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**BIODIVERSITAS**
Volume 23, Number 1, January 2022
Pages: 388-395

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230140

# Comparative diurnal and seasonal variations of ACTH, cortisol and aldosterone in Ouled Djellal and D'Man sheep breeds reared in arid lands

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Manuscript received: 15 October 2021. Revision accepted: 26 December 2021.

**Abstract.** *Amokrane-Ferrah A, Annane A, Ferrouk-Boukenaoui N, Khaldoun M, Amirat Z, Mormede P, Khammar F. 2021. Comparative diurnal and seasonal variations of ACTH, cortisol and aldosterone in Ouled Djellal and D'Man sheep breeds reared in arid lands. Biodiversitas 23: 388-395.* This study aimed to explore the diurnal and seasonal variations of adrenocorticotropic hormone (ACTH), cortisol and aldosterone, main hormones involved in adaptation to the harsh climate of the Sahara Desert in two breeds of sheep (*Ovis aries* Linnaeus, 1758), Ouled Djellal rams (seasonal breeder) and D'Man (no seasonal breeder). Blood samples were taken every hour for 24 hours during equinoxes and solstices. Plasma ACTH levels were similar in the two breeds, with higher levels in the morning and a seasonal rhythm characterized by lower levels and dampened diurnal cycle in winter. In most experimental conditions, plasma cortisol levels showed no circadian rhythm but marked ultradian variations. The seasonal cortisol rhythm was different from the ACTH rhythm and characterized by lowest levels at equinoxes and highest levels in summer. Mean cortisol levels were higher in Ouled Djellal than in the D'Man breed, which may favor better robustness of Ouled Djellal sheep. Plasma aldosterone levels were characterized by diurnal variations with an acrophase around midnight, with large differences across breeds and seasons. In contrast to cortisol, the seasonal rhythm of plasma aldosterone showed the highest levels at equinoxes and lowest at solstices. These changes can be driven by environmental conditions and exceptionally high summer temperatures.

**Keywords:** Biological rhythm, hypothalamic-pituitary-adrenocortical axis, robustness, Sahara Desert, stress

## INTRODUCTION

Living organisms are subject to cyclic environmental changes such as day-night (diurnal variations) and annual (seasonal variations) cycles (Abdoun et al. 2012; Ayo et al. 2014; Vermeulen 2015; Uzal Seyfi and Ilhan 2019; van Dalum et al. 2019; Chakhma et al. 2021; Chergui et al. 2021). They adapt to this cyclic world through behavioral and physiological rhythms for optimal anticipation of changes in activity and food availability (Pickel and Sung 2020). Diurnal rhythms are endogenous biological variations that fluctuate 24h and can be synchronized with the external temporal environment by light and darkness; seasonal rhythms are timed to seasonal environmental signals. The hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine system involved in stress and adaption, is a typical example of the influence of the outside environment on endocrine functioning. Cortisol and aldosterone are common regulators of adrenocorticotropic hormone (ACTH) secretion (Wellman et al. 2021). The diurnal and seasonal plasma concentrations of adreno-corticosteroid hormones are required to maintain the whole organism's homeostasis and respond adequately to environmental changes (Oster et al. 2017).

A diurnal and seasonal periodicity in HPA axis activity has been found in many mammals (Mesbah and Brudieux 1982; Chergui et al. 2017; Chakhma et al. 2021). Therefore, Saharan mammals may be excellent for physiological adaptations to a scorching climate. In particular, sheep (*Ovis aries* Linnaeus, 1758) is an important livestock species that contributes significantly to the agricultural economy, especially in the arid/semi-arid and mountainous areas where crop and/or dairy farming are not so productive. Available sheep breeds display large differences in their adaptability to the local climatic conditions, with an essential impact on production and reproduction (Sejian et al. 2013). Ouled Djellal is the important Algerian ovine breed, by its physical capacities and production level (Saadi et al. 2016). The rusticity and productivity of this breed explain its large distribution in the whole country. On the other hand, D'Man is a rustic breed, with exceptional performances of reproduction, well adapted to the Saharan conditions. Therefore, it could have a great zootechnic and economic interest in the future for breeding as a pure breed and as crossings with meat breeds to improve their prolificacy (Lahlou-Kassi et al. 1989; Kuntjoro et al. 2009). Furthermore, Ouled Djellal is a seasonal breeder (Belkadi et al. 2017), contrary to D'Man (Lahlou-Kassi et al. 1989). This study aimed to analyze the adaptation processes of these two sheep breeds exposed to the environmental conditions of the Algerian Sahara Desert by evaluating the diurnal and seasonal changes in the plasma concentrations of adrenocorticotropic hormone (ACTH), cortisol and aldosterone, the main hormones of the HPA axis, the major neuroendocrine system involved in stress and adaptation processes.

