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Assessment of chronic stress in sheep (part I): The use of cortisol and cortisone in hair as non-invasive biological markers

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ABSTRACT

The ability to evaluate the extent of poor welfare during clinical disease is of great importance for animal welfare. Chronic exposure to stressful situations has a negative impact on animal welfare, and there is a need for valid and reliable biomarkers of chronic stress. The aim of this study was (1) to evaluate hair cortisol (HC) and hair cortisone (HCn) as measures of chronic stress in sheep and (2) to assess stress and pain associated with ovine footrot using different physiological [fecal cortisol metabolites (FCM), HC and HCn] and behavioral measures. The study was performed as a single foot inoculation using a boot. The right hind foot of 24 lambs was inoculated with different strains of *Dichelobacter nodosus*, and the animals were subsequently monitored daily for two weeks to assess lameness and pain. Hair was collected from both hind limbs at the start of the study and before treatment with gamithromycin subsequent to the trial period, and fecal samples were collected weekly for four weeks. Clinical signs of footrot developed in all experimental groups. There was an increase in FCM from week 0 to 2 ($p < 0.001$), and then a tendency of a subsequent decrease from week 2 to 3 ($p = 0.06$), indicating a chronic stress response due to the maceration caused by the boot and the developing infection. FCM decreased to baseline levels after the animals were treated with gamithromycin. Surprisingly, the concentration of HC decreased from week 0 to 3 ($p < 0.001$) in the right and left limb, and significantly more in the right limb ($p < 0.01$). The concentration of HCn increased from week 0 to 3 ($p = 0.05$) in the right limb but decreased in the left limb ($p < 0.05$). Hence, our findings suggest local production and/or metabolism of glucocorticoids in the hair follicles, which should be taken into consideration in studies using HC as a parameter of chronic stress. This study provides a first indication for a potential merit of hair cortisone as a biomarker of stress in sheep.

1. Introduction

Lameness has been a major concern to sheep farmers for centuries, and ovine footrot is one of the most common causes of lameness in sheep. *Dichelobacter nodosus* has been identified as the main causative agent (Roger, 2008). The first stage of the disease is an interdigital dermatitis that may progress to necrotic separation of the claw capsule from underlying tissues. The clinical symptoms of footrot include lameness, reluctance to move and reduced feed intake (Roger, 2008). Footrot has considerable consequences for animal welfare and cause economic losses for the sheep industry. Inflammatory diseases such as footrot are probably the major source of pain in ruminant species (Fitzpatrick et al., 2006). It is therefore vital to evaluate the extent of poor welfare during clinical disease (Broom and Corke, 2002). In this regard, non-invasive,

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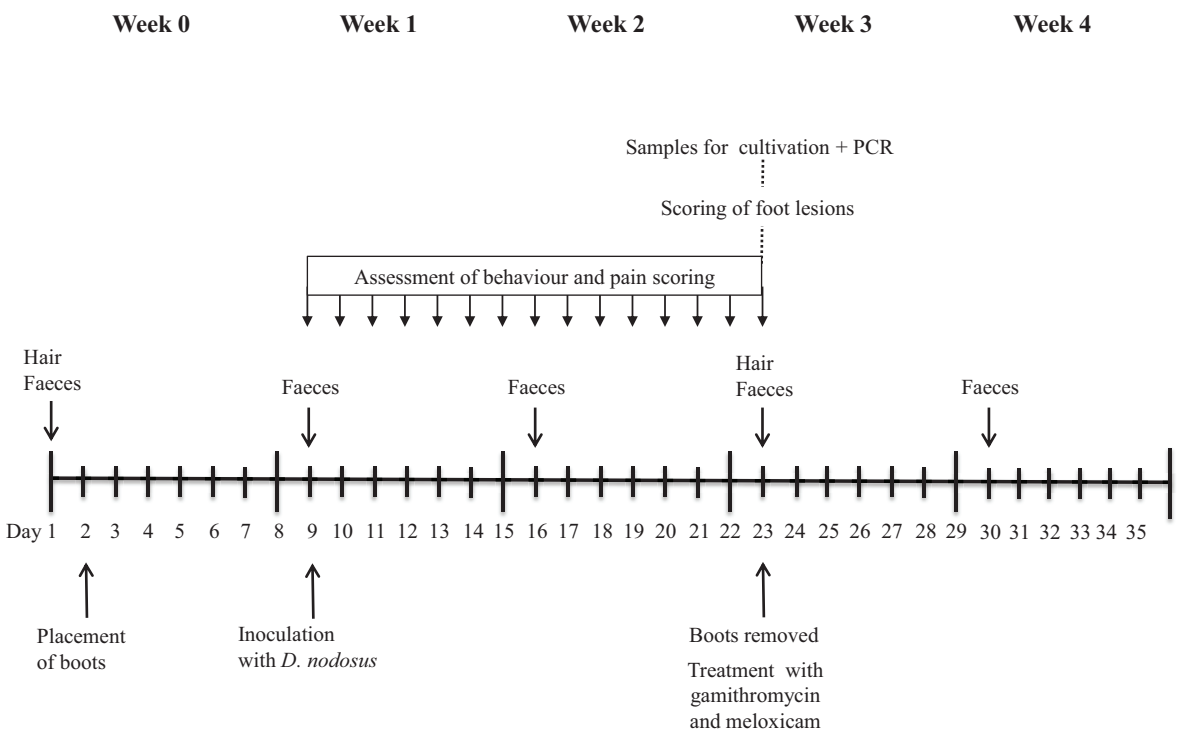

