ORIGINAL ARTICLE

Metabolic response to water shortage in an isolated feral sheep population

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ABSTRACT. To establish metabolic responses for biochemical analytes related to freshwater shortage adaptation, a total of 376 blood samples were collected from feral sheep at the Socorro Island, Revillagigedo Archipelago. Comparisons were made between four sampling periods with repeated measurements at 0, 7, 14, and 21 d (94 blood serums: 84 females and 10 males). During the first week, the sheep received daily water *ad libitum*. During the second and third week, the sheep received 60% daily water in relation to the first 7 days intake. Analysis of variance was used to compare the mean values between sampling days. Stepwise regression analysis was used to evaluate the relationships between the biochemical analytes. The glucose (GLU), total cholesterol (COL-T), triglycerides (TAG), urea, albumin (ALB), total protein (PROT-T), sodium ion (Na$^+$), creatine kinase (CK), arginine vasopressin (AVP), and aldosterone (Aldo), were determined. With the exception of GLU, the COL-T, TAG, urea, ALB, PROT-T, Na$^+$, CK, AVP, and Aldo showed differences between sampling days with the higher values corresponding to 14 d with limited water intake. Negative correlations ($P<0.05$) between ALB with COL-T and TAG, were quantified. Positive correlations ($P<0.05$) between COL-T with TAG, and Aldo; between urea with PROT-T, between CK with ALB and PROT-T, between AVP with COL-T, TAG, urea, PROT-T and Aldo, and between Aldo with Na$^+$, were quantified. Results could help improve the accuracy of metabolic profiles used as a tool for evaluating dehydration indicators and to describe the physiological mechanisms employed by feral sheep to cope with limited availability of freshwater.

Key words: blood biochemistry, metabolic profile, feral sheep, resilience to water shortage.

INTRODUCTION

The limited availability of freshwater for livestock has been a limiting factor in ruminant physiology and productivity (Vosooghi-Postindoz *et al* 2018). Fortunately, small ruminants have several adaptive mechanisms to efficiently use their physiological water reserves (Casamassima *et al* 2016), in this way overcoming the stresses caused by water shortage and dehydration (Berihulay *et al* 2019). These mechanisms include decreasing body water losses in the urine and faeces. The urine water losses can be reduced by promoting water reabsorption at renal tubules (Rotondo *et al* 2016, Trepiccione *et al* 2019), whereas faecal water losses can be decreased by promoting water absorption at gastrointestinal level (Ames *et al* 2019). However, in both cases, the serum volume decreases due to the water uptake by cells (Trepiccione *et al* 2019). Hyperosmolality due to increased solute concentrations is, thus, commonly detected in water-restricted sheep (Pratt *et al* 2016). A remarkable example of these adaptive mechanisms started with the introduction and abandonment of 100 sheep (*Ovis aries* Linnaeus, 1758) by Australian settlers to the Socorro Island of the Revillagigedo Archipelago in 1869 (Salas de León *et al* 2015). In the absence of handling, sheep became feral and adapted to the insular environment (Hernández et al 2017). The prolonged isolation allowed the

sheep population to develop traits adapted to the food and freshwater scarcity (Pickering *et al* 2013). In the absence of natural enemies, the sheep population grew to be about 5,000 individuals and they became the main disturbance agent of the island's ecological conditions (Hernández *et al* 2017). Thus, the Chamber of Deputies in Mexico approved the extermination of the feral sheep on the island (Ortiz *et al* 2016). To preserve the valuable germplasm of the Socorro Island sheep population, the Faculty of Veterinary Medicine of the University of Colima and the National Council of Science and Technology carried out a program of extraction and recovery (Hernández *et al* 2017). A thorough understanding of the adaptive physiological mechanisms to cope with limited water availability should be the starting point of all efforts to improve the resilience of domestic sheep to climate change. In this sense, the glucose (GLU), total cholesterol (COL-T), triglycerides (TAG), urea, albumin (ALB), total protein (PROT-T), sodium ion (Na$^+$), creatine kinase (CK), arginine vasopressin or antidiuretic hormone (AVP), and aldosterone (Aldo) are considered good indicators of dehydration. Thus, this study aimed to establish metabolic responses for biochemical analytes related to freshwater shortage adaptation, in feral sheep at the Socorro Island, Revillagigedo Archipelago, Mexico.

