JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Physiological responses of Rasa Aragonesa ewes to water restriction
Running Title (within 10 words)	Physiological responses of Rasa Aragonesa ewes to water restriction
Author	Sara Pérez-Redondo 1,2, Carlos Calvete 1,2, Margalida Joy 1,2, Andrés Domínguez 1, Jorge Hugo Calvo 1,2,3, Sandra Lobón 1,2.
Affiliation	1Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059, Zaragoza, Spain 2Instituto Agroalimentario de Aragón-IA2 (CITA-Universidad de Zaragoza), C. de Miguel Servet, 177, 50013 Zaragoza, Spain 3Fundación Agencia Aragonesa para la Investigación y el Desarrollo (ARAID), Avda. Ranillas, 1-D, 50018 Zaragoza, Spain.
ORCID (for more information, please visit https://orcid.org)	Sara Pérez-Redondo: https://orcid.org/0000-0002-0666-2289 Carlos Calvete: https://orcid.org/0000-0001-5028-947X Margalida Joy: https://orcid.org/0000-0002-1796-4223 Jorge Hugo Calvo: https://orcid.org/0000-0001-9513-0219 Sandra Lobón: https://orcid.org/0000-0002-7829-1448
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was supported by the Spanish Ministry of Science and Innovation (PID2020-114466RR-I00) and the Research Group Funds of the Aragón Government (A25_23R). S. Pérez-Redondo has a doctoral grant from the AEI (PRE2021-099071).
Acknowledgements	The authors wish to thank the Animal Science Department of CITA-Aragón staff for their technical assistance.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Calvete C, Joy M, Calvo JH, Lobón S. Formal analysis: Pérez-Redondo S, Calvo JH. Methodology: Calvete C, Joy M, Domínguez A, Calvo JH, Lobón S. Software: Pérez-Redondo S, Calvo JH. Investigation: Pérez-Redondo S, Calvete C, Joy M, Calvo JH, Lobón S. Writing - original draft: Pérez-Redondo S. Writing - review & editing: Pérez-Redondo S, Calvete C, Joy M, Domínguez A, Calvo JH, Lobón S.
Ethics approval and consent to participate	Animal manipulations were performed following Spanish Animal Protection Regulation RD53/2013, which is in accordance with European Union Directive 2010/63/EU for experimental animals' welfare and ethical treatment, and was approved by the Animal Ethics Committee of the Research Centre (protocol no. 2021/02).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Sandra Lobón
Email address – this is where your proofs will be sent	slobon@cita-aragon.es
Secondary Email address	
Address	Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059, Zaragoza, Spain
Cell phone number	

Office phone number	+34976716447
Fax number	

6
7

(Unstructured) Abstract (up to 350 words)

Global climate change impacts livestock production, particularly in extensive or semi-extensive systems in semi-arid regions, due to the temperature increases and water scarcity. The aim of this study was to characterize the different physiological responses of Rasa Aragonesa ewes to water restriction. Two hundred and two ewes were challenged to total water restriction for 5 days. Temperature and relative humidity were measured to calculate the temperature-humidity index (THI). According to the THI, ewes were also under heat stress conditions. Daily, dry matter intake (DMI), body weight (BW) and body condition score (BCS) were also recorded. Blood samples were collected on days 0, 1 and 5. Wool samples were collected on days 0 and 28. Blood samples were used for classic hematological and some biochemical parameters (total proteins, glucose, NEFAs, cortisol, dehydroepiandrosterone (DHEA), and its sulphate (DHEA-S)). In the wool cortisol, DHEA and DHEA-S were also measured. Principal component analysis (PCA) and hierarchical clustering (HC) were carried out to classify ewes according to their stress response. DMI, BW and BCS significantly lowered during the water stress period ($p < 0.05$). Most hematological and biochemical parameters were affected ($p < 0.05$), except for blood cortisol and the blood cortisol:DHEA-S ratio ($p > 0.05$). After HC analysis, ewes were classified into three clusters based on their stress tolerance. Cluster 1 (C1, n=168) included the most tolerant ewes, followed by Cluster 2 (C2, n=22) and Cluster 3 (C3, n=12), which was the least tolerant. The C3 ewes had the highest blood cortisol and non-esterified fatty acid mobilization, which were associated with the greatest BW loss. In conclusion, the stress conditions affected hematological and biochemical parameters in blood and wool. The majority of Rasa Aragonesa ewes generally demonstrated good tolerance to these stressors (C1, n=168), with only 34 ewes classified as less tolerant.

Keywords (3 to 6): sheep, performance, hematology, biochemistry, welfare.

Introduction

Climate change is causing rising temperatures and variations in precipitation, which directly impact livestock production, especially in extensive and semi-extensive systems [1]. These changes affect feed crop quality, water availability and overall animal performance, including growth, milk/meat quality and reproductive efficiency. These environmental changes are increasing livestock exposure to environmental stressors, such as heat stress [1] and water stress [2]. All these stresses can induce stress responses in sheep with negative effects on immunity [3] and worse well-being [4]. The physiological response to stress, including water and heat stress, is mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system [5]. This triggers the release of neuropeptides and neurohormones, which activate endocrine and immune responses [6]. The hypothalamus produces arginine vasopressin and corticotropin-releasing hormone, which lead to the secretion of adrenocorticotropic hormone (ACTH) in the pituitary [5,7]. Then ACTH stimulates

the adrenal gland to produce glucocorticoids (cortisol and corticosterone), mineralocorticoids (aldosterone) and androgens (androstenedione and dehydroepiandrosterone [DHEA]/dehydroepiandrosterone sulphate [DHEA-S]) [7].

Cortisol is the gold standard biomarker for assessing physiological response to stress in animals. It plays an important role in the control of carbohydrate metabolism, electrolyte homeostasis, water balance, and anti-inflammatory and immunosuppressive processes [8,9]. DHEA and DHEA-S have been described as neuroprotective hormones, biomarkers of ageing, glucocorticoid antagonists and immunostimulants [10]. They have anti-inflammatory effects and antioxidant properties, and are also involved in lipid metabolism [11]. Some authors suggest that DHEA is a better biomarker for acute stress, while DHEA-S is better for chronic stress in humans [12]. It is worth mentioning that high cortisol levels can reduce lymphocyte and antibody production, while DHEA and DHEA-S enhance T-cell production and counteract inflammation [10]. Some authors suggest measuring cortisol and DHEA/DHEA-S ratios (ratio cortisol:DHEA or cortisol:DHEA-S) to assess glucocorticoid activity and animals' physiological status [7,10]. Although blood is normally used to measure these hormones, other matrices like feces, sweat, urine, hair, wool or saliva can also be employed [8,13]. Thus hair or wool cortisol and DHEA/DHEA-S measurements are being paid more attention because the sampling method is non-invasive [14]. Hair measurements are commonly used to assess chronic or long-term stress because hormones accumulate over weeks and months based on the growth rate and hair length, but are not effective for evaluating acute stress events [14].

