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The influence of echocardiography as a stressful manipulation on substance P, cortisol, and behavior in calves – A pilot study

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ABSTRACT

The aim of this pilot study was to investigate the effects of a stressful stimulus on plasma substance P concentrations (PSPC), salivary substance P (SSPC), plasma cortisol (PCC), and glucose concentrations, behavioral parameters, and milk intake in calves. Animals in STRESS ($n = 12$) were exposed to restrainment in a crush and ultrasonographic examination. Animals in CON ($n = 12$) remained in their igloo. Sampling was done prior (60 min before, 0 min), during (5 to 30 min), and after stimulation (35 min to 2 h). Activity and milk intake were assessed over 24 h; behavior during the stimulus and at sampling times. PSPC and PCC did not differ between groups. In STRESS, PSPC were significantly lower at 5 and 20 min compared with 60 min before. SSPC were below the sensitivity of the ELISA in 34 % of samples. SSPC were significantly higher in CON compared with STRESS at 0, 5, 10, 20, and 30 min, and at 2 h. In STRESS, SSPC were significantly lower at 30 min compared with 60 min before. There was no correlation between PSPC and SSPC. PCC were significantly higher at 20 min compared with 60 min before in STRESS, and significantly lower at 10 and 25 min compared with 60 min before in CON. Glucose concentrations were significantly lower in STRESS compared with CON at 5 and 10 min and differed significantly within groups. "Ear movement", "limb movement", and "urination" was significantly higher in STRESS compared with CON. Milk intake and activity did not differ between groups.

1. Introduction

In bovine medicine, cortisol has been considered as the predominant objective parameter for the assessment of stress and pain for a long time (Coetzee et al., 2008). However, cortisol levels rise due to both pain-related stress as well as distress due to handling and other factors (Karlen et al., 2021). Previous research showed that simultaneous measurements of plasma concentrations of both substance P (SP) and cortisol should be done to differentiate between stress and nociception (Coetzee et al., 2008; Karlen et al., 2021).

SP is a sensory neurotransmitter of the family of tachykinins (DeVane, 2001) and consists of 11 amino acids (Chang et al., 1971). In 2008, SP has been described as an objective biomarker for nociception in cattle (Coetzee et al., 2008). However, research in human medicine showed that SP also has pro-inflammatory effects (O'Connor et al., 2004) and is considered as an inflammatory marker (Xu et al., 2000). Furthermore, SP regulates the excitability of the nociceptive neurons of the dorsal horn and can be found in specific areas of the neuroaxis, which are involved in the integration of anxiety, stress, and pain (DeVane, 2001). Studies showed that post-traumatic stress disorder (PTSD) (Geracioti et al., 2006) and depression (Bondy et al., 2003) were associated with increased SP levels. (Ebner and Singewald, 2006) demonstrated that emotional stressors trigger the in vivo release of SP in brain areas known to be implicated in stress and anxiety mechanisms. According to (DeVane, 2001), animal models have shown that SP is released as a part of the response to stress. However, all the above-mentioned studies either originate from research in humans or have been conducted in laboratory animals. In bovine medicine, SP has been used as a biomarker for nociception, and its course has been assessed during various painful procedures such as castration (Dockweiler et al., 2013), dehorning (Allen et al., 2013), umbilical surgery (Tschoner et al., 2018), and tail docking (Mayer, 2019) in calves, and electroejaculation (Whitlock et al., 2012), ovariectomy (Lauder et al., 2020), and endoscopic abomasopexy (Tschoner et al., 2020) in adult cattle – all of which are pain- as well as stressful, and result in inflammation. No basic research on SP as a biomarker for nociception in the bovine has been done so far.

Echocardiography is described to be the most useful tool for the

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diagnosis of (congenital) heart diseases, and can be easily performed in calves (Mitchell and Schwarzwald, 2016).

The hypothesis of this study was that stress in form of restrainement and an echocardiography result in an increase of cortisol, glucose, heart rate, and respiratory rate, but not substance P. The objectives of this pilot study were to 1) evaluate plasma substance P concentrations (PSPC) in healthy German Simmental calves which were submitted to a stressful stimulus in form of restrainment in a crush and an ultrasonographic examination of the heart, compared with a control group, 2) assess if there is a correlation of SP concentrations in samples taken from blood and saliva (SSPC), and 3) describe plasma cortisol concentrations (PCC), glucose concentrations, and changes to heart rate, respiratory rate, and behavior during and after the trial period.

2. Material and methods

The ethics committee of the government of Upper Bavaria approved all the experimental procedures of the current study (reference number 55.2-2532-Vet_02_20-188). The care and use of all experimental animals complied with local welfare laws, guidelines, and policies. The present study is a pilot study. Therefore, 12 animals were included per group, as recommended by (Hertzog, 2008; Julious, 2005; Van Belle, 2008). Sample collection was done from January 2023 to May 2024. Each animal was subject to a different study day. The experiment was conducted at the Clinic for Ruminants with Ambulatory and Herd Health Services, Bavaria, Germany (48°14′31.202″ N, 11°32′54.469″ E).

2.1. Animals and group assignment

In the present study, a total of 24 calves were included. All calves were male and of the German Simmental breed (at least ≥75 %). Animals were bought from farms in Bavaria, either directly ($n = 2$) or via a market ($n = 22$) in Miesbach, Germany. On the day of purchase, calves were 33 ± 3 (28 to 38) days old and weighing 80.3 ± 6.8 (70 to 105) kg. To be able to assess PSPC concentrations without any interference of nociception or inflammation, all calves needed to be clinically healthy to be included in this study.All animals were clinically examined before being purchased. Immediately after arriving at the Clinic for Ruminants with Ambulatory and Herd Health Services, the calves were subjected to a clinical examination and blood samples were taken for a laboratory blood analysis. Furthermore, all animals where treated with a live vaccine for Bovine respiratory syncytial virus (BRSV) and Bovine Parainfluenza-3 (PI3) infection (2 ml/calf intranasally, Bovilis® Intra-Nasal RSP™ Live, Intervet Deutschland GmbH, Unterschleißheim, Germany) (except animals 4, 5 and 6) and vitamin E and selenium (2–4 ml/calf s.c., Vitamin E + Selen, Bela-Pharm gmbH & Co. KG, Vechta, Germany) (except animals 4 and 5) on the day of arrival, and with Diclazuril (1 mg/kg BW orally, Diacox® 2,5 mg/ml, Virbac Tierarzneimittel GmbH, Bad Oldesloe, Germany) (except animals 4, 5 and 6) on the following day. Exclusion criteria for the trial were acute or chronic diseases and/or treatment with an antibiotic or NSAID within 48 h prior to the trial. Treatment of calves with either antibiotics or analgesics prior to the trial is given in Appendix A. The study was conducted as a randomized controlled trial, calves were either allocated to the trial group (STRESS, $n = 12$) or the control group (CON, n = 12). Randomization consisted of a lottery with equal numbers of lots for CON and STRESS in sealed envelopes, which were kept locked. On the day before each trial, one envelope was randomly chosen by one of two authors (TT or MF) for group assignment. Due to the study setting, researchers knew the group assignment of the animals during the trial.

