Applied Animal Behaviour Science 198 (2018) 1–8

Contents lists available at ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim

Evaluation of visible eye white and maximum eye temperature as non-invasive indicators of stress in dairy cows

Y. Gómez[a,b], R. Bieler[b], A.K. Hankele[c], M. Zähner[a], P. Savary[a], E. Hillmann[b,d,*]

[a] Agroscope, Research Group Work, Building and System Assessment, 8356 Ettenhausen, Switzerland
[b] ETH Zürich, Department of Environmental System Sciences, Ethology and Animal Welfare Unit, 8092 Zurich, Switzerland
[c] ETH Zürich, Department of Environmental System Sciences, Animal Physiology, 8092 Zurich, Switzerland
[d] Humboldt University Berlin, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, 10115 Berlin, Germany

ARTICLE INFO

Keywords:
Cattle crush
Claw trimming
Feed rack
Heart rate variability
Saliva cortisol

ABSTRACT

The aim of this study was to investigate if visible eye white and eye temperature measurements are feasible non-invasive physiological indicators of acute stress in cows when they are exposed to cattle crush treatment for claw trimming.

In the experimental setting, 30 cows of two breeds (Red Holstein and Brown Swiss) were exposed to a non-stressful (feeding) and a stressful situation (claw trimming in a cattle crush) for 10 min each. We took pictures of the eyes at 0, 5 and 10 min after starting of exposure to measure the percentage of visible eye white (from photographs) and the maximum eye temperature (from thermographs). Heart rate and heart rate variability parameters were recorded continuously throughout both situations. Twenty minutes after the beginning of each situation, saliva samples were taken to determine the cortisol concentration.

As expected, sympathetic activity and cortisol concentration were higher in the claw trimming than in the feeding situation. However, neither maximum eye temperature nor percentage of visible eye white differed between treatments. Instead, the results of these measurements differed between the breeds. The maximum eye temperature increased during and after both situations in Brown Swiss cows, whereas in Red Holstein cows, it increased after (but not during) both situations. Furthermore, we found that Red Holstein cows had a lower percentage of visible eye white than Brown Swiss in general. This finding might be due to differences in eye coloration patterns, with Red Holstein cows having more contrast between eye white and iris and Brown Swiss having less contrast because of their darker eye white. This breed effect might have masked potential treatment effects.

1. Introduction

Stressful situations lead to an activation of the hypothalamic-pituitary-adrenal axis (HPA; Selye, 1936) and stimulation of the autonomous nervous system (ANS; Cannon, 1935; Moberg, 2000) with the sympathetic and parasympathetic branches (Stewart et al., 2005). This activation can be measured by means of hormonal secretions after stress exposure (Friend, 1980; Owen et al., 2005; Sheriff et al., 2011; Ursin and Olff, 1993;).

One of the most used stress indicators is the concentration of corticosteroids and catecholamines in plasma. Disadvantages of sampling plasma are well known. It is an invasive method and animals have to be restrained, which in turn may affect hormone concentration (Alam and Dobson, 1986; Möstl and Palme, 2002). Nevertheless, non-invasive methods are available to detect corticosteroid metabolites in saliva, milk, feces or hair (Möstl and Palme, 2002; Palme, 2012; Palme et al., 1996). However, limitations of these methods have been discussed thoroughly (Mormède et al., 2007; Negrão et al., 2004; Sheriff et al., 2011). The concentration of corticosteroids alone does not reflect the full picture because it is increased not only during punishing but also during rewarding situations such as mating or hunting (Mormède et al., 2007; Sapolsky et al., 2000). To overcome this problem, additional indicators can be used to assess the response to a potential stressor, including heart rate variability (such as regulation of sympatho-vagal tone), maximum eye temperature and especially behavioral changes.

However, regulation of the sympatho–vagal tone seems to be context specific and interpretations should thus include other stress-indicating variables (Patt et al., 2016).

Eye temperature as stress indicator has been investigated in several species (humans: Pavlidis et al., 2000, 2002; horses: Bartolomé et al.,

* Corresponding author at: Humboldt-Universität zu Berlin, Albrecht Daniel Thaer-Institute of Agriculutural and Horticultural Sciences, 10115 Berlin, Germany.
E-mail addresses: yamenah.gomez@usys.ethz.ch (Y. Gómez), edna.hillmann@hu-berlin.de (E. Hillmann).

http://dx.doi.org/10.1016/j.applanim.2017.10.001
Received 23 June 2017; Received in revised form 29 September 2017; Accepted 1 October 2017
Available online 07 October 2017

2013; Cook et al., 2001; McGreevy et al., 2012; cows: Schwartzkopf-Genswein et al., 2012; Stewart et al., 2005, 2007, 2008a,b, 2009, 2010). The exact neuroendocrine pattern, however, has not been understood fully.

Artificially injecting exogenous adrenocorticotropic hormone had no immediate effect on maximum eye temperature in calves (Stewart et al., 2007). Therefore, it is thus assumed that in cattle, changes in maximum eye temperature are not due to activation of the HPA axis alone, but that other factors are involved such as a sympathetically mediated response of the ANS (Stewart et al., 2007, 2008b) or emotionally relevant stimuli (Mason, 1971; Stewart et al., 2005, 2007, 2008a,b). The limitations of the non-invasive stress indicators highlight the need for additional indicators to allow a more feasible stress assessment.

Percentage of visible eye white as an indicator of emotional state was shown to cover the entire spectrum of the contentedness axis from frustration to satisfaction (Sandem et al., 2002), with the visible eye white area increasing when cows are exposed to frustration or show startle reactions and decreasing when cows are satisfied and content. Application of the anxiolytic substance diazepam reduced the percentage of visible eye white in frustrated cows (Sandem et al., 2006).

Furthermore, exit speed was positively correlated with cortisol concentration (Curley et al., 2006), and percentage of visible eye white was positively correlated with exit speed (Core et al., 2009).

The proportion of visible eye white is controlled by the sympathetically controlled *Musculus tarsalis* that lifts the eyelid (Patel et al., 2008; Proctor and Carder, 2015; Sandem et al., 2006). Accordingly, Reefmann et al. (2009) found a positive correlation between heart rate and relative eye aperture and a negative correlation between heart rate variability and eye aperture. A reduction of visible eye white thus is assumed to be triggered by a sympathetic deactivation or a parasympathetic activation. Visible eye white might thus allow a reliable assessment of stress responses in cattle.

In modern dairy farming, cows are regularly exposed to potentially acute stressful situations such as regrouping or restraint for artificial insemination, medical treatments and claw trimming. Cows that were squeezed in a cattle crush had higher plasma cortisol levels 1 h after squeezing compared with their baseline values (Szenci et al., 2011; Thun et al., 1998). Furthermore, claw trimming led to higher fecal cortisol concentration in comparison with baseline concentrations before claw trimming (Pesenhofer et al., 2006). Accordingly, treatment with an analgesic and sedative drug led to lower cortisol concentrations, but also to lower heart and respiratory rates during claw trimming (Rizk et al., 2009, 2012).