In addition to cortisol, leukocyte profiles (leukogram) have been used to assess stress in animals [15] as they reflect the physiological status of the animal. Under stress conditions, neutrophilia (increase in the concentration of neutrophils), leukocytosis (increase in the concentration of leukocytes) and lymphopenia or lymphocytopenia (drop in the concentration of lymphocytes) have been shown. A widely used parameter to evaluate stress in animals is the neutrophil-to-lymphocyte ratio (NLR) [16], which shows high values in animals under stress conditions [17] and may be directly related to cortisol and other stress hormones [16]. In addition to these changes, when animals are under water stress conditions widespread physiological disturbances can be observed, such as reductions in body weight and dry matter intake, as well as hemoconcentration and increases in serum proteins due to dehydration [18,19].

Water scarcity significantly impacts small ruminant production, especially in extensive and semi-extensive systems within the Mediterranean basin. However, breeds from these regions are generally more tolerant to water scarcity. Farming systems in these areas, are based on autochthonous breeds, such as the Rasa Aragonesa, which is the most dominant meat breed in Spain, mainly reared in extensive or semi-extensive farming systems. This breed often suffers from water scarcity during certain times of the year, especially in the summer when grazing in stubble fields, hills, or mountain areas. However, limited studies are available about their physiological responses to water restriction. Our hypothesis was that water restriction would affect hematological and biochemical parameters, resulting in different physiological responses in sheep, according to their tolerance to this stress.

Therefore, the aim of this study was to characterize the different stress responses of Rasa Aragonesa ewes subjected to total water restriction for 5 days by measuring hematological and biochemical parameters in blood and wool samples.

Materials and Methods

Ethics approval and consent to participate

Animal manipulations were performed following Spanish Animal Protection Regulation RD53/2013, which is in accordance with European Union Directive 2010/63/EU for experimental animals' welfare and ethical treatment, and was approved by the Animal Ethics Committee of the Research Centre (protocol no. 2021/02).

Experimental design

Two hundred and two Rasa Aragonesa ewes (2-8 years old, BW 60.19±7.10 kg and BCS 3.05±0.32; mean±SD) were used in this study. The physiological state of the ewes was maintenance as they were dry and non-pregnant. All the animals belonged to the experimental flock at the Agrifood Research and Technology Centre of Aragon (CITA-Aragón, Spain). All the ewes were kept in the same indoor facility under any mechanical control of climatic conditions. Ewes were divided into four groups to control feed intake per group. The ewes in each group were allocated together, but there was separation between the groups, so that the experimental unit for the intake control was the group. All animals were managed under homogeneous conditions, including feeding and veterinary treatments.

Ewes were subjected to total water restriction over a 5-day period. One month before the challenge, ewes were dewormed with oral ivermectin (0.2 mg/kg BW) and albendazole (5 mg/kg BW). A second anthelmintic treatment was administered 15 days before the water restriction period. During this time, ewes were always housed indoors, provided with 2 kg/ewe of alfalfa pellets (dry matter: 907.74±2.88 g/kg, crude protein: 203.99±7.29 g/kg; neutral detergent fiber: 330.25±4.30 g/kg, acid detergent fiber: 223.17±5.53 g/kg, acid detergent lignin: 33.21±1.15 g/kg; mean±SD), and had access to straw and water. During the 5-day water restriction period, ewes continued to receive 2 kg/ewe of alfalfa pellets, but had no access to water. Although the flock was accustomed to handling, management intensity was increased during the month before the challenge to avoid stress factors related to human presence.

Data collection

During the water restriction period, temperature and relative humidity were measured every 5 minutes and averaged every 30 minutes with a Vaisala probe. Data were stored in a Campbell CR-800 datalogger to calculate the THI [20] and the time that ewes remained under heat stress:

$$THI = db° C- [(0.31-0.31RH) (db° C-14.4)]$$

where db° C is the dry bulb temperature in Celsius and RH is the relative humidity percentage (RH)/100.

On a daily basis, the amounts of pellets offered and refused were recorded to calculate the DMI per group, ewes' BW was recorded by an electronic balance (0.5 kg precision) and the BCS was estimated by two trained technicians [21]. Blood samples were collected from the jugular vein using 10 mL BD Vacutainer® tubes immediately before water restriction (0d), 24 h later (1d) and at the end of the challenge (5 d). Wool samples (~ 2 cm^2 long) were collected at 0 d and 4 weeks later (28d). Prior to housing, the flock was sheared (-35 d). The 0d samples were taken 2 days before the challenge from the left shoulder (dorsal scapulae side) by shearing with commercially available clippers. The 28-day samples were collected by shearing the regrowth in the same area.

Blood and wool analysis

Blood samples from the jugular vein were obtained and placed inside EDTA tubes (Vaccuette, Madrid, Spain). They were immediately transported to an external laboratory (Albeitar; https://diagnosticoalbeitar.com/servicios/) to analyze the hematological and biochemical parameters: hematocrit, erythrocytes, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), white blood cell count, mean platelet volume (MPV), platelet distribution width (PDW), platelets, total proteins, glucose and NEFAs. The NLR was also calculated by dividing the absolute count of neutrophils by the count of lymphocytes (neutrophils/lymphocytes). Blood samples were also collected in heparin tubes (Vaccuette, Madrid, Spain) and immediately centrifuged (3,500 rpm for 20 min at 4°C). Then plasma was extracted and stored at -80°C until the future analysis of the biochemical parameters (cortisol, DHEA and DHEA-S). Plasma cortisol, DHEA and DHEA-S concentrations were analyzed in CITA's laboratory using quantitative laboratory ELISA tests for plasma. Plasma cortisol was measured using a commercial kit (Arbor Assays, Ann Arbor, MI) with sensitivity of 0.0276 ng/ml, an inter-assay CV from range 7.2 - 10.9% and an intra-assay CV from range 6 – 14.7%. Plasma DHEA was measured using a commercial kit (FineTest, Wuhan, Hubei, China) with sensitivity of 0.094 ng/ml, an inter-assay CV of <10% and an intra-assay CV of <8%. Plasma DHEA-S was measured using a commercial kit (Assay Genie, Dublin, Ireland), with sensitivity of 0.054 ng/ml, an inter-assay CV of $\leq$9.1% and an intra-assay CV of $\leq$5.7%.

In wool, these steroids were extracted by the methods described by Stubsjøen et al. [22] and Ghassemi Nejad et al. [13]. Briefly, 200 mg of wool was cut into small pieces and placed inside a polypropylene tube. Then, 5 ml of isopropanol was added to the tube and the samples were shaken in a rotary tube for 10 minutes. They were centrifuged at 4,000 rpm for 15 minutes at room temperature. The supernatant was discarded, and the samples were dried overnight in a crucible inside a fume hood to evaporate any remaining isopropanol. After drying, 100 mg of wool was weighed, 10 ml of methanol was added, and the samples were shaken again in a rotary tube for 10 minutes. Then, they were subjected to ultrasonication for 10 minutes and left to shake in the rotary tube for 24 hours at room temperature. After this 24-hour period, the samples were centrifuged again at 4,000 rpm for 15 minutes. The supernatant was collected in a 12 ml glass tube and was evaporated at 45°C in a vacuum

evaporator for 95 minutes. The precipitate was resuspended in 1 ml of PBS (if the ELISA analysis was not performed immediately, this extract was stored at -80ºC). Finally, the samples were shaken in a rotary tube for 10 minutes. Next the cortisol, DHEA and DHEA-S wool concentrations were also measured using the same ELISA kits employed for the plasma measurements.