MATERIAL AND METHODS

ANIMALS

This experiment followed institutional and national guidelines for the care and use of animals. All experimental procedures were approved by the Committee of Ethical Review at Colima University (protocol approval number: 06/19; experimental period: October 2019). The animals

belong to the feral sheep extraction and recovery program of the Socorro Island of the Revillagigedo Archipelago. The Socorro Island (figure 1), is a tropical volcanic island located in the Pacific Ocean (18°42'28" N, 111°02'49" W), 540 km south of Cabo San Lucas in Baja California Sur and 720 km west of Manzanillo, Colima, Mexico. It has an area of 132 km^2 and a maximum elevation of 1040 m. Climate is warm, sub-humid (Köppen Cfb) (Peel *et al* 2007). The average temperature is 22-27.5 °C. It is located in the path of tropical cyclones that occur from June to November with an average of 2.75 events/year, with precipitation of more than 400 mm during the season.

Currently, the livestock inventory is located on the animal husbandry experimental unit at the Faculty of Veterinary Medicine, University of Colima, Tecoman, Colima, Mexico (18°56'53" N; 103°53'50" W). The climate is warm, sub-humid (Köppen Cfb) with summer rains (Peel *et al* 2007). The average temperature is 26 °C with precipitation of 750 mm/year (Hernández *et al* 2017). The study was carried out by sampling and analysing 376 blood serums from total livestock inventory (94 feral sheep in maintenance: 84 females and 10 males), with initial body weight (average ± S.D.) of 45 ± 4 kg and 60 ± 6 kg, respectively, body condition score (2.20 ± 0.8) and age (14 ± 2 months). Feeding conditions of sheep in the island were maintained at the experimental housing unit. Animals were able to graze *Conocarpus* scrub, *Croton masonii* scrub, and *Pteridium-Dononaea* scrub, introduced from Socorro Island (Flores *et al* 2009). Previous to the study, the herd was dewormed with Ivermectin (1 mL/25 kg of live weight subcutaneously) followed by immunisations against *Clostridium*, *Pasteurella multocida*

and *Mannheimia haemolytica* (BOBACT 8. SAGARPA B-0273-111; Intervet., Mexico City, Mexico) 2.5 mL/animal subcutaneously.

EXPERIMENTAL PROTOCOL

Comparisons were made between four sampling periods with repeated measurements at 0, 7, 14, and 21 d. During the first week, the sheep received daily water *ad libitum*. To determine the quantity of water for the second and third weeks, water intake was recorded during the first week. In the experiment, the sheep received 60% daily water in relation to the first 7 days intake; this was achieved by restricting water intake by 40% compared to the intake of the first week.

BLOOD COLLECTION AND DETERMINATION OF ANALYTES

Blood samples were collected by puncture of the jugular vein before morning feeding at 0, 7, 14, and 21 d with restricting water intake (figure 2).

