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Sedation With Xylazine Hydrochloride Decreases the Stress Response in Merino Meat Sheep During Routine Hoof Trimming in a Tilt Table

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We hypothesized that the hoof trimming in sheep in dorsal recumbency implicates a short but intensive stress situation and that the sedation with xylazine causes a decrease in the stress response in this situation. Ten healthy female merino meat sheep were randomly divided into two groups receiving either xylazine hydrochloride (0.1 mg/kg body mass (BM) applied intramuscularly) or a placebo treatment with 0.9% NaCl. Routine hoof trimming was performed in a tilt table and vital signs (rectal temperature (RT), heart rate (HR), and respiratory rate (RR)), 33 different behavioral traits and blood cortisol concentrations were recorded throughout the experiment at six different time points (total of 55 min). The procedure itself elicited a clear stress response (increase in the RR, RT, defensive movements, lip twitching, swallowing, and flight behavior). Parallelly, the blood cortisol concentrations were increased, reaching their maximum with 81.5 ng/ml in the control group when the sheep were tilted back into a standing position. In the sedated sheep, no increase in the RR and RT and a decrease in the HR were observed. In addition, the behavioral signs showed a decrease in flight, defensive, and general stress behavior (decrease in licking, movement of head and legs, and sitting on knees), complemented by the serum cortisol concentrations showing 2.28 times reduced concentration at the end of the procedure, compared to the control sheep. The results confirm our hypothesis and support the conduction of future trials investigating the feasibility and benefit of a sedation of sheep prior to routine hoof trimming under practical circumstances.

Keywords: stress, dorsal recumbency, animal husbandry, animal welfare, cortisol, vital signs assessment, behavior, claw trimming

INTRODUCTION

It is widely recognized that stressors can support and increase the risk of developing infectious diseases and of prolongation of illness episodes (Kelley, 1980; Steplewski et al., 1985; Cooper, 1999). Therefore, aside from important and well-acknowledged animal welfare concerns, different zootechnical procedures in animal production, inflicting short-term stress due to handling and restraint, may have an influence on animal productivity (Johnson and Vanjonack, 1976; Miranda-de la Lama et al., 2011, 2012; Marco-Ramell et al., 2016; Grandin, 2021). For these reasons, different protocols to reduce pain and stress in the animal have been developed since studies have shown not only a benefit in this respect, but also in the later animal health status (Ting et al., 2003; Kretschmann et al., 2020).

Hoof trimming is an essential part of cattle, sheep, and goat farm management (Shearer and van Amstel, 2001; Winter, 2008; Anzuino et al., 2010). Most trimming chutes include the fixation of the sheep with a headgate and the subsequent tilting. Hoof trimming, therefore, implicates a short but intensive stress situation due to the transportation, handling, and fixation (Fazio and Ferlazzo, 2003; Rizk et al., 2012a; Janßen et al., 2016; Heinrich et al., 2020). Studies describing the stress response in sheep during routine hoof trimming in dorsal recumbency are however lacking.

Hitherto, stress-reducing methods during hoof-trimming in sheep only include aspects of facility and chute design (Hutson, 2000), but no sedation protocols. In practice, the α2-adrenergic agonist xylazine is widely used as a sedative in wild, production, and domestic animals due to its convenient way of application and cost-effectiveness (Greene and Thurmon, 1988). Earlier studies have shown blood cortisol decreasing effect for xylazine, indicating it decreases the stress response and thereby exerts a positive effect on animal welfare (Brearley et al., 1992; Sanhouri et al., 1992; Cattet et al., 2004; Rizk et al., 2012a).

It is therefore hypothesized that (1) the fixation of sheep in dorsal recumbency for the routine hoof treatment represents a severe stress situation mirrored in behavioral, vital sign, and hormonal stress responses and (2) the sedation with xylazine causes a decrease of this stress response.

MATERIALS AND METHODS

The experiment was approved by the State Administration Office (Landesverwaltungsamt, Referat Verbraucherschutz, Veterinärangelegenheiten Sachsen-Anhalt, Germany) documented with the register 42502-3-734.

Study Animals

Ten healthy female Merino meat sheep between 1- and 2 years of age, in need of routine hoof trimming, without any pathologies or lameness present, and less than 100 days pregnant were selected randomly from a commercial sheep herd. All sheep in this study had not experienced hoof trimming in a tilt table before. The sheep were allocated to the Clinic for Cattle and Swine in Leipzig (Germany) and housed in a group in outdoor stables. The 10 sheep were randomly assigned to two groups: control [CON, $n =$ 5, Body Condition Score (BCS): 2.50 (median, Min.: 2.25, Max.: 2.75), BM: 60.3 ± 4.4 kg (mean ± SD)] and xylazine [XYL, $n =$ 5, BCS: 2.50 (median, Min.: 2.25, Max.: 2.75), BM: 56.2 ± 4.0 kg (mean ± SD); BCS according to Russel (1984)].

Study Design and Procedures

A similar study setup as in Fieseler et al. (2018, 2019) and Heinrich et al. (2020) was chosen (**Figure 1**). On day –2, a permanent residing catheter was inserted in the jugular vein in a priorly shaved, alcohol and iodine disinfected, and anesthetized [2–3 ml 2% Isocaine subcutaneous (s.c.)] location in the middle third of the lateral side of the neck. To facilitate the insertion, the skin was pre-punctured using a Strauss needle (Dispomed Witt e.K, Gelnhausen, Germany), and to ensure solid fixation, the catheters were thereafter fixed with two sutures using non-absorbable suture material (Catgut GmbH Markneukirchen, Germany). Thereafter, the catheters were covered with a gauze, which was attached to the skin using a commercial polyvinylester-based glue. During this procedure, the sheep were fixed by a trained animal keeper. The sheep, the surrounding of the catheters, and the jugular vein were clinically examined, and the catheters were flushed daily with 20 ml of 0.9% NaCl solution (B. Braun SE, Melsungen, Germany) throughout the experimental period and removed at day 0 at the end of the experiment without any complications observed.