Statistical analysis

Statistical analyses were carried out for each phenotype (feed intake, hematologic and biochemical parameters) using a mixed model with the lme4 package in R [23]. The model included sampling day, the initial BW and BCS (at day 0) and age as fixed effects, and animal as a random effect:

$$\text{Phenotype} = \mu + \text{sampling day} + \text{initial BW} + \text{initial BCS} + \text{Age} + e$$

For the BW and BCS phenotypes, the same model was used, but the initial BW and BCS were excluded. Differences between sampling days were estimated by least square means for pairwise comparisons, and Bonferroni correction was applied. The differences between stress and basal conditions (i.e., between days 5 and 0 for the blood samples and between days 28 and 0 for the wool samples) were calculated as percentage increases or decreases from the basal values for all the hematological and biochemical parameters.

A Pearson's pairwise correlation matrix between all the variation traits was calculated using the FactoMineR R package [24]. Principal components analysis (PCA) and hierarchical clustering (HC) were also performed with the FactoMineR package (R) with the percentage changes of 14 hematological and biochemical parameters to identify groups with similar characteristics: Δ erythrocytes, Δ MCV, Δ neutrophils, Δ eosinophils, Δ basophils, Δ lymphocytes, Δ monocytes, Δ platelets, Δ total proteins, Δ glucose, Δ NEFAs, Δ cortisol, Δ DHEA and Δ DHEA-S. Highly correlated parameters, such as hematocrit, hemoglobin, MCH, MCHC and RDW, were removed from further analyses. Age, BW, BCS, leukocytes (because they represent total white blood cells), ratios and wool measurements (due to possible external contamination by urine and feces, see the Discussion), were also removed. The characteristics of each cluster were studied by V-tests [25], which indicate the significant variables in each cluster by comparing the mean of each variable in that cluster to the mean of that variable in the whole data [26]. Finally, differences in age, BW and BCS between clusters were tested using ANOVA and least square means.

Results

Performance

Ewes were under heat stress conditions for 55% of the experiment (66 h), specifically 9.17% (11 h), 18.33% (22 h) and 27.5% (33 h) of moderate, severe and extreme heat stress, respectively (Supplementary Figure 1).

DMI was similar among the four groups, but water restriction caused a significant decrease in intake ($p < 0.05$; Table 1). Immediately before water restriction (0d), DMI was 1.86±0 kg DM/ewe, which dropped as the restriction days advanced. On the first day, the reduction was 17.7%, followed by a

64% reduction on the second day, 88% on the third day, and intake was almost zero thereafter. BW and BCS decreased over the restriction days ($p < 0.05$; Table 1). The reduction observed in BW ranged from 5.2% on day 1 to 15.2% on day 5 ($p < 0.05$). No ewe presented more than a 20% reduction in BW by the end of restriction in compliance with the welfare protocol (protocol no. 2021/02). The reduction in BCS was between 5% and 14% from day 1 to day 5. As expected, no difference was observed among the four groups.

Hematological and biochemical parameters in the blood and wool samples

The hematological and biochemical parameters measured on days 0, 1 and 5 of water restriction are presented in Table 2. The water restriction effect was significant ($p < 0.05$) for all parameters, except blood cortisol and blood cortisol:DHEA-S ratio ($p > 0.05$). As no significant differences were found in blood cortisol between day 0 and day 5, this parameter was not measured on day 1. The most substantial changes appeared between days 0 and 5 of water restriction for most parameters.

Related to the red blood parameters, hematocrit, erythrocytes, hemoglobin and MCV, results showed a consistent increase over the days of water restriction ($p < 0.05$), while MCHC evolved inversely by decreasing over time ($p < 0.05$). In contrast, MCH only increased on day 5, and RDW increased on day 1 of the experiment, remaining steady thereafter. Regarding white blood cells, no differences in leukocytes, neutrophils, lymphocytes, NLR and platelets were observed between days 0 and 1 ($p > 0.05$), but on day 5 of water restriction a significant increase was recorded in leukocytes, neutrophils and NLR ($p < 0.05$). There was a significant decrease in lymphocytes on day 5 ($p < 0.05$). Eosinophils decreased on day 1 ($p < 0.05$), but increased on day 5 to higher levels than on day 0 ($p < 0.05$). Basophils and monocytes rose on day 1, returning basophils to the basal levels (day 0) on day 5, while monocytes kept decreasing, ending at day 5 with lower concentration than day 0. NLR on days 0 and 1 was similar, but increased by the end of the challenge. Finally, platelets remained steady on day 1, but decreased on day 5 ($p < 0.05$).

Regarding the biochemical parameters, glucose levels progressively lowered throughout the water restriction period. Total proteins diminished on day 1, but increased on day 5, whereas NEFAs remained stable between days 0 and 1 and had significantly increased by the end of water deprivation. The levels of both DHEA and DHEA-S rose throughout the water restriction period in blood, the cortisol:DHEA ratio lowered on day 5, whereas no changes were observed for the cortisol:DHEA-S ratio. Of the wool biochemical parameters, cortisol, DHEA, DHEA-S and the cortisol:DHEA-S ratio increased on day 28, whereas the cortisol:DHEA ratio lowered ($p < 0.05$; Table 3).

Wide individual variability was observed for the majority of metabolites, except for hemoglobin and RDW. Correlations between variation traits appear in Supplementary Figure 2 (only the significant correlations are shown; $p < 0.05$). Age correlated positively and slightly with leukocytes and neutrophils, whereas BW correlated slightly and negatively with hematocrit, erythrocytes, hemoglobin and NEFAs. A positive strong correlation was shown among hematocrit, erythrocytes and hemoglobin, and these three hematological parameters correlated highly with RDW.

Principal component analysis and hierarchical clustering

We identified five principal components (PCs) in the blood parameters with eigenvalues of ≥1, which explained 51.92% of total variance. However, hierarchical clustering (HC) was performed using all the 14 identified PCs to avoid losing variability for HC (Supplementary Figure 3). Supplementary Figure 3 shows the contributions of the variables to the different PCs. For example, in Dim1 (13.7% of variance), Δ cortisol (16.35%) and Δ neutrophils (15.11%) contributed the most variability, followed by Δ total proteins (12.93%) and Δ DHEA (11.25%). From Dim6 to Dim14 there were some variables that contributed significantly to overall variability (Δ basophils contributed 33.28% of variability to Dim6, Δ lymphocytes with 45.95% to Dim7, Δ MCV with 37.21% to Dim8, Δ erythrocytes with 32.87% to Dim13 and Δ total proteins with 30.85% to Dim14).