The aim of this study was to validate visible eye white and maximum eye temperature as non-invasive stress indicators. For this aim, we assessed visible eye white and maximum eye temperature in addition to heart beat parameters and saliva cortisol concentration in dairy cows during claw trimming and in a control situation (undisturbed feeding).

2. Materials and methods

2.1. Animal husbandry and management

The study was conducted in March 2015 at the Research Station Agroscope Taenikon, Switzerland. The dairy herd consisted of 52 lactating dairy cows (Brown Swiss and Red Holstein) kept in a cubicle barn with permanent access to an outdoor run. The cows were fed a total mixed ration 7 times daily at a self-locking feed rack. They were locked routinely in the feed rack for approximately 1 h after feed delivery.

On three consecutive days, 10 cows each were selected resulting in a sample of 30 cows (18 Brown Swiss and 12 Red Holstein, parity number 3.38 ± 1.89, 150.93 ± 66.48 days in milk).

2.2. Experimental procedure

All experiments conformed to the Swiss Animal Welfare Legislation and were ethically approved by the cantonal veterinary office (TG 05/2014).

In a cross-over design, we exposed the cows to a claw trimming and to a control situation (undisturbed feeding). In both situations, we took pictures of the cows' eyes, collected saliva samples and recorded heart rate (HR) and heart rate variability (HRV). Measurements were done only during morning hours.

2.2.1. Control treatment

Data collection started at around 7:30 to 8:00 a.m. when the focal cows were self-locked in the feed rack. When the focal cow was feeding calmly, three pictures each were taken at t_0, and approximately 5 min (t_5), and 10 min (t_{10}) later. Saliva was collected approximately 20 min (t_{20}) after t_0. The cows were considered one after the other in a random order.

2.2.2. Claw trimming treatment

Claw trimming was done by two experienced claw trimmers. The cows were guided individually to the cattle crush that was located adjacent to the outdoor run, not visible to the herd. When the cow had entered the crush with all four legs, a girth was attached around her chest, and one hind leg and the opposite front leg were lifted and restrained (Fig. 1). After finishing trimming of the first two claws, these legs were released and the other two legs were lifted. At the end of trimming, all legs were released, the chest girth was opened, and the cow left the crush and returned to the home pen.

As soon as a cow had entered the crush, data collection started with t_0 = cow in crush with all four legs; t_5 = approximately 5 min after legs were lifted, t_{10} = 10 to 15 min after entering crush (claw trimming was finished and all four claws touched the ground again). The cows were released immediately thereafter and returned freely to the home pen for feeding. Saliva was collected in the home pen, approximately 20–30 min after the beginning of claw trimming (= 5–10 min after releasing from the crush (t_{20})).

2.2.3. Picture capturing

Pictures were taken from the left side of the cow, in approximately 1 m distance, with an angle of view of 90° (for details see: Figs. X, Y, Suppl. material), using a fixed focal length (as suggested by Schaefer et al., 2012). Each time (t_0, t_5, t_{10}) three pictures were captured and only clear pictures with an angle of view of 90° were used further.

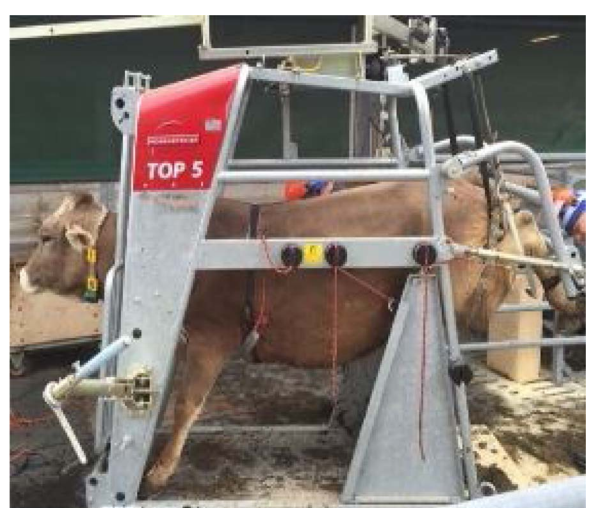

Fig. 1. Walk-in cattle crush used in the study.

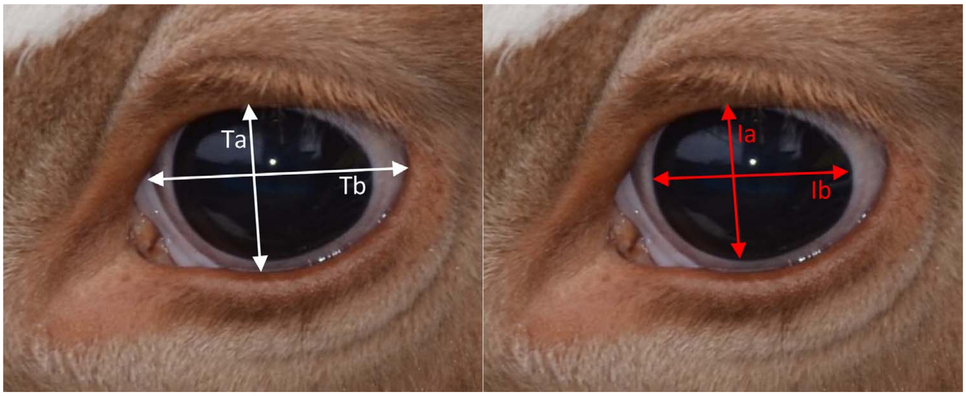

Fig. 2. Semi-major (a/2) and semi-minor (b/) axes measured for total eye (T) and iris (I) area calculations using ellipse equations.

2.2.4. Visible eye white measurements

We used a digital single lens reflex camera (Nikon® D7000 with the Nikon® AF-S DX NIKKOR 35 mm F/1.8 G; Zoom; Nikon, Tokyo, Japan). The images were analyzed using the software ImageJ 1.48 v (Rasband, W.S. [2009] ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), which has been used before to analyze morphometrics (Abràmoff et al., 2004; Doube et al., 2010; Schneider et al., 2012).

The percentage of visible eye white (PW) was calculated as described by Sandem et al. (2002) (Fig. 2).

The person (RB) who was analyzing the pictures was blinded for treatments. To check for intra-observer agreement, she measured all pictures 3 times in different order. Additionally, to test for inter-observer agreement, RB and an additional person measured the axes of the total eye and the iris areas from a selected sample of pictures (n = 80).

2.2.5. Maximum eye temperature measurements

For maximum eye temperature measurements, we used a FLIR® T620 b2.2 thermal imaging camera with a 25° lens and 640 × 480 Resolution per 30 Hz (FLIR Systems, Wilsonville, Oregon, USA), calibrated to ambient temperature and humidity with an accuracy of ± 2 °C and thermal sensitivity of < 0.04 °C. The maximum eye temperature was measured within an oval area covering the entire eye and approximately 1 cm around the eyelids as described by Stewart et al. (2005). We conducted the same procedure as for visible eye white picture capturing by taking three pictures per time point. Because the measurement of eye temperature depends on the distance to the eye, air temperature, wind speed, sunlight and camera setting for humidity and emissivity (Church et al., 2014; Knizkova et al., 2002; Webster et al., 2008), these variables were recorded throughout the experiment and considered for image analysis. Because data collection took place in a sheltered location during morning hours, there was no direct sunlight towards the FLIR® sensor or towards the cows.

