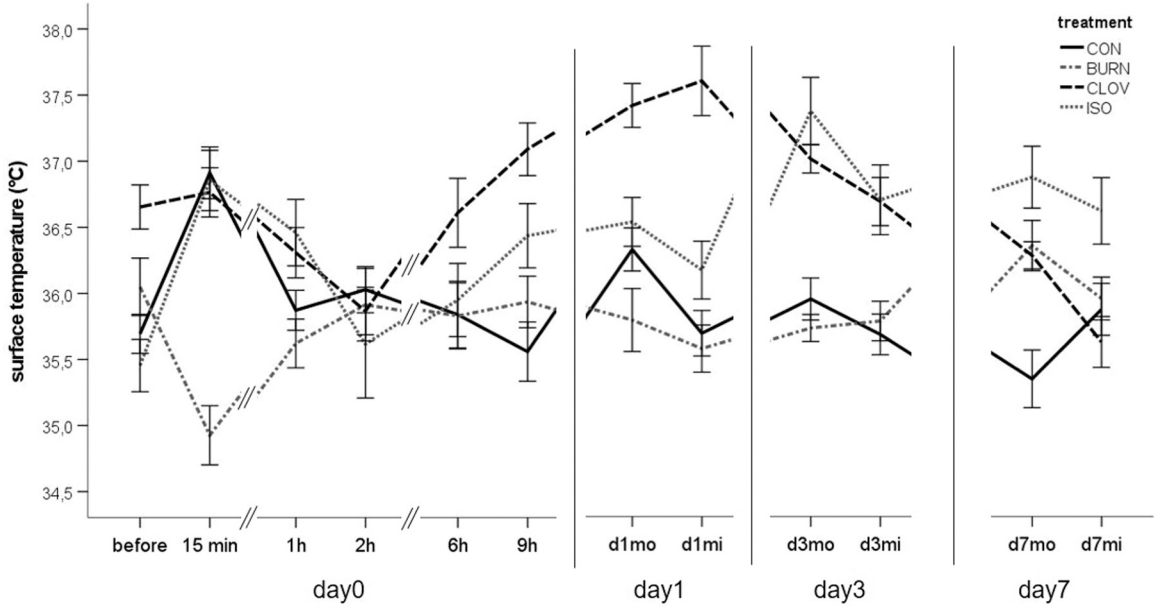

Fig. 5. Development of the surface temperature around both horn buds in the different treatments: injection of clove oil (CLOV). isoeugenol (ISO) or saline (CON). or disbudding with a hot iron (BURN) at the different time points on day 0 (before and 15 min. 1. 2. 6 and 9 h later). day 1 (d1). 3 (d3) and 7 (d7) in the morning (mo) or around midday (mi). Values represent means of the estimated mean of the maximum temperature of the left and right side; error bars show standard errors.

saline.

4.1. Behavioural observations

4.1.1. During the treatment

Behaviour of calves during the treatment suggest that pain and stress during injection of clove oil or isoeugenol under the horn bud is comparable to injection of saline. This contradicts results in goat kids who showed more high-stress vocalisations indicating higher level of pain during injection of clove oil or isoeugenol compared to saline (Frahm, 2022) probably due to the irritating skin sensitizing properties of these substances (Lalko and Api, 2006; Klein et al., 2014). The missing differences between the injection treatments in our trial might be caused by the handling of the calves during the treatment. To allow an injection of the substances as precise as possible, calves were strongly restrained on the floor including the head. This probably has caused (i) already high levels of stress and defence leading to a ceiling effect and/or (ii) some form of freezing behaviour reducing defence behaviour. Being restrained on the ground may clearly enhance stress levels compared to restraint while standing, as shown for alpacas (Waiblinger et al., 2020a).

We used one of the most common anaesthesia for disbudding; the use of local anaesthetics combined with xylazine (Stilwell et al., 2010; Cozzi et al., 2015). Despite waiting sufficiently long (10 min) to allow local anaesthetic reaching effectiveness, BURN calves still showed some defensive movements despite additional sedation, in line with previous studies (Kahrer et al., 2008; Stilwell et al., 2010); suggesting that anaesthesia may not always be sufficient to eliminate pain during burning and/or stress completely (Kahrer et al., 2008). We cannot distinguish if our BURN calves reacted to the handling or to pain during the disbudding or to both.