After HC, ewes were divided into three groups based on the 14 variation traits in the blood parameters (Figure 1). The variation traits that were significant for each cluster and the population mean are shown in Supplementary Table 1. Cluster 1 (C1) included the ewes with a less marked increase in neutrophils, total proteins, NEFAs, cortisol and DHEA, and a less marked decrease in lymphocytes, compared to the population mean. Cluster 2 (C2) included the ewes with more marked increases in erythrocytes, neutrophils and DHEA, and more marked decreases in lymphocytes and platelets. Cluster 3 (C3) included the ewes with the most marked increase in total proteins, NEFAs and cortisol, and the most marked decrease in lymphocytes. Age, BW and BCS were analyzed by clustering to study if these factors influenced the differences among clusters (Table 4). Only significant differences were found for BW. The C3 ewes had the greatest weight loss ($p < 0.05$) compared to those from C1 and C2.

Discussion

However, the period of study ewes were under water stress and under heat stress due to the high environmental temperature registered during the period (which was not expected). Therefore the effects of both stressors cannot be separated. It is worth mentioning that ewes under heat stress conditions often display increased water intake and transpiration to dissipate body heat, increasing body temperature and the respiratory rate [2,18]. In contrast, they reduce feed intake, rumination time and frequency, which can lead to weight loss [2,18]. In this case, water restriction may exacerbate heat stress effects.

Performance

Reduced feed intake is a direct result of water restriction, which causes loss of BW and a decrease of BCS. This highlights the strong correlation between water intake and animal BW, in agreement with previous studies reporting that periods of water restriction lead to a lower DMI and BCS, and to reductions in BW [27,28] in sheep and cattle. For example, in the Awassi and Najdi sheep breeds

subjected to water restriction for 3 days, BW loss was 18% and 21.5%, respectively, with a reduction in feed intake of 96.5% in summer. In our study with Rasa Aragonesa ewes, BW loss was 15.2% with a 99% reduction in feed intake. A drop in DMI may be related to an adaptive mechanism to avoid water loss through feed digestion, and also to a lower metabolic rate during the water restriction period to conserve energy because ruminants' digestive process can account for 40-50% of their metabolic rate [29]. Reduction in BW is also due to body water loss and to lower water fluid mobilization because the rumen acts as a water reservoir [30,31]. At the end of the experiment, the ewes were provided with water *ad libitum*. Rehydration proceeded without complications, and the lost BW was recovered in three days.

Hematological and biochemical parameters in the blood and wool samples

Hemoconcentration is a common consequence of water deprivation in ruminants [19,32] due to less blood water, which can be exacerbated by heat stress [27]. Some authors suggest that an increase in some blood parameters, such as hematocrit, erythrocytes and hemoglobin, and MCHC, might be attributed to a reduced blood volume and their lower hematic concentration due to water loss [33] In line with this, we observed an increase in hematocrit, erythrocytes, hemoglobin and MCV, but controversially a decrease in MCHC. This decrease in MCHC and the increase in MCV might be attributed to the heat stress effect because Autukaitė et al. [34] also observed a decrease in MCHC, while Reddy et al. [35] reported an increase in MCV in ruminants under heat stress. In other studies, no variation in these blood parameters has been observed in different breeds subjected to water restriction [13,19], which suggests that sheep may be well-adapted to water restriction by maintaining blood volume and compensating for other body fluids.

As previously mentioned, stress in ruminants is often associated with an increase in leukocytes, and to marked neutrophilia and lymphopenia, which result in a higher NLR. These patterns have been observed in sheep under water restriction periods [33], and in calves abruptly weaned from their dams [36]. This was evident in our results because we found an increase in leukocytes, neutrophils and the NLR, and a decrease in lymphocytes. In other studies, NLR values of around 0.5 in adults have been recorded in non-stressed sheep, but can reach values of 2-3 under stress conditions [17]. Based on our findings, ewes were not under any significant stress on days 0 and 1 (NLR=0.8), but by day 5 the NLR had increased to 1.8, which indicates stress. In general, all the cellular types were modified on day 5. Literature says that eosinophils typically decrease under stress (eosinopenia) due to glucocorticoid activity [15,16]. Under stress conditions, basophils and monocytes result in basopenia and monocytosis, whereas inflammatory response leads to basophilia and monocytopenia [15]. However, in the present study it was recorded on day 1, eosinopenia, monocytosis and basophilia, and on day 5 eosinophilia, monocytopenia and basopenia. The different response between literature and the present study can be related to the fact that stress is closely related to inflammatory processes [37]. According to former authors, platelet levels increase under water stress conditions [37], which contrasts with the lower platelet count findings in the present. This reduction is aligned with other

studies in cows, in which a lower platelet concentration was observed during hot seasons in association with heat stress, with the lowest platelet count occurring during periods with the highest THI [38].

Regarding other biochemical parameters, protein levels increase in water-deprived sheep and goats due to hemoconcentration, which is consistent with our results [18,32]. In our study, the observed decrease in glucose and the increase in NEFAs on day 5 of water restriction could be related to the reduction of propionate production in the rumen caused by low feed intake and less ruminal activity, which resulted in decreased nutrient availability [39,40]. Additionally, the reduction in BW could be explained by increased fat mobilization, as reflected by high NEFAs levels, to compensate for decreased energy intake [19,41].

Blood cortisol concentration results vary across studies. Some have observed an increase in the cortisol level under stress conditions [11,18], while others have found no significant changes [28,32]. These discrepancies might be related to numerous factors, including environmental stressors and handling procedures [13]. Besides, the variability between ewes' individual tolerance to water restriction is considerable, which could indicate that some ewes require longer water restriction periods to increase the blood cortisol concentration [9]. Lack of significant cortisol changes in the present experiment may also reflect ewes' adaptive response to water and heat stress. It is worth contemplating that many domestic animals like calves can adapt to a rise in the THI (except for extreme THI values) [42]. In that study, an increase in salivary cortisol was observed as the THI increased during the day for 4 days. On day 5, less cortisol release was noted compared to previous days (these values were similar to those obtained on the control day). These authors suggested that a drop in the THI at night would allow the HPA axis to recover from heat stress, which would result in less cortisol released on the following day.

