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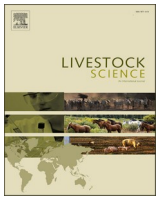

Short Communication

Effect of grazing deprivation as compared to grazing restriction on hair and saliva cortisol levels in pregnant ewes

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HIGHLIGHTS

- When ewes were deprived from grazing, compared to a 5 h daily grazing system, no evidence of chronic stress could be detected through cortisol levels in hair and saliva.
- Cortisol levels increased from the last two months of pregnancy in ewes carrying two lambs compared to ewes carrying one lamb.
- Saliva cortisol variability suggests it should be combined with other biomarkers for accurate stress assessment.

ABSTRACT

Grazing is a natural behaviour of ruminants and when access to pasture is deprivated, a behavioural frustration appears, which can lead to chronic stress. In sheep, no response of stress biomarkers like cortisol has been studied in permanent housed ewes. In the present study, two groups of twenty pregnant Ripollesa ewes were used in a 10-week experiment. The 5h-grazing group (5h-G), had access to pasture five hours daily, and the housed group (H) had no access to pasture and remained permanently in the barn. Hair samples were collected on Weeks 5 and 10 of grazing deprivation (W5, W10), and saliva samples on Weeks 0, 3, 5, 8 and 10 (W0, W3, W5, W8, W10). No significant differences in hair cortisol (HC) or saliva cortisol (SC) were observed between Groups 5h-G and H throughout the study period ($P > 0.05$). However, significant differences were observed in ewes carrying one or two lambs (PROL1 HC=4.97±0.1, PROL2 HC=7.20±0.1, $P < 0.05$). No evidence of chronic stress could be detected through cortisol levels in hair and saliva although abnormal behaviours appeared in previous results published from the same experience. Saliva cortisol showed a significant variability over time and it should be used in a combination with other biomarkers. Further research is needed to analyse pregnancy effect, longer periods of grazing deprivation, and the use of wool samples or other body regions.

1. Introduction

Access to pasture is important for the welfare of grazing ruminants, because they can express their natural behaviour, and grazing deprivation can result in chronic stress (reviewed by Von Keyserlingk et al., 2009). Indeed, access to pasture is considered a positive welfare indicator related to comfort and exploration (Papagiorgiou et al., 2022). Some authors reported that the ability to graze in pasture reduces stress (Higashiyama et al., 2007) and, consequently, animal welfare increases with greater time allocated to grazing (Nejad et al., 2021). On the contrary, when access to pasture is not granted, behavioural frustration occurs, and, consequently, abnormal behaviours appear. In sheep flocks, grazing deprivation induces wool-pulling, which is an abnormal behaviour due to a lack of oral stimulus (Vaseur et al., 2006; Parés et al., 2023). In intensive systems, other abnormal behaviours have also been reported, like repetitive pacing or repetitive cocking, which are more prevalent than in extensive systems (Kanakanja et al., 2024).

When behavioural frustration persists, it can lead to chronic stress, which can be measured through stress biomarkers like cortisol. However, neuroendocrine responses to chronic stress are variable (reviewed by Dwyer et al., 2004). Plasma cortisol has been studied in different species including sheep, goat, cattle, and pig (Mormède et al., 2007). In ewes, plasma cortisol has been used in numerous studies (Nejad et al., 2014; Andanson et al., 2020; Weaver et al., 2021). Cortisol can also be measured from other matrices such as saliva, hair or wool. In ewes, hair cortisol has been used to monitor chronic stress (Stubsjoen et al., 2015 and 2018; Nejad et al., 2021). It has also been used sheep wool (Fürtbauer et al., 2019; Weaver et al., 2021) and salivary cortisol (Fell