For GLU, COL-T, TAG, urea, ALB, PROT-T, Na$^+$, and CK analysis, 8.5 mL of blood were collected into vacuum tubes with clot activator and serum separator gel (BD Vacutainer 367988; Becton-Dickinson Co., Franklin Lakes, United States). For AVP and Aldo, additional samples of 5 mL of blood were collected into vacuum tubes with EDTA-K$_3$ (BD Vacutainer 366352; Becton-Dickinson Co., Franklin Lakes, United States). To collect serum, blood samples were centrifuged at 1500 x *g* for 10 min by using a portable centrifuge (Porta-Spin C828; UNICO, Dayton, United States). Serum samples were separated using
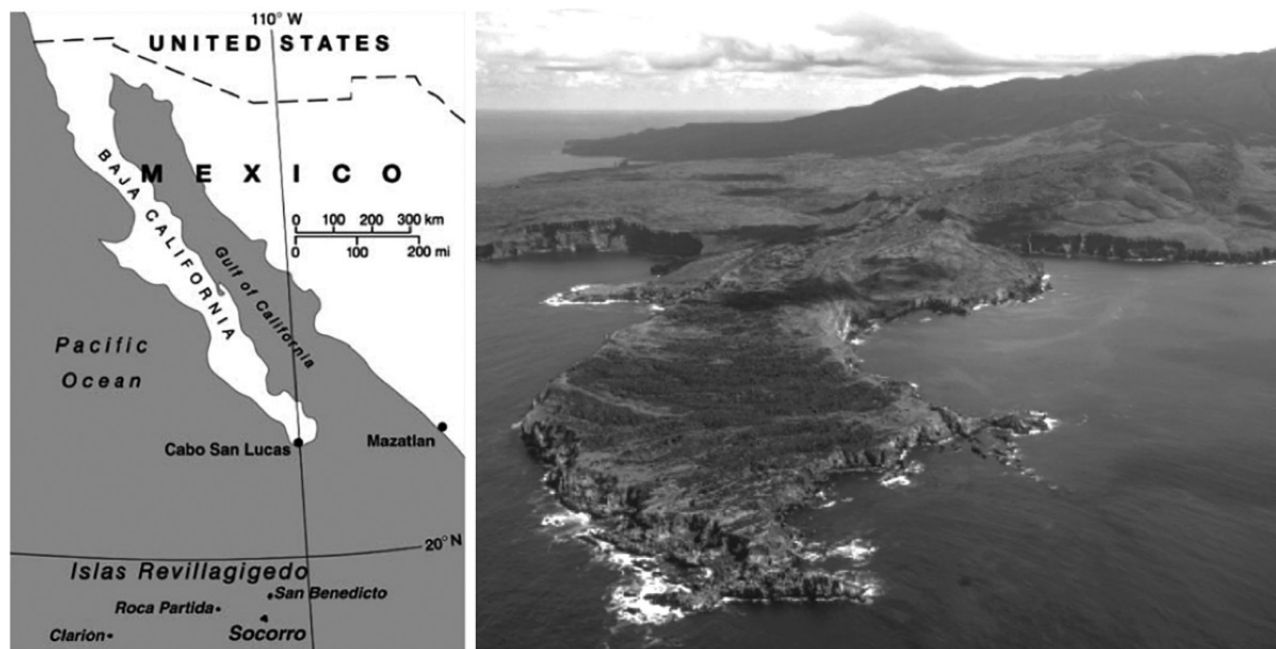

Figure 1. Location of the Socorro Island, Revillagigedo Archipelago, 720 km west of Manzanillo, Colima.

Figure 2. Feral sheep from the Socorro Island, Revillagigedo Archipelago.

1.5 mL tubes with lid (Tubes Safe-Lock 3810X; Eppendorf., Madrid, Spain) and transported at 4 °C in a portable cooler (Thermoelectric Cooler Car/Home M5644-710; Coleman Company, Kansas, United States) to the clinical laboratories at the Autonomous Metropolitan University and the University of Colima, where they were frozen at -20 °C until analysis. The concentration of each analyte was determined with a UV-Vis double beam spectrophotometer (Biochemistry Analyzer; KONTROLab, Guidonia, Italy) and the hormones were determined with a gamma counter (PC-RIA MAS; Stretec, Germany). Biochemical analytes, analytical method for each parameter, units in which the results were expressed, and corresponding commercial reagents, are described in (table 1).

Precision and reliability of the techniques were controlled using lyophilised control serum (SPINTROL NORMAL 1002100; Spinreact, Girona, Spain) and Assayed Multi-Sera AL 1027 (Randox Laboratories, Northern Ireland, United Kingdom). Hemolysis of serum was recorded on a qualitative scale of 0 (none) to 3 (dark). Samples showing haemolysis scores of 2 and above constituted less than 1% of all samples and did not introduce a significant bias in any of the tested models; thus, the influence of serum haemolysis was ignored.

STATISTICAL ANALYSIS

The data were described by average and S.D., and they were tested for normal distribution (Shapiro-Wilk test). The linear relationships between the biochemical analytes were identified by the use of a Pearson Correlation Coefficient matrix, and Stepwise regression analysis (PROC REG, SAS, System, v. 8.2, Cary, NC). Analysis of variance was used to compare the mean values between sampling days. A multiple comparison test of Tukey was performed when the effect of the sampling day was found to be significant ($P<0.05$). A diagnosis for outlier values was performed using robust multivariate outlier detection (OUTLIER; SAS, 2001). This macro calculates the robust Mahalanobis distance for each observation[1]. The following model was tested:

$$d_m(\mathrm{x},\overline{\mathrm{x}}) = \sqrt{(\mathrm{x}-\overline{\mathrm{x}})\sum\nolimits_x^{-1}(\mathrm{x}-\overline{\mathrm{x}})}$$

Where:
$d_m(\mathrm{x}, \overline{\mathrm{x}})$ = robust Mahalanobis distance;
x = vector of the observation;
$\overline{\mathrm{x}}$ = vector average of the observations; and
$\sum\nolimits_x^{-1}$ = variance-covariance matrix of the observations.