For further analysis, the FLIR® Tool + software add-in for Microsoft® Office Word (FLIR® Systems, Wilsonville, Oregon, USA) was used with the measuring person blinded for treatment.

2.2.6. Heart beat measurements

To reduce handling of the cows on the experimental days we fitted the measuring device (thorax belt and an additional elastic girth) on the preceding evening. Application of electrode gel, attaching and activating of the Polar® devices (Polar Electro Oy, Kempele, Finland) took place immediately after the morning milking (finished at 6:00 a.m.). Data collection started when the cows were locked in the feed rack at about 7:30 a.m. All devices were removed after the last saliva collection.

Only intervals of the same length and within ± 2 min before and after picture taking were considered for analysis. The high friction between thorax belt and restraining girth in the crush caused artifacts, limiting the duration of time windows with acceptable error rates. Therefore, we chose time windows of 1 min with ≤5% average error rates in the beat-to-beat measurements and corrected errors using the software Polar® ProTrainer Equine Edition, Polar 32 (Polar Electro Oy, Kempele, Finland). In addition to heart rate (beats per minute), we used the square root of the mean squared differences of successive normal-to-normal R–R intervals (RMSSD) as a short-term HRV measure (Hirsch et al., 1995; Kleiger et al., 1992; former cited in Mohr et al., 2002). The ratio of the standard deviation of the normal-to-normal R–R intervals (SDNN) to RMSSD served as a measure of sympatho–vagal balance (Balocchi et al., 2006; Sollers et al., 2007; both cited in Wang and Huang, 2012), with SDNN as the long-term HRV measure (Hirsch et al., 1995; Kleiger et al., 1992; former cited in Mohr et al., 2002). Analysis of the heart beat parameters was done in a random order across situations, experimental days and cows.

2.2.7. Saliva cortisol measurements

Saliva was collected with a synthetic swap commonly used for cortisol determinations (Salivette® Cortisol, Art. No. 51.1534.500; Sarstedt AG & Co., Nümbrecht, Germany). Sampling took place during restraint in the feed rack before and after claw trimming and in the control situation. The swap was manually maintained in the cow's mouth under the tongue in the region of the diastema (= i.e., the gap between incisors and premolars of the mandibular arcade) for approximately 30 s until the swap was fully soaked with saliva. The swap was then replaced into the Salivette®, and saliva samples were stored immediately at −20 °C until further processing. Saliva was then thawed and collected from the Salivettes® by centrifugation for 2 min at 1000 × *g* and stored again at −20 °C until analysis of the cortisol concentration. Prior to analysis, saliva samples were thawed, centrifuged at 2000 × *g* for 10 min, and the mucin-free supernatant was utilized for saliva cortisol determination by enzyme-linked immunosorbent assays using a commercial kit according to the manufacturer's instructions (Cortisol free in Saliva ELISA, Art. No. DES6611; Demeditec Diagnostics GmbH, Kiel, Germany). The intra- and inter-assay coefficients of variance were 8 and 13%, respectively. The detection limit specified by the manufacturer was 0.024 ng/ml. The analyzing person (AKH) was blinded for treatment. Saliva sampling was not possible in one cow, leading to a sample size of 29.

2.3. Statistical analysis

The analysis was done using RStudio (1.0.136) and R.3.2.2 (R Development Core Team, 2017) with functions of the package "lmerTest" (Kuznetsova et al., 2016). Regarding eye white, intra- and inter-observer agreements were analyzed using the package "Agreement" in R (Yu and Lin, 2012). The error structure was set as constant and the target as random, with concordance correlation coefficient, precision and accuracy as outcomes.

We applied linear mixed-effects models with percentage of eye white, maximum eye temperature, heart rate, RMSSD, and RMSSD/SDNN as outcome variables. Treatment (factor with two levels: control, claw trimming), time (factor with three levels: t_0, t_5 and t_{10}), breed (factor with two levels: Brown Swiss, Red Holstein) and all possible interactions were included as fixed effects in all models.

Time nested in situation, nested in cow identity was included as random effect to consider repeated measures. For visible eye white and maximum eye temperature, this random effect was crossed with picture identity. An additional crossed random effect of measurement order (factor with three levels: 1, 2, 3) was included for visible eye white. For maximum eye temperature analysis, ambient temperature, wind speed and humidity were considered as covariates. Time nested in situation nested in cow identity was added as random effect for heart beat analysis.

The final model was always selected using a stepwise backwards elimination procedure of non-significant ($\alpha > 0.05$) interaction effects. Treatment, time and breed remained as main effects in the final model. Based on residual analysis, log transformation was required for heart beat parameters (heart rate, RMSSD and RMSSD/SDNN) and for maximum eye temperature.

3. Results

3.1. Visible eye white measurements

The inter-observer concordance correlation coefficient for all distances measured (see Fig. Y, Suppl. material) was > 0.95 with high precision (> 0.96) and high accuracy (> 0.98). The concordance correlation coefficient, precisions and accuracies for the intra-observer agreement were also high (> 0.88, > 0.99 and > 0.96, respectively). Thus, intra- and inter-observer agreements were sufficient to provide reliable results. Furthermore, inter-individual variance was very large ranging from 19.5 to 62.3% and 11.3 to 59.5% for the claw trimming and control situation, respectively.

Brown Swiss cows showed a higher percentage of visible eye white than Red Holstein cows (Fig. 3; $F_{1,27.274} = 4.61$, $P = 0.04$). Neither a treatment nor a time effect on the percentage of visible eye white was statistically detectable (treatment: $F_{1,29.457} = 1.66$, $P = 0.21$; time: $F_{2,94.039} = 0.73$, $P = 0.49$). The mean (± standard error) percentage of visible eye white was 37.9% (± 1.10) for the control situation and 40.7% (± 1.20) for claw trimming (Fig. 3).

3.2. Maximum eye temperature

The maximum eye temperature was lowest (35.5 ± 0.07 °C) at the beginning (t_0) and highest (36.0 ± 0.07 °C) at the end (t_{10}) in the claw trimming situation. In the feeding situation the maximum eye temperature was lowest (35.5 ± 0.09 °C) at the beginning (t_0), highest (35.8 ± 0.08 °C) during (t_5) and intermediate (35.7 ± 0.12 °C) at the end (t_{10}; treatment × time: $F_{2,108.591} = 2.95$, $P = 0.06$; Fig. 4). In Brown Swiss cows, the maximum eye temperature was increased at t_5 (35.8 ± 0.06 °C) and t_{10} (35.7 ± 0.09 °C) compared with t_0 (35.4 ± 0.08 °C). In Red Holstein cows, the maximum eye temperature was increased at t_{10} (36.0 ± 0.10 °C) compared with t_0 (35.6 ± 0.08 °C) and t_5 (35.6 ± 0.09 °C; time × breed: $F_{2,110.080} = 3.21$, $P = 0.045$; Fig. 4).