2.2. Housing and husbandry

Calves were housed in individual igloos with visual and hearing contact to other calves. Calves were kept on straw and had free access to water, hay, mineral licks and concentrates during the entire time. Calves bought from local markets were fed with whole milk due to not knowing the feeding management at their original farm, calves originating from the Research- and Teaching Center Achselschwang with milk replacer, which was fed ad libitum at the farm of origin. Depending on the feeding management, all calves were fed 3 (to 6, Achselschwang) times per day (07:00 a.m., 12:00 p.m., 07:00 p.m.) with 3 l (2 l for 6 feeding times) of milk per feeding. Milk intake was determined as volume (liters) of milk. For comparison between groups, milk intake was assessed in percentage. All animals were examined by a veterinarian at least once daily.

2.3. Acclimatization period

Upon request of the government of Upper Bavaria, calves received an acclimatization period of 9.25 ± 2.93 (7 to 15) days until the day of the trial. On the day before the trial day, a clinical examination was carried out and calves were then sedated with xylazine (0.02 mg/kg BM i.v.; XYLAZIN 2 % Bernburg®, Serumwerk Bernburg, DE). The skin was locally infiltrated with 2 ml procaine 2 % (Procasel-2 %, Selectavet, Germany) and a 16-gauge × 15 cm catheter (PUR infusion catheter, Walter Veterinär - Instrumente e. K., Baruth/Mark, Germany) was placed in the right jugular vein. Blood samples were taken to determine PCV, hemoglobin, leukocyte count, total protein, and glucose.

2.4. Experimental setup

On the day of the trial, clinical examination was done at 08:00 a.m. At 08:45 a.m., calves of the STRESS group were brought into the examination room and put into a crush in a standing position, with their heads fixed with a halter. The area around the heart was then clipped. From 09:00 a.m. to 09:30 a.m., animals assigned to STRESS were exposed to a stressful stimulus, which consisted of restrainment in a crush and an ultrasonographic examination of the heart using an ultrasonic device (LOGIQ P6 PRO, GE Healthcare Japan Corporation, Japan) with a cardiac probe. The echocardiography was not done for any diagnostic purposes, as all animals were clinically healthy, but as an additional stressor to the restrainement in the crush. At 09:35 a.m., calves of the control group were moved out of the crush and then led back into the igloo at 09:40 a.m., where they stayed until the end of the trial. Animals of CON remained in their igloo for the whole duration of the trial. The setup of the trial is given in Fig. 1.

2.5. Collection of blood and saliva samples

Schedule of collection of blood and saliva samples is presented in Table 1. Samples are referred to as time from the baseline (60 min before) in hours and minutes. Prior to each blood sampling, the intravenous catheter was flushed with 5 ml of 0.9 % saline. It was then flushed with blood for 3 times, and 5 ml of blood were discarded. Blood samples were then taken with a new syringe and a bloodgas tube, and the catheter was flushed again with 10 ml of 0.9 % saline. Blood samples for assessment of PSPC and PCC were transferred to 2 ml EDTA tubes right after sampling. EDTA tubes for SP samples were spiked with 9 µl aprotinin per tube and were kept in a refrigerator or on ice at all times. Blood samples for both SP and cortisol were kept on ice and brought to the laboratory of the Clinic for Ruminants with Ambulatory and Herd Health Services. All samples were centrifuged within 2 h after blood collection (4 °C, 1600 ×*g* for 15 min). Blood plasma was kept at −80 °C until analysis.

To investigate a less invasive way of assessing SP concentrations in cattle, saliva samples for SSPC were taken using salivettes (Sarstedt AG & Co. KG, Nümbrecht, Germany), which were put in the calf's mouth to be chewed on for 1 min as described by (Mayer, 2019). These samples were immediately transferred to the laboratory for centrifugation (4 °C, 1000 ×*g* for 2 min). Aprotinin was added after centrifugation (Mayer, 2019) Samples were stored at −80 °C until analysis.

Fig. 1. Experimental setup for the assessment of the effect of a stressful stimulus on substance P, cortisol, and glucose concentrations, as well as behavioral parameters, activity, and milk intake in healthy German Simmental calves. The housing and handling was the same for all calves except during the stressful stimulation. Calves in the control group (A, CON, n = 12) were left in their igloo for the whole duration of the trial. Calves in the trial group (B, STRESS, n = 12) were stressed by being submitted to restrainment in a crush and an ultrasonographic examination of the heart.

Table 1
Schedule of sampling to assess substance P, cortisol, and glucose concentrations in the blood plasma, and substance P concentrations in the saliva in $n = 24$ German Simmental calves submitted to either a stressful or a sham stimulus.

Time	Sample	Procedure
08:00 a.m.	+00:00	Baseline, in igloo
09:00 a.m.	+01:00	Standing in crush
09.05 a.m.	+01:05	Ultrasonographic examination of heart in crush
09:10 a.m.	+01:10	
09:15 a.m.	+01:15	
09:20 a.m.	+01:20	
09:25 a.m.	+01:25	
09:30 a.m.	+01:30	Standing in crush
09:35 a.m.	+01:35	Standing next to crush
09:45 a.m.	+01:45	After being brought back to igloo
10:00 a.m.	+02:00	In igloo
10:30 a.m.	+02:30	
11:00 a.m.	+03:00	

2.6. Substance P, cortisol, and glucose analysis

Determination of PSPC and salivatory SP concentrations were done using a Substance P ELISA kit (ENZO®, Enzo Life Sciences GmbH, DE) as done previously by (Landinger et al., 2024; Tschoner et al., 2020). Optical densities were determined in duplicate, and means were used for the calculation of concentrations. Lower and upper limits of quantification for the Substance P ELISA Kit were 9.8 pg/ml and 9,687.6 pg/ml. The sensitivity was 102.3 pg/ml and the intra- and interassay coefficient of variation was calculated to be <28 %; otherwise, a repeat measurement was performed, with an interassay of 26 %.

For determination of PCC, a Cortisol ELISA Kit (DRG Instruments GmbH, DE) was used. Optical densities were determined in duplicate, and means were used for the calculation of concentrations. Lower and upper limits of quantification for the cortisol ELISA Kit were 153.0 pg/ml and 9910.0 pg/ml. The sensitivity was 501.9 pg/ml and the intra- and interassay coefficient of variation was calculated to be <16 %; otherwise, a repeat measurement was performed, with an interassay of 40 %.

Blood samples for assessment of glucose concentrations were analyzed right after sampling using the RAPIDPoint®500e Blood Gas System (Siemens Healthineers International AG, Zürich, Switzerland). Body temperature for glucose analysis was taken rectally with a thermometer prior to the first blood sample of the trial.

2.7. Assessment of physiological parameters and behavior

Behavioral scoring was done at all sampling times and always by the same person (HK). Behavioral scoring included the assessment of physiological parameters (heart rate (**HR**), using a stethoscope), respiratory rate (**RR**, standing on the right side of the animal and counting the breaths), and an ethogram including the evaluation of behavior, dorsal line of the back, and expression of eyes, nose, and ears, modified according to (Feist et al., 2008; Gleerup et al., 2015). Individual aspects of behavior were recorded as either “yes” or “no” (Appendix B). From 0 min to 35 min (09:00 a.m. to 09:35 a.m.) behavior of the calves was recorded using a video camera (Panasonic HC-V785 Camcorder) to assess the frequency of different parameters over a period of 35 min. Behavioral parameters assessed were movement of the ears and head, limb movements, sequence of movements, tail movements, vocalization, teeth grinding, urinating, or defecation, modified as described by (Rizk et al., 2012) (Appendix C). To limit interobserver bias, video recordings were always evaluated by the same person (HK).