As previously mentioned, DHEA and DHEA-S are neuroprotective hormones and glucocorticoid antagonists with anti-inflammatory and antioxidant properties [10]. In ruminants, studies that quantify the effects of stress on DHEA and DHEA-S levels are limited. Almeida et al. [43] reported serum DHEA concentrations of 0.5±0.03 ng/ml in lame cows and of 0.7±0.03 ng/ml in healthy cows. In contrast, in the present study, the blood DHEA levels were substantially higher, with values of 4.0, 5.0 and 7.5 ± 0.20 ng/ml on day 0, 1 and 5, respectively. We also observed an increase in the DHEA and DHEA-S levels in both the blood and wool samples. This finding is consistent with previous studies in humans, which have shown an increase in these hormones when exposed to physical and psychological stressors [10,44]. The contradictory results between the present results and Almeida et al. [43], who found lower DHEA levels in lame cows compared to healthy cows can be explained by the fact that severe infections and chronic inflammatory diseases allow DHEA levels to drop. Hence this decrease in DHEA could be attributed to the chronic inflammatory nature of this disease [43]. Considering the neuroprotective function of DHEA and DHEA-S, it is believed that the levels of these hormones may increase to counteract the negative effects of stressors and cortisol, as an adaptive response to cope with stress [44]. Some authors have suggested measuring cortisol, DHEA and

DHEA-S together, and examining them as a ratio (cortisol:DHEA and cortisol:DHEA-S) to determine an animal's glucocorticoid status and, therefore, its stress response [7,10]. Along these lines, some studies have shown that young bulls under transport stress, and lame cows and cows subjected to deteriorated environmental conditions, exhibit higher cortisol:DHEA and cortisol:DHEA-S ratios, denoting a correlation between high ratios and stress conditions [43]. Therefore, these ratios may serve as potential biomarkers of tolerance to stressors [11], and also as an indicator of immune function and welfare in animals. When ewes were water-deprived, a drop in the cortisol:DHEA ratio was observed in both the blood and wool samples, whereas the blood cortisol:DHEA-S ratio remained unchanged and the wool cortisol:DHEA-S ratio increased. In growing bulls, an increase in salivary DHEA and unchanged salivary cortisol levels have been noted when they were under heat stress (higher THI [45]). This results in these ratios lowering, which aligns with our results. The relation between an increasing THI and DHEA release is not well-known, but some studies in rats suggest the hypothesis that DHEA is implicated in body thermoregulation [46]. With the cortisol:DHEA-S ratios, we suggest that stress affects this ratio, but the blood ratio demonstrates wide variability between ewes.

In our study, ewes were subjected to a total 5-day water restriction, which can be considered chronic stress. Wool measurements are often used to assess chronic stress given their capacity to accumulate hormones, and might not be optimal for acute stress. Therefore, it is possible that the DHEA-S accumulation in wool observed in our study is related to the effect of water and heat stress. However, external factors, such as contamination with feces and urine, may have also influenced the higher detected levels, as it is well-known that cortisol and other glucocorticoids can be excreted in urine and feces [47]. Since the ewes were housed together in four pens, cross-contamination could have occurred regardless of the washing procedure applied prior to analysis. It is important to mention that cortisol and other steroids can easily cross membranes, so diffusion of these hormones from urine into the hair matrix could easily occur [48]. As a result, an ewe with low blood glucocorticoids levels could have high wool glucocorticoids levels due to contamination from another ewe with a higher blood cortisol levels and, consequently, greater glucocorticoid excretion. In our study, the correlation between blood and wool cortisol and DHEA was not significant, but moderate and negative for DHEA-S (-0.28) (Supplementary Figure 2). Therefore, these measurements may not be reliable and were not used for any further analysis in our study. Another important point is the variability observed in some blood and wool parameters, especially in cortisol, DHEA and DHEA-S, and consequently in their ratios. This variability reflects the fact that each animal responds differently to water and heat stress, which makes categorizing animals as tolerant or susceptible challenging when based only on these hormones. For this reason, Rout et al. [49] suggested that identifying contrasting phenotypes is essential for selecting ewes that are susceptible or tolerant to these stresses. Therefore, these variables could be used to identify susceptible and tolerant animals to these stresses.

Principal component analysis and hierarchical clustering

In order to classify ewes according to their response to stress conditions, HC was performed by considering the 14 blood variation traits. After clustering, ewes were divided into three groups according to their different responses to water and heat stress. In general, the mean population defines a typical stress response to these stresses, as shown in other studies. This response is characterized by hemoconcentration and dehydration [19,32], immune system activation [33,36], a decrease in platelets [38] and increases in cortisol [11,18] and DHEA [10,44]. Drops in glucose levels and increases in NEFAs have also been observed under stress conditions [39,40].

C1 could be considered the most tolerant cluster compared to the other two clusters. This was due to lower dehydration (less marked increase in total proteins), less immune system activation (less marked increase in neutrophils and less marked decrease in lymphocytes), lower mobilization of NEFAs, and a lesser increase in cortisol and DHEA. The C2 ewes suffered greater dehydration than those in C1 and C3 (more marked increase in erythrocytes), greater immune system activation than C1 (more marked increase in neutrophils and more marked decrease in lymphocytes), greater decrease in platelets and a more marked rise in DHEA. C2 could be considered less tolerant to heat and water stress than C1. C3 could be considered the least tolerant of the three clusters to these stresses. This could be due to its highest dehydration (greatest increase in total proteins), its most marked immune system activation (most marked decrease in lymphocytes), its greatest increase in cortisol and its marked NEFAs mobilization. As previously mentioned, C3 showed the greatest BW loss of the three clusters. It can be hypothesized that the marked NEFAs mobilization could be a potential strategy that can be applied to cope with the greatest BW loss noted in C3 to compensate for decreased energy intake.

Conclusion

Water restriction and heat stress decreased DMI, which resulted in less BW and a lower BCS in Rasa Aragonesa ewes. These stressors also affected the hematological and wool parameters, which led to the hemoconcentration of blood volume (indicated by increased erythrocytes, hemoglobin, hematocrit and proteins), immune system activation (indicated by an increase in leukocytes, neutrophils and the NLR, and a decrease in lymphocytes), and marked NEFAs mobilization. Although the blood cortisol levels remained unchanged, both the blood and wool concentrations of DHEA and DHEA-S rose with dehydration. Ewes' stress response allowed us to classify them into three clusters: C1 comprised the most tolerant ewes (n=168), C2 included less tolerant ewes (n=22) and C3 contained the least tolerant ewes (n=12). Most Rasa Aragonesa ewes exhibited good tolerance to these stresses, which highlights their adaptability. However, there is still the potential for genetic selection to improve these animals' fitness. In light of our results, it would be interesting to focus on identifying the genetic basis for this adaptation to select better suited ewes to cope with these stressors.

Competing interests

No potential conflict of interest relevant to this article was reported.

Funding sources

This work was supported by the Spanish Ministry of Science and Innovation (PID2020-114466RR-I00) and the Research Group Funds of the Aragón Government (A25_23R). S. Pérez-Redondo has a doctoral grant from the AEI (PRE2021-099071).

Acknowledgments

The authors wish to thank the Animal Science Department of CITA-Aragón staff for their technical assistance.

Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Calvete C, Joy M, Calvo JH, Lobón S.

Formal analysis: Pérez-Redondo S, Calvo JH.

Methodology: Calvete C, Joy M, Domínguez A, Calvo JH, Lobón S.

Software: Pérez-Redondo S, Calvo JH.