The mean (± standard error) ambient temperature, wind speed and humidity were 5.56 ± 0.17 °C, 0.53 ± 0.02 m/s, and 76.65 ± 0.58%, respectively. None of these covariates significantly affected maximum eye temperature (ambient temperature: $F_{1,95.466} = 0.15$, $P = 0.70$; wind speed: $F_{1,136.158} = 1.93$, $P = 0.18$; humidity: $F_{1,11.45} = 0.89$, $P = 0.35$).

3.3. Heart rate and heart rate variability

The heart rate (beats per minute, bpm) was increased in the claw trimming (83.14 ± 2.18 bpm) compared with the control situation (64.19 ± 0.95 bpm; $F_{1,16.73} = 16.85$, $P = 0.001$; Fig. 5). Additionally, there was a slight decrease in the heart rate from t_0 to t_{10} ($F_{2,21.94} = 2.80$, $P = 0.08$). Breed did not significantly affect the heart rate ($F_{1,19.59} = 0.17$, $P = 0.68$).

The RMSSD was reduced during claw trimming (8.91 ± 1.18 ms) compared with the control situation (18.57 ± 2.93 ms; $F_{1,7.95} = 8.77$, $P = 0.02$; Fig. 6). Independent of treatment, RMSSD values were low at t_0 (10.33 ± 1.43 ms) and t_5 (13.95 ± 2.66 ms) and increased towards the end of the situation (t_{10}; 18.96 ± 5.24 ms; time: $F_{2,23.500} = 2.72$, $P = 0.09$). No breed effect was detectable (breed: $F_{1,19.500} = 0.25$, $P = 0.63$).

The ratio RMSSD/SDNN was lower during claw trimming (0.41 ± 0.03) compared with the control situation (0.60 ± 0.04; $F_{1,24.727} = 6.90$, $P = 0.02$; Fig. 7). Neither a time nor a breed effect was detectable (time: $F_{2,71.229} = 0.51$, $P = 0.60$; breed: $F_{1,23.221} = 0.02$,

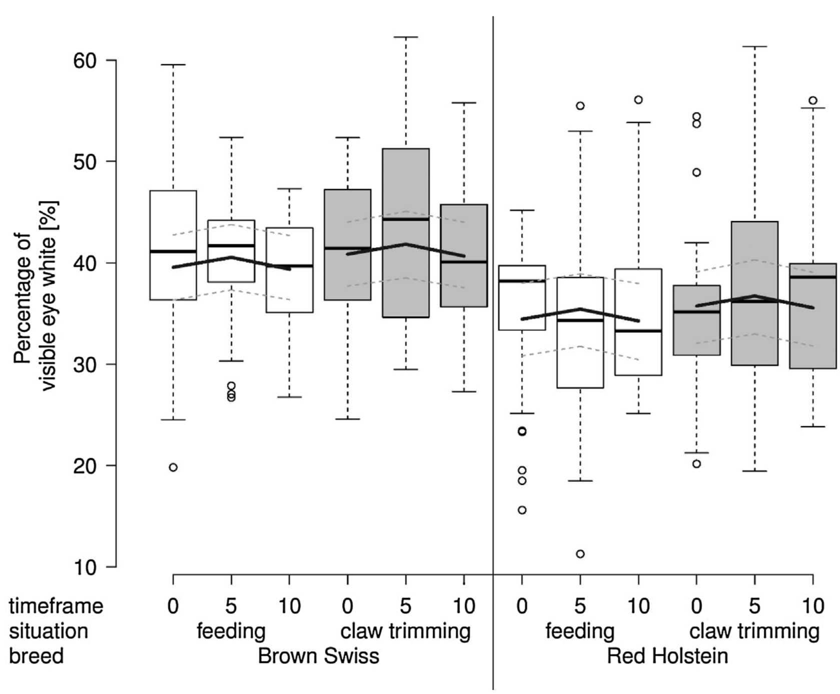

Fig. 3. Effect of treatment situation (claw trimming vs. feeding situation) and timeframe (before treatment [0 = t_0], during treatment [5 = t_5] and after treatment [10 = t_{10}]) on percentage of visible eye white.
Solid lines = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

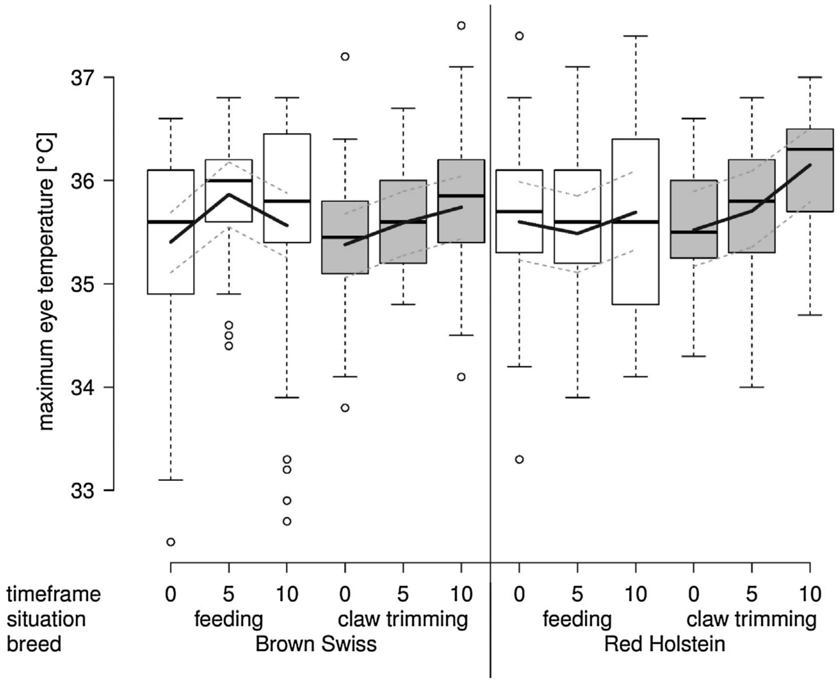

Fig. 4. Effect of treatment situation (claw trimming vs. feeding situation) and timeframe (before treatment [0 = t_0], during treatment [5 = t_5] and after treatment [10 = t_{10}]) on maximum eye temperature.
Solid line = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

P = 0.89).

3.4. Saliva cortisol concentration

Claw trimming led to a clear increase in saliva cortisol concentration (1.94 ± 0.15 ng/ml) compared with the control situation (0.35 ± 0.05 ng/ml), irrespective of breed (treatment: $F_{1,54} = 99.88$, $P < 0.0001$; breed: F1,54 = 0.73, P = 0.40; Fig. 8).