A day prior to the experiment (day –1), blood samples for baseline values were collected. On the experimental day (day 0), the sheep were not withheld from feed and water. The treatment included a duration of 55 min in six positions (first position 5 min, other positions 10 min). Five minutes prior to the tilting into dorsal recumbency (position 1, time point: −15 min), 0.1 mg/kg BM of xylazine hydrochloride (Serumwerk Bernburg AG, Bernburg, Germany) were applied intramuscular (i.m.; right triceps brachii muscle) to the sheep of the XYL group, whereas the CON sheep received a placebo treatment with 0.9% NaCl (treatments were blinded). At time point −10 min, the sheep were tilted into dorsal recumbency (**Figure 2**).

Within the first 2 min of each position, a blood sample was collected from the catheter and vital signs (heart rate (HR), respiratory rate (RR), and rectal temperature (RT)) were recorded (always in this sequence). The behavioral responses were continuously observed and recorded every minute using a 0 (no occurrence) and 1 (occurrence) scoring system according to Fieseler et al. (2018) including the traits depicted in **Table 1**.

After the hoof trimming, the sheep were tilted back into the standing position (position 4, time point: 10 min), resided in the chute (position 5, time point: 20 min), and re-located to their pen thereafter. The sheep arrived in their stable (position 6) at a time point 30 min after the hoof trimming. All sheep were considered healthy and relocated to their farm after the experiment.

Blood Sampling and Cortisol Measurement

Blood samples (8–10 ml each) for cortisol analysis were collected in serum tubes (Sarstedt, Nümbrecht, Germany) and

Abbreviations: HR, Heart Rate; RR, Respiratory Rate; RT, Rectal Temperature; CON, Control Group; XYL, Xylazine Group.

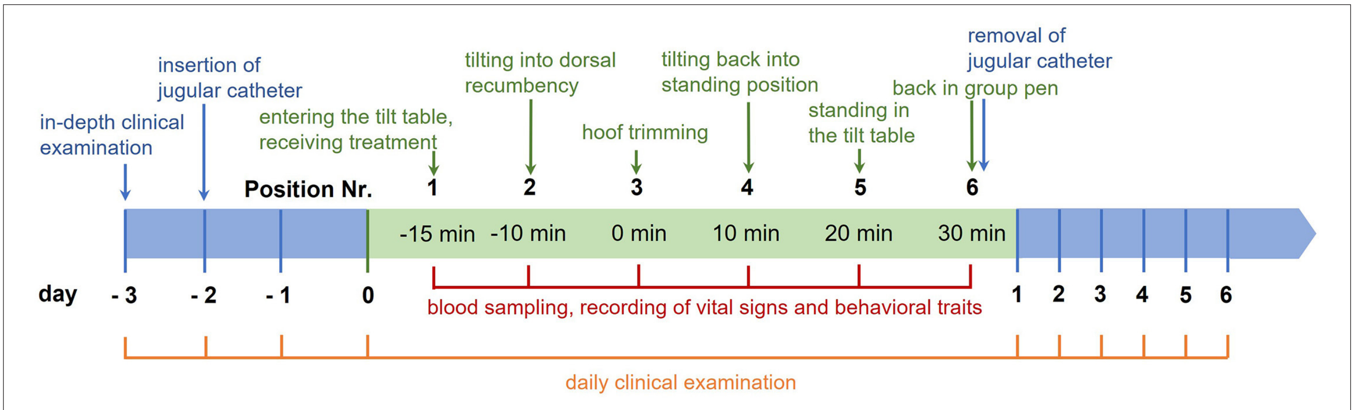

FIGURE 1 | Trial timeline. Ten Merino meat sheep were divided in two groups ($n = 5$), receiving either a sedation with xylazine hydrochloride (0.1 mg/kg BM) or a placebo treatment (0.9% NaCl) prior to hoof trimming in dorsal recumbency. The experimental day is highlighted in green.

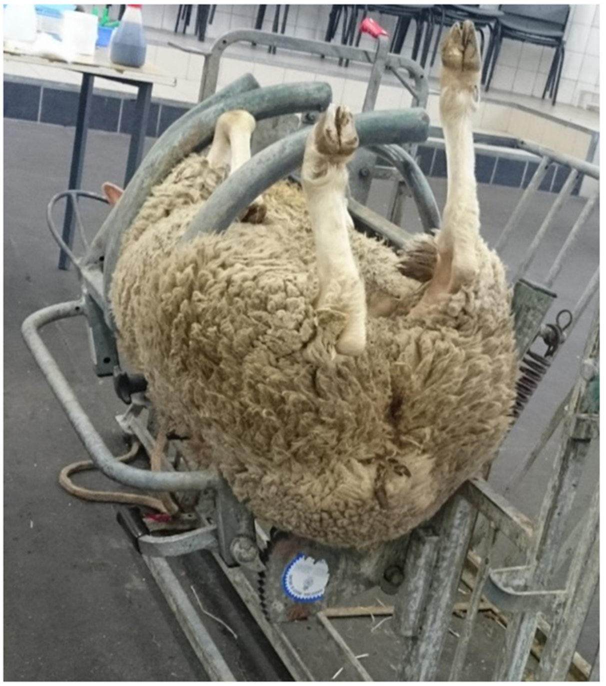

FIGURE 2 | Tilt table and positioning in dorsal recumbency of sheep during the trial.

stored on ice for 1 h (during the sample collection of one sheep) and thereafter in a fridge at 8°C (until samples of all sheep were collected) during 1–4 h before centrifugation (2,000 *g*, 10 min, room temperature) and aliquotation. The samples were stored at −80°C until analysis. Detection of cortisol concentrations was performed by using a solid-phase, competitive chemiluminescence enzyme immunoassay (IMMULITE 1000 systems, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany), previously validated for sheep. The intra-assay coefficient of variation (CV) was 7.6% by analyzing 20 samples within one test routine and the lowest detection limit was <2.0 ng/ml.

Statistical Analysis

Blood Cortisol Concentrations and Vital Signs (HR, RR, and RT)

The trend analysis of cortisol and RR showed a quadratic dependence on the test minutes (positions). Therefore, the dependence of these traits from the positions was described with the help of second-degree polynomials. The regression coefficients are considered to be specific to the two groups. In contrast, for HR and RT no suitable regression function could be derived from the trend analysis. For this reason, the factor treatments and positions and their interactions were represented by fixed effects in the models of these traits.