Investigation: Pérez-Redondo S, Calvete C, Joy M, Calvo JH, Lobón S.

Writing - original draft: Pérez-Redondo S.

Writing - review & editing: Pérez-Redondo S, Calvete C, Joy M, Domínguez A, Calvo JH, Lobón S.

Ethics approval and consent to participate

Animal manipulations were performed following Spanish Animal Protection Regulation RD53/2013, which is in accordance with European Union Directive 2010/63/EU for experimental animals' welfare and ethical treatment, and was approved by the Animal Ethics Committee of the Research Centre (protocol no. 2021/02).

References

1. Rojas-Downing MM, Nejadhashemi AP, Harrigan T, Woznicki SA. Climate change and livestock: Impacts, adaptation, and mitigation. Clim Risk Manag. 2017;16:145–63. https://doi.org/10.1016/j.crm.2017.02.001

2. Chedid M, Jaber LS, Giger-Reverdin S, Duvaux-Ponter C, Hamadeh SK. Review: Water stress in sheep raised under arid conditions. Can J Anim Sci. 2014;94:243–57. https://doi.org/10.4141/cjas2013-188

3. Del Giudice M, Buck CL, Chaby LE, Gormally BM, Taff CC, Thawley CJ, et al. What Is Stress? A Systems Perspective. Integr Comp Biol. 2018;58:1019–32. https://doi.org/10.1093/icb/icy114

4. Broom DM, Fraser AF. Domestic animal behaviour and welfare [Internet]. Broom DM, Fraser AF, editors. Domestic animal behaviour and welfare. UK: CABI; 2007. https://doi.org/10.1079/9781845932879.0000

5. Guilliams TG, Edwards L. Chronic stress and the HPA axis: Clinical assessment and therapeutic considerations. The Standard. 2010;9:1–12.

6. Caroprese M, Albenzio M, Marzano A, Schena L, Annicchiarico G, Sevi A. Relationship between cortisol response to stress and behavior, immune profile, and production performance of dairy ewes. J Dairy Sci. 2010;93:2395–403. https://doi.org/10.3168/jds.2009-2604

7. Whitham JC, Bryant JL, Miller LJ. Beyond glucocorticoids: Integrating dehydroepiandrosterone (DHEA) into animal welfare research. Animals. MDPI AG; 2020. p. 1–25. https://doi.org/10.3390/ani10081381

8. Andrew A, Edward D, Solomon A, Isaac A, Ibrahim Y. The Cortisol Steroid Levels as a Determinant of Health Status in Animals. J Proteomics Bioinform. 2017;10. https://doi.org/10.4172/jpb.1000452

9. Parker AJ, Hamlin GP, Coleman CJ, Fitzpatrick LA. Dehydration in stressed ruminants may be the result of acortisol-induced diuresis. J Anim Sci. 2003;81:512–9. https://doi.org/10.2527/2003.812512x

10. Kamin HS, Kertes DA. Cortisol and DHEA in development and psychopathology. Horm Behav. 2017;89:69–85. https://doi.org/10.1016/j.yhbeh.2016.11.018

11. Peric T, Corazzin M, Romanzin A, Bovolenta S, Prandi A, Montillo M, et al. Cortisol and DHEA concentrations in the hair of dairy cows managed indoor or on pasture. Livest Sci. 2017;202:39–43. https://doi.org/10.1016/j.livsci.2017.05.020

12. Leowattana W. DHEAS as a new diagnostic tool. Clinica Chimica Acta. 2004;341:1–15. https://doi.org/10.1016/j.cccn.2003.10.031

13. Ghassemi Nejad J, Lohakare JD, Son JK, Kwon EG, West JW, Sung KI. Wool cortisol is a better indicator of stress than blood cortisol in ewes exposed to heat stress and water restriction. Animal. 2014;8:128–32. https://doi.org/10.1017/S1751731113001870

14. Heimbürge S, Kanitz E, Otten W. The use of hair cortisol for the assessment of stress in animals. Gen Comp Endocrinol. 2019;270:10–7. https://doi.org/10.1016/j.ygcen.2018.09.016

15. Tornquist SJ, Rigas JD. Interpretation of ruminant leukocyte responses. In: Weiss DJ, Wardrop KJ, editors. Schalm's veterinary hematology. 6th editio. Wiley, Ames, IA; 2010. p. 307–13.

16. Davis AK, Maney DL, Maerz JC. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol. 2008;22:760–72. https://doi.org/10.1111/j.1365-2435.2008.01467.x

17. Wickham SL, Collins T, Barnes AL, Miller DW, Beatty DT, Stockman C, et al. Qualitative behavioral assessment of transport-naïve and transport-habituated sheep. J Anim Sci. 2012;90:4523–35. https://doi.org/10.2527/jas.2010-3451

18. Casamassima D, Vizzarri F, Nardoia M, Palazzo M. The effect of water-restriction on various physiological variables in intensively reared Lacaune ewes. Vet Med (Praha). 2016;61:623–34. https://doi.org/10.17221/144/2015-VETMED

19. Jaber LS, Habre A, Rawda N, Abi Said M, Barbour EK, Hamadeh S. The effect of water restriction on certain physiological parameters in Awassi sheep. Small Ruminant Research. 2004;54:115–20. https://doi.org/10.1016/j.smallrumres.2003.11.004

20. Marai IFM, El-Darawany AA, Fadiel A, Abdel-Hafez MAM. Physiological traits as affected by heat stress in sheep—A review. Small Ruminant Research. 2007;71:1–12. https://doi.org/10.1016/j.smallrumres.2006.10.003

21. Russel AJF, Doney JM, Gunn RG. Subjective assessment of body fat in live sheep. J Agric Sci. 1969;72:451–4. https://doi.org/10.1017/S0021859600024874

22. Stubsjøen SM, Bohlin J, Dahl E, Knappe-Poindecker M, Fjeldaas T, Lepschy M, et al. Assessment of chronic stress in sheep (part I): The use of cortisol and cortisone in hair as non-invasive biological markers. Small Ruminant Research. 2015;132:25–31. https://doi.org/10.1016/j.smallrumres.2015.09.015

23. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;67. https://doi.org/10.18637/jss.v067.i01

24. Lê S, Josse J, Husson F. FactoMineR: An R Package for Multivariate Analysis. J Stat Softw. 2008;25:1–18. https://doi.org/10.18637/jss.v025.i01

25. Husson F, Josse J. Multivariate Data Analysis: Special Focus on Clustering and Multiway Methods. In Proceedings of the UseR!the R User Conference, Gaithersburg, MD, USA, 20–23

July. 2010;

26. Kuivanen KS, Alvarez S, Michalscheck M, Adjei-Nsiah S, Descheemaeker K, Mellon-Bedi S, et al. Characterising the diversity of smallholder farming systems and their constraints and opportunities for innovation: A case study from the Northern Region, Ghana. NJAS: Wageningen Journal of Life Sciences. 2016;78:153–66. https://doi.org/10.1016/j.njas.2016.04.003