## MATERIALS AND METHODS

### Study area

The experiment was conducted in El-Meniaa research station (30°34' N., 02°52' E., 379 m elevation) located in the Algerian Sahara Desert, Algeria. This region was characterized by high temperatures in summer (Figure 1).

### Animals and experimental design

All experiments were carried out according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines, following approval by the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research. Furthermore, the permits and ethical rules were achieved according to the Executive Decrees (n° 10-90 n°04-82) of the Algerian Government, establishing the terms and approval modalities of animal welfare in animal facilities. Furthermore, it was recently supported by the local university ethical committee of the "Association Algérienne des Sciences en Expérimentation Animale" AASEA (www.aasea.asso.dz; Agreement Number 45/DGLPAG/DVA.SDA.14).

The experiment was conducted on Ouled Djellal and D'Man breeds (n = 6 per breed). All animals were two years old, clinically healthy with body weights of 41.50 ± 8.29 kg and 37.89 ± 4.01 kg for Ouled Djellal and D'Man rams, respectively. The two breeds were separated and fed a dairy ration consisting of forage (alfalfa and barley straw) and barley grains (1 kg per sheep), and they had access to water and mineral licks ad libitum.

### Sampling procedure

The blood samples were realized during autumn (September) and spring (March) equinoxes and winter (December) and summer (June) solstices. One day before sampling, animals were housed individually to reduce the stress induced by handling. Blood was collected every hour for 24 hours from the jugular vein, into lithium heparin vacutainer tubes (Venoject®, Terumo, Belgium) for cortisol and aldosterone assays and on EDTA vacutainer tubes (Venoject1®, Becton-Drive, Franklin Lakes, NJ, USA) for ACTH assay. Tubes were immediately centrifuged at 4°C for 15 min at 3000 g and plasma was stored at -20°C until analysis.

### Plasma hormone assays

Plasma ACTH (500 µl) was determined by immunoradiometric assay (IRMA), a "sandwich" type assay (Beckman Coulter, Prague) where ACTH is first modified chemically by succinylation, yielding succinyl ACTH (sACTH). Three mouse monoclonal antibodies, directed against three distinct epitopes of the sACTH molecule and not competing with each other, are used in the kit. After succinylation of the samples, the modified samples are incubated first in tubes coated with two monoclonal antibodies. Then, the contents of the tubes are aspirated carefully. Finally, a solution containing a third monoclonal antibody, 125I-labeled, is added to each tube. The concentration of ACTH in the samples is directly proportional to the radioactivity. Plasma cortisol and aldosterone samples were measured by RIA (Beckman Coulter kit, Prague). Samples (50 µl) are incubated in specific monoclonal antibody-coated tubes with 125I-labeled cortisol and samples (50 µl) are incubated in antibody-coated tubes with 125I-labeled aldosterone.

### Validation of hormone assays

The intra-assay and inter-assay coefficients of variation were less than 10 and 10.8% for ACTH and 5.8 and 9.2% for cortisol and 12.6 and 17.2% for aldosterone. Analytical sensitivity was 0.31 pg/mL, 1.81 ng/mL and 1.44 pg/mL for ACTH, cortisol and aldosterone, respectively. The cross-reactivity of ACTH assay extremely low cross-reactivities was obtained against ACTH 1-24, 1-10, 18-39, 11-24, αMSH and POMC (kit values). The cortisol and aldosterone assays' cross-reactivity was extremely low against other naturally occurring steroids (kit values).