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et al., 1985; Andanson et al., 2020). Hair and wool samples have some advantages, compared to blood samples, because they provide information on cumulative stress over a retrospective period, from weeks to months (Cook, 2012; Nejad et al., 2021). They are easy to extract, less invasive, and there is no effect of a possible stress reaction of the animal during sampling (Poudel et al., 2022). Moreover, in stressful situations like heat stress or water restriction, cortisol in wool has been demonstrated to be a better indicator than blood levels (Nejad et al., 2014). Cortisol in hair correlates with other matrices, but correlation is variable (reviewed by Kalliokoski et al., 2019). Nevertheless, hair cortisol results should be considered with caution. Some authors state that plasma cortisol is partially accumulated in hair, and it could be affected by local cortisol liberation (Keckeis et al., 2012; Salaberger et al., 2016). Furthermore, cortisol in hair does not accumulate permanently but can be eliminated in time (Colding-Jorgensen et al., 2023). In summary, hair and wool samples are considered useful to assess chronic stress (Barrel, 2019), but some cautions should be taken into account. On the other hand, saliva samples have advantatges over plasma samples, and a good correlation has been found with blood cortisol (Fell et al., 1985; Yates et al., 2010).

Grazing deprivation has been demonstrated to affect ewes´behaviour (Parés et al., 2023), and the effects of part-time grazing on feeding behaviour in several species has been analysed in several studies (reviewed by G. Molle et al., 2022). However, although plasma cortisol in dairy ewes has been studied in part-time grazing conditions (G. Molle et al., 2022), the impact of permanent housed system or part-time grazing on cortisol levels in hair and saliva samples has not been analysed yet in pregnant sheep. Ewes are often deprived from grazing in late pregnancy, in order to take better care before lambing. In effect, cortisol levels increase in late pregnancy in other species like cow and pig (Edwards et al., 2018), but in ewes it depends on litter size (Brunet and Sebastian, 1991; Gregula et al., 2021; Santarosa et al., 2022).

The present study shows the results of hair and saliva cortisol levels from a previous study in which wool-pulling and aggressive behaviours increased their prevalence when ewes were managed in a permanent housed system (Parés et al., 2023). The objective was to determine if grazing deprivation produces increased stress response, measured by cortisol levels in hair (HC) and saliva (SC) samples.

2. Material and methods

2.1. Animals and management

A Ripollesa breed flock from the experimental farm of the Autonomous University of Barcelona (UAB) was used. All ewes of the flock were mated at the beginning of September and pregnancy diagnosis was performed by ultrasound 30 days later. During mating, ewes had access to pasture 5 h daily. After pregnancy confirmation, only forty pregnant ewes with an average age of 4.6 ± 1.8 years and an average body condition score 2.54 ± 0.24 were considered. Ewes carrying one lamb (PROL1) or two lambs (PROL2), determined after lambing, were considered during the experiment.

Two groups of twenty ewes were balanced according to age and body condition. No significant differences were found in prolificacy between both groups ($P > 0.05$). Both groups were moved and housed in an open barn in a two-week adaptation period, during which ewes mantained access to pasture five hours daily. Groups were separated by a metal fence, with visual contact between them. After the adaptation period, the groups were randomly assigned to one of the following treatments: 5h-G, group having acces to pasture for five hours daily, from 10:00 to 15:00; H, group permanently housed. The ewes with access to pasture were always guided by a shepherd when entered to pasture, and grazed always in silvopastoral systems around the farm. The experiment lasted 10 weeks between October and December. During the experiment, temperature never exceeded 25 °C. Both groups were fed with alfalfa hay *ad libitum* in the barn, provided in a feed trough of 20 metres long in each pen, located at one side of the barn, allowing simultaneous feeding of all animals (1.0 m of feed trough per ewe). A 1.5-metre filling drinker and one sheep mineral block was allocated in each group. Stocking density in the barn was 2.5 m^2/ewe. Both groups were housed with straw bedding, which was renewed weekly. Experiment design is shown in Fig. 1.