4. Discussion

Although not very high on an absolute scale, heart rate was increased during claw trimming. In addition, RMSSD and the RMSSD/SDNN ratio were decreased during claw trimming. These results are in agreement with earlier findings showing that a decrease in the values of the sympatho–vagal balance (RMSSD/SDNN) indicates a shift towards sympathetic activity due to a decrease of the RMSSD (Mohr et al., 2002; Porges, 1995a,b). Similar patterns were found in previous studies in cows (Gygax et al., 2008; Rushen et al., 2001; Wenzel et al., 2003).

According to the literature, cortisol concentration in saliva peaks at approximately 20 min after exposure to a stressor (Negrão et al., 2004). Our results correspond well with these earlier findings, with saliva cortisol concentration being increased 20 min after claw trimming. Similar claw trimming effects were also shown by Thun et al. (1998) and Szenci et al. (2011), with an increase of plasma cortisol levels in cows after treatment in a cattle crush.

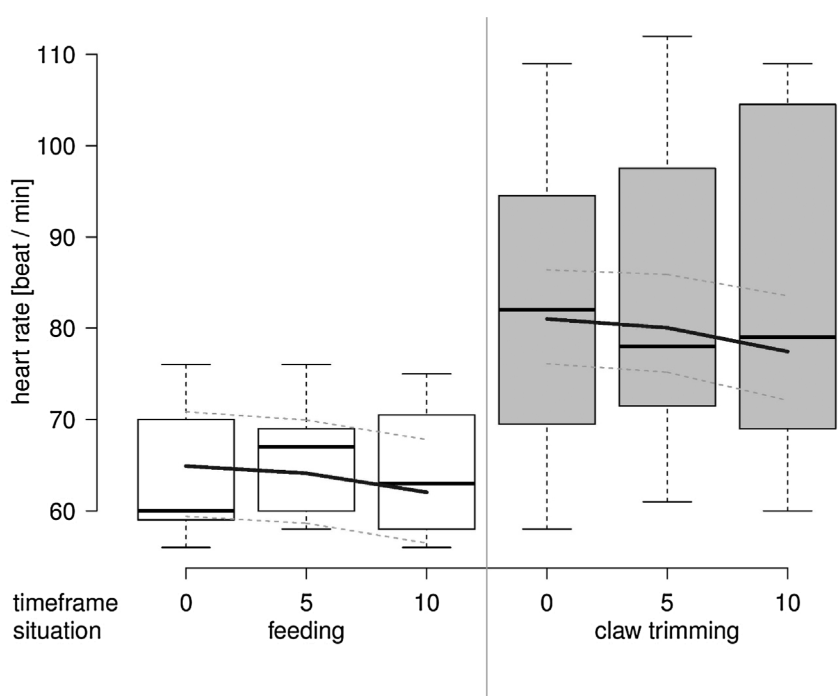

Fig. 5. Effect of treatment situation (claw trimming vs. feeding situation) and timeframe (before treatment [0 = t_0], during treatment [5 = t_5] and after treatment [10 = t_{10}]) on heart rate (beats per minute).
Solid line = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

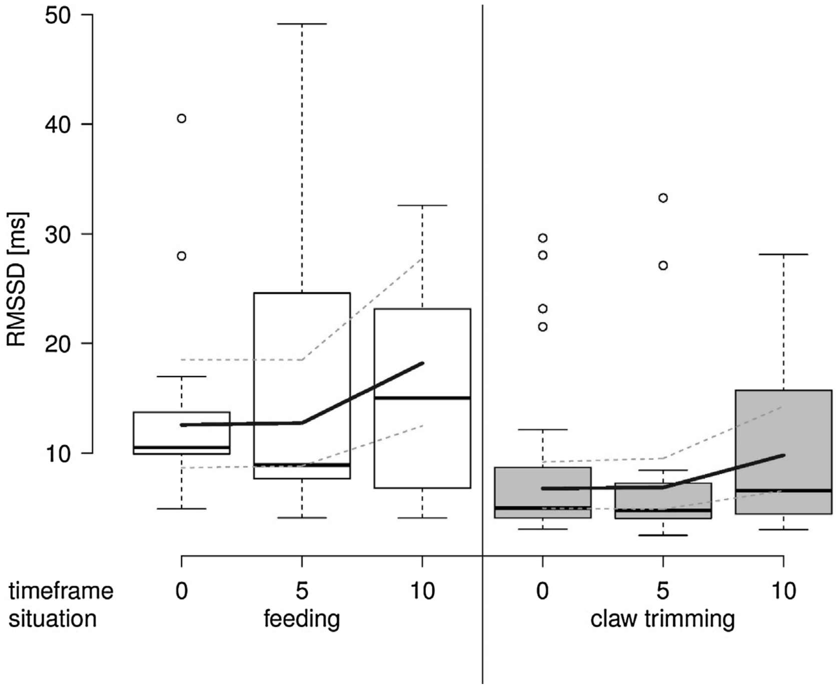

Fig. 6. Effect of treatment situation (claw trimming vs. feeding situation) and timeframe (before treatment [0 = t_0], during treatment [5 = t_5] and after treatment [10 = t_{10}]) on RMSSD (ms).
Solid line = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

However, we found no treatment effect for percentage of visible eye white. This finding contradicts previous studies. In food-deprived cows that experienced frustration through visual denial of food, the percentage of visible eye white was found to increase up to 60% within four minutes after food denial and did not reach baseline values (25% of visible eye white) six minutes thereafter (Sandem et al., 2002). In another experiment, the percentage of visible eye white increased up to 45% within one minute when cows were exposed to food denial (Sandem et al., 2006). Furthermore, the highest (30%) and the lowest (15%) percentages of visible eye white were detected four minutes after separation and reunion between cow and calf, respectively (Sandem and Braastad, 2005).

In our study, we used restraint in a cattle crush and claw trimming as stressful situation. This procedure may be perceived differently by the animals than food deprivation or cow-calf separation. All cows in our experiment were used to being restrained in the crush and to the claw trimming itself on a half-yearly basis. Nevertheless, heart beat parameters as well as cortisol concentrations revealed a stress reaction due to the procedure. Although the percentage of visible eye white in the cattle crush treatment was high (41%), eye white during feeding (considered as relaxed situation) was also higher (38%) than the baseline values found in previous studies (16–18% in Proctor and Carder, 2015; 25% in Sandem et al., 2002, 2004). During feeding at the feed rack, as used in our study, cows might express some arousal due to restraint and food competition.