The repeated measurements within an animal were taken into account by using random animal effects and (or) by the variance-covariance structure of the residual effects. The following competing structures were tested: compound symmetry (CS), heterogeneous compound symmetry (CSH), first order autoregressive (AR (1)), heterogeneous first order autoregressive (ARH (1)), unstructured (UN), toeplitz with two, four, six bands resp (TOEP (2), TOEP (4), and TOEP (6)). The final structure was chosen according to the lowest value of the Akaike Information Criterion (AIC). To obtain the required estimates, we used the SAS procedure MIXED. This procedure provides the Least Square Means and statistical inference of their differences (*t*-test, Tukey-test respectively).

The studentized residuals of the evaluation models were tested for normal distribution using the Shapiro-Wilk test. For cortisol, a normal distribution was attained after logarithmic transformation.

Behavioral Responses

For every five animals in two treatments and six positions, different ethological traits are recorded within position over a period of 5 min (position "In tilt table") or 10 min (other positions). The recording was carried out according to a 0, 1

TABLE 1 | Recorded behavioral traits.

Abbreviation	Description
Backst	Back step, moves or tries to move backward
Chew	Chewing
Def	Defecation
Earmov	Ear movement
Eat	Eating
Ext1leg, Ext2leg, Ext3leg, Extallleg	Extending 1, 2, 3, or all legs
Fleh	Flehming, pulling and curling-up of the upper lip with open mouth and lifting of the head
Frontst	Front step, moves or tries to move forward
Flex1leg, Flex2leg, Flex3leg, Flexallleg	Flexing 1, 2, 3, or all legs
Groan	Groaning, long lingering soft growling/call
HeadDev	Head deviation sideways to the flank region
HeadLow	Head lowering
HeadMov	Head movement toward the noise
Integ	Integration, directed movement to join other sheep in the pen
Lacr	Lacrimation
Lay	Laying
Lick	Licking its lips
Liptwitch	Lips twitching
LegMovFour	Movement of one or more of the unaffected legs or all four legs
LegMovOne	Movement of the affected limb
MuscleMov	Muscle twitching, tremor of different muscle groups
Regur	Regurgitation
Restlene	Restlessness
Earrising	Rising of the ears
Rum	Rumination
Saliv	Salivation
KneeSit	Sitting on knees, resting on both carpi by flexion of carpal joints
Swalo	Swallowing
TailMov	Tail movement
TeethGrn	Teeth grinding
Trysca	Trying to escape
Urin	Urination
Vocal	Vocalization

scheme (0: ethological trait does not occur, 1: ethological trait occurs) every minute.

Let y_{ijk} be a random variable whose realizations represent the numbers of occurrences of an ethological trait on the k-th animal, from the i-th treatment in the j-th position. Designate N_{ijk} the number of measurements on the k-th animal from the i-th treatment in the j-th position. Then y_{ijk} is assumed to be binomially distributed with the number of measurements N_{ijk} and probability of occurrence p_{ijk}. The relationship between the probabilities, given the random animal effect $u_{ik} \sim N(0,\ \sigma_u^2)$, and the influencing factors are modeled using the logit link function:

$$logit\left(p_{ijk}\right) = \eta_{ij} + u_{ik} with\ \eta_{ij} = u + \alpha_{ij} \quad (1)$$

In the formula above, α_{ij} are the fixed effects of combination treatment*position.

Marginal probabilities, i.e., the probabilities averaged over the animals, can be calculated approximately in the following way:

$$p_{ij} = \exp\left(a \cdot \eta_{ij}\right) / \left(1 + \exp\left(a \cdot \eta_{ij}\right)\right) \quad (2)$$

with $a = 1/\sqrt{(1 + c^2 \cdot \sigma_u^2)}$ und $c = \frac{16}{15} \cdot \frac{\sqrt{3}}{\pi}$

Derived from p_{ij} we are able to calculate a mean probability per treatment (p_i) and per position (p_j).

The estimation of the model parameters was carried out with the maximum likelihood method implemented in the SAS procedure NLMIXED.

The estimated values express the probability with which the relevant ethological trait can be expected per minute in the classification unit (treatment, position, treatment*position).

The results are presented in the form of estimated probabilities, their CIs, and the statistical tests of the respective differences (*t*-test, Tukey-test, respectively).

In both groups of traits, results were considered statistically significant at $p \leq 0.05$, and a trend was declared at $0.05 < p \leq 0.10$.

RESULTS

Vital Signs

The HR decreased in the XYL group between being tilted over in dorsal recumbency (position 2) and hoof trimming (position 3) and stayed lower thereafter, whereas it remained unaltered in the CON group (**Table 2**). A difference between the groups was observed at position 5 ($p = 0.017$).

The RR remained unaltered in the XYL group and increased in the CON group between positions 2 and 3 and decreased again between standing in the chute and being back in their box (positions 4 and 5), with differences between groups at positions 2–5 ($p < 0.02$; **Table 3** and **Figure 3**).

For the RT, a greater value was observed in the CON group after the hoof trimming procedure at position 5 ($p = 0.044$; **Table 4**).

Behavioral Responses

Regardless of factors for treatment and period, the overview of the traits under investigation shows a very differentiated variability. Whereas certain traits exhibited frequently the behavioral score 1 (e.g., Lick, Eat, Earrising, and Earmov) and other traits show with very low-frequency score 1 and therefore could not be evaluated (e.g., Teethgrn, Tailmov, Fleh, and Lacr). It was therefore decided to exclude the following traits with only little variability from the further statistical analysis: Frontst, Chew, TeethGrn, Def, Saliv, Lacr, Fleh, Tail Mov, Integ, Restlene, Rum, Ext1leg, Ext2leg, Ext3leg, Extallleg, Flex1leg, Flex2leg, Flex3leg, and Flexallleg.

TABLE 2 | Treatment (CON, XYL) comparison at different positions for the heart rate.