2.8. Activity and number of steps

Activity and number of steps was recorded using a pedometer and analyzed using the associated software (RumiWatch Systems®, Itin and Hoch GmbH, Liestal, Switzerland). The pedometers continuously recorded lying, standing, and walking activity, including number of steps, and have been described for use in calves in recent literature (Mayer, 2019). All calves were fitted with the pedometer at 08:00 a.m. on the trial day. Pedometers were attached on either the left or right hind limb proximal to the fetlock joint. Data recording with the pedometer was done for 24 h until 08:00 a.m. on the day following the trial.

2.9. Statistical analysis

Data analysis was performed using R 4.4.0 (2024-04-24) statistical software. Linear mixed effects models were used to study PSPC, SSPC, PCC, glucose concentrations, HR, and RR. The predictors “time” (60 min before:2 h) and “group” (CON vs. STRESS) were used as fixed effects with an interaction between them and with a random effect of an individual animal due to the repeated measures of every animal over time. Normality and homoscedasticity of residuals were assessed via visual residual-diagnostics after the models fit. Due to the not-normally distributed and heteroskedastic residuals data for PSPC, SSPC and PCC was log-transformed. The generalized linear and robust linear mixed-effects models were conducted for every analysis and compared via Akaike's Information Criterion (AIC) and R^2. The best model was used

for further analysis and post-hoc tests where timepoint and groups were compared. The correlation between PSPC and PCC (across all groups—CON and PAIN—and all timepoints) was assessed using Spearman's correlation on logarithmically transformed data (both PSPC and PCC were logarithmized).

The differences in behavioral and activity parameters and milk intake between unpaired control and stress groups were assessed via the unpaired two sample *t*-test in case of normally distributed data and via Wilcoxon rank sum (Mann-Whitney U) test in case of not normally distributed data. Difference between groups for categorical behavior and activity predictors and milk intake were studied via Fisher's exact tests.

Results with a p-value < 0.05 were considered statistically significant. Due to the exploratory approach of the study and small sample size, correction of the P value for multiple comparisons was not performed, in order to decrease the probability of Type 2 error (missing a discovery).

3. Results

3.1. Clinical examination and laboratory findings on the day before trial

Age of calves on the day prior to the trial was 41 ± 3 (35–46) days. Findings of clinical examination and selected blood parameters are presented in Appendix D.

3.2. Plasma substance P concentrations

A total of 2 analysis of concentrations had to be repeated due to duplicates of optical densities varying more than 15 % ($n = 2$ for CON), and one sample needed to be diluted 1:20 ($n = 1$ for STRESS) because PSPC were exceeding 10,000 pg/ml. Detailed information about missing/repeated data is presented in Appendix E.1.

Mean PSPC (with lower and upper CI) were 828.8 (639.1–1074.9) pg/ml in CON and 757.5 (584.1–982.4) pg/ml in STRESS 60 min before intervention. Course of PSPC concentrations is presented in Fig. 2. In STRESS, PSPC were significantly lower at 5 min (632.7 pg/ml, $p = 0.0400$) and 20 min (632.7 pg/ml, $p = 0.0416$) compared with 60 min before. There were no significant differences between groups at any time point. Mean PSPC concentrations are presented in Appendix F, and further significant differences within groups are given in Appendix G.

3.3. Substance P concentrations in saliva

SSPC were below the sensitivity of the ELISA kit in 106 samples in 12 animals ($n = 22$ for CON and $n = 83$ for STRESS). A total of 10 analysis of concentrations had to be repeated due to duplicates of optical densities varying more than 15 % ($n = 7$ for CON and $n = 4$ for STRESS). Detailed information about missing/repeated data is presented in Appendix E.2.

Mean SSPC (with lower and upper CI) were 38.1 (22.6–64.7) pg/ml in CON, and 22.4 (13.2–38.1) pg/ml in STRESS 60 min before intervention. Course of SSPC concentrations is presented in Fig. 3. In STRESS, SSPC were significantly lower at 30 min (11.8 pg/ml, $p = 0.0223$) compared with 60 min before intervention. SSPC were significantly higher in CON compared with STRESS at 0 min (32.5 and13.5 pg/ml, $p = 0.0211$), 5 min (41.3 and. 13.1 pg/ml, $p = 0.0027$), 10 min (39.3 and 13.3 pg/ml, $p = 0.0047$), 20 min (40.4 and 13.5 pg/ml, $p = 0.0041$), 30 min (40.4 and 11.8 pg/ml, $p = 0.0012$), and 2 h (41.3 and 17.8 pg/ml, $p = 0.0276$). There was no correlation between PSPC and SSPC ($P = 0.12$). Mean SSPC concentrations are presented in Appendix F, and further significant differences within groups are given in Appendix G.

3.4. Correlation between plasma substance P and cortisol concentrations

There was a positive correlation between PSPC and PCC, which was significant ($p < 0.001$, rho = 0.21). In CON, there was a positive correlation with a trend for significance ($p = 0.05$, rho = 0.15). In STRESS, the positive correlation was significant ($p = 0.007$, rho = 0.21).

3.5. Plasma cortisol concentrations

A total of 69 samples in 10 animals needed to be diluted 1:20 ($n = 31$ for CON and $n = 38$ for STRESS). For PCC, a total of 9 analysis of concentrations had to be repeated due to duplicates of optical densities varying more than 15 % ($n = 9$ for CON). Detailed information about missing/repeated data is presented in Appendix E.3.

Mean PCC (with lower and upper CI) were 2059.1 (1032.8–4105.2) pg/ml in CON, and 2038.6 (1022.5–4064.3) pg/ml in STRESS 60 min

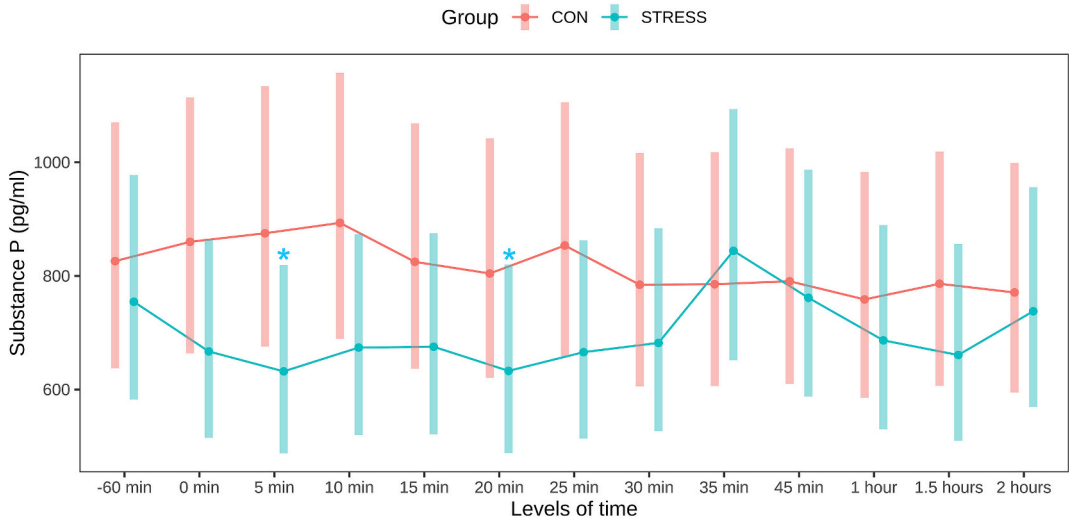

Fig. 2. Course of plasma substance P concentration in calves in CON (n = 12, without stimulus, in red) and STRESS ($n = 12$, stressful stimulus, blue). Points represent mean values with bars representing the 95 % confidence intervals. Basal blood samples were taken at the igloo 60 min (min) before intervention. No stimulus while remaining in the igloo (CON)/stressful stimulation (STRESS) was done from 09:00 a.m. (0 min) to 09:30 a.m. (30 min). (45 min) onwards, animals of STRESS were back in their igloos. There were no significant differences between groups. Significant differences ($p < 0.05$) within groups compared with 60 min before intervention are indicated either with a red (CON) or blue (STRESS) asterisk *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