4.1.2. Behaviour before and after the treatment

The increase and thus highest frequencies of ear flicking in the hours after the treatment in BURN calves equates findings in the literature where it was elevated up to 24 h after disbudding without analgesia and suggest higher level of pain on the day of treatment in BURN compared to CON, CLOV and ISO calves. Anaesthesia during and analgesia post-treatment largely reduces this behaviour (e.g. Faulkner and Weary, 2000; Stilwell et al., 2012; Braz et al., 2012) confirming its validity as indicator of disbudding pain. Ear flicking duration observed from videos was higher not only for BURN but also for ISO and CLOV compared to CON in the morning of day 1, i.e. about 22 h post-treatment; at day 7 ISO had highest ear flicking frequencies, differing from CON and BURN. This delayed response in CLOV and ISO may be explained by transient anaesthetic and anti-inflammatory effects in the first hours after injection of these substances before irritating and cytotoxic effects prevail (Frahm et al., 2020) which may contribute to lower ear flicking in CLOV in the afternoon of day 0 (but see also below discussion on self-grooming). The same calves as described in this paper showed also lowest mechanical nociceptive tresholds, that is highest sensitivity, in the morning of day 1 after the treatment (Juffinger et al., 2021), fitting to the increases in ear flicking and suggesting pain at these time points. At day 7, all CLOV and ISO calves showed tissue damage (Juffinger et al., 2021) that likely cause pain and may elicit increased frequency of ear flicking, which was significant for ISO, while CLOV only numerically were higher than CON.

Also the highest number of head scratching, differing from all injection treatments, and of head shaking, differing from CON and ISO, on day1morning as well as the strong decrease of self grooming in the afternoon of day 0 (d0af) in BURN calves and the according differences to CON and ISO, but not CLOV, suggest higher level of pain after hot-iron disbudding as compared to the injection with saline and isoeugenol, and to a lesser degree clove oil. Calves show increased head shaking and head scratching and reduced self-grooming in the 4 h after disbudding (Morisse et al., 1995); if calves receive anaesthetic or/and analgesic treatment they show less head shaking and head scratching after disbudding (Graf and Senn, 1999, Faulkner and Weary, 2000), indicating the value of these behaviours for assessing disbudding pain.

Results were less consistent in the afternoon of day 1, i.e. 30 h after the treatment: then, CLOV had lower number of ear flicking than CON and ISO, and ISO higher number of head scratching than BURN, while no differences were seen in head shaking and self-grooming. The higher number of head scratching in ISO fits to the aforementioned delayed reactions due to a transient anaesthetic effect. All CLOV calves were treated with anti-inflammatory and analgesic drugs due to moderate to severe swellings of the upper eye lids (Juffinger et al., 2021) after the morning observation of day 1 explaining the low ear-flicking values in the afternoon observation of day 1, while no such treatment was

necessary for ISO calves.