Fig. 1. The time line of the performed experiment. Baseline measures were conducted on Day 1 and 2 (Week 0). The lambs were inoculated with different strains of *D. nodosus* on day 9 (Week 1). The boots were removed on Day 23 (Week 3), and a range of samples and measures were collected before the lambs were treated for footrot. Fecal samples were also collected in Week 4 to assess the effects of treatment with gamithromycin.

objective methods to quantify stress and pain are indispensable tools to evaluate the welfare impact of diseases.

Cortisol has traditionally been measured in various body fluids and excreta in order to assess stress and pain in animals (Palme, 2012). Especially, the quantification of glucocorticoid metabolites in feces has been used as a non-invasive method to assess stress in a variety of species (Palme, 2012; Keckeis et al., 2012; Touma and Palme, 2005; Kleinsasser et al., 2010; Lepschy et al., 2010; Arias et al., 2013). The results remain unaffected by GC secretion in response to handling stress (Möstl and Palme, 2002) and the samples are easy to collect. This technique has previously been validated in sheep (Palme and Möstl, 1997; Palme et al., 1999; Möstl et al., 2002). However, measured cortisol (metabolites) in body fluids and excreta do not reflect the overall stress response over longer periods of time (Palme, 2012).

In recent years, the use of hair cortisol as a biomarker of chronic stress has attracted much attention (Accorsi et al., 2008; Gow et al., 2010; Russell et al., 2012; Ghassemi Nejad et al., 2014). Hair cortisol is insensitive to the impact of acute stress, including that caused by handling during sampling procedures. The procedure of collecting hair is simple, and the samples can be stored at room temperature and sent through the mail (Gow et al., 2010). Hair is a biomaterial that may accumulate glucocorticoid (GC) hormones over weeks to months (Gow et al., 2010). Three main models for compound incorporation into hair have been proposed: (1) active or passive diffusion from blood into growing cells in hair follicle, (2) diffusion from body secretion (sweat, sebum) during formation of hair shaft and (3) external environmental sources after hair shaft formation (Gow et al., 2010). Local cortisol production may participate as well. Indeed, Ito et al. (2005) demonstrated that hair follicles contain a functional equivalent of the hypothalamic–pituitary–adrenal (HPA) axis and can synthesize cortisol after stimulation by corticotrophin-releasing hormone. This finding has been supported by other studies (Sharpley et al., 2009; Keckeis et al., 2012) and may contribute to differences in hair cortisol concentrations found between different body locations (Moya et al., 2013). The mediators of this peripheral HPA axis are proposed to regulate the cutaneous response to local stressors (Slominski et al., 2007).

In addition, cortisol can be inactivated locally by an 11β-hydroxysteroid-dehydrogenase resulting in the formation of cortisone (Vanaelst et al., 2013). Although hair cortisone has received little attention so far, previous studies have indicated that cortisone may be a useful additional biomarker for stress research in hair (Raul et al., 2004; Perogamvros et al., 2010; Stalder et al., 2013; Vanaelst et al., 2013). Hence, further studies elucidating the incorporation of cortisol and cortisone into hair, and the usefulness of these glucocorticoids as parameters of chronic stress, are urgently needed.

The aim of this study was therefore: (1) to evaluate hair cortisol (HC) and cortisone (HCn) as measures of chronic stress in sheep and (2) to assess stress and pain associated with ovine footrot using different physiological [fecal cortisol metabolites (FCM), HC and HCn] and behavioral measures.

2. Material and methods

This study was conducted as part of a larger study which aimed to generate knowledge about ovine footrot in Norway by studying the effect of experimental infection with different strains of *D. nodosus* (Knappe-Poindecker et al., 2014). The protocol and conduct of this study was approved by the National Animal Research Authority in Norway (protocol number 11/3554).