The maximum eye temperature was only slightly affected by the claw trimming procedure. This result is in contrast to the clear evidence of stress-induced changes in maximum eye temperature found in

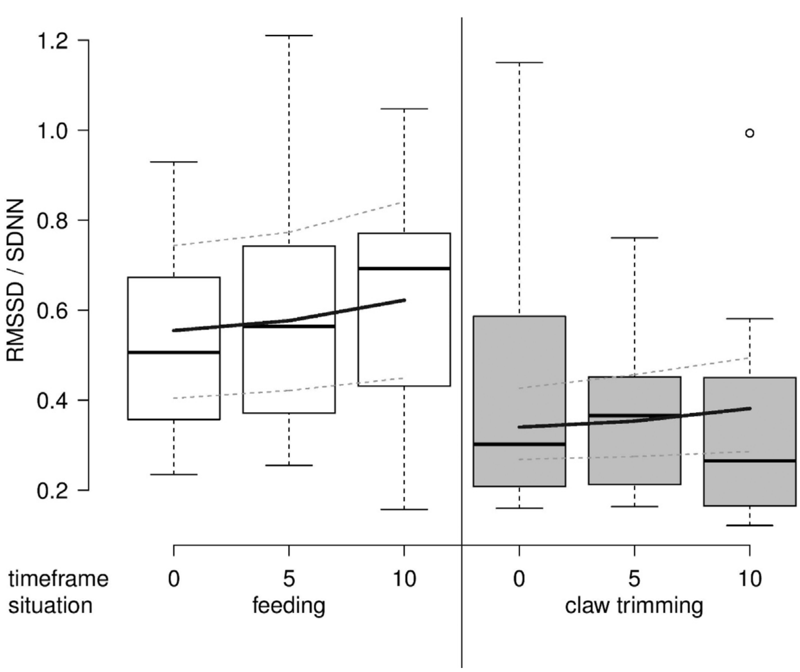

Fig. 7. Effect of treatment situation (claw trimming vs. feeding situation) and timeframe (before treatment [0 = t_0], during treatment [5 = t_5] and after treatment [10 = t_{10}]) on sympatho–vagal balance (ratio RMSSD/SDNN).
Solid line = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

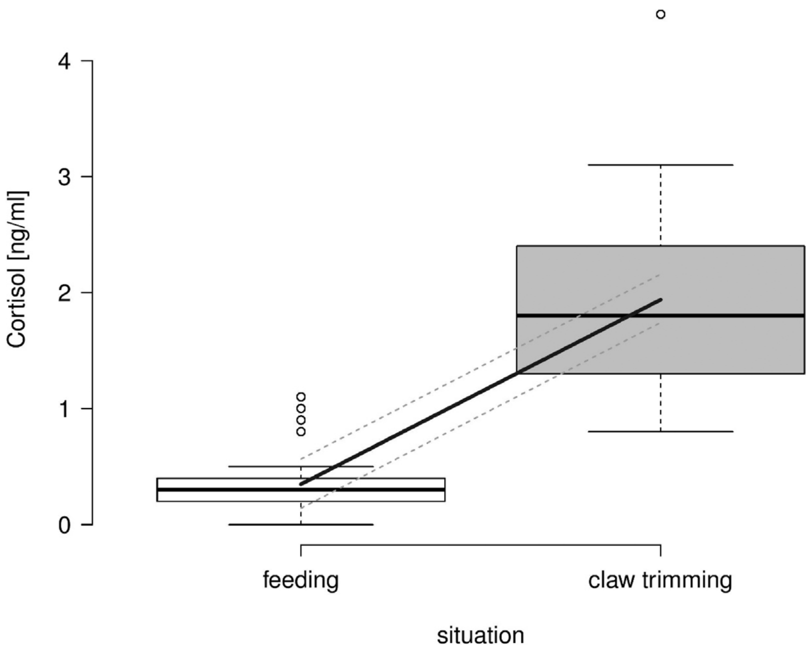

Fig. 8. Effect of treatment situation (claw trimming vs. feeding situation) on cortisol concentration (ng/ml) found in saliva samples.
Solid line = model estimate, dashed lines = 95% upper and lower confidence intervals of model estimate.

previous studies with exposure to pain, fear or other stressors (elk and reindeer: Cook et al., 2005; calves: Schaefer et al., 2003; cows: Castro, 2014; Stewart et al., 2007). Stewart et al. (2008a, b) found a sudden drop after hitting and startling within 1 min or disbudding within 2 min, respectively, with an increase thereafter. Although not reflected in a low *p*-value, stress induced changes in maximum eye temperature, as found in our study, were similar to stress-induced changes in maximum eye temperature in studies by Stewart and co-workers (0.23–0.66 °C; Stewart et al., 2008a,b, 2009).

In our study, both the duration of the stimulus itself and the latency between stimulus onset and picture capturing (it was not possible to take pictures while the stockmen were handling the animals) were considerably longer. We might have missed sudden changes in visible eye white or eye temperature immediately after the beginning of trimming. Nevertheless, we found an increase in the maximum eye temperature several minutes after the onset of claw trimming while the cows were still restrained.

We found breed-specific changes in the percentage of visible eye white and maximum eye temperature. Eyes of Brown Swiss cows have a darker sclera and thus less contrast between the white and the iris area than Holstein cows (see Fig. Z in Suppl. material). This phenotype might have masked a potential treatment effect. Furthermore, the breed-specific difference found here might reflect genetic differences in temperament of cattle breeds (Gutiérrez-Gil et al., 2008; Murphey et al., 1981; reviewed in Haskell et al., 2014). In dairy cows, physiological and behavioral responses to milking under stress, such as novel surrounding, varied depending on the temperament score (Sutherland et al., 2012; Van Reenen et al., 2002). In a previous study, Holstein dairy cattle were more sound- and touch-sensitive than beef cattle and this sensitivity was positively correlated with temperament score (Lanier et al., 2000). And dairy cows had less flight distance than beef cattle (Murphey et al., 1981). However, these studies compared dairy cows with beef cattle and not between different breeds of dairy cows (in our case: Holstein versus Brown Swiss).

5. Conclusions

Despite the clear support that claw trimming triggered a physiological stress response, the percentage of visible eye white was not found to reflect this stress response in a reliable way.

In addition, we detected only weak changes in the maximum eye temperature. Follow-up studies with shorter intervals between stimulus onset and picture capturing under different treatment situations might help to conclusively assess the suitability of these measures as non-invasive stress indicators.

Acknowledgments

Special thanks go to Lorenz Gygax for his statistical support, and Sarah Bieler for measuring eye white to allow testing for inter- and intra-observer agreements. We are also grateful to Marianne Cockburn and Melissa Terranova for their support in handling the animals and assistance in taking pictures and saliva samples, and to Christoph Bühler and Stephan Mathys for the professional claw trimming. The project was funded by the Federal Food Safety and Veterinary Office FSVO (project no 2.15.05).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.applanim.2017.10.001.