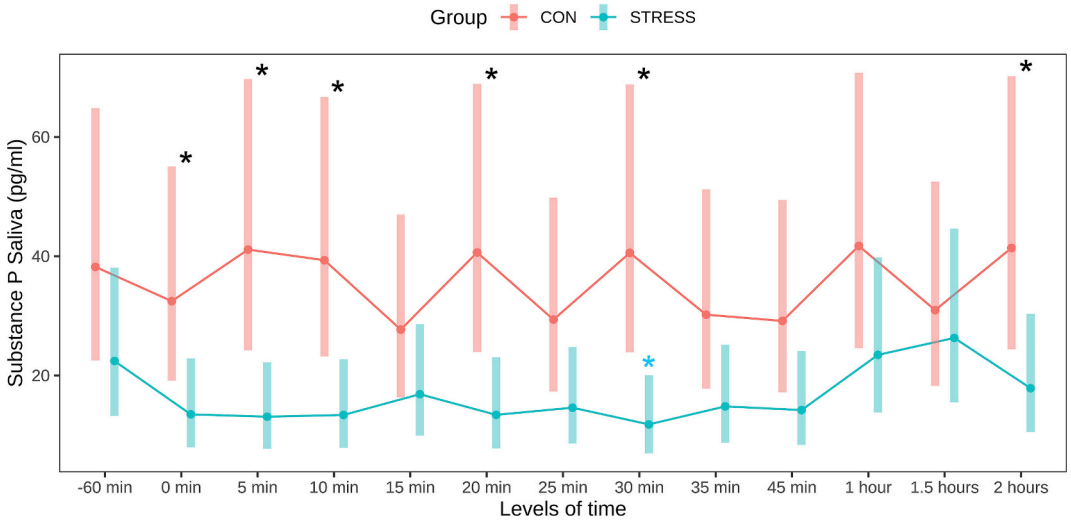

Fig. 3. Course of substance P saliva concentration in calves in CON (n = 12, without stimulus, in red) and STRESS (n = 12, stressful stimulus, blue). Points represent mean values with bars representing the 95 % confidence intervals. Basal blood samples were taken at the igloo 60 min (min) before intervention. No stimulus while remaining in the igloo (CON)/stressful stimulation (STRESS) was done from 09:00 a.m. (0 min) to 09:30 a.m. (30 min). From 09:45 a.m. (45 min) onwards, animals of STRESS were back in their igloos. Significant differences ($p < 0.05$) between groups are indicated with asterisk *, and within groups compared with 60 min before intervention with a red (CON) or blue (STRESS) asterisk *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

before intervention. Course of SSPC concentrations is presented in Fig. 4. In CON, PCC were significantly lower at 10 min (1236.5 pg/ml, $p = 0.0342$) and 25 min (1164.4 pg/ml, $p = 0.0171$) compared with 60 min before intervention. In STRESS, PCC were significantly higher at 20 min (3294.5 pg/ml, $p = 0.0460$) compared with 60 min before intervention. There were no significant differences between PCC between groups at any time point. Mean PCC concentrations are presented in Appendix H, and further significant differences within groups are given in Appendix G.

3.6. Glucose concentrations in the blood

Mean glucose concentrations (with lower and upper CI) were 6.03 (5.57–6.48) mmol/lt in CON and 6.03 (5.57–6.48) mmol/lt in STRESS 60 min before intervention. Course of glucose concentrations is presented in Fig. 5. In CON, mean glucose concentrations were significantly higher at 0 min (6.51 mmol/lt, $p = 0.0118$) and 5 min (6.42 mmol/lt, $p = 0.0408$) compared with 60 min before intervention. In STRESS, blood glucose concentrations were significantly lower at 20 min (5.62 mmol/lt, $p = 0.0367$), 25 min (5.64 mmol/lt, $p = 0.0452$), 1.5 h (5.57 mmol/lt, $p = 0.0168$), and 2 h (5.47. mmol/lt, $p = 0.0042$) compared with 60 min before intervention. Glucose concentrations were significantly higher in CON compared with STRESS at 5 min (6.42 and 5.74 mmol/lt, $p = 0.0403$) and 10 min (6.33 and 5.67 mmol/lt, $p = 0.0480$). Mean glucose concentrations are presented in Appendix G, and further significant differences within groups are given in Appendix I.

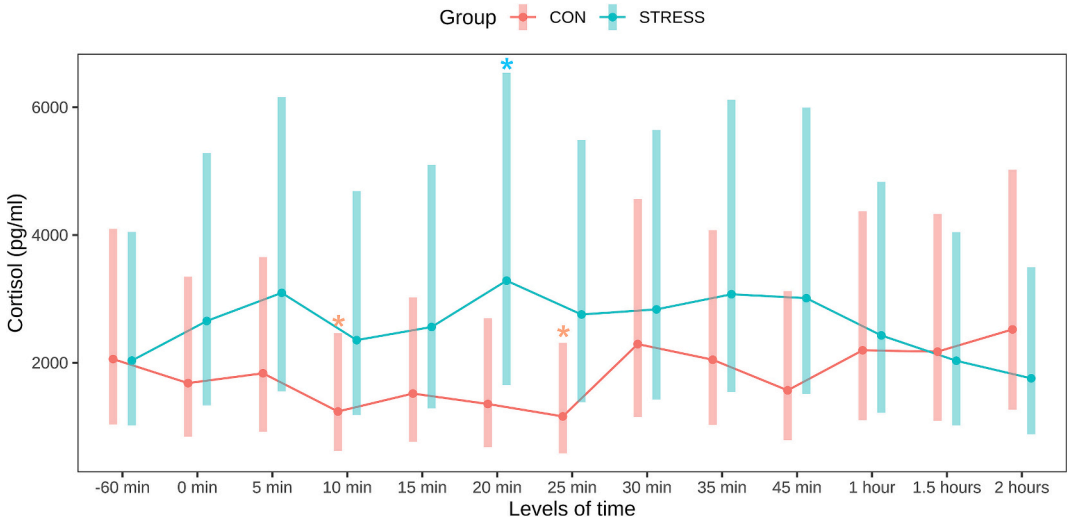

Fig. 4. Course of plasma cortisol concentration in calves in CON (n = 12, without stimulus, in red) and STRESS (n = 12, stressful stimulus, blue). Points present mean values with bars representing the 95 % confidence intervals. Basal blood samples were taken at the igloo 60 min (min) before intervention. No stimulus while remaining in the igloo (CON)/stressful stimulation (STRESS) was done from 09:00 a.m. (0 min) to 09:30 a.m. (30 min). From 09:45 a.m. (45 min) onwards, animals of STRESS were back in their igloos. There were no significant differences between groups. Significant differences ($p < 0.05$) within groups compared with 60 min before intervention are indicated either with a red (CON) or blue (STRESS) asterisk *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