2.1. Animals and housing

Twenty seven lambs of the breed Norwegian White, aged 4–5 months, with a mean body weight of 44 kg (range: 33–54 kg) were used in this study. Treatment groups of six lambs were used, and two cattle strains and two sheep strains (benign and virulent) of *D. nodosus* were tested. There were 3 males and 3 females in each treatment group, while the 3 lambs in the control group were males. The lambs were selected randomly from the experimental sheep flock free of footrot and *D. nodosus* at the Norwegian University of Life Sciences. The claws were clinically examined and samples were analyzed using PCR with regards to *D. nodosus* 8 weeks before the

Table 1
Description of the behavioral and postural indicators assessed in 24 lambs from Day 9 to 23.

Variable	0	1	2	3
Mental assessment	Normal and alert	NC	NC	Signs of depression
Respiratory rate	Normal	NC	Abnormal (slow or panting)	NC
Recumbency	Normal	Slightly delayed rising	Requires encouragement to stand	Unwilling or unable to stand
Shifting weight	Normal	Mild or occasional	Moderate	Constant
Appetite	Normal	Mildly reduced interest	Moderately reduced interest	Inappetent
Palpation of foot	No signs of pain	Mild signs of pain	Moderate signs of pain	Severe signs of pain

NC: No criteria applicable for this category.
Table modified after Ahern et al. (2009).

start of the experiment. The animals were housed two weeks prior to the start of the experiment. The selected lambs underwent a clinical examination by a veterinary surgeon to exclude any diseased or injured animals prior to the study. There were nine boxes in the sheep isolate (mean size of 3 m^2, range: 2.1–3.6 m^2) with slatted metal flooring, permitting three lambs of the same sex in each box. Each animal was identified by the ear tag number and lambs were randomly allocated to one of the four treatment groups. The lambs were fed hay and concentrate twice daily and had access to water ad libitum.

2.2. Experimental design

Fig. 1 shows the time line of the experimental design. The experimental design is described in Knappe-Poindecker et al. (2014). Briefly, on Day 1 (Week 0), hair was collected using a shave and re-shave method. Old hair had been removed 2 weeks previously from the dorsal aspect of the metatarsus on both hind limbs by shaving close to the skin with commercially available clippers. A sample of new hair was collected within the same region, being careful not to cut any old hair. Fecal material was carefully removed from each animal directly from the rectal ampulla, immediately stored on ice and thereafter frozen (−20°). Fecal samples were collected every week until the end of the study (i.e., one week after the lambs were treated for footrot (Week 4)).

On Day 2, canine rubber boots (Nordströms rubber boots) were placed on the right hind foot of each lamb (including the control group) for maceration, and the boots were left in place for seven days.

On Day 9 (Week 1, seven days after placing the boots on the feet) the boots were infused with 10 ml bacterial suspension (1.75×105 CFU/ml of *D. nodosus*). 10 ml tap water was added to the boots instead of bacterial suspension in the control group. The animals were monitored daily for two weeks to assess lameness, and behavior and pain (adapted from Ahern et al., 2009) was scored every day by a veterinary surgeon (see Table 1). Lameness was scored using the following scale (Morck et al., 1994): 0 = no limp; 1 = slight limp; 2 = moderate limp; 3 = nonweight bearing. If any animal scored ≥1 on the lameness score and/or ≥1 on either of two