27. Alamer M, Al-hozab A. Effect of water deprivation and season on feed intake, body weight and thermoregulation in Awassi and Najdi sheep breeds in Saudi Arabia. J Arid Environ. 2004;59:71–84. https://doi.org/10.1016/j.jaridenv.2004.01.003

28. Burgos MS, Senn M, Sutter F, Kreuzer M, Langhans W. Effect of water restriction on feeding and metabolism in dairy cows. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2001;280:R418–27. https://doi.org/10.1152/ajpregu.2001.280.2.R418

29. Webster AJF. The energetic efficiency of metabolism. Proceedings of the Nutrition Society. 1981;40:121–8. https://doi.org/10.1079/PNS19810017

30. Silanikove N. The physiological basis of adaptation in goats to harsh environments. Small Ruminant Research. 2000;35:181–93. https://doi.org/10.1016/S0921-4488(99)00096-6

31. Pérez S, Calvo JH, Calvete C, Joy M, Lobón S. Mitigation and animal response to water stress in small ruminants. Animal Frontiers. 2023;13:81–8. https://doi.org/10.1093/af/vfad049

32. Ghanem AM, Jaber LS, Abi Said M, Barbour EK, Hamadeh SK. Physiological and chemical responses in water-deprived Awassi ewes treated with vitamin C. J Arid Environ. 2008;72:141–9. https://doi.org/10.1016/j.jaridenv.2007.06.005

33. Serrano JO, Villares-Garachana A, Correa-Herrera N, González-Morales A, Pérez-Bonachea L, Hernández L, et al. Impacts of short-term water restriction on Pelibuey sheep: physiological and blood parameters. Trop Anim Health Prod. 2022;54:39. https://doi.org/10.1007/s11250-022-03050-9

34. Autukaitė J, Poškienė I, Juozaitienė V, Antanaitis R, Žilinskas H. The impact of temperature-humidity index on blood morphology and β-hydroxybutyrate in different sheep breeds. Acta Veterinaria Brno. 2020;89:247–54. https://doi.org/10.2754/avb202089030247

35. Reddy PR, Kumar BR, Prasad ChS, Venkataseshiah Ch, Hyder I. Erythrocyte fragility based assessment of true thermal resilience in tropical small ruminants. Biol Rhythm Res. 2022;53:234–45. https://doi.org/10.1080/09291016.2019.1629087

36. Lynch EM, Earley B, McGee M, Doyle S. Characterisation of physiological and immunological responses in beef cows to abrupt weaning and subsequent housing. BMC Vet Res. 2010;6:37. https://doi.org/10.1186/1746-6148-6-37

37. D'Ambrosio C, Sarubbi F, Scaloni A, Rossetti C, Grazioli G, Auriemma G, et al. Effect of short-term water restriction on oxidative and inflammatory status of sheep (Ovis aries) reared in Southern Italy. Small Ruminant Research. 2018;162:77–84. https://doi.org/10.1016/j.smallrumres.2018.03.008

38. Casella S, Scianò S, Zumbo A, Monteverde V, Fazio F, Piccione G. Effect of seasonal variations in Mediterranean area on haematological profile in dairy cow. Comp Clin Path. 2013;22:691–5. https://doi.org/10.1007/s00580-012-1468-8

39. Jaber L, Chedid M, Hamadeh S. Water Stress in Small Ruminants. Responses of Organisms to Water Stress. InTech; 2013. https://doi.org/10.5772/53584

40. Kumar D, De K, Singh AK, Kumar K, Sahoo A, Naqvi SMK. Effect of water restriction on physiological responses and certain reproductive traits of Malpura ewes in a semiarid tropical environment. Journal of Veterinary Behavior. 2016;12:54–9. https://doi.org/10.1016/j.jveb.2015.11.006

41. Adeniji YA, Sanni MO, Abdoun KA, Samara EM, Al-Badwi MA, Bahadi MA, et al. Resilience of Lambs to Limited Water Availability without Compromising Their Production Performance. Animals. 2020;10:1491. https://doi.org/10.3390/ani10091491

42. Kovács L, Kézér FL, Ruff F, Szenci O, Bakony M, Jurkovich V. Effect of artificial shade on saliva cortisol concentrations of heat-stressed dairy calves. Domest Anim Endocrinol. 2019;66:43–7. https://doi.org/10.1016/j.domaniend.2018.09.001

43. Almeida PE, Weber PSD, Burton JL, Zanella AJ. Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions. Domest Anim Endocrinol. 2008;34:89–99. https://doi.org/10.1016/j.domaniend.2006.11.006

44. Charney DS. Psychobiological Mechanisms of Resilience and Vulnerability. Focus (Madison). 2004;2:368–91. https://doi.org/10.1176/foc.2.3.368

45. Giaretta E, Mongillo P, Da Dalt L, Gianesella M, Bortoletti M, Degano L, et al. Temperature and humidity index (THI) affects salivary cortisol (HC) and dehydroepiandrosterone (DHEA) concentrations in growing bulls following stress generated by performance test procedures. Front Vet Sci. 2023;10. https://doi.org/10.3389/fvets.2023.1237634

46. Catalina F, Milewich L, Frawley W, Kumar V, Bennett M. Decrease of Core Body Temperature in Mice by Dehydroepiandrosterone. Exp Biol Med. 2002;227:382–8. https://doi.org/10.1177/153537020222700603

47. Otten W, Heimbürge S, Kanitz E, Tuchscherer A. It's getting hairy – External contamination may affect the validity of hair cortisol as an indicator of stress in pigs and cattle. Gen Comp Endocrinol. 2020;295:113531. https://doi.org/10.1016/j.ygcen.2020.113531

48. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. Clinica Chimica Acta. 2006;370:17–49. https://doi.org/10.1016/j.cca.2006.02.019

587 49. Rout PK, Kaushik R, Ramachandran N, Jindal SK. Identification of heat stress-susceptible and -
588 tolerant phenotypes in goats in semiarid tropics. Anim Prod Sci. 2018;58:1349.
589 https://doi.org/10.1071/AN15818

590

591

Tables and Figures

Table 1

Effect of water restriction on dry matter intake, body weight and the body condition score in Rasa Aragones ewes (n=202).

	Sampling day							
Variable	day 0	day 1	day 2	day 3	day 4	day 5	SE[1]	*p*-value
DMI[2] (kg/ewe)	1.86[a]	1.53[b]	0.67[c]	0.22[d]	0.08[e]	0.03[f]	0.004	< 0.001
BW (kg)	62.5[a]	59.3[b]	56.8[c]	55.1[d]	53.8[e]	53[f]	0.48	< 0.001
BCS	2.86[a]	2.72[b]	2.66[c]	2.59[d]	2.48[e]	2.45[e]	0.014	< 0.001

[1]Standard error; [2]dry matter intake; [a,b,c,d,e,f]Different letters in a row indicate significant differences between days ($p < 0.05$).

