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Original Research Article

Influence of previous plane of nutrition on molecular mechanisms regulating the expression of urea and water metabolism related genes in the rumen and kidney of finishing crossbred Angus steers

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ABSTRACT

This study aimed to understand how molecular mechanisms controlling water and urea metabolism at the finishing phase can be affected by previous plane of nutrition of crossbred Angus beef steers. Twenty-four ($n = 24$) animals were randomly distributed into either a moderate (MP) or high plane of nutrition during the background phase for 85 d. Animals were then blocked by their previous plane and were moved onto a 105-d finishing phase in a 2×2 factorial arrangement. The forage-finished group received only high-quality alfalfa hay, whereas the grain-fed group received a high grain diet (80% whole corn and 20% alfalfa hay). By the end of the finishing phase, animals were harvested and tissue samples from the rumen and kidney were collected. Changes in gene expression of aquaporins (*AQP*)-*2*, *-3*, *-4*, *-7*, *ATP1A1*, *ATP1B1*, *SGK1*, *CLIC1* (kidney and rumen), *UT-A1* (kidney only) and *UT-B* (rumen only), were assayed via real-time qPCR; *18S* rRNA was used as an endogenous control. One-way ANOVA followed by Tukey's post hoc analysis was conducted. When animals were from MP, forage-finishing increased the relative abundance of *AQP3* ($P \leq 0.05$), *AQP7* ($P \leq 0.05$), *ATP1B1* ($P \leq 0.05$), and *SGK1* ($P \leq 0.05$) in the kidney when compared to grain-fed animals. In the rumen, for the MP group, *AQP7* was differentially expressed in both treatments at the finishing phase ($P \leq 0.01$), with forage-finished steers having the highest expression of *AQP7*. For the MP group, *UT-B* had a tendency of presenting a higher expression on grain-fed animals ($P = 0.075$). Overall, these results suggest that previous plane can impact expression of genes associated with water and urea metabolism during the finishing phase, namely *AQP3*, *AQP7*, *ATP1B1*, and *SGK1* in the kidney, and *AQP7* and *UT-B* in the rumen. The greatest impact observed on gene expression changes of investigated genes at the finishing phase was reflective of animals backgrounded on the moderate previous plane.

1. Introduction

Stocker/backgrounding production occurs year-round in various forage systems, which inherently vary widely in both quality and availability throughout the year (Brown, 1985). Therefore, there are very few locations where stocker grazing systems are available and offer high-quality forage year-round. Once animals transition into the finishing phase, they are mainly fed a high-energy diet composed mainly of grains that have a high environmental footprint (i.e. water) (Mekonnen and Hoekstra, 2011). Due to the increasing concern over the environmental impacts of conventional beef, grass/forage-fed beef is often perceived by consumers as a more sustainable alternative (Xue et al., 2010) for the cattle

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industry. Klopatek et al. (2022), has shown that grass-fed beef had a 150% greater water footprint than grain-fed animals, indicating a great influence of diet on water metabolism of those animals.

In cattle, water contained in the intracellular space constitutes, on average, two-thirds of total body water pool, whereas the remaining one-third consists of water surrounding the cells and connective tissue, water in the blood plasma and the gastrointestinal tract (Woodford et al., 1984). Once consumed, the rumen serves as a giant water reservoir that could be utilized when water is scarce (Shkolnik et al., 1980). Synchronously, the regulation of extracellular fluid volume and composition is controlled by the kidney (Choshniak et al., 1984).

At the cellular level, the maintenance of the correct molality of fluids and proper distribution of water within body compartments will depend upon the environment, where cells will adapt to changes by altering their patterns of gene transcription and protein modification as well as their cytoskeletal structure (Bell et al., 2000). Some of the most important water-related cell components that will be prone to modification upon hydric stress are the aquaporins (AQP), a specialized group of water channels that allow the passage of water and other small molecules (Michalek, 2016) to and from the cytosol. Water transport also affects the balance of water in the body, being driven by the creation of an osmotic gradient across an epithelium through active ion/solute transport (Verkman, 2008). Therefore, other cellular components related to solute transport are also important and involved in the balance of body water, such as Na^+/K^+-ATPase, sodium channels, and chloride channels. All those channels are regulated by several genes including ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*) and beta 1 (*ATP1B1*), serum/glucocorticoid regulated kinase 1 (*SGK1*), and chloride intracellular channel 1 (*CLIC1*), which are related to the ability of the cell to sense and appropriately respond to environmental changes in osmotic balance through an integrated network of intracellular signaling pathways (Bell et al., 2000). In ruminants, another factor that plays a role in osmotic balance is the level of urea in the blood due to their ability of recycling dietetic nitrogen as urea (Lapierre and Lobley, 2001). High levels of blood urea, which is associated with high protein diets, need to be excreted in the urine, which will require proper regulation of ion/solute transport to avoid excessive loss of water through the urine (Bankir et al., 1996).

Altogether, taking in consideration the molecular mechanisms that might regulate water pool in the body and how dietary adaptations may change its control, we hypothesize that previous plane of nutrition could modulate expression of water and nitrogen related genes later in their life cycle, at the finishing phase. Therefore, the objective of this study is to understand how the previous plane of nutrition can affect the expression of water and urea metabolism related genes in the kidney and rumen of cattle backgrounded in a moderate or a high plane of nutrition, and subsequently finished in a grain- or forage-based system.

2. Material and methods

2.1. Animal ethics statement

All experimental and animal husbandry procedures conducted were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno, NV (protocol #00845) and complied with the ARRIVE guidelines.

2.2. Experimental design, treatments, and animals

Twenty-four crossbred Angus steers (298.01 ± 10.17 kg) were housed in the research feedlot area of the Main Station Field Laboratory at the University of Nevada, Reno. The experimental trial lasted 220 d, consisting of two phases: backgrounding and finishing phase. During the backgrounding phase (85 d), animals were randomly assigned to one of the two treatments ($n = 12$ per treatment; Table 1): moderate plane of nutrition (MP) or high plane of nutrition (HP). By the end of the background phase, steers were restrictly randomized by previous plane of nutrition (MP or HP) and transitioned to the finishing phase (50% of animals of each backgrounding group were randomly reallocated in each finishing treatment resulting on an equal number of steers for each combination of previous plane of nutrition and finishing diets). The finishing phase included a 30-d adaptation period and a 105-d finishing period. The finishing period consisted of either alfalfa hay only (forage-fed, $n = 12$) or predominantly whole corn (grain-fed, $n = 12$) (Table 2). Therefore, we had a factorial composed of four treatments: MP + grain-fed (animals from MP and finished on grains), MP + forage-fed (animals from MP and finished on forages), HP + grain-fed (animals from HP and finished on grains), HP + forage-fed (animals from HP and finished on forages) following recommendations of biological replicates provided by Schurch et al. (2016). All animals were individually fed, and had free access to feed and water, and a balanced mineral mix throughout

Table 1
Ingredients and nutrient composition of diets fed to crossbred Angus steers during background phase (% DM basis).

Item	Treatment	
	Moderate plane	High plane
Ingredients		
Alfalfa	–	85
Beardless wheat	–	15
Triticale	100	–
Mineral mix[1]	Ad libitum	Ad libitum
Chemical analysis		
Dry matter, % as-is	93.70	93.88
Crude protein	9.10	12.62
Organic matter	90.28	92.38
Soluble protein	4.80	5.78
Soluble protein, % CP	52.80	45.62
Rumen degradable protein	7.00	9.20
Rumen degradable protein, % CP	76.40	72.81
Acid detergent fiber	29.28	39.97
NDICP	1.28	1.52
aNDFom[2]	47.78	46.92
apNDFom[3]	46.50	45.40
Lignin	4.07	6.91
Sugar	12.80	7.46
Starch	0.40	0.98
Ash	9.72	7.62
Ca	0.35	1.20
P	0.19	0.21
Mg	0.15	0.32
K	1.41	1.49
Na	0.08	0.16
Fe, mg/kg	297.00	112.15
Mn, mg/kg	35.00	27.35
Zn, mg/kg	22.00	21.40
Cu, mg/kg	11.00	11.55
Total digestible nutrients	53.00	57.59
Net energy for maintenance, Mcal/kg	0.25	0.25
Net energy for gain, Mcal/kg	0.10	0.13
Non-fiber carbohydrates	25.99	30.40

DM = dry matter; CP = crude protein; NDICP = neutral detergent insoluble crude protein.