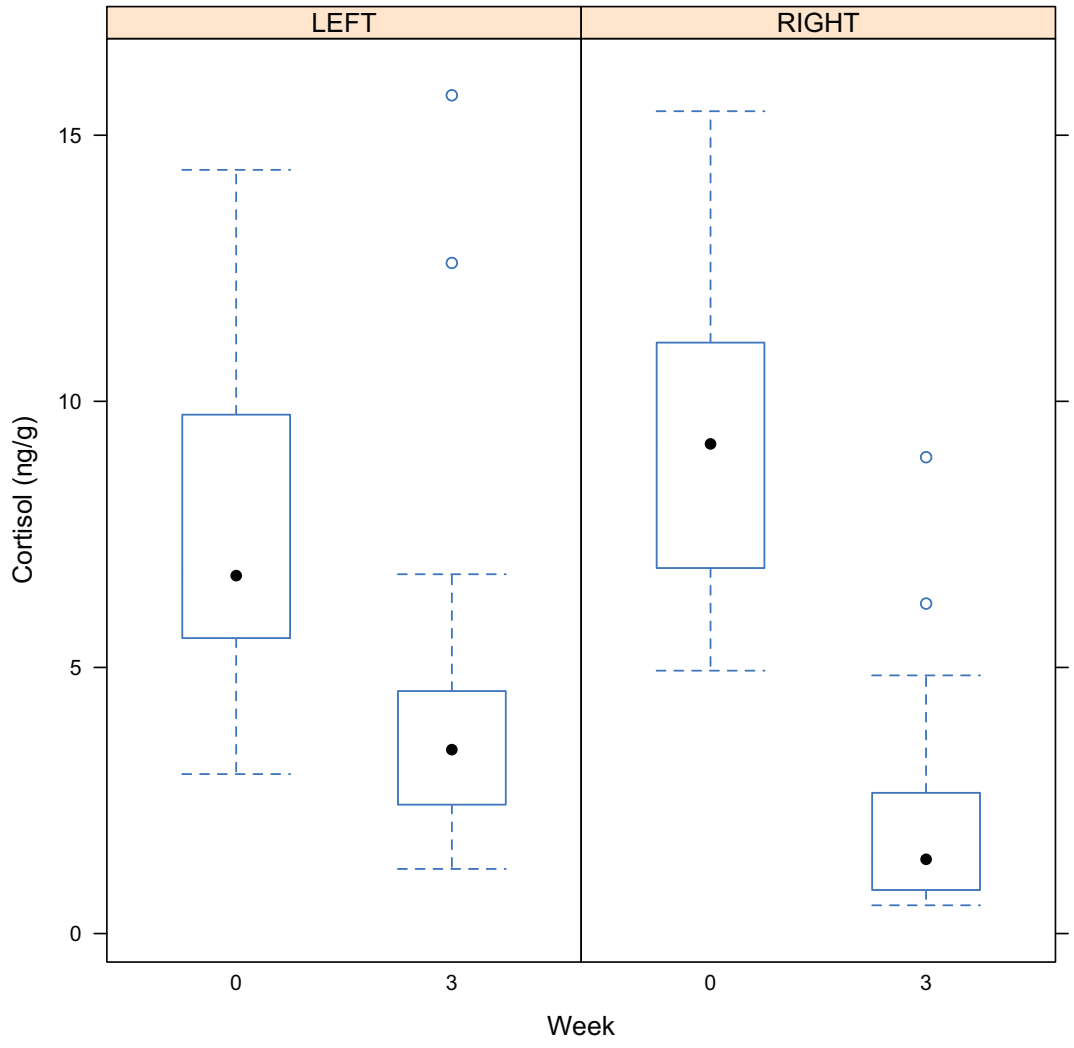

Fig. 2. Box and whiskers plot of the concentrations (ng/g) of cortisol in hair of 24 lambs measured in the right and left hind limb in week 0–3.

predefined pain score variables (shifting weight and/or palpation of foot) it would receive meloxicam (0.5 mg/kg SC) which would be administered every 48 h until the symptoms resolved.

On Day 23 (Week 3, two weeks post inoculation) the boots were removed and the foot lesions were scored on a scale from 0 (normal) to 4 (severe interdigital dermatitis and underrunning of the horn of the heel, sole and wall) (Knappe-Poindecker et al., 2014). All the lambs were examined for evidence of lameness. Samples were collected from the interdigital skin for culturing and PCR of *D. nodosus*. Hair was collected from the dorsal aspect of the metatarsus using commercially available clippers. Each animal received a single injection of gamithromycin (Zactran 6 mg/kg SC, Merial). Lambs which had clinical signs of footrot, blisters or were lame received meloxicam (Metacam 0.5 mg/kg SC, Boehringer Ingelheim Vetmedica GmbH). The lambs were monitored daily until fully recovered.

2.3. Extraction and measurement of steroids from hair

The hair samples were stored in tin foil bags at room temperature until analysis. An aliquot of 200–400 mg of each hair sample was washed with 5 ml 100% *n*-hexane for 10 min (Thermomixer, stage 5 of 10, RT; Eppendorf, Germany) in order to remove dust and sebum. The *n*-hexane fraction was discarded and the hair samples were left over night in a fume hood for complete dryness. 100 mg aliquots of washed hair samples were extracted with 5 ml 100% methanol and incubated at RT for 24 h with gentle rotation (Thermomixer, stage 5 of 10, RT; Eppendorf, Germany). After centrifugation (15 min at 2500 × *g*), the methanol extracts were removed and evaporated to dryness and dissolved in 1 ml of 80% methanol. The immunoreactivity of the samples was then determined in a cortisol enzyme immunoassay (EIA) (Palme and Möstl, 1997) and a cortisone EIA (Rettenbacher et al., 2004).

2.4. Extraction and determination of fecal cortisol metabolites (FCM)

Extraction of all fecal samples was performed as described by Palme et al. (2013). To determine the amount of FCM, we used an 11-oxoaetiocholanolone EIA described by Palme and Möstl (1997). Details of all used EIAs including the respective antibody cross-reactivities with relevant steroids are provided in Palme and Möstl (1997) and Rettenbacher et al. (2004).