Table 2

Blood hematological and biochemical parameters on days 0, 1 and 5 of water restriction in Rasa Aragonesa ewes (n=202).

	Sampling day				
Variable	day 0	day 1	day 5	SE[1]	*p*-value
Hematocrit (%)	37.4[c]	38.3[b]	45.8[a]	0.28	< 0.001
Erythrocytes [10^6/µl]	9.5[c]	9.6[b]	10.6[a]	0.07	< 0.001
Hemoglobin [g/dl]	10.7[c]	10.9[b]	12.0[a]	0.07	< 0.001
MCV [µm]	39.6[c]	40.0[b]	43.5[a]	0.21	< 0.001
MCH [pg]	11.4[b]	11.4[b]	11.4[a]	0.04	0.002
MCHC [g/dl]	28.8[a]	28.5[b]	26.3[c]	0.1	< 0.001
RDW (%)	25.7[b]	26.0[a]	26.0[a]	0.13	< 0.001
Leukocytes [/mm^3]	5,831.0[b]	5,712.0[b]	6,251.0[a]	102	< 0.001
Neutrophils [/mm^3]	2,236.0[b]	2,186.0[b]	3,130.0[a]	71.2	< 0.001
Eosinophils [/mm^3]	235.0[b]	178.0[c]	283.0[a]	11.7	< 0.001
Basophils [/mm^3]	19.1[b]	21.4[a]	18.4[b]	0.85	0.001
Lymphocytes [/mm^3]	3,093.0[a]	3,046.0[a]	2,618.0[b]	53	< 0.001
Monocytes [/mm^3]	248.0[b]	280.0[a]	201.0[c]	9.42	< 0.001
NLR	0.8[b]	0.8[b]	1.3[a]	0.03	< 0.001
Platelets [10^3/mm^3]	414.0[a]	392.0[a]	323.0[b]	9.45	< 0.001
Total proteins [g/dl]	8.1[b]	8.0[c]	9.4[a]	0.04	< 0.001
Glucose [mg/dl]	69.6[a]	63.2[b]	59.7[c]	0.72	< 0.001
NEFAs [mmol/L]	0.2[b]	0.1[b]	1.6[a]	0.03	< 0.001
Cortisol [ng/ml]	9.3		8.9	0.66	0.67
DHEA [ng/ml]	4.0[c]	5.0[b]	7.5[a]	0.2	< 0.001
DHEA-S [ng/ml]	1.4[b]	1.3[c]	1.6[a]	0.05	< 0.001
Cortisol:DHEA ratio	3.2[a]		1.7[b]	0.42	< 0.001
Cortisol:DHEA-S ratio	7.7		7.5	0.64	0.79

[1]Standard error. [a,b,c]Different letters in a row indicate significant differences between days ($p < 0.05$). MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red cell distribution width, NLR: Neutrophil-to-lymphocyte ratio, NEFAs: Non-esterified fatty acids.

609 **Table 3**

610 Wool biochemical parameters on day 0 of water restriction and 28 days before in Rasa Aragonesa
611 ewes (n=202).

	Sampling day			
Variable	day 0	day 28	SE[1]	*p*-value
Cortisol [pg/mg]	3.5[b]	9.1[a]	0.14	< 0.001
DHEA [pg/mg]	0.8[b]	2[a]	0.05	< 0.001
DHEA-S [pg/mg]	9.4[b]	21.1[a]	0.36	< 0.001
Cortisol:DHEA ratio	7.6[a]	5.4[b]	0.35	< 0.001
Cortisol:DHEA-S ratio	0.39[b]	0.44[a]	0.01	< 0.001

612 [1]Standard error. [a,b]Different letters in a row indicate significant differences between days ($p < 0.05$).
613
614

Table 4

Age, Δ Body weight (BW) and Δ body condition score (BCS) in each cluster (Mean ± SE) in Rasa Aragonesa ewes (n=202).

	Cluster 1 (*n*=168)	Cluster 2 (*n*=22)	Cluster 3 (*n*=12)	*p*-value
Age (months)	61.3 ± 1.23	66.0 ± 3.41	61.8 ± 4.62	0.43
Δ BW (%)	-14.9 ± 0.19[b]	-15.8 ± 0.52[b]	-18.4 ± 0.70[a]	< 0.001
Δ BCS (%)	-13.9 ± 0.57	-15.1 ± 1.57	-13.8 ± 2.13	0.78

[a,b]Different letters in the same row indicate significant differences between clusters ($p < 0.05$). Δ Variation between stress and basal conditions (days 5 and 0).

Figure captions

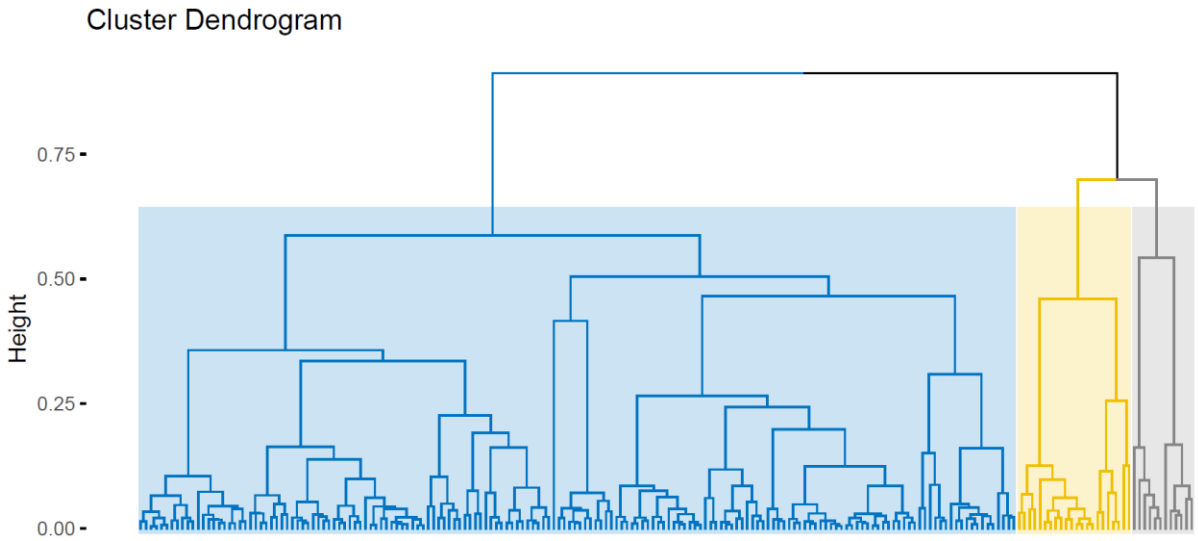

Figure 1. Dendrogram resulting from the hierarchical clustering analysis with 14 variation traits in blood (5d-0d) samples of Rasa Aragonesa ewes (n=202). Cluster 1 (n=168), 2 (n=22) and 3 (n=12) are represented in blue, yellow and grey, respectively.