	Heart rate (beats per minute)						
Position	**LSM$_{XYL}$**	**95% CI**		**LSM$_{CON}$**	**95% CI**		***P*-value**
1. In tilt table	95.0[ab]	75.7	114.3	102.4[a]	81.2	123.6	0.579
2. Dorsal recumbency	98.4[a]	79.1	117.7	104.8[a]	83.6	126.0	0.631
3. Hoof trimming	82.4[bc]	63.1	101.7	95.2[a]	74.0	116.4	0.343
4. Tilting back	76.0[c]	56.7	95.3	93.6[a]	72.4	114.8	0.199
5. Standing	73.6[c]	54.3	92.9	108.8[a]	87.6	130.0	**0.017**
6. In box	78.4[bc]	59.1	97.7	96.0[a]	74.8	117.2	0.199

Least Square Means (LSM) with 95% CI with P-values (t-test) of the estimated with observations on 10 sheep (n = 5 per treatment).
[a–c]LSM within a column with different superscript letters indicate significant differences (p ≤ 0.05; Tukey-test).
All P-values that are significant or show a trend (P ≤ 0.10) are bold.

TABLE 3 | Treatment (CON, XYL) comparison at different positions for the respiratory rate.

	Respiratory rate (breaths per minute)						
Position	**LSM$_{XYL}$**	**95% CI**		**LSM$_{CON}$**	**95% CI**		***P*-value**
1. In tilt table	33.0[a]	27.4	38.7	33.0[d]	27.3	38.6	0.982
2. Dorsal recumbency	32.8[a]	26.6	39.1	44.1[bc]	37.9	50.3	**0.016**
3. Hoof trimming	31.8[a]	23.4	40.1	52.7[a]	44.5	61.0	**0.002**
4. Tilting back	29.6[a]	20.3	38.9	54.9[a]	45.7	64.2	**0.001**
5. Standing	26.3[a]	16.7	36.0	49.3[ab]	39.7	59.0	**0.002**
6. In box	22.0[a]	8.7	35.3	35.9[cd]	22.2	49.5	0.140

Least Square Means (LSM) with 95% CI with P-values (t-test) of the estimated with observations on 10 sheep (n = 5 per treatment).
[a–d]LSM within a column with different superscript letters indicate significant differences (p ≤ 0.05; Tukey-test).
All P-values that are significant or show a trend (P ≤ 0.10) are bold.

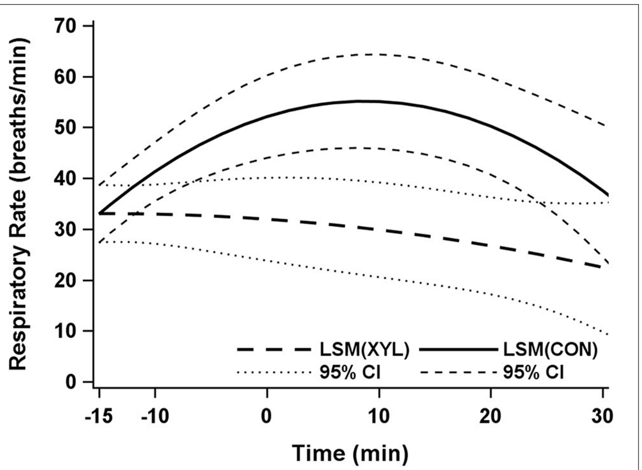

FIGURE 3 | Least Square Means (LSM) curves (polynomial regression) with 95% CI of the groups (XYL, CON) for the respiratory rate estimated with observations on 10 sheep ($n = 5$ per treatment). The sheep were placed in the chute and the XYL group was injected with 0.1 mg/kg body mass xylazine hydrochloride (−15 min), brought into dorsal recumbency (−10 min), hoof trimmed (0 min), tilted back (10 min), left to stand in the chute (20 min), and brought back to their box (30 min).

Probabilities per Treatment

A difference between treatments was observed on the traits HeadMov, Lick, KneeSit, and Liptwitch ($p < 0.03$). In addition, the traits LegMovFour and Groan exhibited a trend ($p = 0.059$ and $p = 0.074$; **Table 5**). With the probability of observance increasing in the traits Liptwitch and Groan and decreasing in HeadMov, Lick, KneeSit, and LegMovFour by the treatment XYL, regardless of the positions.

Probabilities per Positions

For the traits HeadMov, LegMovOne, Earrising, Earmov, Liptwitch, Swalo, and Backst, an influence of the position, regardless of the treatment, was observed ($p < 0.05$; **Table 6**). The probability of occurrence of HeadMov and LegMoveOne increased when the sheep were in dorsal recumbency and decreased thereafter again, whereas Earrising and Earmov exhibited a vice-versa development. Liptwitch and Swalo increased at positions 2 and 3, respectively, to reach their maximum at position 5 (standing in the chute after tilting back) and decreased again when the sheep were back in their box, whereas the probability of occurrence of Backst was increased when the sheep were inside the chute and after exposing to dorsal recumbency and hoof trimming.

Probabilities of Treatment Within Positions

For the traits HeadMov and Lick, a difference between the treatment groups was observed at one of the positions ($p = 0.001$ and $p = 0.025$). The traits Regur and Liptwitch exhibited a trend at one and two different positions, respectively (Regur: $p = 0.090$; Liptwitch: $p = 0.082$ and $p = 0.052$; **Table 7**). The traits HeadMov, Regur, and Lick exhibited a 30, 2, and 32% greater

TABLE 4 | Treatment (CON, XYL) comparison at different positions for the rectal temperature.

	Rectal temperature (°C)						
Position	**LSM_{XYL}**	**95% CI**		**LSM_{CON}**	**95% CI**		***P*-value**
1. In tilt table	39.36	38.80	39.92	39.64	39.08	40.20	0.457
2. Dorsal recumbency	39.40	38.84	39.96	39.78	39.22	40.34	0.317
3. Hoof trimming	39.42	38.86	39.98	40.02	39.46	40.58	0.125
4. Tilting back	39.42	38.86	39.98	39.94	39.38	40.50	0.179
5. Standing	39.40	38.84	39.96	40.22	39.66	40.78	**0.044**
6. In box	39.54	38.98	40.10	39.88	39.32	40.44	0.369

Least Square Means (LSM) with 95% CI with P-values (t-test) of the estimated with observations on 10 sheep (n = 5 per treatment).
All P-values that are significant or show a trend (P ≤ 0.10) are bold.