2.5. Statistical analysis

All statistical analyses were carried out using the statistical package R (www.r-project.org). All models were estimated using standard linear regression/ANOVA, and carefully checked to conform approximately to normality using residual and goodness-of-fit plots. The R^2 statistic was used as the goodness-of-fit statistic. As no significant differences were found between the experimental groups inoculated with different challenge strains, the measures were pooled prior to the statistical analysis. If not otherwise indicated, all values are given as mean ± SE.

3. Results

3.1. Steroids in hair

The concentration of cortisol in hair decreased between Week 0 and 3 in the right limb, where the boot was placed, and the left limb ($p < 0.001$). A significant difference was also detected between right and left limb in Week 3 ($p < 0.01$; Fig. 2). Cortisone increased between Week 0 and 3 in the right limb ($p = 0.05$) and decreased between Week 0 and 3 in the left limb ($p < 0.05$; Fig. 3). Further, a

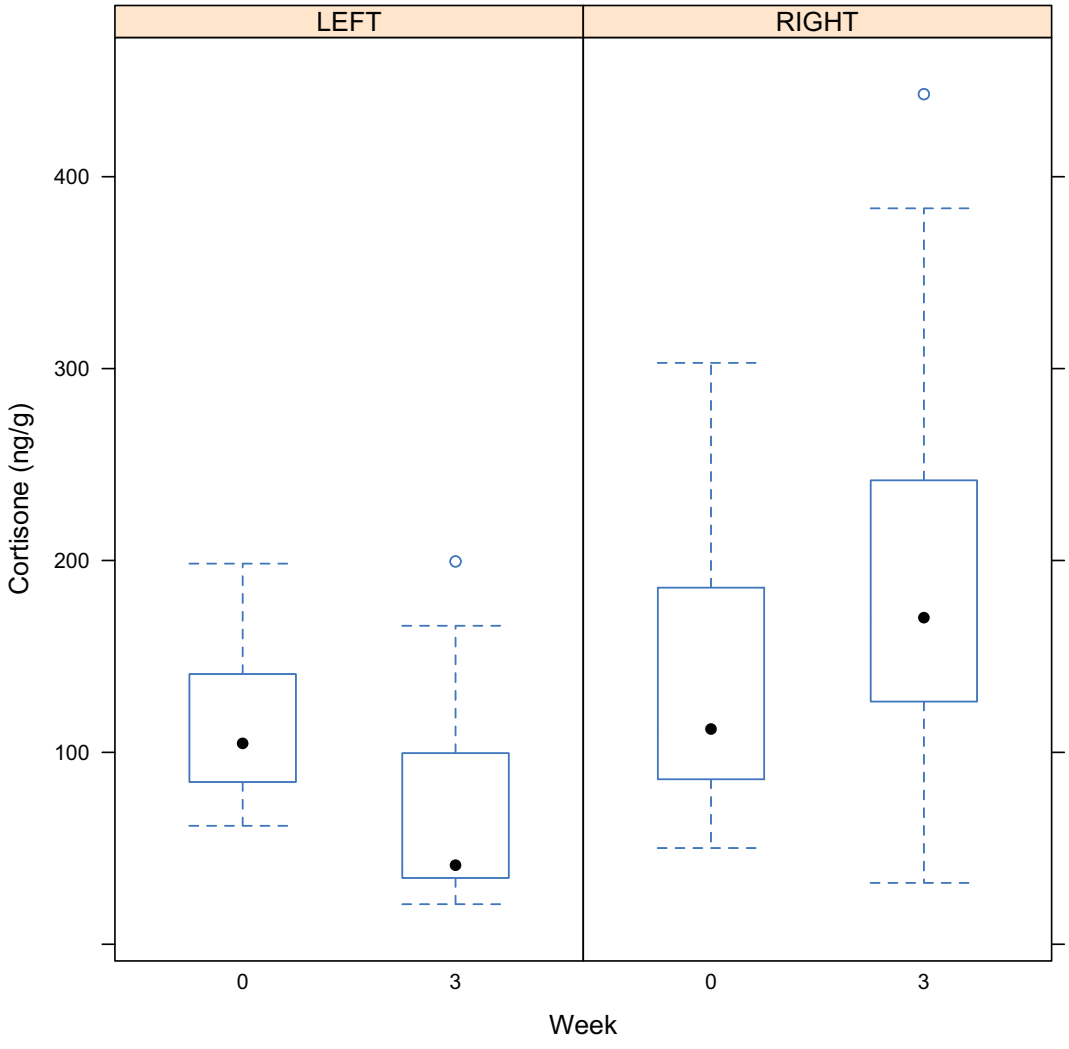

Fig. 3. Box and whiskers plot of the concentrations (ng/g) of cortisone in hair of 24 lambs measured in the right and left hind limb in week 0–3.