References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophoton. Int. 11 (7), 36–42.
Alam, M.G.S., Dobson, H., 1986. Effect of various veterinary procedures on plasma concentrations of cortisol, luteinizing hormone and prostaglandin E2 metabolite in the cow. Vet. Rec. 118 (1), 7–10.
Balocchi, R., Cantini, F., Varanini, M., Raimondi, G., Legramante, J.M., Macerata, A., 2006. Revisiting the potential of time-domain indexes in short-term HRV analysis. Biomed. Tech. 51 (4), 190–193.
Bartolomé, E., Sánchez, M.J., Molina, A., Schaefer, A.L., Cervantes, I., Valera, M., 2013. Using eye temperature and heart rate for stress assessment in young horses competing in jumping competitions and its possible influence on sport performance. Animal 7 (12), 2044–2053.
Cannon, W.B., 1935. Stresses and strains of homeostasis. Am. J. Med. Sci. 189, 1–14.
Castro, J., 2014. Measure of Stress in Hérens Cows Kept in Loose Housing Using Infrared Thermography Thesis. University of Bern, Switzerland.
Church, J.S., Hegadoren, P.R., Paetkau, M.J., Miller, C.C., Regev-Shoshani, G., Schaefer, A.L., Schwartzkopf-Genswein, K.S., 2014. Influence of environmental factors on infrared eye temperature measurements in cattle. Res. Vet. Sci. 96 (1), 220–226.
Cook, N.J., Schaefer, A.L., Warren, L., Burwash, L., Anderson, M., Baron, V., 2001. Adrenocortical and metabolic responses to ACTH injection in horses: an assessment by salivary cortisol and infrared thermography of the eye Abstract. Can. J. Anim. Sci. 81 (4), 621.
Cook, N.J., Church, J.S., Schaefer, A.L., Webster, J.R., Matthews, L.R., Suttie, J.M., 2005. Stress and pain assessment of velvet antler removal from elk (*Cervus elaphus canadensis*) and reindeer (*Rangifer tarandus*). J. Vet. Sci. 9 (1), 13–25.