[1] Mineral mix composition: 18% Ca, 6% P, 18% NaCl, 4% Mg, 0.5% K, 0.36% Mn, 0.0012% Co, 0.12% Cu, 0.006% I, 0.0027% Se, 0.36% Zn.

[2] aNDFom: neutral detergent fiber (NDF) assayed with a heat stable amylase and expressed exclusive of residual ash.

[3] apNDFom: NDF assayed with a heat stable amylase and expressed exclusive of residual ash and protein.

Table 2
Ingredients and nutrient composition of diets fed to crossbred Angus steers during finishing phase (% DM basis).

Item	Treatment	
	Grain-fed	Forage-fed
Ingredients		
Alfalfa (21% CP)	–	100
Alfalfa (16% CP)	80	–
Corn	20	–
Mineral mix[1]	Ad libitum	Ad libitum
Nutrient composition (chemical analysis)		
Dry matter, % as-is	90.28	94.00
Organic matter	90.8	96.44
Crude protein	10.8	21.3
Soluble protein	3.5	8.2
Soluble protein, % CP	30.46	38.4
Rumen degradable protein	5.07	14.7
Rumen degradable protein, % CP	41.82	69.2
Acid detergent fiber	11.02	26.2
NDICP	0.746	1.93
aNDFom[2]	17.12	32.2
apNDFom[3]	16.374	30.27
Lignin	3.176	5.72
Sugar	3.26	9.5
Starch	56.92	2.2
Ash	3.56	9.2
Ca	0.344	1.82
P	0.288	0.19
Mg	0.168	0.32
K	0.758	1.62
Na	0.092	0.2
Fe, mg/kg	1,283	387
Mn, mg/kg	33.4	47
Zn, mg/kg	1,107.2	34
Cu, mg/kg	9.6	13
Total digestible nutrients	80.52	64.8
Net energy for maintenance, Mcal/kg	0.966	0.7
Net energy for gain, Mcal/kg	0.658	0.43
Non-fiber carbohydrates	65.12	36.6

DM = dry matter; CP = crude protein; NDICP = neutral detergent insoluble crude protein.

[1] Grain-fed mineral mix composition: 26.17% Ca, 10.52% P, 3.35% Na, 2.95% Mg, 6.80% K, 0.17% Mn, 0.0006% Co, 0.06% Cu, 0.003% I, 0.002% Se, 0.17% Zn, 0.18% Fe. Forage-fed mineral mix composition: 18% Ca, 6% P, 18% NaCl, 4% Mg, 0.5% K, 0.36% Mn, 0.0012% Co, 0.12% Cu, 0.006% I, 0.0027% Se, 0.36% Zn.

[2] aNDFom: neutral detergent fiber (NDF) assayed with a heat stable amylase and expressed exclusive of residual ash.

[3] apNDFom: NDF assayed with a heat stable amylase and expressed exclusive of residual ash and protein.

the experimental period. Water and feed intakes were measured daily.

2.3. Feedstuff chemical analysis

Feed samples were collected weekly for bromatological analysis. Feedstuffs were composited into one representative sample for each experimental phase, and a 200-g subsample was shipped to Cumberland Valley Analytical Services (CVAS; Waynesboro, PA). The samples were analyzed for the chemical composition of dry matter (method # 930.15; AOAC, 2000), crude protein (CP; method # 990.03; AOAC, 2000), soluble protein (Krishnamoorthy et al., 1982), rumen degradable protein (Krishnamoorthy et al., 1983), acid detergent fiber (method # 973.18; AOAC, 2000), acid detergent insoluble CP using acid detergent fiber residue in a Leco FP-528 nitrogen combustion analyzer (Leco Corporation, St. Joseph, MO), neutral detergent fiber (Van Soest et al., 1991) corrected for protein (Leco Corporation, St. Joseph, MO) and ash (method # 942.05; AOAC, 2000), lignin (Goering and Van Soest, 1970), sugar (Dubois et al., 1956), starch (Hall, 2009), ash (method # 942.05; AOAC, 2000) and a complete mineral panel (method # 985.01; AOAC, 2000) in an inductively couple plasma spectrometers (PerkinElmer 5300 DV ICP, PerkinElmer, Shelton, CT). Values for total digestible nutrients and net energy were obtained by empirical equations (Weiss, 1998).

2.4. Sample collections

By the end of the finishing phase, all steers were transported to a USDA-inspected commercial abattoir (CS Beef Packers, Kuna, Idaho, UT), where all the animals were harvested. Steers were stunned and exsanguinated immediately. Kidney and ventral sacs' rumen wall tissue samples were collected from each steer immediately upon evisceration (within 10 min from slaughter). Collected samples were placed in a 2-mL cryotube and flash frozen in liquid nitrogen. Samples were then transferred to a −80 °C freezer for storage and subsequent RNA extraction and analysis.

2.5. Real-time qPCR

Total RNA was extracted from kidney and rumen samples using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were diluted to 100 ng/μL (500 ng) and then converted to complementary DNA (cDNA) using the Verso cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA). Gene expression was examined via quantitative real-time polymerase chain reaction (qPCR) using Apex qPCR Master Mix (Genesee Scientific Corp., San Diego, CA; 42-120) and samples read on a BioRad CF96X qPCR instrument (Bio-Rad Laboratories, Hercules, California). Primers were purchased from IDT (Coralville, IA) (Table 3). Target genes (Table 4) included: aquaporin-2 (*AQP2*), -3 (*AQP3*), -4 (*AQP4*), and -7 (*AQP7*), *ATP1A1* and *ATP1B1*, *SGK1*, *CLIC1*, solute carrier family 14 member 2 (kidney only; *SLC14A2*; codes for urea transporter A1 [*UT-A1*]) and solute

Table 3
Primer sequences for gene transcripts analyzed by quantitative real-time reverse transcription polymerase chain reaction (qPCR).

Gene[1]	Primer design[2]	Primer sequence
Gene control		
18S	FWD	5′-GCC GCT AGA GGT GAA ATT CTT A-3′
	REV	5′-CTT TCG CTC TGG TCC GTC TT-3′
Target genes		
AQP2	FWD	5′-CAA TGC CCT CAA CAA CAA CTC-3′
	REV	5′-GTC AGT GGA GGC GAA GAT AC-3′
AQP3	FWD	5′-GTC CAG GTA CAG GCA TTT CTC-3′
	REV	5′-CCT CCT CCT AGC CCT ACT TAT ATT-3′
AQP4	FWD	5′-TTC GGT GCT AGG AAA GGA ATG-3′
	REV	5′-CCA AAG GGA CCT GGG ATT TAG-3′
AQP7	FWD	5′-CTC TTA GCC ATC GCA GAC AA-3′
	REV	5′-GAG TTC ATG CCC AGG GAT ATT-3′
ATP1A1	FWD	5′-GGA GAT CTG GTG GAA AAA G-3′
	REV	5′-TCC CGT GAG TGA GGA GTT AT-3′
ATP1B1	FWD	5′-GAA CTC GGA GAA GAA GGA GTT T-3′
	REV	5′-TGG ATG GTT CCG ATG AAG ATG-3′
SGK1	FWD	5′-TCT CCT GGC AAG ACA CAA AG-3′
	REV	5′-AAC ATT CCG CTC CGA CAT AAT A-3′
CLIC1	FWD	5′-CAG CTG GGC TGG ACA TAT T-3′
	REV	5′-ACT TTC AGG GCT TTC AGG AG-3′
SLC14A1	FWD	5′-CTC CTT CAG ACT CCA GAA CAT C-3′
	REV	5′-CTT AGT GCC AAT GCC CTA CT-3′
SLC14A2	FWD	5′-GCT GGA CTT CAC GGC TAT AA-3′
	REV	5′-GGA GTA GAA GCC ACC AGA AAT AG-3′

[1] Eukaryotic 18S ribosomal (*18S*); aquaporin-2 (*AQP2*), -3 (*AQP3*), -4 (*AQP4*), and -7 (*AQP7*); ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*) and beta 1 (*ATP1B1*); serum/glucocorticoid regulated kinase 1 (*SGK1*); chloride intracellular channel 1 (*CLIC1*); solute carrier family 14 member 1 (*SLC14A1*; codes for urea transporter B [*UT-B*]); solute carrier family 14 member 2 (*SLC14A2*; codes for urea transporter A1 [*UT-A1*]).