Regression diagnosis for main assumptions was performed (PROC UNIVARIATE; SAS, 2001). The linear functional form was visually checked by a normal plot. Shapiro-Wilk test was used to check the normality of residuals. Homoscedasticity was checked by plotting

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[1] http://www.datavis.ca/sasmac/outlier.html, accessed May 10, 2020.

Table 1. Biochemical analytes, units, analytical methods, and corresponding commercial reagents.

Analyte[*]	Unit	Method	Reagent
Glucose (GLU)	mM	Colorimetric. Trinder[a]	1001190[1]
Total cholesterol (COL-T)	mM	Colorimetric. Liquid[b]	41020[1]
Triglycerides (TAG)	mM	Colorimetric. Liquid[c]	41032[1]
Urea	mM	Enzymatic[d]	1001333[1]
Albumin (ALB)	g/dL	Colorimetric. Bromocresol green	1001020[1]
Total protein (PROT-T)	g/dL	Colorimetric. Biuret	1001291[1]
Sodium ion (Na^{+})	mM	Enzymatic. Galactosidase	1001385[1]
Creatine kinase (CK)	U/L	Enzymatic[e]	41250[1]
Arginine vasopressin (AVP)	pg/mL	Radioimmunoassay[f]	KIPERB319[2]
Aldosterone (Aldo)	pg/mL	Radioimmunoassay[g]	KIPZ0100[2]

[*]Official abbreviation of the International Union of Pure and Applied Chemistry (IUPAC); [a]Glucose Oxidase-Peroxidase; [b]Cholesterol Oxidase-Peroxidase; [c]Glycerol Phosphate Dehydrogenase-Peroxidase; [d]Urease-Glutamate Dehydrogenase; [e]Phosphocreatine NADPH; [f]sensitity 20 pmol/L and intra-assay and inter-assay coefficient of variations were 5.6% and 6.1% respectively; [g]sensitivity 6 pg/mL and intra-assay and inter-assay coefficient of variations were 9.5% and 10.4% respectively; [1]Spinreact, Girona, Spain; [2]DIAsource ImmunoAssays, Ottignies-Louvain-la-Neuve, Belgium.

residual versus predicted values, and the Durbin-Watson test was employed to check for error uncorrelation.

RESULTS

The reference value, descriptive statistics for GLU, COL-T, TAG, urea, ALB, PROT-T, Na^{+}, CK, AVP, Aldo, and their comparison between sampling days with limited drinking water availability in feral sheep from Socorro Island, Revillagigedo Archipelago are shown in table 2.

As shown in table 2, the biochemical analytes quantified in feral sheep from Socorro Island, presented no differences between males and females. However, their concentrations were above the reference range of the sheep. Except for GLU, the serum concentration of the other biochemical analytes (combined males and females) showed differences (*P*<0.05) between sampling days, with the higher values corresponding to 14 d with limited water intake. Negative correlations (*P*<0.05) between ALB with COL-T and TAG, were quantified. Positive correlations (*P*<0.05) between COL-T with TAG, and Aldo; between urea with PROT-T, between CK with ALB and PROT-T, between AVP with COL-T, TAG, urea, PROT-T and Aldo, and between Aldo with Na^{+}, are shown in table 3.

DISCUSSION

Assessment of biochemical analytes sensitive to water shortage revealed that feral sheep from Socorro Island exhibited changes in serum concentrations of COL-T, TAG, urea, ALB, PROT-T, Na^{+}, CK, AVP, and Aldo under 60% water restriction. All sheep needed 14 d to adapt to the water shortage and the concentration of biochemical analytes was closer to the value recorded during the first week, at 21 d. This drop in biochemical analytes concentration