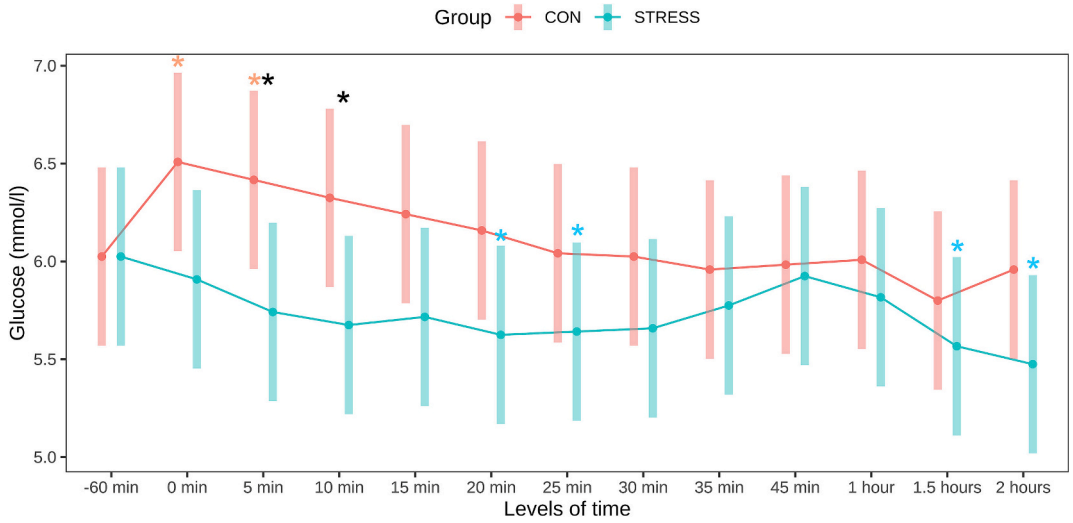

Fig. 5. Course of glucose concentration in calves in CON (n = 12, without stimulus, in red) and STRESS (n = 12, stressful stimulus, blue). Mean glucoses concentrations are given with 95 % confidence intervals. Basal blood samples were taken at the igloo 60 min (min) before intervention. No stimulus while remaining in the igloo (CON)/stressful stimulation (STRESS) was done from 09:00 a.m. (0 min) to 09:30 a.m. (30 min). From 09:45 a.m. (45 min) onwards, animals of STRESS were back in their igloos. Significant differences ($p < 0.05$) between groups are indicated with asterisk *, and within groups compared with 60 min before intervention with a red (CON) or blue (STRESS) asterisk *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. Behavioral scoring during stressful stimulus

Data for behavioral assessment during the stressful stimulus is missing in one animal (18, STRESS) due to technical issues with the camera.

Median frequencies of behavioral parameters evaluated for the duration of either the stressful stimulus (STRESS) or in animals remaining in their igloo (CON) with significant differences are presented in Table 2. Frequency of "ear movements", "movements of limbs", and "urination" was significantly higher in STRESS compared with CON ($p = 0.03$, $p = 0.00343$, and $p = 0.00434$, respectively).

3.8. Physiological parameters on the day of trial

RR assessed for behavior is missing for 18 timepoints due to animals sniffling and investigators not being able to count a RR ($n = 9$ for CON and n = 9 for STRESS). Detailed information about missing/repeated data is presented in Appendix E.4.

Mean HR (with lower and upper CI) was 115.1 (104.8–125) beats/min in CON and 122.6 (112.3–133) beats/min in STRESS. In CON, HR was significantly lower at 10 min (105.5 beats/min, $p = 0.0166$), 25 min (106.1 beats/min, $p = 0.0246$), 35 min (105.4 beats/min, $p = 0.0159$), 45 min (104.6 beats/min, $p = 0.0086$), 1 h (99.5 beats/min, $p = 0.0001$), 1.5 h (104.6 beats/min, $p = 0.0091$), and 2 h (98.6 beats/min, $p \leq 0.0001$) compared with 60 min before intervention. In STRESS, HR was significantly higher at 20 min (131 beats/min, $p = 0.0367$) and 45 min (139.8 beats/min, $p \leq 0.0001$), and significantly lower at 1.5 h (110.6 beats/min, $p = 0.0028$) and 2 h (111.6 beats/min, $p = 0.0058$) compared with 60 min before intervention. HR was significantly higher in. STRESS compared with CON for all time points expect 60 min before, 1.5 h, and 2 h. Mean frequencies of HR with significant differences between groups are presented in Table 3.

Mean RR (with lower and upper CI) was 42.2 (37.1–47.4) breaths/min in CON and 39.7 (34.5–44.8) breaths/min in STRESS. In CON, RR was significantly lower at 2 h (36.8 breaths/min, $p = 0.0164$) compared with 60 min before intervention. In STRESS, RR was significantly higher at 0 min (44.6 breaths/min, $p = 0.0287$), 20 min (44.6 breaths/min, $p = 0.0294$), 25 min (45.8 breaths/min, $p = 0.0067$), 30 min (45.9 breaths/min, $p = 0.0059$), 45 min (47.7 breaths/min, $p = 0.0011$), and 1.5 h (44.3 breaths/min, $p = 0.0380$) compared with 60 min before intervention. There were no significant differences between groups. Mean frequencies of RR with significant differences within groups are presented in Table 3. Further significant differences within groups for HR and RR are given in Appendix I.

Table 2
Behavioral parameters evaluated in animals submitted to a stressful stimulus (STRESS, $n = 12$), compared with animals undergoing no stressful stimulus but remaining in their igloo (CON, $n = 12$). Due to the low number of occurrences within the groups, differences in vocalization and defecation could only be characterized in descriptive terms. The Mann-Whitney *U* test produced *p*-values based on rank comparisons, while medians are presented in the table for better understanding.

Parameter	Group		p-Values
	CON (medians)	STRESS (medians)	
Ear movements	116.9	167.2	**p = 0.03**
Movement of the head	3.3	4.4	$p = 0.59$
Movement of the limbs	31.8	65.9	**p = 0.00343**
Sequence of movements	62.3	43.8	p = 0.06
Tail movements	14.3	23.0	p = 0.12
Teeth grinding	0	0.3	p = 0.09
Urination	0.3	1.5	**p = 0.00434**

Significant differences (<0.05) are presented in bold letters.

3.9. Behavior on the day of trial

Mixed effects models for each of the other assessed parameters (activity, position of head, dorsal line, eyes, nose, ears) did not show any significant differences in dorsal line and nose at the different time points of the trial. Animals in STRESS tended ($p = 0.092$) to show "looks at observer, head held high, ears to front" more often compared with animals in CON. For position of the head, animals in STRESS showed "same height as dorsal line" significantly ($p = 0.049$) more often than animals in STRESS, whereas animals in CON tended to show "below dorsal line" significantly ($p = 0.057$) more often than animals in STRESS. Animals in CON tended ($p = 0.084$) to show "lids half closed" more often than animals in STRESS, whereas animals in STRESS tended ($p = 0.062$) to show "normal, wide eyes" more often compared with animals in CON. Animals in STRESS tended ($p = 0.073$) to show "ears held in normal

Table 3
Mean values and lower and upper confidence intervals (**CI**) of heart rate and respiratory rate in n = 24 calves of the German Simmental Breed, which were either submitted to a sham (CON, n = 12) or a stressful (STRESS, n = 12) stimulus. Significances (**p**) within groups are indicated, compared with baseline 60 min before intervention (*$p < 0.05$, **$p < 0.01$). Significant differences between groups are indicated.