[2] FWD = forward primer (anti-sense strand); REV = reverse primer (sense strand).

Table 4
Target genes related to water and urea metabolism and its respective functions.

Target genes[1]	Function	Reference
AQP2	Located in the cytosol, but when in need of increased water absorption, it will migrate to the membrane and allow free passage of water.	Kwon et al. (2013)
AQP3	Transports water, urea, ammonia, and glycerol, and represent exit pathways for water reabsorbed via *AQP2*	Rojek et al. (2008); Ikeda and Matsuzaki (2015)
AQP4		
AQP7	Allows movement of water, glycerol ammonia, and urea across cell membranes down a gradient concentration	Rojek et al. (2008)
ATP1A1	Encodes the large catalytic α subunit of Na^+/K^+-ATPase pump	Zouzoulas and Blostein (2006)
ATP1B1	Encodes a smaller highly glycosylated β subunit of Na^+/K^+-ATPase pump, which is necessary for the proper folding, insertion and maturation of the α subunit in the plasma membrane	
SGK1	Phosphorylated in response to aldosterone, and it stimulates sodium transport by the epithelial sodium channels (including Na^+/K^+-ATPase pump and ENaC) and increase transport of sodium to the cell (decreases sodium urinary excretion). This will also lead to an increase in water uptake (concentration gradient).	Feraille and Dizin (2016)
CLIC1	Chloride channel—carry out transepithelial transport of salt and water according with the concentration gradient.	Ulmasov et al. (2007)
SLC14A1	This gene will code for urea transporter B (*UT-B*) in the rumen, but its exact pathway in the rumen is still being studied. However, it is believed that the *UT-Bs* located on the luminal and basolateral membrane of the ruminal epithelium are responsible for facilitating urea transport from the blood to the rumen epithelium.	Zhong et al. (2022)
SLC14A2	*SLC14A2* codes for urea transporter A (*UT-A*), a urea renal transporters in the kidney nephron that facilitates the reabsorption and recycling of urinary urea, and with that increases medullary urea concentration.	Stewart (2011)

[1] Aquaporin-2 (*AQP2*), -3 (*AQP3*), -4 (*AQP4*), and -7 (*AQP7*); ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*) and Beta 1 (*ATP1B1*); serum/glucocorticoid regulated kinase 1 (*SGK1*); chloride intracellular channel 1 (*CLIC1*); solute carrier family 14 member 1 (*SLC14A1*); Solute carrier family 14 member 2 (*SLC14A2*).

carrier family 14 member 1 (rumen only; *SLC14A1*; codes for urea transporter B [*UT-B*]). The PCR amplification protocol consisted of enzyme activation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s combined with annealing/extension at 60 °C for 30 s. Expression levels of target genes were normalized to 18S ribosomal RNA (*18S*), which was validated as a suitable reference gene under these experimental conditions. The $2^{-\Delta\Delta CT}$ method was used to determine relative abundance of mRNA (Livak and Schmittgen, 2001; Ferguson et al., 2010) and expressed as fold change relative to HP + grain-fed treatment when both previous planes were considered or MP + grain-fed when only MP was considered.

2.6. Statistical analyses

When comparing the four treatment groups, two-way ANOVA followed by multi comparison mean separation through Bonferroni tests. When only MP animals were compared, differences were analyzed through Student's *t*-test. Statistical significance was declared at $P \leq 0.05$, whereas statistical tendency was declared at $0.05 < P \leq 0.10$. Identification of outliers was performed by plotting the studentized residuals against the predicted values as well as by Cook's D. Observations with studentized residuals exceeding a coefficient of 2.5 were considered outliers and removed from the data (Neter et al., 2004). In total, 3 observations were removed from *AQP7* (HP + grain-fed, MP + forage-fed, and MP + grain-fed), 1 from *AQP3* (MP + forage-fed), and 1 from *AQP4* (MP + grain-fed) for kidney samples, while 1 observation for *AQP2* (HP + forage-fed), 2 observations for *AQP4* (MP + forage-fed and HP + forage-fed), and 1 observation for *ATP1A1* (HP + grain-fed) were removed for rumen samples. Linear model assumptions were examined on the residuals. GraphPad Prism software (GraphPad InStat Software, San Diego, CA) was used to analyze data and produce graphs.

Data were analyzed as a linear mixed model under a 2 × 2 factorial arrangement following a completely randomized design following the statistical model:

$$Y_{ij} = \mu + T_i + P_j + TP_{ij} + e_{ij} \ ,$$

where Y_{ij} is the dependent variable for the *i*th finishing diet, and *j*th previous plane of nutrition, μ is the mean, T_i is the fixed effect of finishing diet *i*th, P_j is the fixed effect associated with the *j*th previous plane of nutrition, TP_{ij} is the fixed interaction associated with the interaction between finishing diet *i*th and previous plane *j*th, random intercepts and slopes for animal within treatments was used as random effects for the models, e_{ij} is the random error associated with *ij*th data value assuming that e_{ij} are independently identically N(0, σ^2). Initial body weight was tested as a covariate and was later removed due to a lack of statistical significance.

3. Results

3.1. Performance, feed, and water intake

No differences were observed on the final body weight and average daily gain among treatments (Table 5). Steers backgrounded on MP and finished on a forage diet had the greatest dry matter intake among treatments ($P < 0.01$), whereas water intake was higher for forage-fed animals ($P < 0.01$; Table 5).

3.2. Aquaporins

The mRNA expression of *AQP2*, *AQP3*, *AQP4*, and *AQP7* in the rumen and kidney are presented in Figs. 1 and 2. Previous plane of nutrition had no effect on *AQP* expressed in the kidney at the finishing phase (Fig. 1A). However, in the rumen (Fig. 1B), animals from the MP + forage-fed diet had a higher expression of *AQP7* than HP + forage-fed ($P \leq 0.05$), HP + grain-fed ($P \leq 0.01$) and MP + grain-fed ($P \leq 0.01$). Next, since most of the differences were observed only on animals from MP, we further analyzed differences on relative mRNA abundance from MP animals only. Interestingly, in the kidneys, the expression of *AQP3* and *AQP7* were both higher for forage-fed animals when compared to the grain-fed animals ($P = 0.029$, $P = 0.026$, respectively; Fig. 2A), whereas for the rumen, the only differences found were still for *AQP7*, which was still higher for the forage-fed animals ($P \leq 0.01$; Fig. 2B).

3.3. Na^+/K^+ ATPase subunits

As shown in Fig. 3A, relative abundance of Na^+/K^+ ATPase subunits were only different for *ATP1B1* in the kidney for animals backgrounded in a MP. For MP + forage-fed animals, *ATP1B1* had a higher expression ($P = 0.029$) when compared to the MP + grain-fed animals. The same behavior can also be observed in Fig. 4A.

Table 5
Effect of previous plane of nutrition on performance, feed and water intake of crossbred Angus beef steers backgrounded on a moderate or high plane of nutrition and subsequently finished on grain or forage-fed systems.