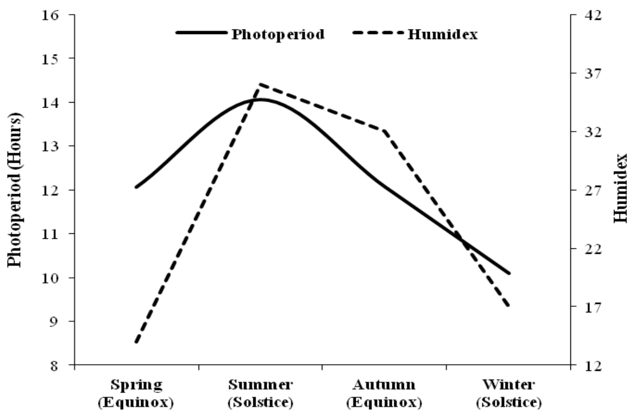


**Figure 1.** Seasonal variations of the photoperiod (hours) and the humidity index (humidex) in Meniaa. Climatic data are obtained from the Meniaa meteorological station (30°34' N., 02°52' E., elevation: 379 m). The humidity index (Humidex) combines temperature and humidity (Sirangelo et al. 2020).

**Statistical analysis**

All statistical analyses were performed with the R software, version 3.4.0 (R Core Team. 2015). Normality of distributions was analyzed with the Shapiro-Wilk test and all variables were transformed to their logarithmic scores for normalization before analysis. Hormone concentrations were analyzed using linear mixed-effects models (package 'nlme' of R) with the animal as a random factor to account for repeated measures and time of the day, season, and breed as fixed factors. The Tukey method was used for post-hoc comparisons. Statistical significance was set at $P < 0.05$. Diurnal variations were also analyzed by cosinor with the R package 'season' (Engert et al. 2018). The figures show the values as means ± SEM on a log10 scale.

## RESULTS AND DISCUSSION

**ACTH**

ACTH levels were not different between breeds but differed across seasons ($P < 0.001$) and times of the day ($P < 0.001$), with a significant interaction between season and time ($P < 0.05$) (Figures 2 and 3a). ACTH levels show the existence of a distinct diurnal pattern with a peak in the early morning and lowest levels in the late afternoon early evening. However, this diurnal cycle was absent in winter, with low levels maintained over the day (Figure 2). It is worth noting that the magnitude of the diurnal rhythm varies with hormonal levels and disappears when plasma levels are at their lowest in winter (Figures 2 and 3a). Bell et al. (1991) did not find any diurnal changes in plasma ACTH, cortisol and progesterone in ewes with different reproductive statuses.

The two sheep breeds' seasonal pattern of ACTH concentration showed the highest values in summer and lowest in winter with flat low levels across the day (Figures 2 and 3a). A similar pattern was found in adult Soay rams (Ssewannyana et al. 1990) and in Saharan goats (*Capra aegagrus hircus*) (Chakhma et al. 2021).

**Cortisol**

Cortisol levels were higher ($P < 0.05$) in Ouled Djellal than in the D'Man breed and differed across seasons ($P < 0.001$) and times of the day ($P < 0.001$) with significant interactions between breed and time ($P < 0.05$), season and time ($P < 0.001$) and breed, season and time ($P <0.001$) (Figures 3b and 4). Analysis of individual data (not shown) and of group means (Figure 4) shows numerous bursts of secretion during the day but no clear circadian rhythm in most cases. The cosinor analysis validates the absence of a diurnal rhythm when all data are considered (Table 1). However, a significant rhythm occurred in some breed x season combinations, the most significant in the Ouled Djellal breed in spring, with an acrophase around midnight. This varied pattern explains the significant second and third-level interactions of time of the day with breed and season.