TABLE 5 | Estimated probabilities $\hat{p}_i$ of occurrence of behavioral traits for the treatments (CON, XYL), their 95% CI, and significance test of differences between treatments (*P*-value, *t*-test).

Trait[a]	**$\hat{p}_{CON}$ (95% CI)**[b]	**$\hat{p}_{XYL}$ (95% CI)**	***P*-value**
Backst	0.125 (0.063; 0.188)	0.070 (0.024; 0.115)	0.136
Earmov	0.307 (0.223; 0.391)	0.395 (0.289; 0.501)	0.176
Earrising	0.206 (0.132; 0.279)	0.197 (0.123; 0.270)	0.850
Eat	0.074 (0.016; 0.131)	0.037 (0.000; 0.080)	0.277
Groan	0.000 (0.000; 0.001)	0.044 (0.000; 0.093)	**0.074**
HeadDev	0.062 (0.018; 0.106)	0.057 (0.013; 0.101)	0.865
HeadMov	0.087 (0.052; 0.121)	0.003 (0.000; 0.011)	**0.001**
KneeSit	0.027 (0.004; 0.050)	0.000 (0.000; 0.001)	**0.029**
Lay	0.083 (0.005; 0.156)	0.016 (0.000; 0.045)	0.104
LegMovFour	0.059 (0.019; 0.100)	0.017 (0.000; 0.036)	**0.059**
LegMovOne	0.037 (0.016; 0.057)	0.023 (0.006; 0.040)	0.319
Lick	0.155 (0.081; 0.230)	0.006 (0.000; 0.024)	**0.002**
Liptwitch	0.044 (0.009; 0.079)	0.215 (0.122; 0.308)	**0.004**
MusclMov	0.074 (0.000; 0.156)	0.028 (0.000; 0.067)	0.273
Regur	0.059 (0.013; 0.104)	0.042 (0.006; 0.077)	0.521
Swalo	0.112 (0.053; 0.170)	0.088 (0.040; 0.136)	0.495
Trysca	0.029 (0.000; 0.080)	0.049 (0.000; 0.118)	0.600
Urin	0.023 (0.004; 0.043)	0.007 (0.000; 0.017)	0.121
Vocal	0.084 (0.000; 0.202)	0.045 (0.000; 0.122	0.532

[a] Trait's abbreviations explained in the "Material and methods" section.
[b] CI in brackets, lower interval limits < 0 were set to 0.
All P-values that are significant or show a trend (P ≤ 0.10) are bold.

probability to be observed in the untreated sheep in positions 2 (dorsal recumbency), 2 and 4 (tilting back), respectively. The trait Liptwitch was observed more prominent in xylazine-treated sheep with a chance for observance of ∼30% during dorsal recumbency and hoof trimming.

Blood Cortisol Concentrations

The average cortisol concentration at day 1 was 22.5 ng/ml (mean, Min: 2.5, Max: 41.4). On an experimental day, the cortisol concentrations increased continuously throughout the experimental procedure, with the greatest values time point 10 min (4. tilting back) in the CON group and at time point 0 min (3. hoof trimming) in the XYL group and decreased thereafter (**Table 8** and **Figure 4**). A difference between groups was observed at time point 30 min, when the sheep were back in their box, with the CON group exhibited 2.28 times greater serum cortisol concentrations than the XYL group ($p = 0.042$).

DISCUSSION

Position Effect

Our analysis of the effects attributable to the trimming procedure itself clearly illustrates that hoof trimming in a tilt table induces a stress response in sheep, thereby confirming our first hypothesis. On the level of the vital signs, we observed an increase in the RR, being highest at the end of the period in dorsal recumbency. These results are in line with a study by Meyer et al. (2010) investigating cardiopulmonary effects of dorsal recumbency in calves, with or without high-volume caudal epidural anesthesia. The authors suggest that the tachypnoea is a direct stress response due to the unphysiological positioning or a compensatory increase for reduced tidal volume caused by the formation of atelectasis. Parallelly, we observed an increase in RT, being in line with different studies showing an interrelation between psychological stress and hyperthermia in different species (Kündig et al., 1996; Pedernera-Romano et al., 2010; Oka, 2018; Scherf et al., 2020). We furthermore suggest that the tachypnoea observed in dorsal recumbency might be induced by an increase in internal body temperature (Hayashi et al., 2006). Similar to Rizk et al. (2012a), investigating the effect of lateral recumbency on stress traits in dairy cows, and Meyer et al. (2010), we did not observe any alteration in the HR. Meyer et al. (2010) furthermore observed a concurrent decrease in the cardiac index (=cardiac output/BM) and suggest that this can be ascribed to a decreased venous pre-load caused by the compression of the caudal vena cava by the abdominal viscera (mainly fore-stomachs).

The behavioral traits complement the observations made in the vital signs, by the observation of defensive movements of head and legs (increase in HeadMov and LegMoveOne), increased facial expression (Liptwitch and Swalo) when the sheep were lying on their back, and flight behavior when standing back on their legs (an increase of Backst; Henry, 1992; Dodd et al.,

TABLE 6 | Estimated probabilities $\hat{p}_j$ of occurrence of behavioral traits in the j-th position (j = 1,…,6), their 95% CI and significance test of differences between positions (Tukey-test).