Item	Treatment				SEM	*P*-value[1]
	Grain-fed group		Forage-fed group			
	Moderate plane	High plane	Moderate plane	High plane		
Final body weight, kg	553.51	586.39	565.84	578.38	15.270	0.306
Average daily gain, kg	1.86	1.71	1.95	1.71	0.119	0.306
Dry matter intake, kg	10.87^{a}	11.11^{ab}	13.07^{c}	12.76^{bc}	0.469	0.005
Water intake, kg	41.78^{a}	37.72^{a}	71.48^{b}	64.15^{b}	3.052	< 0.001

SEM = standard error of the mean.
[a,b,c] Means followed by the same letter are not significantly different at $P \leq 0.05$.
[1] *P*-value < 0.05 is statistically significant; 0.05 ≤ *P*-value 0.10 indicates a trend.

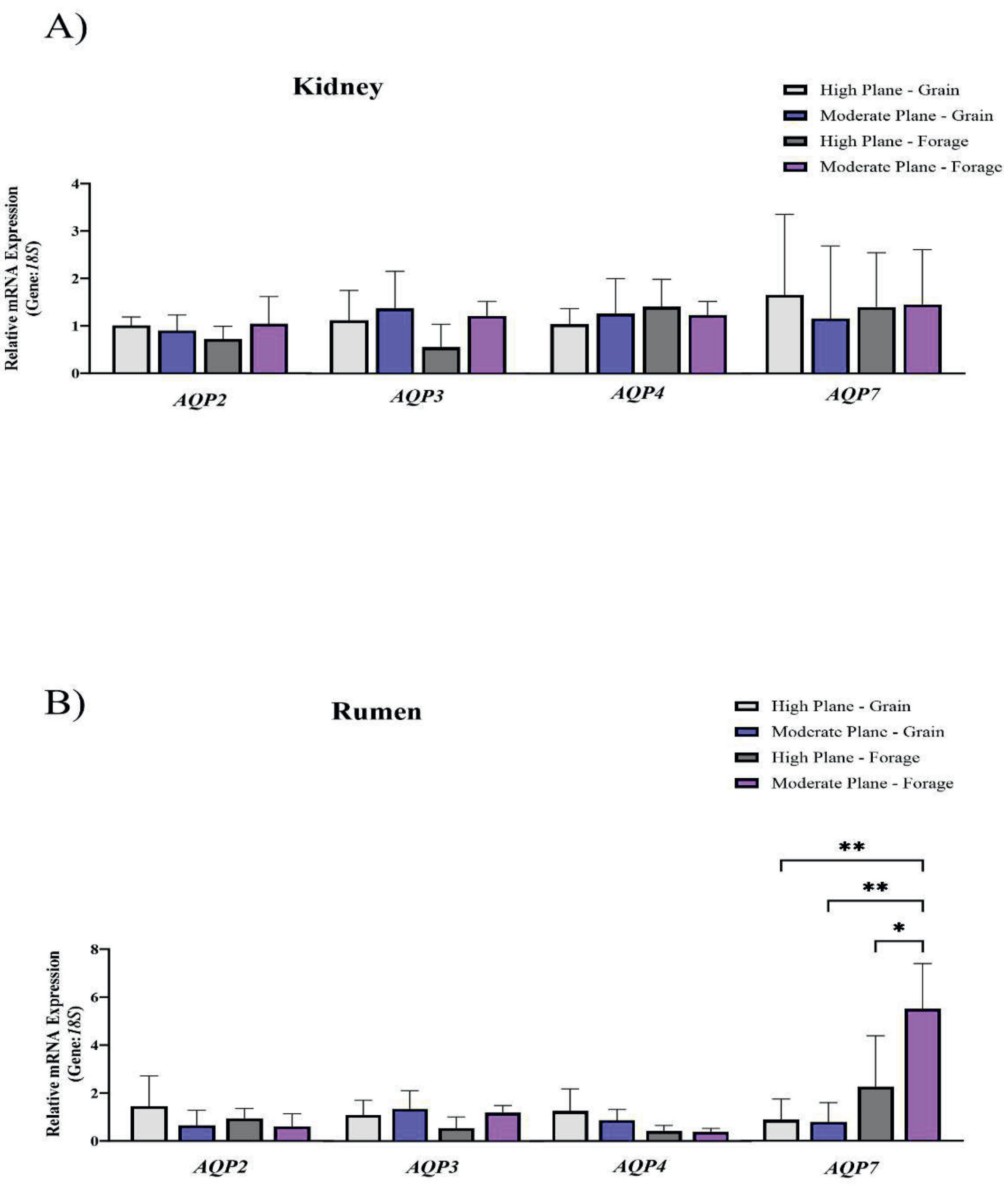

Fig. 1. Gene expression of aquaporins (*AQP*)-2, -3, -4 and -7 in the kidney and rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in either a moderate or high plane of nutrition. During the finishing phase animals were either grain-fed ($n = 12$) or forage-finished ($n = 12$). (A) Aquaporins expression in the kidney; (B) aquaporins expression in the rumen. Asterisks indicate statistical significance (*: $P \leq 0.05$; **: $P \leq 0.01$) between groups indicated by brackets. Error bars show the standard error of the mean.

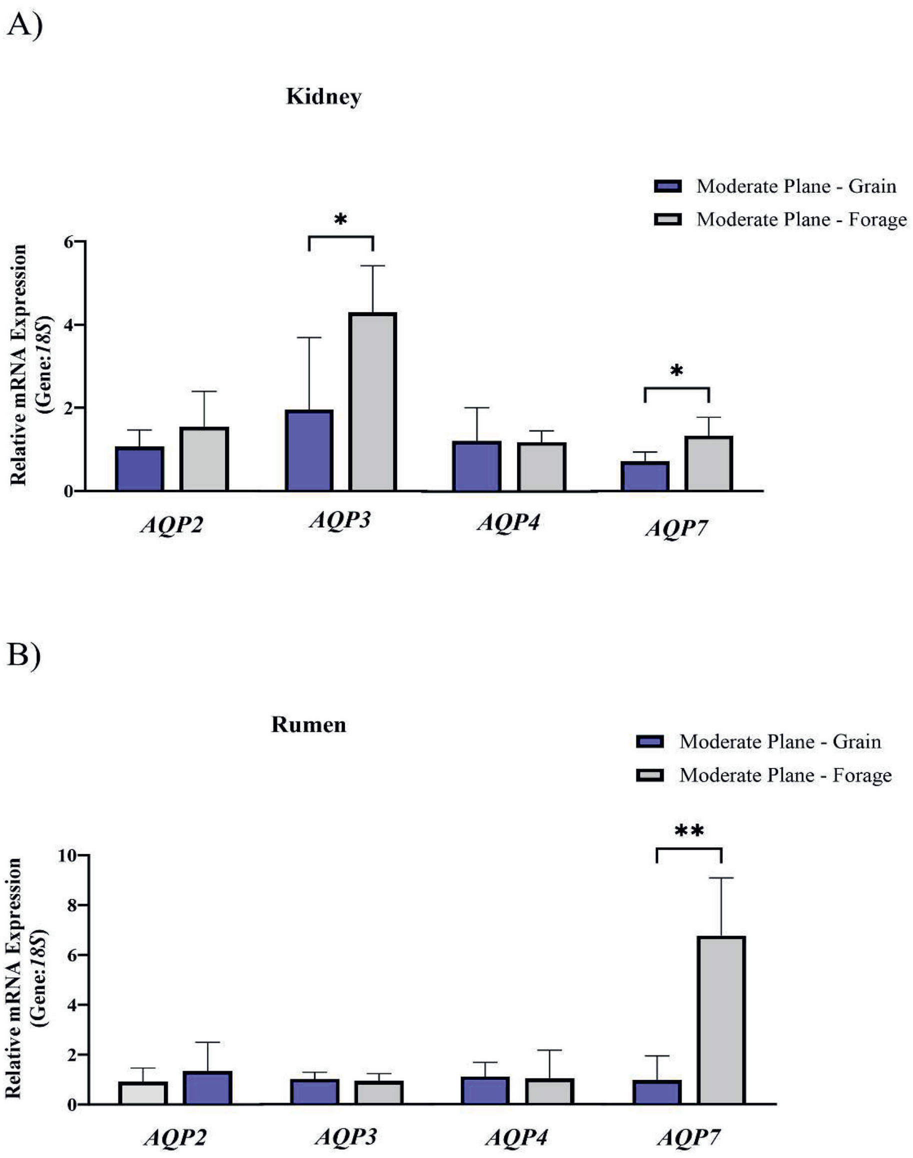

Fig. 2. Gene expression of aquaporins (*AQP*)-2, -3, -4 and -7 in the kidney and rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in a moderate plane of nutrition prior to the finishing phase animals when animals were either grain-fed ($n = 6$) or forage-fed ($n = 6$). (A) Aquaporins expression in the kidney; (B) aquaporins expression in the rumen. Error bars show the standard error of the mean. Asterisks indicate statistical significance (*: $P \leq 0.05$; **: $P \leq 0.01$) between groups indicated by brackets.