2.2. Measurement of cortisol from hair

In the first week of the experiment, hair from the left forelimb was shaved in all ewes. The shaved surface was from the cranial part of the forearm, approximately 5 × 5cm in the middle point between the carpus and humeroradial joint. Later, the same surface-hair samples were obtained twice (Fig. 1), in Week 5 (W5), when ewes were in the first half of pregnancy and in Week 10 (W10), when ewes were in the second half of pregnancy. Samples were collected using an electric hair clipper (X3 ceramic-titanium hair clipper, Palson® Trading España S.L.; Collbató, Spain) and were stored in paper bags at room temperature before laboratory analysis. Samples were processed following a methanol-based extraction protocol previously described (Davenport et al., 2006; Tallo-Parra et al., 2015). A total of 250 mg of each sample was weighed into a 15-ml polypropylene tube. Samples were washed with 2.5 ml of isopropanol and vortexed for 2.5 min. The isopropanol was eliminated by decantation and the process was repeated twice to perform three washes in total. Samples were left to dry at room temperature for 48h approximately. Once dried, they were mechanically minced with a ball mill (Retsch, MM2 type; Germany). Fifty mg of each powdered sample was incubated with 1.5 ml of methanol for 18h at 30 °C in an incubator shaker with continuous mixing (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc.; Edison, USA). Following extraction, samples were centrifuged, and 0.750 ml of the supernatant were transfered to a new microtube and evaporated in an oven. Once completely evaporated, samples were reconstituted with 0.2 ml of EIA buffer provided by the assay kit and stored frozen at −20 °C until analysis. Cortisol concentrations in hair samples were determined by using a competitive cortisol enzyme immunoassay (EIA) kit.

2.3. Measurement of cortisol from saliva

Saliva samples were taken in all ewes on Weeks 0, 3, 5, 8 and 10 (W0, W3, W5, W8, W10), and sampling was always performed between 16:00 and 17:30. Samples were obtained from a sponge of Salivette® tubes (Sarstedt, Aktiengesellsschaft & Co.; Nümbrecht, Germany) by gently introducing it into the ewe mouth to be chewed during approximately one minute. Each sheep was individually restrained to obtain saliva samples. The sponge was introduced into the ewe's mouth using an arterial clamp to the sides of the mouth to chew the sponge. After sampling all ewes (n = 40), samples were subsequently centrifuged at 3000 rpm (J20 XPI, Beckman Avanti®, Beckman Coulter, Inc.; Brea, CA, USA) for 10 min and then stored at −20 °C until analysis. Measurement of cortisol concentrations in saliva samples and the assay validation tests were performed using a commercial Enzyme-Linked ImmunoSorbent-Assay (ELISA) kit (Neogen Corporation©; Ayr, UK). According to the manufacturer, cross-reactivity of the EIA antibody with other steroids is as follows: prednisolone 47.4 %, cortisone 15.7 %, 11-deoxycortisol 15.0 %, prednisone 7.83 %, corticosterone 4.81 %, 6-hydroxycortisol 1.37 %, 17-hydroxyprogesterone 1.36 %, deoxycorticosterone 0.94 %. Steroids with a cross-reactivity of <0.06 % are not presented. Salivary samples were analysed in a 1:10 dilution. For the assay, 50 µl of standards and 50 µl of the 1:10 diluted salivary samples were added to the appropriate wells in duplicate. Immediately after, 50 ul of the diluted enzyme conjugate was added and incubated for 1 h. After incubation, the plate was washed three times and incubated for 30 min in 150 ul of substrate. Then, the optical density was read using a microplate reader (SunriseTM basicmicroplate reader, Tecan Austria GmbH; Grödig/Salzburg, Austria) at 450 nm. The assay was validated for the analysis of cortisol in saliva of