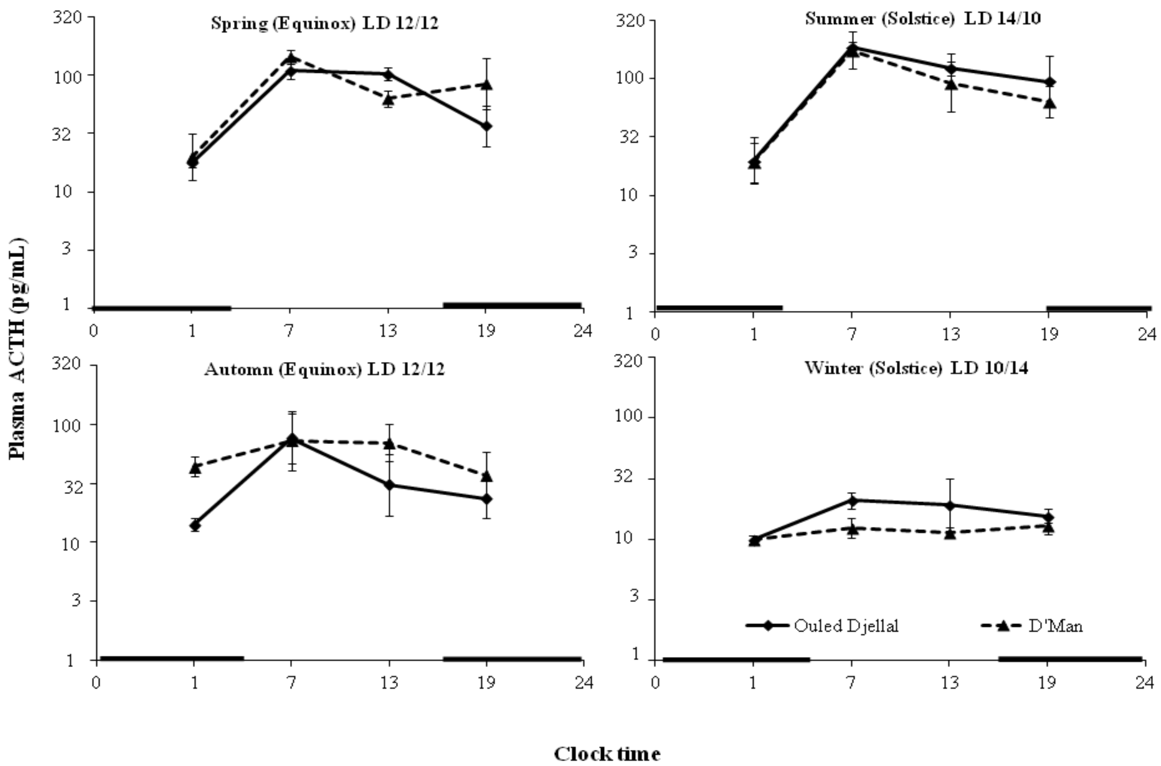


**Figure 2.** Diurnal plasma ACTH levels profiles in Ouled Djellal and D'Man sheep breeds measured in solstices and equinoxes (means ± SEM, n = 6 for each breed and season). Black bars on the x-axis denote hours of darkness, y axes in log scale. LD: light/dark cycle

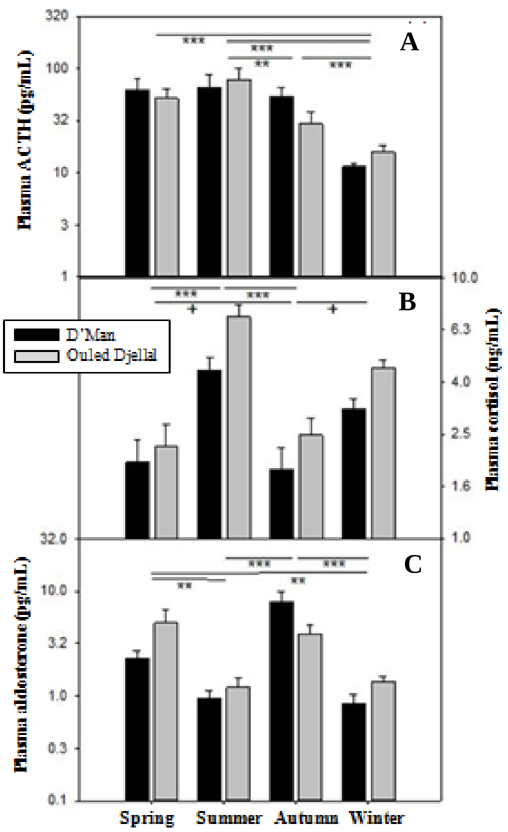


**Figure 3.** Seasonal changes in mean plasma ACTH (A), cortisol (B) and aldosterone (C) levels in Ouled Djellal and D'Man sheep breeds. n = 6 for each season and breed. Values are means ± SEM, y axes in log scale. Comparisons among breeds: * $P < 0.10$, ** $P < 0.01$, *** $P < 0.001$

However, when comparing these studies in different sheep breeds, it must be taken into consideration that ACTH and corticosteroid hormone levels are strongly influenced by genetics (Mormede et al. 2011) and many other factors like reproductive status (Bell et al. 1991) and environmental conditions. For example, higher cortisol levels in the Ouled Djellal breed may positively affect traits related to robustness and adaptation (Mormede and Terenina 2012).