suggests adaption to drinking water shortage at the end of the experimentation period. When small ruminants become dehydrated during water restriction, the serum volume decreases due to water uptake by tissue cells (Trepiccione *et al* 2019). In this sense, the urea molecules generate osmotic traction of water in the urinary tract (Weiner *et al* 2015), as water moves from areas of low concentration to those of high concentration to dilute urea in urine (Weiner 2017). Small ruminants such as the Awassi sheep, a Middle Eastern breed, have the ability to decrease urine volume and increased urea concentration, after being deprived of water for 5 d (Jaber *et al* 2004). Under conditions of drinking water shortage, however, the kidneys function is altered in Marwari sheep (Kataria and Kataria 2007) with slower glomerular filtration and higher urea reabsorption (Weiner and Verlander 2016), leading to increased blood concentrations of CK and urea (Weiner *et al* 2015), but without affecting GLU concentration (Abdelatif *et al* 2010), results similar to those found in this study (table 2). Reduced serum volume also increases concentrations of certain biochemical analytes (Berihulay *et al* 2019), and forces sheep to activate physiological mechanisms to adapt to dehydration and cope with limited drinking water availability (Mengistu *et al* 2016). Comisana sheep, a central and northern Sicily breed, under 60% water restriction for 40 d, increased their concentrations of ALB, COL-T, PROT-T, and TAG, without affecting your concentrations of GLU (Casamassima *et al* 2008). In another study by the same group, the Lacaune sheep, a South of France breed, under 60% water restriction for 28 d, increased their concentrations of CK and PROT-T (Casamassima *et al* 2016). Hamadeh *et al* (2006) suggested that concentrations of ALB, COL-T, CK, PROT-T, and urea are influenced by the consumption of water. In their study, they offered water to Awassi sheep every 3 d under

Table 2. Reference value, mean ± standard deviation, and comparison of different biochemical analytes of feral sheep from the Socorro Island, Revillagigedo Archipelago, during restricting water intake.

Analyte	Reference	Males n = 40 blood serums[1]	Females n = 336 blood serums[1]
Glucose (mM)	3.80 ± 0.33[†,a]	3.89 ± 0.28[a]	3.74 ± 0.24[a]
Total cholesterol (mM)	1.66 ± 0.31[†,a]	2.50 ± 0.11[b]	2.47 ± 0.09[b]
Triglycerides (mM)	0.33 ± 0.01[§,a]	0.40 ± 0.01[b]	0.39 ± 0.01[b]
Urea (mM)	7.70 ± 0.43[§,a]	8.63 ± 0.06[b]	8.61 ± 0.05[b]
Albumin (g/dL)	2.70 ± 0.11[†,a]	3.74 ± 0.48[b]	3.51 ± 0.37[b]
Total protein (g/dL)	7.20 ± 0.52[†,a]	8.87 ± 0.37[b]	8.71 ± 0.33[b]
Sodium ion (mM)	141.5 ± 4.1[†,a]	172.47 ± 22.11[b]	161.58 ± 17.29[b]
Creatine kinase (U/L)	10.3 ± 1.6[†,a]	13.20 ± 0.11[b]	13.17 ± 0.09[b]
Arginine vasopressin (pg/mL)	3.30 ± 1.37[¥,a]	8.53 ± 0.12[b]	8.50 ± 0.11[b]
Aldosterone (pg/mL)	20.10 ± 2.21[¶,a]	28.14 ± 1.15[b]	27.82 ± 1.01[b]

	Combined males and females n = 94 blood serums/sampling days Restricting water intake (d)			
	0	7	14	21
Glucose (mM)	3.75 ± 0.24[a]	3.76 ± 0.25[a]	3.76 ± 0.26[a]	3.75 ± 0.24[a]
Total cholesterol (mM)	2.37 ± 0.07[a]	2.49 ± 0.05[b]	2.58 ± 0.07[c]	2.45 ± 0.05[d]
Triglycerides (mM)	0.38 ± 0.01[a]	0.40 ± 0.01[b]	0.41 ± 0.01[c]	0.39 ± 0.01[d]
Urea (mM)	8.55 ± 0.03[a]	8.63 ± 0.02[b]	8.68 ± 0.04[c]	8.60 ± 0.02[d]
Albumin (g/dL)	3.04 ± 0.16[a]	3.63 ± 0.08[b]	4.04 ± 0.22[c]	3.42 ± 0.10[d]
Total protein (g/dL)	8.30 ± 0.15[a]	8.84 ± 0.12[b]	9.16 ± 0.14[c]	8.62 ± 0.14[d]
Sodium ion (mM)	140.00 ± 7.77[a]	167.02 ± 3.82[b]	186.27 ± 10.22[c]	157.68 ± 4.75[d]
Creatine kinase (U/L)	13.07 ± 0.07[a]	13.19 ± 0.05[b]	13.28 ± 0.07[c]	13.15 ± 0.05[d]
Arginine vasopressin (pg/mL)	8.40 ± 0.10[a]	8.52 ± 0.05[b]	8.62 ± 0.09[c]	8.49 ± 0.05[d]
Aldosterone (pg/mL)	26.78 ± 0.68[a]	28.09 ± 0.64[b]	28.96 ± 0.69[c]	27.59 ± 0.63[d]