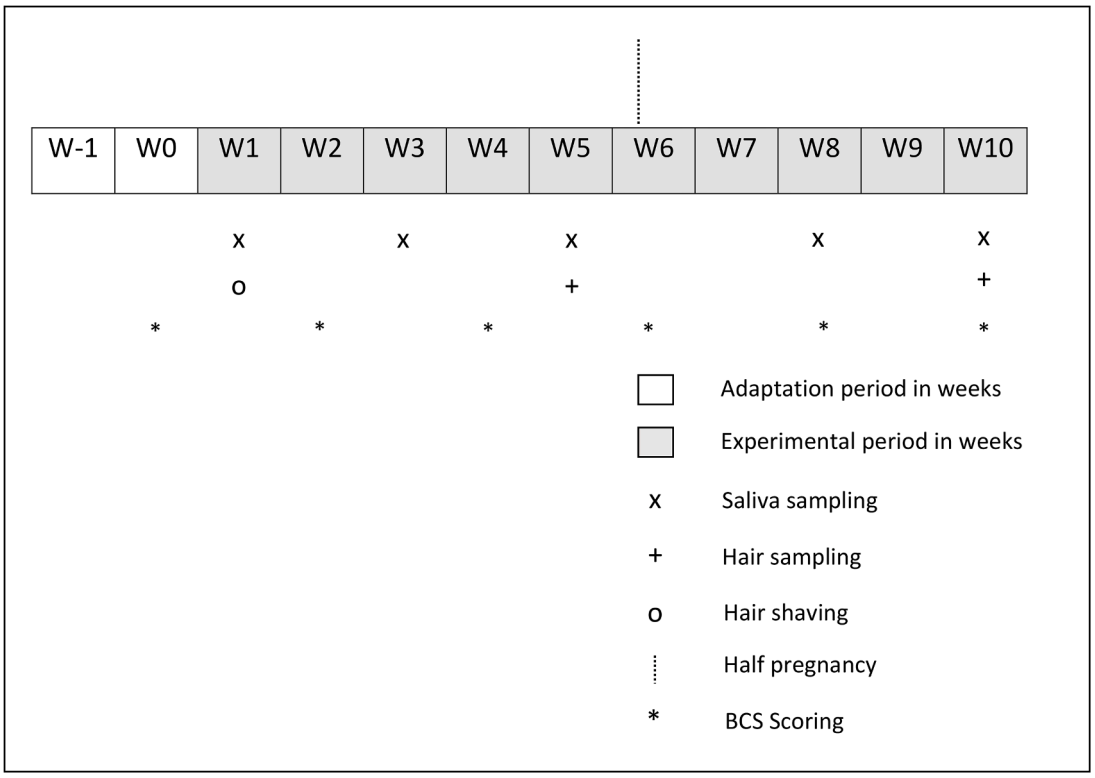

Fig. 1. Experimental design, indicating hair shaving, hair sampling, saliva sampling, BCS scoring and half pregnancy.

Ovis aries following the criteria for an immunological validation (Midgley et al., 1969; Reimers and Lamb, 1991). Saliva samples from 20 different individuals were pooled for the validation assay. Intra-, and inter-assay coefficients of variation (CV) from duplicated samples analysed were calculated for precision assessment. Specificity was tested with the linearity of dilution, determined by using 1:1, 1:2, 1:5, and 1:10 dilutions of the pool with EIA buffer. Mean intra- and interassay coefficients of variation were 5.81 % and 11.23 %, respectively, indicating good repeatability of the test. The dilution test showed an $R^2 = 98.54$ % and a mean recovery rate of 105.90 % suggesting high specificity of the test.

2.4. Statistical analysis

HC and SC of the same individual were analysed as repeated measures with a mixed-effect analysis of the variance test using the MIXED procedure of SAS (version 9.1.3, SAS Institute Inc.; Cary, NC, USA), containing Weeks '5' and '10' of the experimental period to analyse hair cortisol and weekly values to analyse saliva cortisol. Fixed effects were 'treatment' (5h-G or H), 'week of the experimental period', prolificacy