Time Point	Heart rate (beats/min)			Respiratory rate (breaths/min)		
	CON	STRESS	p	CON	STRESS	p
60 min before	115 (104.8–125)	122.6 (112.3–133)	0.3105	42.2 (37.1–47.4)	39.7 (34.5–44.8)	0.4964
0 min	108.3 (98–119)	125.1 (114.8–135)	**0.0238**	42 (47.2–)	44.6* (39.4–49.8)	0.4859
5 min	108.5 (98.2–119)	124.9 (114.7–135)	**0.0267**	42 (36.7–47.2)	44.1 (38.9–49.2)	0.5776
10 min	105.5* (95.2–116)	130.3 (120.1–141)	**0.0008**	43.6 (38.4–48.8)	44.0 (38.9–49.2)	0.9050
15 min	109.8 (99.5–120)	125.1 (114.8–135)	**0.0394**	42.3 (37–47.5)	43.7 (38.4–48.9)	0.7097
20 min	109.6 (99.3–120)	131* 120.7–141	**0.0039**	44.4 (39.1–49.8)	44.6* (39.4–49.7)	0.9726
25 min	106.1* (95.8–116)	125.8 115.6–136	**0.0078**	42.1 (36.7–47.4)	45.8** (40.6–50.9)	0.3283
30 min	109.8 (99.6–120)	127.5 (117.2–138	**0.0175**	40.4 (35.1–45.6)	45.9** (40.7–51)	0.1425
35 min	105.4* (95.1–116)	129.8 (119.6–140	**0.0010**	40.1 (34.9–45.2)	34.5 (38.2–48.9)	0.3627
45 min	104.6** (94.3–115)	139.8** (129.5–150)	**<0.0001**	41.3 (36–46.5)	47.7** (42.2–53.1)	0.0987
1 h	99.5** (89.2–110)	120.9 (110.6–131)	**0.0039**	38.1 (32.7–43.5)	42.6 (37.3–47.8)	0.2422
1.5 h	104.6** (94.4–115)	110.6** (100.4–121)	0.4191	41.4 (36.3–46.6)	44.3* (39.2–49.5)	0.4318
2 h	98.6** (88.4–109)	111.6** (101.3–122)	0.0818	36.8* (31.7–42)	41.3 (36.1–46.6)	0.2315

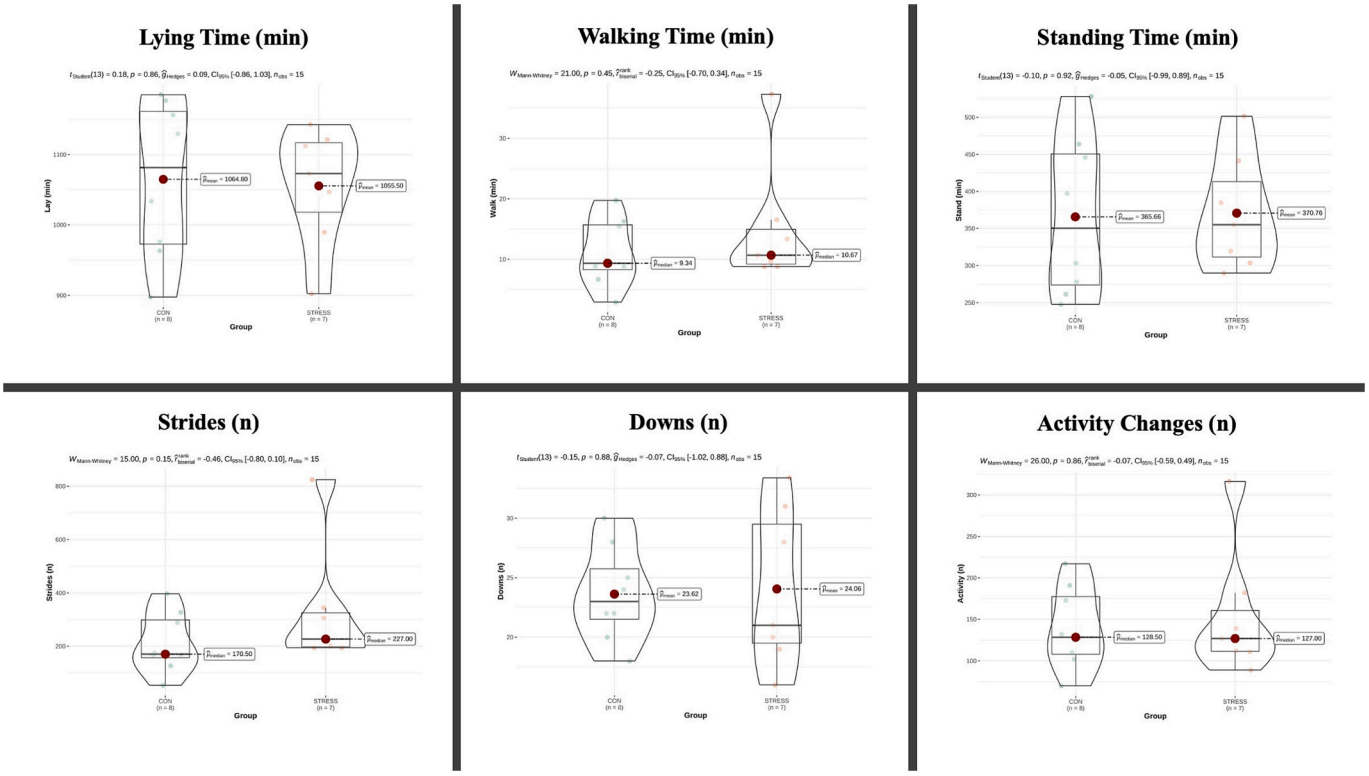

Fig. 6. Activity parameters in calves in CON (n = 12, without stimulus) and STRESS (n = 12, stressful stimulus). Activity was recorded for 24 h, starting at 08:00 a.m. on the day of the trial. Values are given as mean with 95 % Confidence Interval. The box represents 50 % of the data, with the midline being the mean value. The lowest horizontal line (whisker) is the first quartile (Q1) and the highest horizontal line (whisker) is the third quartile (Q3). Dots represent outliners. There were no significant differences in activity parameters between groups. A Student's *t*-test was employed to compare the means between two groups, due to a normally distributed data and equal variances.

position, facing front" more often than animals in CON.

3.10. Activity and number of steps

Activity data is missing in 9 animals ($n = 4$ for CON and $n = 5$ for STRESS), due to technical problems with the pedometer. Detailed information about missing/repeated data is presented in Appendix E.5. Activity data and number of steps is given in Fig. 6. There were no significant differences in activity parameters between CON and STRESS.

3.11. Milk intake

Mean milk intake was 8.7 ± 1.4 (5.6–11.7) liters in CON and 9.1 ± 0.5 (8.5–10.5) liters in STRESS. Percentage of total milk intake did not differ significantly between groups.