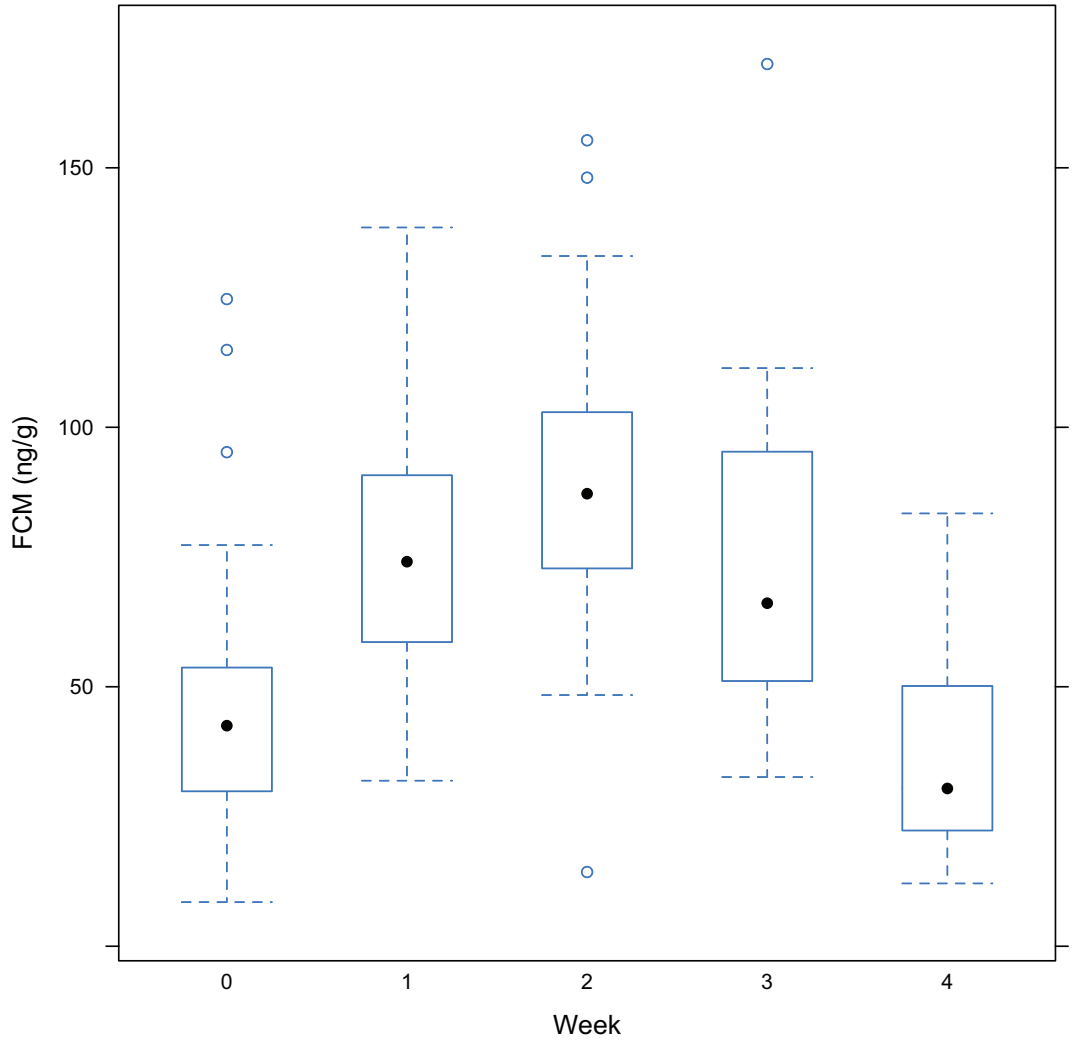

Fig. 4. Box and whiskers plot of the concentrations (ng/g) of fecal cortisol metabolites (FCM) of 24 lambs in week 0–4.

significant difference between the right and left limbs was observed with respect to cortisone in Week 3 ($p < 0.001$). While a strong correlation was found between cortisone and cortisol in Week 3 for the left limb ($R^2 = 0.84$, $p < 0.001$; Fig. 3), no significant association was detected between cortisone and cortisol in Week 3 for the right limb. The controls were removed from the dataset used in the regression models. However, including the controls did not substantially change the results. No significant difference in relation to sex was found.