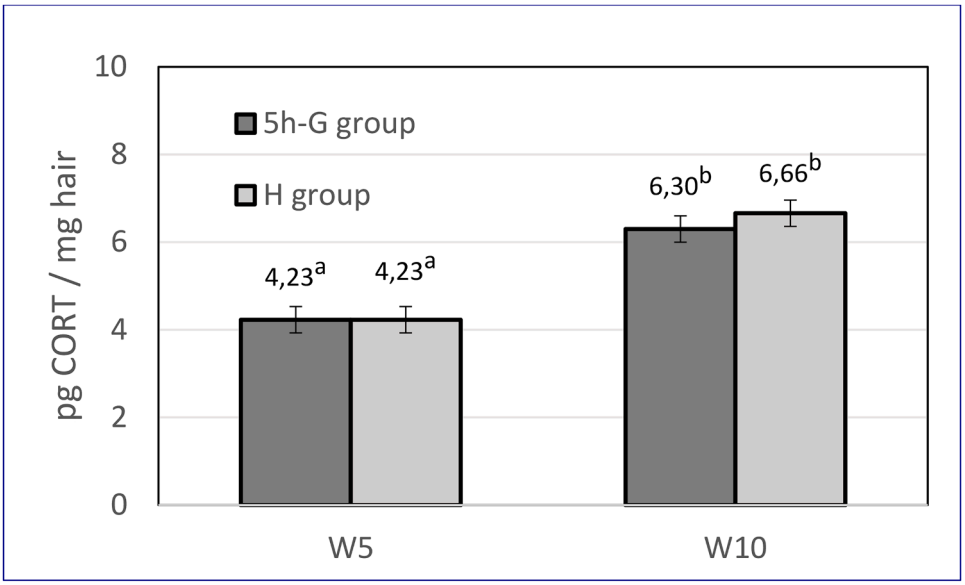

Fig. 2. Cortisol concentration in ewes hair samples in weeks 5 and 10 for each treatment group (5h-G and H). Different superscripts indicate significant statistical differences.

(PROL1 or PROL2) and interactions (TrxW, TrxPROL, WxPROL). The individual animal was considered a random effect. Statistical differences were considered significant at $P < 0.05$.

3. Results and discussion

Mean hair cortisol results are shown in Fig. 2. HC of the entire flock (5h-G and H groups) showed a significant difference between W5 and W10 (W5 HC=4.23±0.1, W10 HC=6.48±0.1, $P < 0.05$). No significant differences were observed between Groups 5h-G and H throughout the study period. Both groups maintained the same body condition score (BCS) during the experiment (5h-G BCS=2.6 ± 0.1, H BCS=2.7 ± 0.1, $P > 0.05$). Nevertheless, significant differences were observed in W10 in ewes carrying one or two lambs (PROL1 HC=4.97±0.1, PROL2 HC=7.20±0.1, $P < 0.05$). No significant interactions were detected between treatment and prolificacy. Mean hair cortisol results according to prolificacy are shown in Fig. 3.

HC from W5 reflected cortisol levels during the first five weeks of grazing deprivation. During this period, no differences were observed between groups 5h-G and H, indicating that stress response was not different between 5h-G and H, and thus a period of a 5-week grazing deprivation had no effect on cortisol release or it is not a long enough period to be detected in hair.

From Weeks 5 to 10 there was a higher cortisol release in both groups (5h-G and H), but no effect of grazing restriction was detected. The higher HC in both groups could be attributed to advanced pregnancy, although the pregnancy effect in ewes is different, depending on the studies (Brunet and Sebastian, 1991; Alon et al., 2021). Somes studies report increased cortisol in late pregnancy in ewes carrying twins (Gregula et al., 2021; Alon et al., 2021) and other in ewes carrying one lamb (Santarosa et al., 2022). In studies performed with ewes carrying one lamb no pregnancy effect was found (Brunet and Sebastian, 1991). Other studies report an increased wool cortisol during pregnancy, but suggest other variables to affect it, like environmental factors (Sawyer et al., 2019).

In cows, different hair and plasma cortisol levels had been found between housed animals, 12 h access to pasture and 24 h access (Nejad et al., 2021). In our study was only used a housed group and a 5 h access to pasture, however, a 10-week period of grazing deprivation compared to 5 h access to pasture has been previously described to affect ewes' aggressive and wool-pulling behaviours (Parés et al., 2023). Nevertheless, increased HC concentrations in housed group were not found in our study. A possible explanation could be that abnormal behaviours appeared in a low prevalence, which makes it difficult to detect possible cortisol increases. Some authors suggest that abnormal behaviours will not always be accompanied by an increase in stress indicators (Aguayo-Ulloa et al., 2019). In pigs, redirected behaviours have been found without cortisol increases (Peeters et al., 2006). On the other hand, some of the abnormal behaviours appeared progressively throughout the 10-week restriction period, and perhaps longer periods of grazing restriction could be needed to detect effects on HC.

Some aspects of sampling should also be considered. Age, body region, hair colour, hair segment and season can affect hair cortisol (Fürthbauer et al., 2019; Heimburgue, 2021). In our experiment the body region selected for sampling could have been the most important one. However, as reported in previous studies, hair cortisol must be evaluated with caution because of the local effects on cortisol release. (Salaberger et al., 2016). Other authors appoint that cortisol in hair can be eliminated in time, however in our experiment the stress effect (grazing deprivation) was maintained throughout the experimental period.