However, no differences were observed in the rumen for *ATP1A1* and *ATP1B1* ($P > 0.05$; Figs. 3B and 4B).

3.4. Genes related to osmotic balance

No differences were observed for kidney or rumen ($P > 0.05$; Fig. 5). However, the expression of *SGK1* in the kidney was higher (Fig. 6) for MP + forage-fed animals than MP + grain-fed animals ($P = 0.041$; Fig. 6A). No differences were observed in the gene expression of osmotic balance related genes in the rumen of the animals (Figs. 5B and 6B).

3.5. Urea transporters

Differences in *UT-B* were observed only for animals backgrounded in a MP (Fig. 7B). Animals that were MP + grain-fed tended to have a higher gene expression of *UT-B* ($P = 0.075$) in the rumen compared to MP + forage-fed animals. No statistical differences were observed among treatments for *UT-A1* (Fig. 8A and B).

4. Discussion

With the increasing concern on water scarcity worldwide, understanding the factors that regulating the water in cattle is crucial. Although the effect of previous plane of nutrition on gene expression has been widely investigated on lactation, reproduction and growth traits (Loor et al., 2006; Gutierrez et al., 2014; Chen et al., 2015), this is the first paper to investigate how the previous plane of nutrition of finishing cattle could further affect water and nitrogen metabolism at the gene level. Modifications in gene expression are the first step towards answering what happens at the animal level and could allow further understanding on how we could mitigate water use on beef cattle systems.

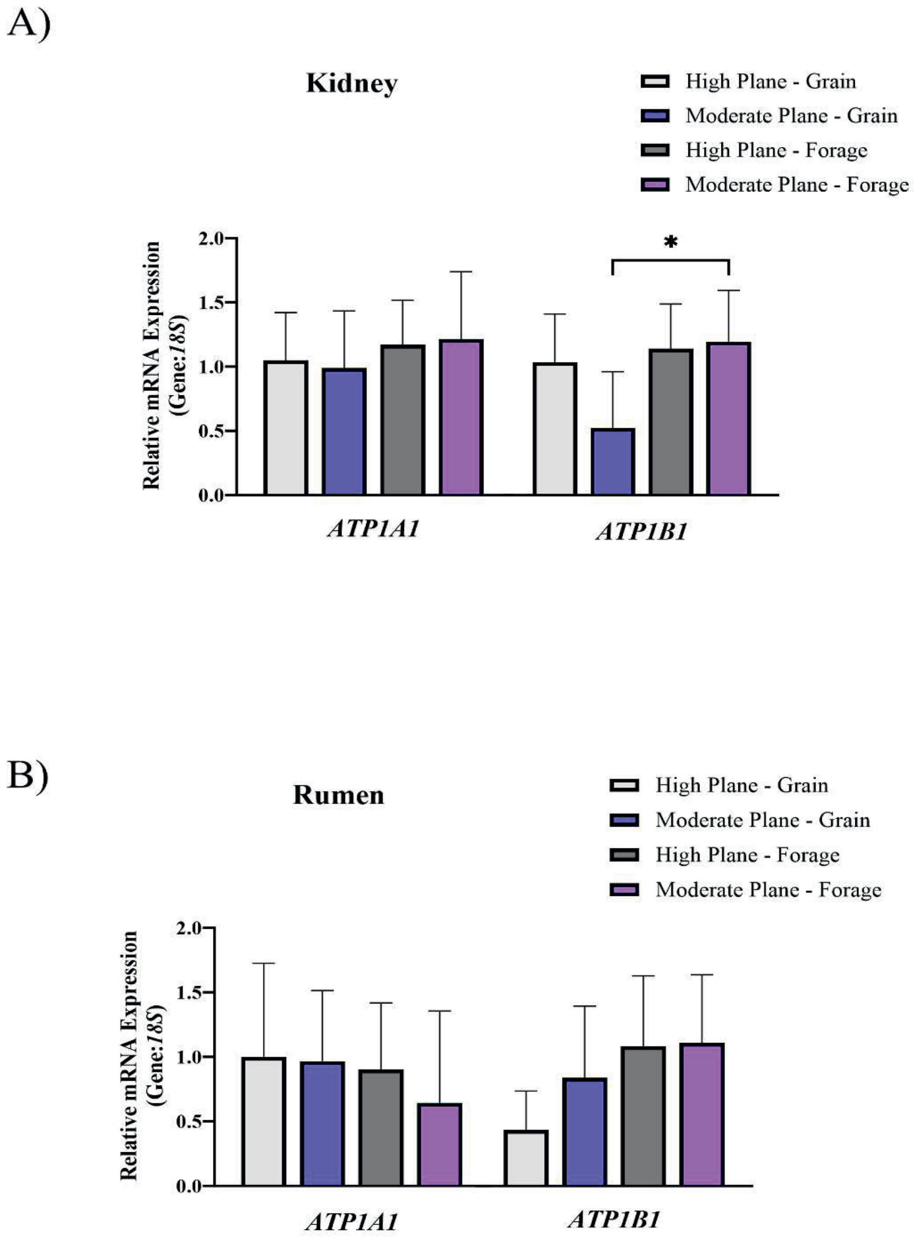

Fig. 3. Gene expression of Na^+/K^+ ATPase subunits A1 (*ATP1A1*) and B1 (*APT1B1*) in the kidney and rumen of crossbred Angus beef steers previously backgrounded in either a moderate or high plane of nutrition. During the finishing phase animals were either grain-fed ($n = 12$) or forage-fed ($n = 12$). (A) *ATP1A1* and *ATP1B1* expression in the kidney; (B) *ATP1A1* and *ATP1B1* expression in the rumen. Error bars show the standard error of the mean. Asterisks (*) indicate statistical significance ($P \leq 0.05$) between groups indicated by brackets.

Among the factors that can affect water intake, dry matter intake is one of the most cited, where a positive relationship is usually observed (Meyer et al., 2004; Kume et al., 2010). The observed decrease in feed and water intake for grain-fed animals could be in response to the higher energy concentration in the diet which might have constrained intake due to chemostatic mechanisms (Dulphy and Demarquilly, 1994; Montgomery and Baumgardt, 1965).

One of the main proteins required in the process of regulating water balance and proper acid-base balance are the AQPs (Michalek, 2016). The model of finishing diets that we utilized in this study were inherently different in protein levels (10.8% vs. 21.3% for grain-fed and forage-finished, respectively). Previous studies have shown increased water intake when animals were fed diets with increased protein levels (Ritzman and Benedict, 1924; Holter and Urban Jr, 1992); however, no differences were found in mRNA fold change expression of *AQP* in the kidney of those animals. These results suggest that the differences in dietary protein levels at the finishing diet, independent of the previous nutrition plane, did not affect the capacity of the kidney in concentrating the urine and excreting excess of solutes without losing massive amounts of water.