There were fewer and less consistent differences in these behaviours (ear flicking, head shaking, head scratching, self-grooming) between the injection of clove oil or isoeugenol compared to saline injection and some differences to BURN, which sugest that the injection of clove oil and especially isoeugenol is less painful as compared to BURN. However, it might be that clove oil and isoeugenol activate different nociceptive receptors and nerve fibres thus triggering different pain sensations which may manifest in different behavioural changes. While Aδ-fibres respond to mechanical and thermal stimuli resulting in a pricking type of pain, C-fibres are polymodal and can be activated by mechanical, thermal and chemical stimuli, and their activation leads to a dull kind of pain (Kidd and Urban, 2001; Yam et al., 2018). The use of a hot iron might activate Aδ- and C-fibres, whereas clove oil and isoeugenol might only activate C-fibres. This may explain the longer duration of lying in ISO and especially CLOV calves as compared with BURN, in line with previous findings comparing clove oil injection and hot-iron disbudding (Sutherland et al., 2018), while the similar levels of lying for CON and BURN are in accordance with another study that found no difference in lying duration on day 3 and 10 after hot iron disbudding, but only on day 17 (Adcock et al., 2023). Further, the very low duration of play behaviour of CLOV calves within the first one and a half days and of self grooming in the afternoon of day0, differing from CON and ISO, indicate impaired welfare of CLOV calves that correlate to the swellings of the eye lid and MNT results in the same animals (Juffinger et al., 2021) mentioned above. The low duration of ear flicking at this time point (d0af) thus may also point at a general low activity of CLOV calves at this time point coinciding with higher levels of lying as shown in Sutherland et al. (2018) for the day of injection and in our study on day 1, 3 and 7. Lower level of play behaviour (running) in a test arena were observed 18 h after applying caustic paste for disbudding (Rushen and De Passillé 2012) in line with reduced play in CLOV in our study. We did only find numerically longer play in CON vs. BURN; this partly confirms results in hot-iron disbudded calves without post-treatment analgesia that clearly played less than sham disbudded controls or calves that had received analgesic treatment in an arena 3 h post-procedure but no differences were seen 27 h post-procedure (Mintline et al., 2013). Results regarding the duration of play on day 7 are, in principle, contrary to our expectations: BURN calves were playing the most followed by ISO and CLOV calves, while CON calves played the least, while we expected the reverse order. The occurrence of play behaviour is on the one hand thought to be associated with positive well-being (Held and Špinka, 2011) and, besides pain, conditions compromising calf welfare are associated with recuded play in calves, e.g. low space allowance, lack of social contact, separation from the dam or low milk allowance (Jensen et al., 1998, Krachun et al., 2010, Waiblinger et al., 2020b). On the other hand, young animals are highly motivated to play, and they play more after a phase of deprivation (Jensen, 2001). Such a ŕebound-effecť may explain why the duration of play was higher in BURN compared to CON in our study on day 7. In addition, play leads to the release of neuromodulators that can lower stress and suppress pain (Boissy et al., 2007), which may enhance play in animals experiencing pain and which contribute to, numerically, lowest play duration in CON at day 7. The duration of play of our control calves seems comparable to the one of two-week old calves kept in single pens in the study of Jensen et al. (1998), which supports enhanced levels due to aforementioned reasons in BURN and, to a lesser degree, CLOV and ISO calves.

On day 7 the calves were more active in general, which is reflected by a lower duration of lying, more head shaking, a longer duration of exploration and self grooming. Because this was the case also for control calves these findings reflect age effects (Kiley-Worthington and de la Plain, 1983; regarding play behaviour: Held and Špinka, 2011; Jensen, 2011; Whalin et al., 2021). Accordingly, age at treatment was a significant variable in all models. Calves disbudded at the age of one week compared to four weeks older calves, were less active as well (Caray et al., 2015).

Except for ear flicking, differences in behaviours were present only during the video observations but not during the real-time observations; several aspects may have contributed to this: (i) The selected time points differed being balanced for time of the day in the video observations. (ii) Slight changes in the definition of some variables (which was necessary due to the camera angle) may also have contributed. (iii)The presence of the observer during the direct observation might also have influenced the behaviour of the calves (Metcalfe et al., 2022), although we tried to keep the impact as low as possible. (iv) The inclusion of the confounding factor “flies present” in the video observations while flies were taken into account only for the behaviour ear flicking during live observation. The importance for considering this factor is supported by the results: “flies present” was highly significant in the models and had a large increasing effect on the behaviours known as insect-defence behaviour (ear flicking, head shaking, tail flicking; Mooring et al., 2007) besides their value as indicator of pain after disbudding (see above), but had no effect in the models for head scratching or self-grooming. During real-time observations we only considered ear flicks when there was no fly present/leaving the ear. This may contribute to the lower frequency of ear flicking in our study compared to some others (e.g. Stilwell et al., 2012, Adcock et al., 2020) besides effects of age as our calves were neonate as compared to several week old calves in the other studies (see discussion above), potential breed effects (Caray et al., 2015) or potential effects due to differences in definitions of bout length. These results underline the importance of considering presence of flies and other potential confounders if a completely balanced design such as randomized block is not possible. Also ambient temperature and sex had an effect on some of the behaviours. By considering the potential confounding factors we could confirm overall expected treatment effects on behaviours in most of the behaviours of the video observations. However, post-hoc pairwise comparisons did not always confirm (numerical) differences likely due to the relatively low sample size given the complexity of the models.

4.2. Heart rate variability

Heart rate and heart rate variability are suitable indicators for pain and stress in animals (Hagen et al., 2005; von Borell et al., 2007), also in calves disbudded with a hot iron (Stewart et al., 2008, 2009; Byrd et al., 2019). Our sample size was considerably reduced due to technical problems, our limited time frame for sampling (see below) and our strict selection criteria excluding phases with potential disturbances. This decrease in statistical power may have caused the lack of treatment differences. Our sampling period was limited to 2 h per day, to have comparable times of the day during baseline (day 0 before the treatment) and observations after the treatment (day 1 and 7), reducing the effect of daytime

The age of the animals might also have interfered with effects of the treatment, further complicating treatment differences. The numerical higher RMSSD on day 7 as compared to day 0 or 3 is in line with a previous study where HRV increased with age in calves (Longin et al., 2005).