Pregnancy showed an effect on HC. In fact, increased cortisol levels have been described in other species like cow and pig, but it was found in the last month of pregnancy (Edwards et al., 2018). In our experiment, samples on W10 were before the last month of pregnancy and no effect was expected, as previously mentioned. . The effect of pregnancy and the effect of grazing deprivation could have overlapped. On the other hand, our results showed that in both Groups 5h-G and H, the increased cortisol level only appeared in ewes carrying two lambs (PROL2), which agree with other authors (Alon et al., 2021; Zeinstra et al., 2023). Other authors reported higher cortisol levels in ewes carrying one lamb compared with ewes carrying twins (Santarosa et al., 2022).

The pattern of saliva cortisol through the experimental period is shown in Fig. 4. SC showed no differences between both groups throughout the experiment ($P > 0.05$). The average level of the entire flock (5h-G and H groups) increased progressively from the first sampling in W1 to W8. In W10, saliva cortisol showed a significant decrease

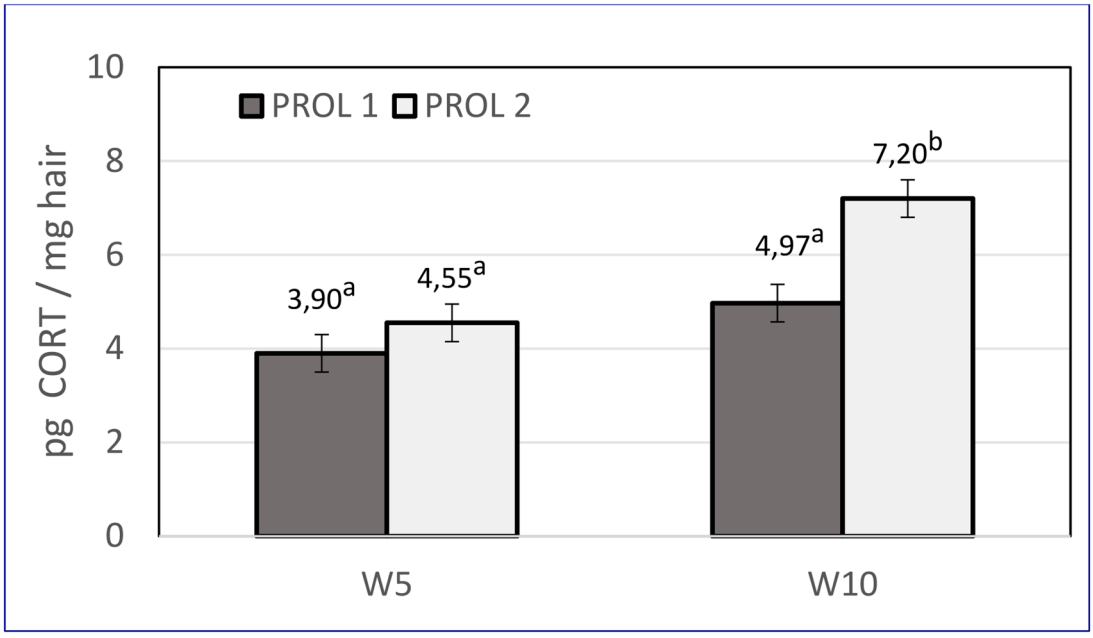

Fig. 3. Cortisol concentration in hair samples in weeks 5 and 10 in ewes carrying one (PROL1) or two lambs (PROL2). Different superscripts indicate significant statistical differences.