In this study, no diurnal rhythm in cortisol levels was apparent in either sheep breed and at any season, despite the existence of an ACTH diurnal rhythm. The absence of diurnal variation of cortisol levels with marked ultradian variations were found in sheep (Bell et al. 1991) and other ruminant species (Chergui et al. 2017; Focke and Iremonger 2020). The ultradian rhythm of glucocorticoid hormone secretion, characterized by rapid pulses of a near-hourly frequency with increasing amplitude towards the start of the active period, was found in numerous species (Focke and Iremonger 2020). Different pathways are involved in these rhythms. Circadian rhythms in mammals are generated in the suprachiasmatic nucleus (SCN) of the hypothalamus. Light input from the retina into the SCN is the main external synchronizer of the central circadian pacemaker that monitors the timing of numerous physiological functions, including food intake and fasting (Oster et al. 2017). Ultradian pulsatility does not depend on the SCN like the diurnal cycle but results from the interplay between ACTH and corticosteroids at the pituitary and adrenal levels (Lightman et al. 2020). These rhythms of cortisol release differ largely among species, being ultradian only in sheep (Bell et al. 1991) and other ruminant species (Chakhma et al. 2021), ultradian and circadian in the rat (*Rattus norvegicus*) and humans (Qian et al. 2012), and circadian only in pigs (*Sus scrofa scrofa*) (Zupan and Zanella 2017).

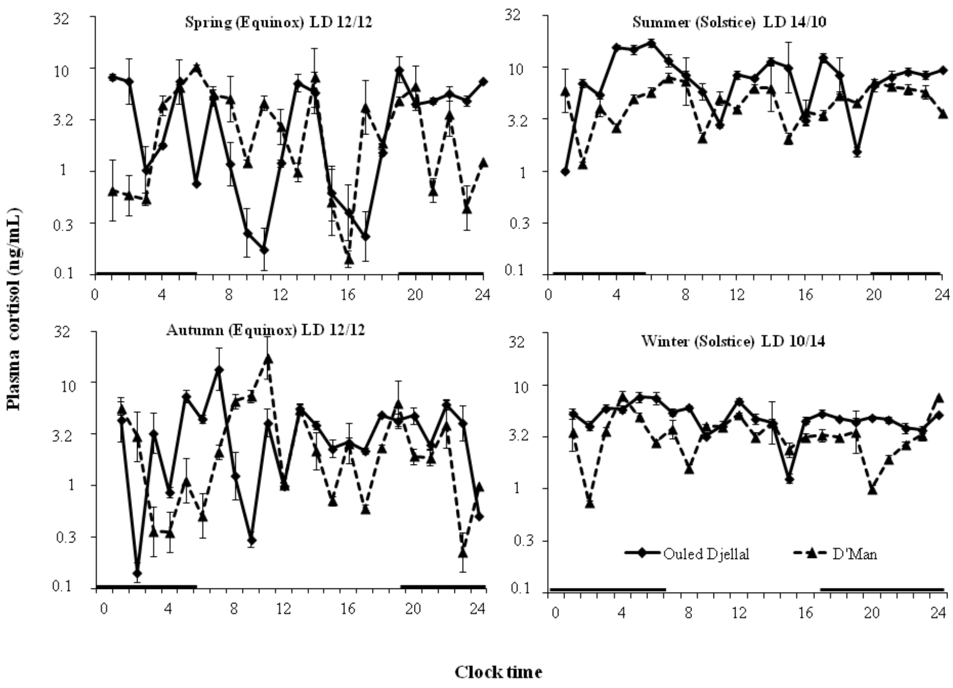


**Figure 4.** Diurnal plasma cortisol levels profiles in Ouled Djellal and D'Man sheep breeds measured at solstices and equinoxes (means ± SEM, n = 6 for each breed and season). Black bars on the x-axis denote hours of darkness, y axes in log scale. LD = light/dark cycle