Trait[a]	$\hat{p}_j$ (95% CI)[b]					
	1. In tilt table	2. Dorsal recumbency	3. Hoof trimming	4. Tilting back	5. Standing	6. In box
Backst	0.158 (0.036; 0.280)[ab]	0.000 (0.000; 0.001)[b]	0.000 (0.000; 0.001)[b]	0.219 (0.113; 0.324)[a]	0.207 (0.103; 0.313)[a]	0.000 (0.000; 0.001)[b]
Earmov	0.461 (0.292; 0.630)[abc]	0.279 (0.167; 0.391)[bde]	0.179 (0.088; 0.270)[ce]	0.460 (0.331; 0.588)[abd]	0.570 (0.444; 0.696)[a]	0.159 (0.072; 0.246)[e]
Earrising	0.279 (0.134; 0.424)[abc]	0.120 (0.043; 0.196)[bde]	0.070 (0.011; 0.129)[ce]	0.279 (0.170; 0.388)[abd]	0.339 (0.220; 9,458)[a]	0.120 (0.043; 0.196)[bde]
Eat	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.334 (0.118; 0.550)
Groan	0.000 (0.000; 0.001)	0.066 (0.000; 0.147)	0.066 (0.000; 0.147)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)
HeadDev	0.119 (0.001; 0.227)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.119 (0.037; 0.021)	0.119 (0.037; 0.200)	0.000 (0.000; 0.001)
HeadMov	0.000 (0.000; 0.002)[a]	0.150 (0.077; 0.223)[b]	0.010 (0.000; 0.032)[a]	0.030 (0.000; 0.007)[ab]	0.010 (0.000; 0.032)[a]	0.070 (0.014; 0.125)[ab]
KneeSit	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.025 (0.003; 0.116)	0.020 (0.000; 0.005)	0.000 (0.000; 0.001)
Lay	0.208 (0.032; 0.383)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.025 (0.000; 0.069)	0.065 (0.000; 0.148)
LegMovFour	0.000 (0.000; 0.001)	0.129 (0.042; 0.216)	0.089 (0.018; 0.161)	0.000 (0.000; 0.001)	0.011 (0.000; 0.034)	0.000 (0.000; 0.001)
LegMovOne	0.000 (0.000; 0.001)[a]	0.140 (0.070; 0.209)[b]	0.020 (0.000; 0.048)[a]	0.020 (0.000; 0.048)[a]	0.000 (0.000; 0.001)[a]	0.000 (0.000; 0.001)[a]
Lick	0.040 (0.000; 0.102)	0.119 (0.040; 0.199)	0.060 (0.070; 0.248)	0.159 (0.070; 0.247)	0.079 (0.014; 0.144)	0.041 (0.000; 0.086)
Liptwitch	0.041 (0.000; 0.104)[bc]	0.149 (0.058; 0.239)[abc]	0.169 (0.074; 0.264)[ab]	0.139 (0.051; 0.226)[abc]	0.259 (0.144; 0.373)[a]	0.020 (0.000; 0.053)[c]
MusclMov	0.000 (0.000; 0.001)	0.134 (0.005; 0.264)	0.154 (0.011; 0.296)	0.000 (0.000; 0.001)	0.015 (0.000; 0.051)	0.000 (0.000; 0.001)
Regur	0.041 (0.000; 0.105)	0.011 (0.000; 0.036)	0.021 (0.000; 0.056)	0.108 (0.028; 0.188)	0.119 (0.035; 0.203)	0.000 (0.000; 0.001)
Swalo	0.022 (0.000; 0.067)[b]	0.061 (0.004; 0.118)[b]	0.130 (0.047; 0.212)[ab]	0.109 (0.033; 0.184)[ab]	0.248 (0.135; 0.361)[a]	0.029 (0.000; 0.067)[b]
Tryesca	0.000 (0.000; 0.012)	0.000 (0.000; 0.010)	0.000 (0.000; 0.009)	0.091 (0.000; 0.215)	0.142 (0.000; 0.317)	0.000 (0.000; 0.010)
Urin	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.040.(0.000; 0.083)	0.020 (0.000; 0.052)	0.030 (0.000; 0.068)
Vocal	0.080 (0.000; 0.198)	0.000 (0.000; 0.001)	0.000 (0.000; 0.001)	0.119 (0.000; 0.026)	0.165 (0.000; 0.339)	0.022 (0.000; 0.073)

[a−−e], *different letters indicate significant difference between positions within respective behavioral trait with $p \leq 0.05$ (Tukey-test).*

2012). We furthermore suggest that the observed decrease in ear movement (Earrising and Earmov) can be attributed to the decrease in optical and acoustic stimuli in this position and the inverted gravity on these extremities. The serum cortisol concentrations confirm the observations made in the other traits, showing a continuous increase between the beginning of the procedure until the sheep are tilted back in a standing position. Several studies, showing a strong interrelation between the degree of severity of a stimulus and the blood cortisol concentration, have established the measurement of blood cortisol as reference for the amount of stress sheep are exposed to Fell et al. (1985) and Hargreaves and Hutson (1990a,b). Other studies have shown a lag of ∼20–30 min until the peak level of blood cortisol is reached after exposure to stress (Lay et al., 1992; Wagner et al., 2021), which is in agreement with our results.

The control group in our study exhibited similar maximum blood cortisol concentrations of 70–80 ng/ml to the sheep in the studies of Hargreaves and Hutson (1990b) during shearing (however using a different assay for analysis). Notable is that at position 1, the blood cortisol concentrations were double the concentrations measured on day 1 in the box. This illustrates that the short transport to the location of the tilt table comprises a stressful situation for the sheep.

Extrapolating from a peripheral measurement of glucocorticoids to the stress level of an animal is difficult when it is determined as a stand-alone trait. It is not possible to determine what stimulus exactly activated the paraventricular nucleus, since blood cortisol levels can increase due to positive and negative conditions (e.g., fear, reward, and hypoglycemia; Ralph and Tilbrook, 2016). By combining three different levels of stress assessment (vital signs, behavior, and serum cortisol concentrations), we were able to illustrate that the hormonal response observed in these sheep was most likely due to fear and distress.

In summary, these results therefore show that our procedure, recorded traits, and analysis methods are an adequate model for stress and to investigate possible stress-reducing methods during routine hoof trimming in sheep.

Treatment Effect

In several studies with different species, it was shown that a sedation with xylazine causes a cardio-respiratory depression, characterized by a reduction of the HR and RR (St Jean et al., 1990; Sanhouri et al., 1992; Meyer et al., 2010; Rizk et al., 2012a). In our study likewise, a decrease in HR and no increase in the RR was observed. Furthermore, in the current study, the RT was not altered in the sheep that received xylazine, whereas it increased in the control sheep. Rizk et al. (2012a) reported a decrease in RT in cattle treated with xylazine in lateral recumbency, with the control group exhibiting no alterations. Different other studies across several species show similar results (Ponder and Clarke, 1980; Rector et al., 1996) or no influence of xylazine on RT (Livingston et al., 1984; Rector et al., 1996; Ullah et al., 2017).