On the other hand, when we analyzed animals that were backgrounded as most animals are (MP), we observed a higher mRNA expression of *AQP3* and *AQP7* in the kidney at the finishing phase when animals were forage-fed, namely representing an overload of CP intake that would exceed recommended requirements (NASEM, 2016). Once ingested, protein is degraded by

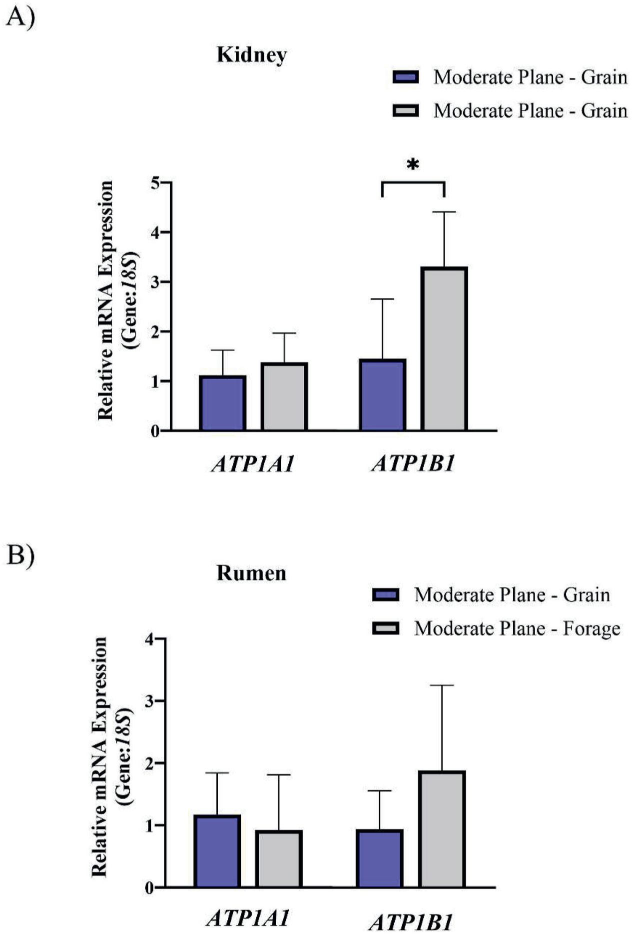

Fig. 4. Gene expression of Na$^+$/K$^+$ ATPase subunits A1 (*ATP1A1*) and B1 (*APT1B1*) in the kidney and rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in a moderate plane of nutrition prior to the finishing phase animals when animals were either grain-fed ($n = 6$) or forage-fed ($n = 6$). (A) *ATP1A1* and *ATP1B1* expression in the kidney; (B) *ATP1A1* and *ATP1B1* expression in the rumen. Error bars show the standard error of the mean. Asterisks (*) indicate statistical significance ($P \leq 0.05$) between groups indicated by brackets.

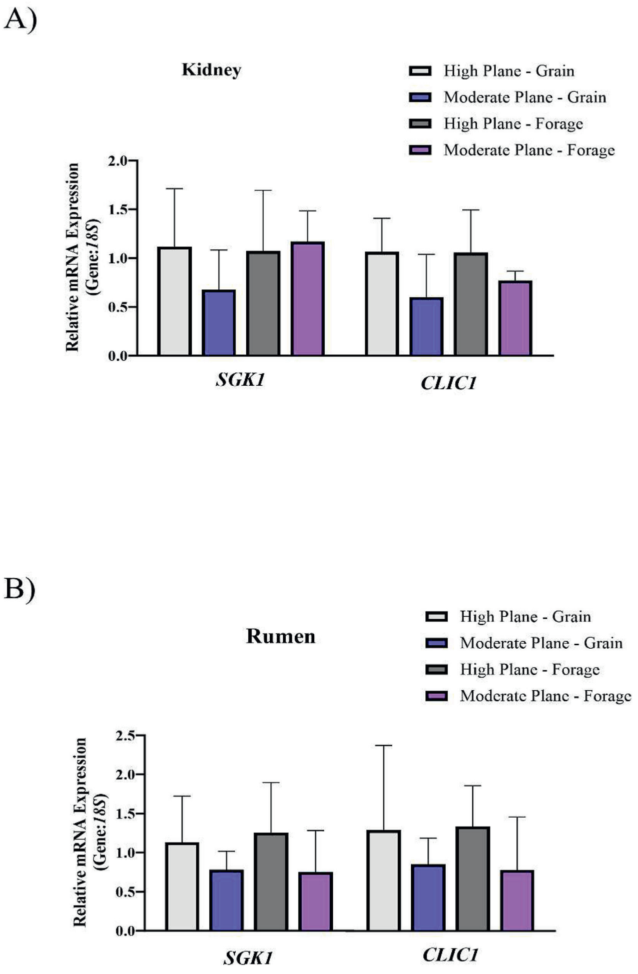

Fig. 5. Gene expression of serum/glucocorticoid regulated kinase 1 (*SGK1*) and chloride intracellular channel protein 1 (*CLIC1*) in the kidney and rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in either a moderate or high plane of nutrition. During the finishing phase animals were either grain-fed ($n = 12$) or forage-fed ($n = 12$). (A) *SGK1* and *CLIC1* expression in the kidney; (B) *SGK1* and *CLIC1* expression in the rumen. Error bars show the standard error of the mean.

ruminal bacteria into ammonia (Abdoun et al., 2007). This ammonia will be absorbed through the rumen wall and go to the liver, where it will be metabolized into urea and either recycled back to the rumen or transported to the kidneys (Lapierre and Lobley, 2001). When levels of dietary CP are below animal's requirements, it creates a need for the reabsorption of urea arriving in the kidneys, which would then decrease its urinary excretion and increase its recycling back to the rumen (Marini et al., 2004). Conversely, as dietary protein levels increase, urinary excretion of urea also increases (Huhtanen et al., 2008). In the kidneys, reabsorption of urea can also be done through aquaglyceroporins (AQGP), which are *AQP*s that are not only permeable to water, but also to glycerol, ammonia, as well as urea (Rojek et al., 2008). Aquaporin-3 and -7 are both considered AQGP. Thus, the observed increase in their expression in the kidneys of animals backgrounded on MP and subsequently finished on forage-based diet, indicates that after a period of reduced CP supply, a subsequent CP overload could increase the reabsorption of urea, even if those animals were no longer suffering from a dietary CP limitation. Reabsorbed urea would be expected to go back to the rumen. In the rumen, bacterial urease hydrolyzes urea into ammonia, which can be excreted in the feces or, once more, absorbed by the rumen wall and subsequently metabolized into urea in the liver (Lapierre and Lobley, 2001). However, this process of producing urea repeatedly can be a major energy consuming event (McBride and Kelly, 1990), each mole of urea produced in the liver has an energetic cost of 4 moles of ATP. Furthermore, Huntington and Archibeque (2000) estimated that 2.5% to 5% of whole-body oxygen consumption was attributable to ureagenesis in the liver. Similarly, Jennings et al. (2018) noted that animals fed high protein diets increased their energy requirements by 3% to 4.5%. Therefore, energy available for tissue deposition would be expected to decrease for MP + forage-fed animals when compared to MP + grain-fed animals.