**Table 1.** Cosinor analysis of diurnal variations of plasma cortisol (upper table) and aldosterone (lower tables) levels measured in D'Man and Ouled Djellal rams at solstices and equinoxes

| Seasons | Breed | Mesor | se_mesor(1) | Amplitude | se_amp(2) | p_amp (3) | Acrophase(4) |
|---|---|---|---|---|---|---|---|
| **Cortisol** | | | | | | | |
| Spring equinox | D'Man | 0.2847 | 0.0917 | 0.2037 | 0.131 | 0.1199 | 0805 |
| | Ouled Djellal | 0.3177 | 0.0821 | 0.4231 | 0.1183 | 0.0003 | 2326 |
| Summer solstice | D'Man | 0.6476 | 0.047 | 0.0275 | 0.0652 | 0.6732 | 1719 |
| | Ouled Djellal | 0.8541 | 0.0431 | 0.0956 | 0.0608 | 0.1159 | 0753 |
| Autumn equinox | D'Man | 0.2655 | 0.0835 | 0.2369 | 0.1213 | 0.0508 | 1216 |
| | Ouled Djellal | 0.3988 | 0.0661 | 0.1187 | 0.0921 | 0.1976 | 1533 |
| Winter solstice | D'Man | 0.5005 | 0.0379 | 0.0789 | 0.0539 | 0.1434 | 0833 |
| | Ouled Djellal | 0.6535 | 0.0321 | 0.111 | 0.0441 | 0.0119 | 0550 |
| All data | | 0.4959 | 0.0233 | 0.0315 | 0.0325 | 0.3336 | |
| **Aldosterone** | | | | | | | |
| Spring equinox | D'Man | 0.3575 | 0.071 | 0.2504 | 0.1005 | 0.0127 | 0053 |
| | Ouled Djellal | 0.6881 | 0.1228 | 0.2502 | 0.1737 | 0.1498 | 0218 |
| Summer solstice | D'Man | -0.0249 | 0.0704 | 0.1384 | 0.0996 | 0.1645 | 0356 |
| | Ouled Djellal | 0.0856 | 0.0811 | 0.5428 | 0.1148 | 0.0144 | 2335 |
| Autumn equinox | D'Man | 0.8994 | 0.0987 | 0.0802 | 0.1396 | 0.5656 | 2027 |
| | Ouled Djellal | 0.5969 | 0.0768 | 0.2619 | 0.1086 | 0.159 | 1933 |
| Winter solstice | D'Man | -0.0654 | 0.721 | 0.2694 | 0.102 | 0.083 | 0125 |
| | Ouled Djellal | 0.1401 | 0.498 | 0.1333 | 0.0704 | 0.0582 | 1654 |
| All data | | 0.3319 | 0.0348 | 0.1422 | 0.0492 | 0.004 | 2346 |

Note: [1]Standard-error of mesor, [2]Standard-error of amplitude, [3]Probability of amplitude being different from zero (no significant diurnal cycle). Bold numbers indicate the values of amplitude significantly different from zero, [4]Acrophase in hours and minutes (hhmm)

Although the mechanisms underlying these variations of HPA axis rhythms among species have not been extensively analyzed, specific features of HPA axis regulation in sheep are worth mentioning. The parvocellular neurons of the hypothalamic paraventricular nucleus release the neurohormones CRH and AVP, regulating ACTH secretion in a pulsatile manner (Roelfsema et al. 2017). This may be linked to the daily regulation mode of ACTH secretion controlled by CRH and AVP. These neurohormones are under the control of medullary adrenergic cell groups (C1, C2 and C3) and the C1 group is present in the rat but appears to be absent from the sheep brain (Tillet 1988).

In sheep, these data show that the regulation of ACTH is controlled by AVP more than by CRH (Hassan et al. 2003). However, in the Sprague-Dawley rat (*Rattus norvegicus*), CRH and AVP in the physiological range cause a marked increase in ACTH secretion and potentiate the effect of each other on ACTH secretion (Deng et al. 2017), and in the pig, CRH is the only neuropeptide known to increase POMC biosynthesis (Abraham and Minton 1996). Therefore, more work will be necessary to explore further the role of these specific features in regulating diurnal rhythms in these species.