3.2. Fecal cortisol metabolites (FCM) levels

Concentrations of fecal cortisol metabolites increased between Week 0 and 2 with approximately 23.1 (± 3.91, $R^2 = 0.32$, $p < 0.001$) ng/g per week and decreased with approximately 27.3 (± 4.0, $R^2 = 0.38$, $p < 0.001$) ng/g per week between Week 2 and 4 (Fig. 4). There was a tendency toward a decrease in FCM between Week 2 and 3 ($p = 0.06$). It should be noted that the controls were removed from the dataset used in the regression models. Including the controls did not substantially change the results (21.3 ± 3.8 ng/g per week, $R^2 = 0.28$, $p < 0.001$ and -26.5 ± 3.8 ng/g per week, $R^2 = 0.38$, $p < 0.001$ for Week 0–2 and Week 2–4, respectively). No significant difference in relation to sex was found.

3.3. Footrot lesions and lameness

Symptoms of footrot developed in all experimental groups (Knappe-Poindecker et al., 2014). *D. nodosus* was isolated from 5 out of 24 lambs, and 15 out of 24 lambs tested positive for *D. nodosus* by PCR. All lambs tested negative for *D. nodosus* by PCR within six weeks after treatment with gamithromycin. Four lambs developed lameness during the experiment. One lamb was scored as 2 (on Day 17) using the described lameness score. Upon removal of the boots, three lambs were lame. All four lambs were treated with meloxicam (Metacam 0.5 mg/kg SC, Boehringer Ingelheim Vetmedica GmbH). Six lambs had blisters upon removal of the boots on Day 23.

3.4. Pain scoring

The lambs were scored as alert during the whole experiment, and their appetite and respiratory rate was scored as normal. All animal's appeared to rise from a recumbent position, stand and move around the box normally, with exception of the one lamb scored as 2 on the lameness score (on Day 17). This lamb was also evaluated to have moderate pain by palpation of the right hind limb and evaluation of the response.

4. Discussion

Symptoms of footrot developed in all experimental groups, but the proportion of infected lambs was lower than observed in other studies and the symptoms were mainly mild (Knappe-Poindecker et al., 2014). The low prevalence of lameness and few signs of altered behavior and pain are in agreement with the mild symptoms of footrot recorded. However, the systemic stress response evaluated by the concentrations of FCM indicates that the animals experienced chronic stress due to maceration and the development of the infection. Sheep are prey animals that will try to mask the signs of stress and pain (Fitzpatrick et al., 2006), and we may therefore have missed more subtle behavioral signs expressed by the lambs. Also, the size of the pens was smaller than ideal for lameness assessment, and slight lameness may therefore have been undiscovered.

In our study, we found a rise in FCM between Week 0 and 2 and then a tendency of a subsequent decrease from Week 2 to 3. FCM is in general expected to increase during clinical disease, and an increase in FCM has been described in cows with abomasal displacement (Kahrer et al., 2006) and in horses with colic (Merl et al., 2000). The rise in FCM in response to the developing infection was therefore as expected. However, a reduction in HPA-axis activity has previously been observed in response to chronic stress in different species (Fordham et al., 1991; Fisher et al., 1997). Cortisol levels have been found to be depressed below baseline values due to severe footrot in sheep (Ley et al., 1991). These findings may be due to the adaptation of the HPA system to repeated or long-term exposure to a particular stressor (Dwyer and Bornett, 2004). This process may be viewed as an adaptive biochemical down-regulation of areas of the HPA response rather than a psychological adaptation. This is achieved by alteration of the control systems for stress responses and ensures a protective response to novel stressors (Jensen et al., 1996; Terlouw et al., 1997). The decrease in FCM between Week 2 and 3 may therefore be due to alterations in internal regulation systems due to chronic stress induced by the infection. Heart rate variability (HRV) was also measured in this study (Stubsjøen et al., part II). The results indicated an inverse relationship between FCM and the time domain HRV parameters in response to chronic stress caused by maceration and the developing infection. This finding is in accordance with other studies which have reported inverse relationships between HRV and cortisol responses to stressors (Schmidt et al., 2010a,b; Johnsen et al., 2012). FCM levels returned to baseline level in Week 4 subsequent to the animals being treated with gamithromycin in Week 3, which suggests an effect of treatment.

We found a decrease in hair cortisol in both limbs from Week 0 to 3. Passive (or active) diffusion from blood has generally been accepted as the primary mechanism for the incorporation of (systemic) cortisol into the hair. The concentrations of FCM increased in our study, and therefore the HC concentration would also be expected to increase if the primary mechanism for incorporation was diffusion from blood. Keckeis et al. (2012) investigated whether they could differentiate between systemic glucocorticoids and those produced in the hair follicle after intraperitoneal administration of radiolabeled cortisol in guinea pigs. They found that only small amounts of systemically administered radioactive cortisol were deposited in hair of guinea pigs. However, they also found large amounts of unlabelled cortisol in the hair. Their findings agree with our results and indicate that the predominant way of incorporation of GC into the hair shaft is not via the blood capillaries, but more likely they originate from local production in the hair follicles and subsequent sequestration in the hair shaft (Keckeis et al., 2012).