From a water balance perspective, *AQP3*-deficient mice were shown to be severely polyuric, demonstrating that basolateral membrane water transport can also be a rate-limiting factor for water reabsorption (Ma et al., 2000). However, *AQP7*-null mice appeared to lack clear defects in urinary concentration abilities or in the regulation of water balance abilities (Sohara et al., 2005), indicating that *AQP7* might have a bigger role on absorption of solutes (such as glycerol, ammonia, and urea) rather than just water. Therefore, when compared to MP + grain-fed animals, Ma et al. (2000) and Sohara et al. (2005) suggest that the overload of protein observed at finishing from MP + forage-fed animals leads to

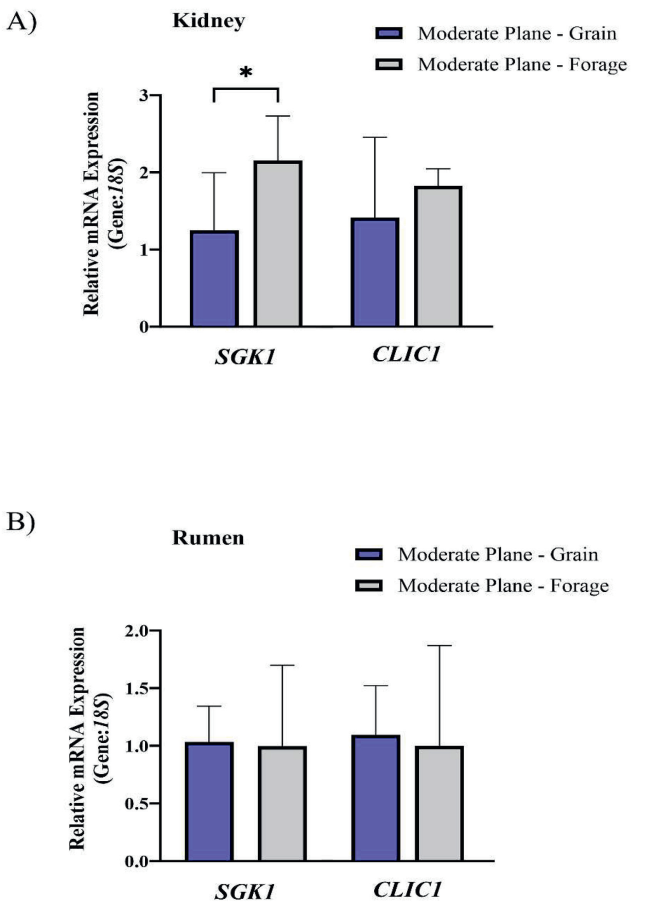

Fig. 6. Gene expression of serum/glucocorticoid regulated kinase 1 (*SGK1*) and chloride intracellular channel protein 1 (*CLIC1*) in the kidney and rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in a moderate plane of nutrition prior to the finishing phase animals when animals were either grain-fed ($n = 6$) or forage-fed ($n = 6$). (A) *SGK1* and *CLIC1* expression in the kidney; (B) *SGK1* and *CLIC1* expression in the rumen. Error bars show the standard error of the mean. Asterisks (*) indicate statistical significance ($P \leq 0.05$) between groups indicated by brackets.

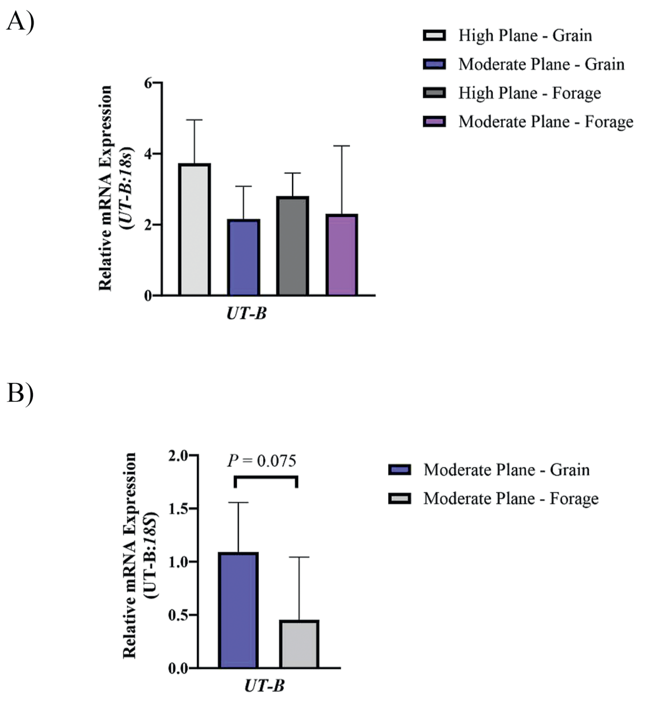

Fig. 7. Gene expression of urea transporter B (*UT-B*) in the rumen at the end of finishing phase of crossbred Angus beef steers previously backgrounded in either a moderate or high plane of nutrition. During the finishing phase animals were either grain-fed ($n = 12$) or forage-fed ($n = 12$). (A) *UT-B* gene expression of animals from a moderate or high plane of nutrition finished on a grain versus forage diet; (B) *UT-B* gene expression of animals from a moderate plane of nutrition finished on a grain versus forage finishing diet. Error bars show the standard error of the mean.

an increase in filtration of water by the kidneys, mainly due to *AQP3*.

Røjen et al. (2011) investigated the mRNA expression of *AQP* in the rumen and observed that mRNA abundances of *AQP3*, *AQP7*, and *AQP10* were significantly upregulated when lactating Holstein cows were fed 17% CP compared to cows fed 12.9% CP. Our results indicate that animals on MP + forage-fed diets had the highest expression of *AQP7* in the rumen when compared to HP + forage-fed, HP + grain-fed and MP + grain-fed animals, but no differences were observed for *AQP3*. Although there is still limited information regarding the function and location of *AQP7* within the rumen epithelium, its location in the brush border cells of the intestine make up for a higher expression at the apical side of brush border membranes (Tritto et al., 2007). Ultimately, these data indicate that *AQP7* could act in transporting excessive ammonia from the rumen to the bloodstream.

Despite large variations of feed and water intake, body fluid homeostasis can be maintained mostly due to reabsorption and secretion processes that happens on the kidney tubules (Summa et al., 2001; Feraille and Dizin, 2016). In the kidney, reabsorbed solutes first cross the apical membrane and are then extruded from intracellular medium to the interstitium, whereas secreted solutes are taken from the interstitium across the basolateral membrane and are then extruded into the lumen after crossing the apical membrane (Feraille and Dizin, 2016). Both processes preserve the balance between the intake and loss of water and ions and can be energized by the Na^+ gradient generated by the Na^+/K^+-ATPase, a Na^+/K^+ pump required for the establishment of electrochemical gradients driving cellular transport and substrate flow across epithelia (Zouzoulas et al., 2005; Feraille and Dizin, 2016). The Na^+/K^+-ATPase comprises two subunits, a large catalytic α subunit (coded by the gene *ATP1A1*) and a smaller highly glycosylated β subunit (coded by the gene *ATP1B1*) necessary for the proper folding, insertion, and maturation of the α subunit in the plasma membrane (Zouzoulas and Blostein, 2006). *ATP1A1* and *ATP1B1*, are two distinct, differentially regulated genes, where expression of α1 subunit is usually present in excess when compared to β1 subunits, which might limit the formation of the αβ heterodimer that will compose the Na^+/K^+-ATPase (Taub, 2018). Interestingly, in this current study, only *ATP1B1* was higher for the MP + forage-fed animals. This might be related to an overload of urea in the blood caused by the higher content of CP in the finishing diet, which might have increased only *ATP1B1* since it is the limiting subunit for the formation of Na^+/K^+-ATPase. In ruminants, excessive dietary protein is degraded into ammonia in the rumen and metabolized to urea in the liver (Lu et al., 2014). Excess of blood urea needs to be excreted through urine to avoid toxicity; however, when protein intake is higher than the requirements, in attempting to avoid massive water loss, a huge amount of plasma needs to be filtered. Such filtration is driven by the sodium chloride gradient in the kidneys that would allow for water to be conserved (Knepper and Roch-Ramel, 1987; Bankir et al., 1996). In rats, Bouby and Bankir (1988) observed that diets with higher concentration of protein