Core, S., Widowski, T., Mason, G., Miller, S., 2009. Eye white percentage as a predictor of temperament in beef cattle. J. Anim. Sci. 87, 2168–2174.
Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006. Technical note: exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. J. Anim. Sci. 84 (11), 3100–3103.
Doube, M., Kłosowski, M.M., Arganda-Carreras, I., Cordelières, F.P., Dougherty, R.P., Jackson, J.S., Schmid, B., Hutchinson, J.R., Shefelbine, S.J., 2010. BoneJ: free and extensible bone image analysis in ImageJ. Bone 47 (6), 1076–1079.
Friend, T.H., 1980. Stress: what is it and how can it be quantified? Int. J. Study Anim. Probl. 1 (6), 366–374.
Gutiérrez-Gil, B., Ball, N., Burton, D., Haskell, M., Williams, J.L., Wiener, P., 2008. Mapping quantitative trait loci for temperament traits in cattle. J. Hered. 99, 629–638.
Gygax, L., Neuffer, I., Kaufmann, C., Hauser, R., Wechsler, B., 2008. Restlessness behaviour, heart rate and heart-rate variability of dairy cows milked in two types of automatic milking systems and auto-tandem milking parlours. Appl. Anim. Behav. Sci. 109 (2–4), 167–179.
Haskell, M.J., Simm, G., Turner, S.P., 2014. Genetic selection for temperament traits in dairy and beef cattle. Front. Genet. 5 (368), 1–18.
Hirsch, M., Karin, J., Akselrod, S., 1995. Heart rate variability in the fetus. In: Malik, M., Camm, A.J. (Eds.), Heart Rate Variability. Futura Publishing, Armonk (NY), pp. 517–531.
Kleiger, R.E., Stein, P.K., Bosner, M.S., Rottman, J.N., 1992. Time domain measurements of heart rate variability. Cardiol. Clin. 10 (3), 487–498.
Knizkova, I., Kunc, P., Koubkova, M., Flusser, J., Dolezal, O., 2002. Evaluation of naturally ventilated dairy barn management by a thermographic method. Livest. Prod. Sci. 77 (2–3), 349–353.
Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2016. lmerTest: Tests in Linear Mixed Effects Models. R package version 2. pp. 0–33. URL: https://CRAN.R-project.org/package=lmerTest.
Lanier, J.L., Grandin, T., Green, R.D., Avery, D., McGee, K., 2000. The relationship between reaction to sudden, intermittent movements and sounds and temperament. J. Anim. Sci. 78, 1467–1474.
Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23 (1–2), 67–74.
Mason, J.W., 1971. A re-evaluation of the concept of non-specificity in stress theory. J. Psychiatr. Res. 8 (3), 323–333.
McGreevy, P., Warren-Smith, A., Guisard, Y., 2012. The effect of double bridles and jaw-clamping crank nosebands on temperature of eyes and facial skin of horses. J. Vet. Behav. 7 (3), 142–148.
Moberg, G.P., 2000. In: Moberg, G.P., Mench, J.A. (Eds.), Biological Response to Stress: Implications for Animal Welfare. CABI Publishing, Wallingford, UK.
Mohr, E., Langbein, J., Nürnberg, G., 2002. Heart rate variability: a noninvasive approach to measure stress in calves and cows. Physiol. Behav. 75 (1–2), 251–259.
Mormède, P., Andanson, S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., Van Reenen, C.G., Richard, S., Veissier, I., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92 (3), 317–339.
Murphey, R.M., Moura Duarte, F.A., Torres Penedo, M.C., 1981. Responses of cattle to humans in open spaces: breed comparisons and approach-avoidance relationships. Behav. Genet. 11 (1), 37–48.
Negrão, J.A., Porcionato, M.A., de Passillé, A.M., Rushen, J., 2004. Cortisol in saliva and plasma of cattle after ACTH administration and milking. J. Dairy Sci. 87 (6), 1713–1718.
Owen, D., Andrews, M.H., Matthews, S.G., 2005. Maternal adversity, glucocorticoids and programming of neuroendocrine function and behavior. Neurosci. Biobehav. Rev. 29 (2), 209–226.
Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43 (1), 43–63.
Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welfare 21 (3), 331–337.
Patel, B.C.K., Gupta, R., Joos, Z.P., 2008. Eyelid anatomy. eMedicine. http://emedicine.medscape.com/article/834932-overview (last Accessed: 14.03.2017).
Patt, A., Gygax, L., Wechsler, B., Hillmann, E., Langbein, J., Keil, N.M., 2016. Context specificity of the ANS stress response during two regrouping experiments in goats. Front. Vet. Sci. 3 (article 58).
Pavlidis, I., Levine, J.A., Baukol, P., 2000. Thermal imaging for anxiety detection. IEEE 2, 315–318.
Pavlidis, I., Eberhardt, N.L., Levine, J.A., 2002. Human behavior: seeing through the face of deception. Nature 415, 35.
Pesenhofer, G., Palme, R., Pesenhofer, R.M., Kofler, J., 2006. Comparison of two methods of fixation during functional claw trimming—walk-in crush versus tilt table—in dairy cows using faecal cortisol metabolite concentrations and daily milk yield as parameters. Vet. Med. Austria 93 (1), 288–294.
Porges, S.W., 1995a. Cardiac vagal tone: a physiological index of stress. Neurosci. Biobehav. Rev. 19 (2), 225–233.
Porges, S.W., 1995b. Orienting in a defensive world: mammalian modifications of our evolutionary heritage. A polyvagal theory. Psychophysiology 32 (4), 301–318.
Proctor, H.S., Carder, G., 2015. Measuring positive emotions in cows: do visible eye whites tell us anything? Physiol. Behav 147, 1–6.
R Development Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria URL: https://www.R-project.org/.
Reefmann, N., Wechsler, B., Gygax, L., 2009. Behavioural and physiological assessment of positive and negative emotion in sheep. Anim. Behav. 78 (3), 651–659.
Rizk, A., Meyer, H., Zaghloul, A., Rehage, J., 2009. Use of Rompun during orthopaedic treatment of dairy cows in lateral recumbency. In: Proceedings of European Buiatrics Forum, Palais Du Pharo. Marseille, France. p. 116.
Rizk, A., Herdtweck, S., Offinger, J., Meyer, H., Zaghloul, A., Rehage, J., 2012. The use of xylazine hydrochloride in an analgesic protocol for claw treatment of lame dairy cows in lateral recumbency on a surgical tipping table. Vet. J. 192 (2), 193–198.
Rushen, J., Munksgaard, L., Marnet, P.G., DePassillé, A.M., 2001. Human contact and the effects of acute stress on cows at milking. Appl. Anim. Behav. Sci. 73 (1), 1–14.
Sandem, A.I., Braastad, B.O., 2005. Effects of cow–calf separation on visible eye white and behaviour in dairy cows—a brief report. Appl. Anim. Behav. Sci. 95 (3–4), 233–239.
Sandem, A.I., Braastad, B.O., Bøe, K.E., 2002. Eye white may indicate emotional state on a frustration–contentedness axis in dairy cows. Appl. Anim. Behav. Sci. 79 (1), 1–10.
Sandem, A.I., Janczak, A.M., Braastad, B.O., 2004. A short note on effects of exposure to a novel stimulus (umbrella) on behaviour and percentage of eye-white in cows. Appl. Anim. Behav. Sci. 89 (3–4), 309–314.
Sandem, A.I., Janczak, A.M., Salte, R., Braastad, B.O., 2006. The use of diazepam as a pharmacological validation of eye white as an indicator of emotional state in dairy cows. Appl. Anim. Behav. Sci. 96 (3–4), 177–183.
Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21 (1), 55–89.
Schaefer, A.L., Cook, N.J., Tessaro, S.V., Deregt, D., Desroches, G., Dubeski, P.L., Tong, A.K.W., Godson, D.L., 2003. Early detection and prediction of infection using infrared thermography. Can. J. Anim. Sci. 84 (1), 73–80.
Schaefer, A.L., Cook, N.J., Bench, C., Chabot, J.B., Colyn, J., Liu, T., Okine, E.K., Stewart, M., Webster, J.R., 2012. The non-invasive and automated detection of bovine respiratory disease onset in receiver calves using infrared thermography. Res. Vet. Sci. 93 (2), 928–935.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9 (7), 671–675.
Schwartzkopf-Genswein, K.S., Faucitano, L., Dadgar, S., Shand, P., González, L.A., Crowe, T.G., 2012. Road transport of cattle, swine and poultry in North America and its impact on animal welfare, carcass and meat quality: a review. Meat Sci. 92 (3), 227–243.
Selye, H., 1936. A syndrome produced by diverse nocuous agents. Nature 138, 32–33.
Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166 (4), 869–887.
Sollers, J.J., Buchanan, T.W., Mowrer, S.M., Hill, L.K., Thayer, J.F., 2007. Comparison of the ratio of the standard deviation of the r–r interval and the root mean squared successive differences (SD/rMSSD) to the low frequency-to-high frequency (LF/HF) ratio in a patient population and normal health controls. Biomed. Sci. Instrum. 43 (1), 158–163.
Stewart, M., Webster, J.R., Schaefer, A.L., Cook, N.J., Scott, S.L., 2005. Infrared thermography as a non-invasive tool to study animal welfare. Anim. Welfare 14 (4), 319–325.
Stewart, M., Webster, J.R., Verkerk, G.A., Schaefer, A.L., Colyn, J.J., Stafford, K.J., 2007. Non-invasive measurement of stress in dairy cows using infrared thermography. Physiol. Behav. 92 (3), 520–525.
Stewart, M., Schaefer, A.L., Haley, D., Webster, J.R., 2008a. Infrared thermography as a non-invasive method for detecting fear-related responses of cattle to different handling procedures. Anim. Welfare 17 (4), 387–393.
Stewart, M., Stafford, K.J., Dowling, S.K., Schaefer, A.L., Webster, J.R., 2008b. Eye temperature and heart rate variability of calves disbudded with or without local anaesthetic. Physiol. Behav. 93 (4–5), 789–797.
Stewart, M., Stookey, J.M., Stafford, K.J., Tucker, C.B., Rogers, A.R., Dowling, S.K., Verkerk, G.A., Schaefer, A.L., Webster, J.R., 2009. Effects of local anesthetic and a nonsteroidal antiinflammatory drug on pain responses of dairy calves to hot-iron dehorning. J. Dairy Sci. 92 (4), 1512–1519.
Stewart, M., Verkerk, G.A., Stafford, K.J., Schaefer, A.L., Webster, J.R., 2010. Noninvasive assessment of autonomic activity for evaluation of pain in calves, using surgical castration as a model. J. Dairy Sci. 93 (8), 3602–3609.
Sutherland, M.A., Rogers, A.R., Verkerk, G.A., 2012. The effect of temperament and responsiveness towards humans on the behavior, physiology and milk production of multi-parous dairy cows in a familiar and novel milking environment. Physiol. Behav. 107, 329–337.
Szenci, O., Karen, A., Bajcsy, Á.C., Gáspárdy, A., de Sousa, N.M., Beckers, J.F., 2011. Effect of restraint stress on plasma concentrations of cortisol, progesterone and pregnancy associated-glycoprotein-1 in pregnant heifers during late embryonic development. Theriogenology 76 (8), 1380–1385.
Thun, R., Kaufmann, C., Janett, F., 1998. The influence of restraint stress on reproductive hormones in the cow. Reprod. Domest. Anim. 33 (3–4), 255–260.
Ursin, H., Olff, M., 1993. Psychobiology of coping and defence strategies. Neuropsychobiology 28 (1–2), 66–71.
Van Reenen, C.G., Van der Werf, J.T.N., Bruckmaier, R.M., Hopster, H., Engel, B., Noordhuizen, J.P.T.M., Blokhuis, H.J., 2002. Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking. J. Dairy Sci. 85, 2551–2561.
Wang, H.-M., Huang, Sh.-Ch., 2012. SDNN/RMSSD as a surrogate for LF/HF: a revised investigation. Model. Simul. Eng. 2012, 1–8.
Webster, J.R., Stewart, M., Rogers, A.R., 2008. Assessment of welfare from physiological and behavioural responses of New Zealand dairy cows exposed to cold and wet conditions. Anim. Welf. 17 (1), 19–26.
Wenzel, C., Schönreiter-Fischer, S., Unshelm, J., 2003. Studies on step–kick behavior and stress of cows during milking in an automatic milking system. Livest. Prod. Sci. 83 (2-3), 237–246.
Yu, Y., Lin, L., 2012. Agreement: Statistical Tools for Measuring Agreement. R package version 0. pp. 1–8. URL: https://CRAN.R-project.org/package=Agreement.