4. Discussion

Calves included in this study were 4–6 weeks of age. The age of the calves is one limitation of this study. A study by (Hasan et al., 2017) showed that calves under 6 months of age have a significantly higher risk of contracting diseases. Diarrhea and respiratory diseases were observed as the most frequently occurring diseases in our study population, which is in accordance with (Johnson et al., 2021; Svensson and Liberg, 2006). Nonetheless, this age group was selected for the present study, as most zootechnical procedures such as (surgical) castration and dehorning are normally performed in that age group and lead to pain and stress (Stafford and Mellor, 2005), making basic research work about pain parameters necessary for that age group.

One major limitation of the present study is that calves needed to be clinically healthy to be included in the trial to exclude the influence of nociception, inflammation, or diseases on PSPC. Although all calves were clinically examined before purchase at the market, there was no information about other risk factors for diseases, e.g. colostrum intake, hygiene, temperature and draught, grouping and housing of the animals, and management, and others (Johnson et al., 2021; Lundborg et al., 2005). Further risk factors for diseases were infection pressure due to selling via calf market (Dubé et al., 2010) as well as transportation (Creutzinger et al., 2021). During the acclimatization period at the clinic, animals were initially not subjected to any treatment, but after the first 3 calves were excluded from the trial due to being diagnosed with acute diarrhea on the day of the trial, a standardized prophylactic treatment protocol was implemented. Additionally, blood samples were taken one day before the trial to assess leucocyte count, which has been found to be significantly and positively correlated with PSPC (Landinger et al., 2024). Animals with a leukocyte count well above the reference range would have been excluded from the study. Another exclusion criteria was treatment with an antibiotic or NSAIDs within 48 h prior to the trial, as treatment with analgesics such as metamizole (Tschoner et al., 2018) or Meloxicam (Allen et al., 2013) influence the PSPC in the blood.

Stressful stimulation in our study consisted of restrainment in a crush and an ultrasonographic examination of the heart. According to (Pesenhofer et al., 2006) restraining animals in a crush in a standing position increases cortisol concentrations in adult cows. The ultrasonographic examination of the heart was done as an additional stress stimulus. To keep the stress stimulation constant during our trial, the number of people in the room was always the same, and the ultrasonographic examination was always performed by the same two people (MF, TT).

In the present study, PSPC decreased during the stressful event, with significant differences between 60 min before and 5 min and 20 min, whereas there were no differences in CON. These findings are contrary to those of (Van Engen et al., 2014; Van Engen et al., 2019) who described that SP concentrations in calves increased due to transportation stress. Calves were either transported for 8 (Van Engen et al., 2019) or 16 h (Van Engen et al., 2014), which is a longer exposure to stress compared with the present study. Also, as blood samples were taken immediately after transportation and 5 days later (Van Engen et al., 2014), and before transportation and immediately after transportation (Van Engen et al., 2019), respectively, with no control groups, comparison with our study is not possible. It is possible that the stress stimulus in our study was not strong enough to induce a more pronounced reaction in the animals. (Hwang et al., 2005) showed that stressing rats by restraining them for 2 h increases SP receptor binding in the central amygdala 24 and 48 h after the exposure to stress. Therefore, it is likely that the duration of stress exposure and the severity of the stressor are important factors in the observed effects on SP transmission, at least in the amygdala (Ebner and Singewald, 2006). In other bovine studies, stronger or chronic stress stimuli were used and evaluated. In a work by (Amberger, 2009), calves were restrained in a lateral position for ultrasonic examination, which could be considered a more acute stress stimulus compared with an ultrasonic examination in a crush. However, as (Amberger, 2009) did neither assess PSPC nor PCC, a direct comparison is not possible. (Geracioti et al., 2006) found elevated SP concentrations in the central nervous system in human patients with major depression and PTSD. It is possible that sampling of the cerebrospinal fluid could have provided different results in our study. However, in another human study, an increased B-endorphin plasma concentration, but no changes in PSPC in blood were found after acute stress (Schedlowski et al., 1995). There is evidence that SP modulates physiological and behavioral stress responses in the brain, and that exposure to a variety of emotional, physical, and painful stressors leads to altered SP tissue levels or SP immunoreactivity in different brain regions (Ebner and Singewald, 2006). Therefore, studies of SP concentrations in other matrices than blood and saliva, or analysis of different brain areas during stressful stimulation could provide different results in cattle. Nevertheless, our findings are somewhat in accordance with (Tschoner et al., 2020), who found no differences in PSPC between animals treated with either a placebo or xylazine hydrochloride to reduce stress prior to a laparoscopic fixation of the abomasum. Overall, PSPC in our study were higher compared with PSPC in 14 to 21 days old calves (Landinger et al., 2024). These differences may be age-related, as higher PSPC were found both in older calves (Dockweiler et al., 2013) and adult cattle (Landinger et al., 2024).

There was a positive and significant correlation between PSPC and PCC. Our findings are not in accordance with a recent study by (Tschoner et al., n.d.) who found a significant and negative correlation between PSPC and PCC in calves submitted to repeated painful stimulation. However, a positive and significant correlation has been described for neonatal humans (Dionysakopoulou et al., 2023). (Rohleder et al., 2006) found a trend towards significance between the area under the curve for SP and cortisol following stress in male humans. Another study in humans found a significant correlation between lower cortisol ratios, which was seen as indicative for chronic stress, and the number of SP positive inflammatory cells (Remröd et al., 2007).

Collecting saliva samples is less invasive (Pfaffe et al., 2011) compared with taking blood samples. In the present study, there was no correlation between SSPC and PSPC using the samples which could be analyzed. Comparison with previous research is difficult, as in the only other study assessing SSPC in calves, only two saliva samples were collected (Mayer, 2019). Study results from human medicine differ. (Jasim et al., 2018) found significant differences between SP concentrations in blood and saliva, whereas (Kallman et al., 2018) could not identify a correlation between saliva and plasma concentrations of SP in patients with chronic neuropathic pain. The results of the present study need to be considered with care, as SSPC were below the sensitivity of the ELISA kit in 16 animals and 106 out of 312 samples. As the SSPC samples were analyzed as described by (Mayer, 2019), where all samples were analyzable, it is possible that SP was simply not excreted sufficiently into the saliva in our study. In CON, there was a higher number of analyzable results, which suggests a reduced saliva excretion in stressed

animals. Human medical studies show that stress (Gholami et al., 2017), anxiety, and depression (Gholami et al., 2017) can influence salivary flow rate and lead to xerostomia. Additionally, it is possible that calves chewed the salivettes in different ways, or that the measurement method – using a salivette that was designed for use in humans – was ineffective, even if it had been described by (Mayer, 2019) before. Furthermore, another possible factor influencing SSPC in the present study is that aprotinin, which stabilizes SP, could only be added after sampling and centrifugation of saliva, and had to be adjusted to the quantity of the collected saliva. Fast transfer of samples to the laboratory and the addition of aprotinin was a priority, but human factors may have also led to time differences.

In ruminants, immediate changes in cortisol concentrations are mainly caused by acute pain-related stress, but also by handling (Molony and Kent, 1997), human presence, and restraint (Karlen et al., 2021), as well as by different external conditions and management techniques (Ogino et al., 2013), and individual and fear-related behavior (Bristow and Holmes, 2007). Simultaneous measurements of the plasma concentrations of SP and cortisol has been found to be useful for the differentiation of these two states (Coetzee et al., 2008). In the present study there were no significant differences in PCC between groups, indicating that either the stimulus was not strong enough to stress the calves, or that calves in CON experienced a similar amount of stress as calves in STRESS. (Stilwell et al., 2008) found that cortisol levels are not affected up to 1 min if blood samples are taken immediately after restraining calves by gently pressing them against the barn. As calves in CON sometimes had to be restrained in a similar way to prevent injuries during sampling, restraining could have influenced our results. On the other hand, the lower PCC could also be due to habituation to sampling and human presence. In STRESS, efforts were made to keep the stress stimulus constant; in CON, the aim was to shield the calves from possible external stressors as best as possible. However, as the trial took place during routine clinical work days, various stressors, such as passing personnel or vehicles (CON) and laboratory personnel collecting the SP samples (STRESS) represent a limitation of the study, as the increase in PCC in calves is always dependent on the stressor (Stilwell et al., 2010). As housing, feeding times, daily general examinations, and number of people present during the trial remained constant, the influence of these factors on our study results should be small.