[1]Data summarised by sheep across all the sampling days; [†](Kaneko *et al* 2008); [§](Lotfollahzadeh *et al* 2016); [¥](Mengistu *et al* 2016); [¶](Kataria and Kataria 2007); *significant differences were obtained between groups indicated with different letters; *P< 0 .05.

Table 3. Pearson Correlation Coefficients for different biochemical analytes of feral sheep from the Socorro Island, Revillagigedo Archipelago, during restricting water intake (n = 376 blood serums).

	COL-T[b]	TAG[c]	urea	ALB[d]	PROT-T[e]	Na[+f]	CK[g]	AVP[h]	Aldo[i]
GLU[a]	0.04	0.03	0.04	0.03	0.03	0.03	0.04	0.06	0.03
	COL-T	0.33*	0.26	-0.75*	0.24	0.14	0.10	0.90*	0.33*
		TAG	0.14	-0.80*	0.09	0.13	0.11	0.84*	0.09
			urea	0.14	0.80*	0.09	0.20	0.82*	0.19
				ALB	0.07	0.09	0.60*	0.11	0.10
					PROT-T	0.10	0.79*	0.71*	0.12
						Na+	0.19	0.13	0.76*
							CK	0.13	0.11
								AVP	0.84*
									Aldo

Official abbreviation of the International Union of Pure and Applied Chemistry (IUPAC); [a]Glucose; [b]Total cholesterol; [c]Triglycerides; [d]Albumin; [e]Total protein; [f]Sodium ion; [g]Creatine kinase; [h]Arginine vasopressin; [i]Aldosterone; *P<0.05

two physiological conditions, lactating and dry, over a 3-week-period, and an increase ALB (3.08 vs. 3.43 g/dL), COL-T (1.7 vs. 2.4 mM), PROT-T (7.39 vs. 7.86 g/dL), and urea (8.29 vs. 10.01 mM) was observed. Jaber *et al* (2004) also reported that water restriction increased COL-T (1.90 vs. 2.14 mM), PROT-T (7.59 vs. 8.03 g/dL), and urea (5.96 vs. 7.71 mM), and Ghanem *et al* (2008) reported that COL-T increased (1.61 vs. 2.06 mM).

The negative correlations ($P<0.05$) between ALB with COL-T and TAG presented in table 3, relate to the availability of acetyl-CoA. After its synthesis, the ALB resides in the smooth endoplasmic reticulum and Golgi bodies (Menzies *et al* 2016). The smooth endoplasmic reticulum is the compartment for lipid synthesis (Black *et al* 2002). There, the acetyl-CoA molecules are joined together to form COL-T and TAG (Kumar *et al* 2018). Thus, nascent ALB is expected to cleave the thioester bond of acetyl-CoA (Menzies *et al* 2016). It is plausible to think that this non-enzymatic acetylation of ALB is possible in the intracellular compartment using acetyl-CoA (Kumar *et al* 2018) and that this limits the availability of acetyl-CoA for the synthesis of COL-T and TAG (Morgan *et al* 2016). Positive correlations ($P<0.05$) between the different biochemical analytes presented in table 3 relate to two main mechanisms for conserving body water. The first mechanism involves osmoreceptors that detect increases in solute concentrations in the blood during dehydration (Prager-Khoutorsky 2017). These osmoreceptors send a neural signal to the hypothalamic supraoptic nucleus (Gizowski and Bourque 2018), to translate AVP from nine amino acids (aa) (Rotondo *et al* 2016). AVP stored and released by the neurohypophysis travels to the kidneys, binds to receptors in the distal or collecting tubules, and promotes antidiuresis (retention of water) (Pratt *et al* 2016), in the loop of Henle and the proximal tubule of

the nephron (Bankir *et al* 2017). Water reabsorption varies, depending on the concentration of circulating AVP, which is usually determined by plasma osmolality, but also intracellular fluid volume (Trepiccione *et al* 2019). Hence, the serum volume decreases due to water uptake by cells (Trepiccione *et al* 2019) and COL-T, TAG, urea, PROT-T, and Aldo increase positively in relation to the AVP concentration (table 3).