Besides its cardio-vascular effects, xylazine influences the consciousness and behavior of the animal (Rizk et al., 2012a; Flecknell et al., 2015). We interpret the decrease in the behavioral traits HeadMov, Lick, KneeSit, and LegMovFour as an expression of decrease in consciousness and defensive and flight behavior

TABLE 7 | Estimated probabilities $\hat{p}_{ij}$ of occurrence of behavioral traits for the treatments (CON, XYL) within the j-th position (j=1,…,6 and significance test of differences between treatments within positions (Tukey-test; for reasons of clarity, the CI of the estimates are not given here).

Trait[a]	Estimated probabilities	1. In tilt table	2. Dorsal recumbency	3. Hoof trimming	4. Tilting back	5. Standing	6. In box
Backst	CON	0.197	0	0	0.318	0.237	0
	XYL	0.119	0	0	0.120	0.179	0
Earmov	CON	0.400	0.221	0.062	0.460	0.639	0.062
	XYL	0.520	0.337	0.297	0.459	0.500	0.256
Earrising	CON	0.159	0.178	0.060	0.378	0.358	0.099
	XYL	0.399	0.061	0.080	0.180	0.319	0.140
Eat	CON	0	0	0	0	0	0.444
	XYL	0	0	0	0	0	0.224
Groan	CON	0	0	0	0	0	0
	XYL	0	0.132	0.132	0	0	0
HeadDev	CON	0.079	0	0	0.136	0.156	0
	XYL	0.160	0	0	0.101	0.081	0
HeadMov	CON	0	0.300[a]	0.020	0.040	0.020	0.140
	XYL	0	0[b]	0	0.020	0	0
KneeSit	CON	0	0	0	0.119	0.041	0.000
	XYL	0	0	0	0	0	0
Lay	CON	0.365	0	0	0	0	0.130
	XYL	0.050	0	0	0	0.050	0
LegMovFour	CON	0	0.216	0.118	0	0.021	0
	XYL	0	0.041	0.060	0	0	0
LegMovOne	CON	0	0.160	0.040	0.020	0	0
	XYL	0	0.120	0	0.020	0	0
Lick	CON	0.080	0.218	0.119	0.318[a]	0.158	0.041
	XYL	0	0.021	0	0[b]	0	0.041
Liptwitch	CON	0.041	0.021[B]	0.021[B]	0.061	0.119	0
	XYL	0.041	0.277[A]	0.317[A]	0.217	0.398	0.040
MusclMov	CON	0	0.170	0.241	0	0.031	0
	XYL	0	0.099	0.067	0	0	0
Regur	CON	0.043	0.022[A]	0.043	0.083	0.161	0
	XYL	0.039	0[B]	0	0.134	0.077	0
Swalo	CON	0.042	0.102	0.182	0.102	0.241	0
	XYL	0	0.020	0.077	0.116	0.255	0.058
Tryesca	CON	0	0	0	0.124	0.051	0
	XYL	0	0.001	0.001	0.059	0.234	0.001
Urin	CON	0	0	0	0.080	0.020	0.040
	XYL	0	0	0	0	0.020	0.020
Vocal	CON	0.065	0	0	0.158	0.236	0.044
	XYL	0.095	0	0	0.080	0.095	0

[a]Trait's abbreviations explained in the "Material and methods" section.
Different letters indicate significant difference between treatments within position with $p \leq 0.05$ (small letters) and $0.5 < p \leq 0.10$ (big letters, Tukey-test).

(the difference in probability of 2% between treatment groups in the case of Regur was considered not significant from a biological standpoint). In line with this, England et al. (1992) reported head lowering in horses under the influence of a xylazine sedation and Vickers et al. (2005) noticed that xylazine prevents the appearance of head rubbing or shaking after dehorning in calves. In the study of Fieseler et al. (2019) the moving of the head appeared to be primarily stress-related in association with dorsal recumbency. Furthermore, Sanhouri et al. (1992) describe a decrease in flighting behavior during the transportation of male goats under the influence of xylazine. An increase in vocalization under xylazine sedation is well-known in practice and described in a study by Stilwell et al. (2010), investigating the influence of a xylazine sedation on the stress response during disbudding in calves, as "long lingering call." In addition in our study, an increase in the probability of observance of twitching with the lips and groaning was observed, which can most likely be ascribed to the compounds central nervous interference (Ruiz-Colón et al., 2014) and might also be interpreted as a vocal expression of mood and emotion (Manteuffel et al., 2004).

TABLE 8 | Treatment (CON, XYL) comparison at different positions for the blood cortisol concentrations.

Position	Serum cortisol concentrations (ng/ml)						
	LSM_{XYL}	95% CI		LSM_{CON}	95% CI		*P*-value (*t*-test)
1. In tilt table	49.5[bd]	37.2	65.8	50.9[bf]	38.3	76.7	0.873
2. Dorsal recumbency	56.8[ac]	41.2	78.3	61.3[cd]	44.5	84.6	0.710
3. Hoof trimming	63.2[ab]	42.4	94.2	77.4[ae]	51.9	115.5	0.449
4. Tilting back	56.3[abe]	35.5	89.2	81.5[abd]	51.4	129.1	0.242
5. Standing	40.2[cd]	24.4	66.2	71.4[abc]	43.3	117.8	0.103
6. In box	22.9[e]	13.2	40.0	52.1[def]	29.9	90.8	**0.042**

Least Square Means (LSM) with 95% CI with P-values of the estimated with observations on 10 sheep (n = 5 per treatment).
[a–f] Different letters indicate significant difference between treatments within position with p ≤ 0.05 (Tukey-test).
All P-values that are significant or show a trend (P ≤ 0.10) are bold.