The “fight or flight response” is considered an important mechanism by which an organism reacts to stress or danger (Cannon, 1929), with glucose concentrations rising in the course of the flight response (Mudron et al., 2005). In the present study, the flight reaction and the associated increase in glucose concentration could have been a result of the repeated examinations as well as of the tight sampling schedule. It is noticeable that glucose concentrations were significantly lower in STRESS compared with CON at the beginning of the trial (5 min and 10 min). However, glucose concentrations should be viewed with caution in terms of assessing stress intensity, as they can also be easily influenced by several other factors (Scholz, 1990), such as milk intake, which has an effect on the postprandial glucose kinetics (Gerrits, 2019), and therefore on the postprandial plasma glucose in dairy calves (Ghaffari et al., 2017). The aspect of feeding should also be considered for this study. Variations in concentrate intake could be a reason for the different glucose levels both between and within groups, as concentrates were available ad libitum except for animals in STRESS during the duration of the ultrasonographic examination in the crush. Furthermore, differences in glucose concentrations could be related to the feeding which was set for 07:00 a.m. not always being adhered to in daily clinical routines, resulting in feedings prior or after 07:00 a.m.

In the present study, the HR in STRESS was significantly higher compared with CON at nearly all time points. This result could reflect the increased stress level in STRESS. In CON, the decrease in HR could be related to an adaptation to the human presence and the sampling procedure. HR can also change as a result of individual temperament and reactivity to humans (Kovács et al., 2015), which could have influenced our results.

An increase in RR is an important indicator of stress (Gaughan et al., 2000) and an indirect tool to asses pain (Saeed et al., 2008). The temperature-humidity-index (Thom, 1959), the posture of an animal (Pinto et al., 2019), and diseases (Gaeta et al., 2018), can also have an influence on the RR. In STRESS, the RR was above the basal value, especially during placement in the crush and ultrasonographic examination, indicating that the calves were indeed experiencing stress. Frequency of the other assessed behavioral parameters between groups only showed significant differences in “position of the head”, whereby calves of STRESS showed “same height as dorsal line” significantly more often compared with animals in CON. Neck posture in cattle has been poorly investigated (De Oliveira and Keeling, 2018), but (Gleerup, 2017) found that pain often leads to a lower head posture. In our study, this posture and the horizontal position of the head could likely be due to the restriction of movement caused by the crush and being restrained.

STRESS calves showed a significant higher frequency of “ear movements” compared with CON calves. According to (Proctor and Carder, 2014) the relaxed position of the ears is indicative of a positive emotional state in cows. (Reefmann et al., 2009) found a reduced number of ear position changes during positive and an increased number during negative experiences in sheep, which suggests that the calves in STRESS experienced a higher level of negative experiences. “Movement of the limbs” was found significantly more often in STRESS compared with CON, which is in accordance with (Grandin, 1993), who stated that increased movement in cattle is considered as a sign of agitation. However, other factors influencing movement such as social isolation (Boissy and Le Neindre, 1997) and placement of cows in new environments (Hopster, 1998) could also apply to the results of our study. According to (Rushen et al., 1999) social isolation in unfamiliar surroundings increases the incidence of defecation, urination, and/or vocalization. In the present study, we found an increase in urination while standing in the crush in STRESS. One limitation of behavioral assessment was the fact that the video assessment in animals in CON was significantly more difficult compared with animals in STRESS, as calves in CON could retreat into their igloo. Therefore, not all behavioral changes could be recorded and assessed. Constantly forcing the calves out of the igloo would have resulted in stress for the calves, thereby negatively influencing our results. The presence of an observer may also have an impact on the animals' behavior and, consequently, the outcomes of ethograms (Fraser and Broom, 1990). In our case, the trial setup for calves in STRESS required the presence of 3 people. The same amount of people was always present at this stage of the trial to reduce the impact of number of observers on calves' behavior. Since the saliva samples needed to be processed as quickly as possible, the regular collection of the samples by the laboratory staff and the associated repeated entry into the trial room or the approach to the igloo was essential but could have influenced animals' behavior, which is a limitation. Videos and behavior frequency were always evaluated by the same observer (HK). A major limitation to behavior assessment in the present study is,that blinding of group allocation towards the observer was not possible due to the setup of the experimental design. To avoid bias during assessment, blinding is recommended (Fraser and Broom, 1990).

Stress, e.g. following weaning, can influence the number of steps in calves (Haley et al., 2005) and according to (Džermeikaitė et al., 2023) there could be a link between distance traveled by cattle, and stressful and unpleasant procedures. Furthermore, (Boissy and Bouissou, 1995) suggest that reduced locomotor activity in heifers indicates high fearfulness. The activity and number of steps did not differ significantly between groups. As already discussed, this could be related to insufficient stress stimulation. However, differences between the individual animals could also be due to potential diseases that had not yet been diagnosed at the day of trial. A study by (Duthie et al., 2021) found that differences in activity were already evident on the days prior to the peak day of disease. Apart from the ultrasonographic examination where

calves were restrained in the crush, and the moving to and from the igloo to the trial room, all calves were housed in the same way, which makes the influence of environmental factors as described by (Theurer et al., 2012) unlikely. Restrainment and movement of calves in STRESS could be a possible influence on our study results.

Total milk intake did not differ significantly between groups. Stress (Sutherland et al., 2018), as well as heat stress (Dado-Senn et al., 2020) can result in changes of milk intake, and feeding a higher amount of milk increases the sensitivity of feeding behavior as a measure of stress in calves (Sutherland et al., 2018). As feed and milk intake did not differ between control and trial groups, it can be assumed that the stimulus in STRESS was not stressful enough to negatively influence milk intake. Due to the daily work routine in the clinic, different caretakers were responsible for feeding the calves on the individual trial days, which could have influenced milk intake, with some caretakers being more patient and enthusiastic in feeding the calves compared with others.

5. Conclusion

According to the present study, an acute short term stressful event (restrainment in a crush and ultrasonographic examination of the heart for 30 min) results in a decrease in PSPC, with no differences between a trial and a control group. Saliva samples do not appear to be a suitable matrix for assessing SP concentrations in calves, as 34 % of samples could not be analyzed correctly. Using an ethogram, significant increases in ear and limb movement, as well as in frequency of urination in stressed animals could be assessed, indicating that observation of animals is an important and valuable tool to assess an animal's state. Further research in animals submitted to chronic stress should be done to assess the effect of stress on PSPC in the bovine.

CRediT authorship contribution statement

H. Kerber: Writing – original draft, Visualization, Investigation, Data curation. **M. Feist:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Y. Zablotski:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis, Data curation. **G. Knubben-Schweizer:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **T. Tschoner:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

Appendix A. Supplementary data

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