The seasonal pattern of cortisol concentration in the two sheep breeds showed the highest values in summer, intermediate in winter solstice and lowest in autumn and spring equinoxes (Figure 3b). The same rhythm was found in bedouin goats living in the same environment (Chergui et al. 2017) and in adult Soay rams (Ssewannyana et al. 1990). Other seasonal rhythms were described as well. In Syrian Awassi rams, cortisol levels were low from March to October and highest in December (Alomar et al. 2016). As mentioned previously, these differences may be due to breed differences and to varying environmental conditions. Significantly increased serum cortisol levels were found in autumn for all breeds (Jezersko-Solchava, Bovec and Istrian) were probably associated with the onset of puberty and low environmental temperature (Snoj et al. 2014).

The seasonal rhythms are driven by environmental factors, particularly temperature and photoperiod. Indeed, our results show that both ACTH and cortisol plasma levels are highest in summer, which can result from heat stress; the second peak in winter may also be related to cold temperatures. Nazifi et al. (2003) showed in experimental conditions that both hot (40°C) and cold (4°C) temperatures increased plasma cortisol in Iranian fat-tailed sheep. In this context, HPA axis hormone concentrations changes are considered biomarkers of stress (Gaete 2016). On the other hand, glucocorticoid hormones have a significant role in thermoregulation in animals (Shaji et al. 2016). As shown in pigs (Marple et al. 1972; Campos et al. 2014), adaptation to heat reduces and adaptation to cold increases plasma cortisol levels. Marple et al. (1972) studied the influence of temperature and humidity in pigs. They showed that both parameters changed ACTH and cortisol circulating levels, but both hormones did not follow each other as could be expected from the direct ACTH-cortisol relationship. Again, these results point to the role of factors other than ACTH in regulating plasma cortisol concentration. Indeed, in environmental conditions similar to those of the present study, Chergui et al. (2017) reported a distinct circannual rhythm of adrenal sensitivity to ACTH, with a maximum in winter and spring, while the highest cortisol levels were measured in summer. The change in day length is an essential cue for seasonal physiological changes. Indeed, short photoperiod sensitizes the adrenal gland to ACTH to generate a robust glucocorticoid rhythm (Otsuka et al. 2012).

Other endogenous factors, including androgens, significantly affect the modulation of variations in the HPA axis activity (van Lier et al. 2014). Alomar et al. (2016) showed previously that seasonal changes of cortisol and testosterone were opposite in the Awassi rams. In addition, androgens can act directly on steroidogenic enzymes of the adrenal cortex to suppress cortisol production (Handa et al. 2013). However, despite the difference in the reproductive behavior of the breeds studied (seasonal and non-seasonal), both breeds exhibit similar cortisol response patterns to cyclic environmental changes, suggesting the existence of other, more potent factors that regulate the cycles of plasma cortisol concentration.

### Aldosterone

Aldosterone levels did not differ between breeds. The primary sources of variation were the seasons ($P < 0.001$) and times of the day ($P < 0.001$), with a significant interaction between breed, season and time ($P < 0.05$) (Figures 3c and 5). Plasma aldosterone levels are lower than those found in Tadmit and Romanov-Ouled Djellal crossed rams (Mesbah and Brudieux 1982). With an acrophase around midnight, the cosinor analysis validates the diurnal rhythm when all data are considered (Table 1). A significant rhythm occurred in some breed x season combinations only, the most significant in the Ouled Djellal breed in March, as for cortisol. This varied pattern explains the significant second and third-level interactions of time of the day with breed and season.

This circadian pattern is similar to that reported in dromedaries (*Camelus dromedarius*) living in the same Desert area (Khaldoun et al. 2002). However, different results were found in Tadmit and Romanov-Ouled Djellal crossed rams reared in the Algiers region, showing a mineralocorticoid activity characterized by high values in the morning and early afternoon and lower values at night (Mesbah and Brudieux 1982). This morning increase in aldosterone would be due to the combined circadian rhythm and morning activities (Thosar et al. 2019).

Aldosterone levels in both sheep breeds were lowest in summer and winter equinoxes, highest in autumn and intermediate in spring solstice (Figures 3c and 5). To our knowledge, no study has been undertaken on the seasonal variations of aldosterone in sheep. Our results are similar to those obtained in animals living in arid areas like the dromedary (Khaldoun et al. 2002). The main effect of aldosterone is on renal collecting ducts to reabsorb sodium ions and water and excrete potassium ions. Serum potassium concentrations are the most potent stimulator of aldosterone production, together with angiotensin II and ACTH. We could expect that animals are subjected to dehydration during heat stress conditions with an activation of the renin-angiotensin-aldosterone pathway to restore the fluid and electrolyte balance (Sejian et al. 2013). Still, due to the availability of water ad libitum, the response of the renin-angiotensin system is not accompanied by a high plasma concentration of aldosterone. Indeed, Aleena et al. (2020) reported that under heat stress, the level of plasma aldosterone was significantly lower in Malabari goats, which had ad libitum access to water.