The second mechanism involves baroreceptors that detect low blood pressure and low blood volume (Burlando *et al* 2019). In response, neurons send a signal to the juxtaglomerular cells of the kidneys to translate renin also called angiotensinogenase (Ames *et al* 2019), which hydrolyses angiotensinogen to angiotensin I (Patel *et al* 2017). Angiotensin I, is hydrolysed by the angiotensin-converting enzyme (ACE) forming angiotensin II (Burlando *et al* 2019), which stimulates the conversion of COL-T to Aldo (mineralocorticoid) in the adrenal gland (Ames *et al* 2019). Aldo conserves Na^+ in the renal distal convoluted tubule (Patel *et al* 2017), as can be seen in figure 3, where the results indicate that Aldo was positively correlated with Na^+, triggering reabsorption of water through osmosis. Figure 3 shows the linear regression model for these variables.

The Na^+ is the dominant extracellular cation and the main determinant of osmolality (Berihulay *et al* 2019), and therefore of water distribution between the extracellular and intracellular fluid compartments (Burlando *et al* 2019). This has also been reported by Hamadeh *et al* (2006) in Awassi sheep. In particular, the progressive increase in Aldo, in response to water restriction, leads to an increase in Na^+ concentration. Hence, many of the extrinsic regulators of renal function are intended to alter Na^+ excretion, preserve Na^+ balance, and regulate blood pressure (Trepiccione *et al* 2019).
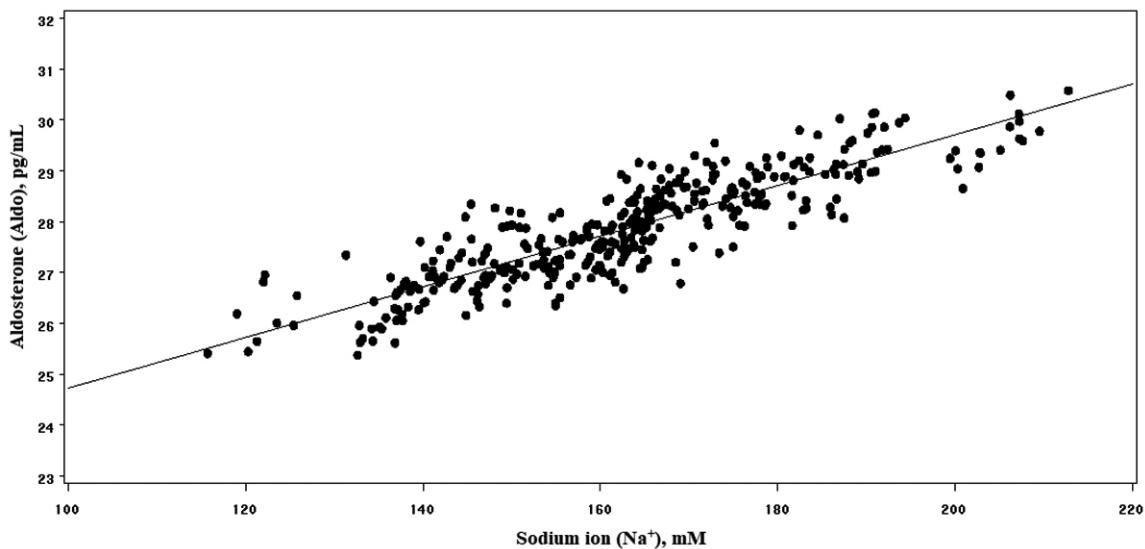

Figure 3. Relationship between aldosterone and sodium ion. Aldosterone (●); predicted response (—).

The feral sheep from Socorro Island of the Revillagigedo Archipelago needed 21 d to adapt to water shortage treatment. Results suggested that the feral sheep had high resilience to limited water availability, expressed in the concentration of different biochemical analytes considered as good indicators of dehydration. These results show the important role of water, as a limiting factor for sheep in low-water availability environments. The Pearson Correlation Coefficients could be used at the herd level, to detect physiological mechanisms to cope with water restriction. Understanding the physiological mechanisms associated to these values would help researchers to select animals that maintain their productive ability under environmental stressors such as drought.

ACKNOWLEDGEMENTS

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