Our recordings of the serum cortisol concentrations furthermore confirm the stress-reducing effect of a sedation with xylazine, with a 2.28 times lower concentration in the treated (position 6: back in a box, 22.9 ng/ml) compared to the untreated sheep (52.1 ng/ml). These concentrations were lower than the initial level measured at position 1 (standing in tilt table prior to tilting, 49.5 ng/ml) and similar to the levels measured on day 1 in rest (22.5 ng/ml). Our results are in line with a study by Sanhouri et al. (1992) investigating the possible application of xylazine to reduce stress during transport in goats and of Stafford et al. (2003) and Stafford and Mellor (2005) and Lepková et al. (2007) showing a positive effect during the dehorning of calves and confirm our initial hypothesis.

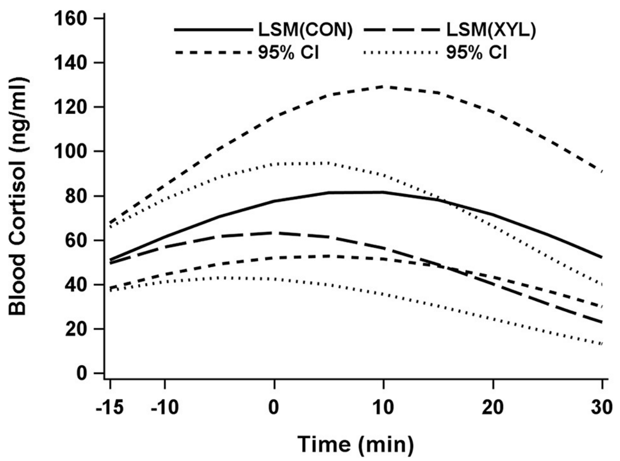

FIGURE 4 | Least Square Means (LSM) curves (polynomial regression) with 95% CI of the groups (XYL, CON) for the blood cortisol concentrations estimated with observations on 10 sheep (*n* = 5). The sheep were placed in the chute and the XYL group was injected with 0.1 mg/kg body mass xylazine hydrochloride (−15 min), brought into dorsal recumbency (−10 min), hoof trimmed (0 min), tilted back (10 min), left to stand in the chute (20 min), and brought back to their box (30 min).

Implications and Limitations

Stress is defined as a state in which the body homeostasis is disturbed over a short- to long-termed period and needs to be reinstated by adaptive responses [reviewed in Turner et al. (2012) and Ralph and Tilbrook (2016)]. Different studies have illustrated that even acute or short-termed stress responses can have impacts on animal health and welfare, if occurring during a critical time [reviewed in Ralph and Tilbrook (2016)]. Ralph and Tilbrook (2016) summarize several studies in this context describing a negative impact on ewe reproduction on the level of the production of the luteinizing hormone required for ovulation (Pierce et al., 2009; Wagenmaker et al., 2009) and sexual behavior (Pierce et al., 2008; Papargiris et al., 2011). They furthermore state that there is also evidence from human medicine that an acute stress response may result in a detrimental effect on essential body functions (e.g., ischemic heart injury and failure; Sapolsky, 2000). Professional and stress-free animal handling is therefore a central aspect in the husbandry of livestock and in the interest of every farmer. It is granted by the construction of a facility that allows gentle and calm handling of the animals (Grandin, 2021), training personnel in adequate handling methods (allowing habituation), and the development and application of suitable treatment protocols when indicated.

Our results show that a sedation with xylazine prior to hoof trimming decreases the stress response on behavioral and physiological levels. It can therefore be considered beneficial for animal health and welfare. Our results support its consideration for practical application, especially in very stressful situations (e.g., hoof treatment), due to its effectiveness, convenient way of administration, and low costs. The sedation furthermore allows a more feasible handling of the animal by decreasing defensive and flight behavior and thereby reduces the risk for injury of the sheep, and the handling person. However, careful handling of the animals is still a prerequisite since it is well-known in practice that the effect of xylazine is much dependent on the initial stress level of the animal (Clarke and England, 1989; Sanhouri et al., 1992; Lamont et al., 2001). Further studies are needed to investigate the feasibility and benefit of our sedation protocol under practical circumstances, and the possible positive effects of such a routine application on health and production traits, e.g., meat and wool quality, fertility, susceptibility for different diseases, life span and lifetime production, and take into account different fixation methods (see Introduction).

Xylazine hydrochloride is registered in the European Union (EU) and other countries for application in food-producing animals, but is however not available for livestock in many other parts of the world (e.g., United States; Coetzee, 2013; Smith, 2013). Our study adds to other publications that clearly show the beneficial effect of a sedation during stressful procedures (Sanhouri et al., 1992; Rizk et al., 2012a,b), thereby strongly recommending the legalization of this substance in livestock in the respective countries.

Furthermore, a focus should be laid on the development of improved sedation protocols with other and/or more modern compounds (e.g., romifidine, medetomidine, detomidine, and acepromazine) and possible emergency protocols in case of life-threatening side effects. Thompson et al. (1991) have for example shown the positive effect of atipamezole in calves suffering from xylazine-induced sedation, bradycardia, and ruminal atony. Atipamezole and yohimbine are two antidotes for α2-agonists that are widely used in veterinary medicine for non-food-producing animals (Kim et al., 2004; Mees et al., 2018). Their usage is however forbidden in food-producing animals in the EU and other countries, not due to direct evidence of the danger of these substances for consumers, but since the procedure of approval is too expensive for pharmaceutical companies in respect to the small sales market (Richter et al., 2014).

CONCLUSION

It was confirmed that the fixation of sheep in dorsal recumbency for routine hoof treatment represents a severe stress situation. The results show that a sedation with xylazine prior to hoof trimming decreases the behavioral and physiological stress response. Further studies are needed to investigate the feasibility and benefit under practical circumstances.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by State Administration Office (Landesverwaltungsamt, Referat Verbraucherschutz, Veterinärangelegenheiten Sachsen-Anhalt, Germany). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AS contributed to project acquisition. AS, RW, HF, SA, and AR contributed to trial and project design. SA, RW, and HF contributed to trial implementation and sample collection. MS contributed to sample analysis. JS, NM, SA, and MS-B contributed to data analysis and data interpretation. SA and MS-B contributed to writing of manuscript. WB, AS, RW, HF, AR, MS, JS, and NM contributed to revision of manuscript. All authors contributed to the article and approved the submitted version.

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