4.3. Infrared thermography

The increase of surface temperature can indicate an ongoing inflammation (Schaefer et al., 2004; 2022), as well as stress and pain (Stewart et al., 2008; Rekant et al., 2015; Scherf et al., 2020). The increase of the surface temperature in ISO and, numerically, in CON and even less so in CLOV calves 15 min after start of the treatment is likely caused by stress or pain due to the handling of the animals and the injection itself, as the animals did not get any sedation or analgesia. Similar findings have been made by Scherf et al. (2020), who observed an increase in surface temperature of about 1.5°C from 30 min to 2 h after sham disbudding of calves that further had received one

intramuscular and three subcutaneous saline injections 30–5 min before. This likely is more aversive than for our control treatment and may explain our shorter and lower, only numerical, result in our study. The lack of a difference in ST between injection treatments at the time point 15 min conform to the behavioural results.

The decrease of the surface temperature in CLOV and ISO calves 1 h and 2 h after the treatment and continuous, though partly only numerical, increase thereafter might reflect the aforementioned transient anaesthetic, analgesic and anti-inflammatory properties (Atsumi et al., 2005; Chaieb et al., 2007; Taher et al., 2015) and inflammation reflecting the cytotoxic effects thereafter. This development of surface temperatures are in accordance with the calves' behaviour and with the aforementioned findings regarding the mechanical nociceptive threshold (MNT) in the same animals (Juffinger et al., 2021) and in animals of previous studies of our working group (Frahm et al., 2020). The decrease of the temperature in CLOV calves after a peak on day 3 might be explained by the anti-inflammatory treatment of the eye lid swellings of all CLOV animals(see above).

There was a decrease of the surface temperature 15 min after the treatment in BURN calves, likely caused by the sedation with xylazine known to reduce body temperature (e.g. in rats: Livingston et al., 1984; Tarahovsky et al., 2020; in calves: Vasseur et al., 2014). In addition, the spray used to treat the wounds in BURN calves right after disbudding might have cooled the surface. From 1 h onwards the surface temperature of BURN calves stayed at the baseline level, confirming previous studies starting surface temparature assessment one hour (Mirra et al., 2018) or three days after the treatment (Adcock and Tucker, 2018). Due to the occurrence of third-degree burns (Caray et al., 2015) we expected associated inflammation and increased surface temperatures in BURN calves, but the occurrence of crusts that developped in all BURN calves within the first week after disbudding (described in Juffinger et al., 2021) might have shielded the temperature of subjacent inflammated tissue. Additionally, in humans deep burn wounds are significantly cooler than normal surface temperature in IRT images, due to the destruction of blood vessels (Hardwicke et al., 2013; Paul et al., 2015).

5. Conclusion

In comparison with hot-iron disbudding without post-operative analgesia the injection of isoeugenol and, to a lower extent, clove oil seems to elicit lower level of pain as reflected in less behavioural changes. However, behaviour and surface temperature of animals injected with clove oil or isoeugenol suggest painful processes compared to control animals. There are some indications for stronger reactions after clove oil injections compaired to isoeugenol. Together with the results of the previous paper on MNT, tissue alterations and horn growth (Juffinger et al., 2021), we conclude that the use of clove oil is not a recommendable alternative to hot-iron disbudding. The use of isoeugenol seems to be more promising, although the question of the currently insufficient effectiveness (Juffinger et al., 2021, Schoiswohl et al., 2022) needs further consideration. In addition, post-treatment pain management is recommended and the potential need for anaesthetic drugs during the injection need to be investigated further.

CRediT authorship contribution statement

Reinhild Krametter-Frötscher: Writing – review & editing, Project administration. **Thomas Wittek:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Andreas Futschik:** Writing – review & editing, Methodology, Formal analysis. **Susanne Waiblinger:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anna Juffinger:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Sophie Bramberger:** Writing – review & editing, Investigation. **Anna Stanitznig:** Writing – review & editing, Investigation. **Julia Schoiswohl:** Writing – review & editing, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2024.106290.

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