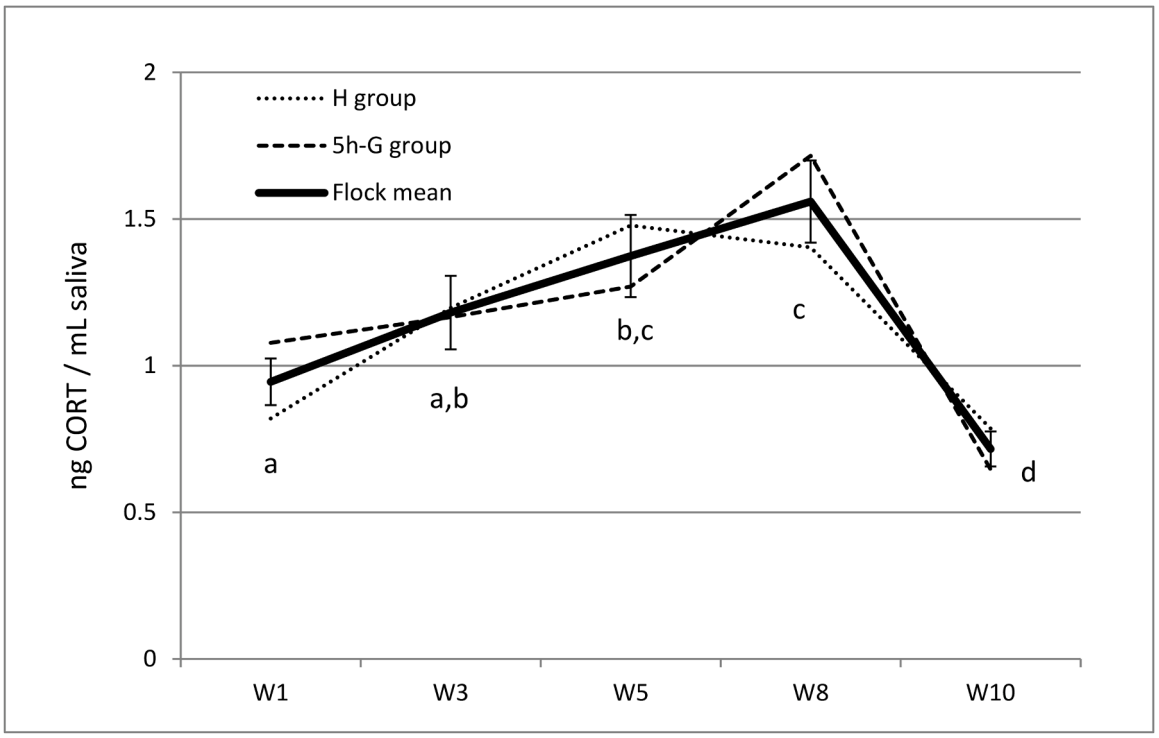

Fig. 4. Cortisol concentration in ewes saliva samples throughout the experimental period. Different letters indicate significant statistical differences.

($P < 0.01$), reaching the lowest level of the experimental period.

According to our results, SC showed great variability over time. Other authors previously reported saliva samples as optimal (Fell et al., 1985; Yates et al., 2010) but according to our results it is not a valid measure to evaluate the effect of chronic stress caused by grazing restriction. There is not a clear explanation for the pattern of SC levels, which could be affected by sampling or weather conditions. Saliva samples were always taken same time af day to avoid circadian variability, however, an automatic system to restrain the animals, like self-blocking feeders, was not available, and ewes were restrained inside the pen while the rest of the animals were moving, which could affect SC release. According to our results and previous experiments in other species, we suggest that saliva cortisol should be used in combination with other biomarkers from other stress responses, such as chromogranin A or other biomarkers like alpha-amylase and lipase (Fuentes-Rubio et al., 2016).

4. Conclusion

When ewes were deprived from grazing, no evidence of chronic stress could be detected through cortisol levels in hair and saliva compared to 5 h daily grazing, although abnormal behaviours appear. However, to confirm that grazing restriction does not induce a stress response, longer periods of grazing restriction should be monitored. Also, wool samples or other body regions to sample hair should be analysed. Saliva cortisol showed a significant variability over time and it should be used in combination with other biomarkers. Hair cortisol levels increased in all ewes from W5 to W10 of grazing deprivation, and in the latter period tey were higher in sheep carrying twins than singletons.

CRediT authorship contribution statement

Ricard Parés: Writing – original draft. **Pol Llonch:** Validation, Supervision, Conceptualization. **Manel López-Béjar:** Methodology. **Anaïs Carbajal:** Methodology. **Xavier Such:** Supervision. **Xavier Manteca:** Supervision, Conceptualization.

Declaration of competing interest

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

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