A hypercortisolism-induced negative feedback in the "peripheral" HPA-axis in the hair follicles may have caused the cortisol production to decline. The degree to which the "peripheral" HPA-axis in the hair follicles are affected by, or contributes to, the regulation of the central HPA-axis is still unknown. The hair cortisol response may therefore be independent of the central HPA-axis response, and hence, responses of hair cortisol and fecal cortisol metabolites to the same stressor (i.e., the inoculation of *D. nodosus*) could be independent of each other (i.e., hair cortisol reflects local production, whereas FCM reflect adrenocortical activity). This may explain the differences seen in our study between the FCM and the hair cortisol responses.

We also found that the hair cortisol and cortisone responses were different in the limb that was inoculated with *D. nodosus* (right hind limb) compared to the opposite control limb (left hind limb). In the affected right limb, cortisol concentrations decreased while the cortisone concentration increased between week 0 and 3. In the left limb, the concentration of both cortisol and cortisone decreased. However, cortisol decreased significantly more in the right limb compared to the left limb. Moreover, there was no correlation found between cortisone and cortisol in week 3 for the right limb, but a strong correlation was found for the left limb. These site-specific responses indicate a localized reponse in the right limb. Local stressor demands (as induced by wet, anaerobic conditions due to the rubber boot and inoculation with *D. nodosus*) may have changed the local GC production in hair follicle cells regulated by locally expressed HPA mediators, which may explain the localized effects seen in the right hind limb of the lambs in this study.

A local conversion of cortisol to cortisone by the 11β-hydroxysteroid-dehydrogenase (HSD) enzyme could explain the change in cortisone/cortisol ratio in the right hind limb (Raul et al., 2004). This enzyme is a potent dehydrogenase that rapidly inactivates glucocorticoids converting cortisol to cortisone, and Raul et al. (2004) suggested that there was a conversion of cortisol to cortisone before its incorporation in hair by type 2HSD. Our study showed, in accordance with Raul et al. (2004), that cortisone concentrations were higher than cortisol concentrations in hair. This is in contrast to the blood ratio in sheep where cortisone concentrations were reported to be lower than cortisol concentrations (Clements and Newsome, 1973). The observed difference could be due to the different affinities of cortisol and cortisone to the corticoid binding protein. Also, cortisone is less polar than cortisol and a better incorporation of cortisone into hair from the bloodstream would therefore be expected (Raul et al., 2004). Keckeis et al. (2012) found radiolabeled cortisone in the hair of guinea pigs, which suggests that radiolabeled cortisol administered intraperitoneally had been metabolized prior to incorporation into the hair shaft. Local stressor demands may also have enhanced the conversion of cortisol to cortisone in the right hind limb.

The three control lambs tested negative for *D. nodosus*, but all three had lesion score 1 at day 23. Score 1 is considered a limited mild dermatitis, which was probably caused by irritation to the skin as a result of the maceration. This finding is in accordance with Egerton et al. (1969), who found histopathological alterations in the interdigital skin after only four days of maceration. This may explain why the control group was not found to be significantly different from the experimental groups. A pitfall in our study is therefore the use of only one control group. The use of two control groups (one with no boot applied and the other with the boot but no inoculation) would have been beneficial to determine the effect of wearing the boot. Therefore, a chronic stress response due to both maceration and the infection with *D. nodosus* was assessed in this study.

Footrot is a common cause of ovine lameness, and lameness is under emphasized as a cause of pain and distress in sheep. It cannot be ruled out that the lambs did not feel any significant pain in the present study. However, although the clinical signs in general were found to be mild, the measurement of FCM levels indicated a

chronic stress response due to the maceration and infection with *D. nodosus*.

5. Conclusions

In accordance with previous studies, higher concentrations of hair cortisone were found compared to hair cortisol. Our results indicate local production and/or metabolism of glucocorticoids in the hair follicles. To which degree local cortisol production and/or metabolism have been affected by the systemic stress response of the lambs in this study is unknown. Therefore, more studies evaluating influences on local glucocorticoid production and metabolism are needed. Although this study provides a first indication for a potential merit of hair cortisone as a stress biomarker in sheep, further research has to be conducted to elucidate the importance and relevance of hair cortisone analysis as a measure of chronic stress.

Conflict of interest

There is no conflict of interest associated with this article.

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