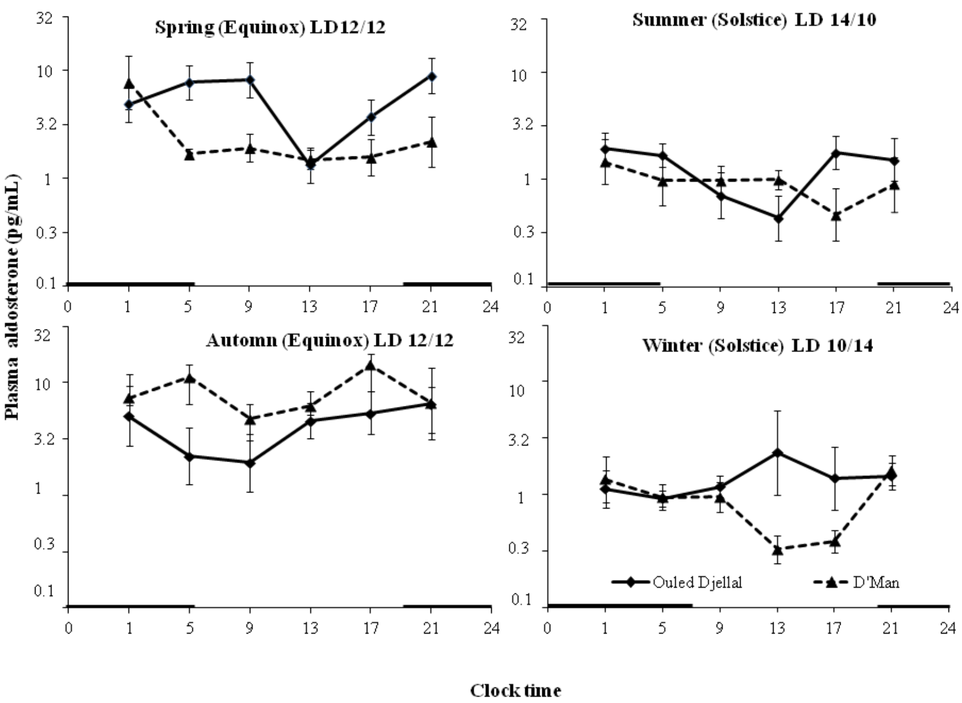


**Figure 5.** Diurnal plasma aldosterone levels profiles in Ouled Djellal and D'Man sheep breeds measured at solstices and equinoxes (means ± SEM, n = 6 for each breed and season). Black bars on the x-axis denote hours of darkness, y axes in log scale. LD = light/dark cycle

However, in our study, the highest aldosterone concentration coincides with the autumn and spring equinoxes and not with periods of high temperature. ACTH also activates aldosterone production, but McDougall et al. (1980) reported that ACTH induces a decrease in aldosterone concentration in sheep. This regulation of aldosterone production by ACTH in sheep may explain the original finding of our study that is the opposite seasonal cycle of aldosterone and cortisol.

In conclusion, clear-cut diurnal and seasonal rhythms in circulating HPA axis hormones levels are present in rams raised in the extreme environmental conditions of the Sahara Desert. Differences between the two breeds studied were found in mean cortisol levels and interaction with the diurnal and seasonal cycles and could help understand the robustness of these breeds. Despite the well-documented effects of androgens on HPA axis activity, the large difference in the seasonality of reproduction between these breeds could not explain HPA axis changes across seasons.

## ACKNOWLEDGEMENTS

The authors thank all the staff of the experimental station of El Meniâa (W. Ghardaïa, Algeria). Special thanks to Hakoum Bourdjila and Arezki Bentahar for their help with a blood sample. Special thanks to Dr. Mostapha Ferrouk (Veterinary Sciences Institute, University Saad Dahlab Blida 1).

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