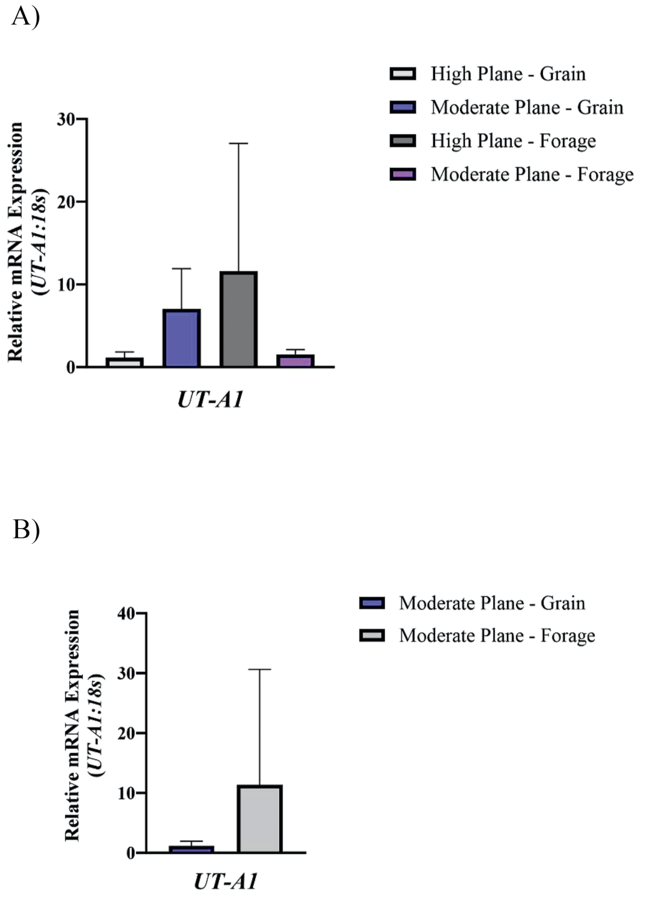

Fig. 8. Gene expression of urea transporter A1 (*UT-A1*) in the rumen at the end of the finishing phase of crossbred Angus beef steers previously backgrounded in either a moderate or high plane of nutrition. During the finishing phase animals were either grain-fed ($n = 12$) or forage-fed ($n = 12$). (A) *UT-A1* gene expression of animals from a moderate or high plane of nutrition finished on a grain versus forage diet; (B) *UT-A1* gene expression of animals from a moderate plane of nutrition finished on a grain versus forage finishing diet. Error bars show the standard error of the mean.

increased Na^+/K^+-ATPase activity enabling an enhanced NaCl transport pipeline. We did not observe differences among the animals that were backgrounded in a HP of nutrition, which might suggest that adequate levels of protein during the background phase will decrease the effect of an overload of protein in the subsequent phases.

Although the rumen epithelium has a high expression of *ATP1A1* (Graham et al., 2005; Albrecht et al., 2008), no differences were observed in the rumen level for either *ATP1A1* or *ATP1B1.* According to Lopez et al. (1994), water exchange between the rumen contents and the plasma can occur in both directions depending on the osmolality pressure, where net movement of this water will define the balance in the rumen pool. However, when studying the flux of water in the rumen, the authors noticed that the rumen seemed not very permeable to water since the net extent of the transepithelial movement of water into or out of the rumen observed by them was not very high. Lopez et al. (1994) explained that to keep the animal hydrated, most of the water seems to be absorbed and recycled post-ruminally. Thus, since not much water is absorbed in the rumen, an increase on Na^+/K^+-ATPase activity might not be required in order to create a gradient for water absorption in the rumen.

Besides the Na^+/K^+-ATPase, the epithelial sodium channel (ENaC) is another important transporter of sodium. Regulation of sodium channels can be done by *SGK1.* Upregulation of *SGK1* is usually stimulated by aldosterone when blood sodium levels are low, *SGK1* will then stimulate sodium transport by the ENaC and Na^+/K^+-ATPase and increase transport of sodium to the cell (decreases sodium urinary excretion), leading to increased water uptake (concentration gradient), and thereby inducing a regulatory cell volume increase (Hills et al., 2008). In the current study, downregulation of *SGK1* expression was observed for MP + grain-fed animals when compared to MP + forage-fed animals. This result corroborates with previous data, indicating that water is shifted to urinary excretion and the kidney increases the transport of sodium as an alternative to save water from urinary excretion.

Lastly, since the levels of dietary CP appear to play a role in the mRNA expression of the aforementioned genes, we investigated the expression of urea transporters (*UT-B*) in the rumen. Once ammonia is converted into urea in the liver, it can be excreted in the urine or recycled back to the rumen (Lapierre and Lobley, 2001). Blood urea can then cross the rumen mucosa by simple diffusion, AQGP or via facilitative UT-B. Nonetheless, *UT-B* mediates the movement of urea down a concentration gradient (Stewart et al., 2005; Abdoun et al., 2007; Walpole et al., 2015). In this current study, a trend was observed for animals backgrounded in a lower plain of nutrition, where MP + grain-fed diet tended to have a higher expression of *UT-B* as compared to their forage-fed counterparts. This result indicates that due to the lower levels of CP in the diet during the backgrounding phase and subsequent balanced levels of CP in the finishing phase— which also corresponds to the conventional beef production in the U.S.— animals had to recycle more urea back to the rumen to optimize microbial growth and maximize nutrient utilization. Furthermore, increased recycling of urea back to the rumen may also play a role in buffering the rumen epithelial microclimate by removing protons, which will further decrease the acidity caused by the increased levels of bacterially derived short chain fatty acids that are commonly observed on grain-based diets (Lu et al., 2014). Although previous studies have reported no effect of dietary protein levels on the expression of *UT-B* in the rumen (Ludden et al., 2009; Røjen et al., 2011; Saccà et al., 2018), the differences between CP levels in these studies ranged from 1.5% to 5%, whereas in ours the levels of CP were approximately doubled between groups during the finishing period.

Different from the rumen, in the kidney UT-As are the main urea transporter present. As urea concentration increases in the nephrons, an increased transport of water to form urine is required and therefore increased water loss on the animal end (Sands and Layton, 2009). Urea transporters A1 are specially important whenever urea needs to be recycled since they allow for rapid reabsorption of urea as urine flows along the collecting duct (Stewart, 2011). Although differences on *UT-A1* expressions were expected among treatments, we were unable to detect changes in expression probably due to the high variation on the results obtained. Most UT-A isoforms are acutely regulated via phosphorylation and trafficking of the glycosylated transporters to the plasma membranes, which is induced by the antidiuretic hormone vasopressin (Stewart, 2011). Therefore, the results herein highlight the importance of protein abundance rather than only mRNA expression, since it would be a more accurate measure of changes on urea transporters along the nephron.

5. Conclusion

In conclusion, this study is the first to show that changes in the diet from earlier ages can influence the fate of water and urea metabolism of animals as strategies that may allow cattle nutritionists to fine tune their recommendations that would mitigate the use of water by the cattle industry. Those effects can be further evidenced between different finishing systems, and when and how the water mitigation conundrum should be tackled. In the kidney, mRNA expression of *AQP3*, *AQP7*, *ATP1B1* and *SGK1* were higher for animals backgrounded in a lower quality plane of nutrition and subsequently finished in a forage-fed diet, whereas in the rumen only *AQP7* was different between groups. The expression of *UT-B* tended to be higher for animals backgrounded in the lower plain of nutrition and finished in a grain-fed diet. In the U.S. most animals are backgrounded to some extent in a low-quality forage and then finished either on a grass/forage-based or grain-based diet, and both would imprint different water footprints. Our results suggest that the decreased supply of protein earlier in life might cause some adaptative mechanism to cope with the lower nitrogen supply. However, further differences will also depend on the finishing diets of those animals. If animals are finished on a diet that matched their requirement, such as the grain-fed diet, they will tend to be better recyclers of nitrogen; whereas if they have an overload of protein in the next phase, due to a more efficient reabsorption of nitrogen by the kidneys, those animals might have a higher energy and water cost related to urea recycling and excretion. Therefore, the molecular mechanisms regulating gene expression of urea and water metabolism on those animals are dependent on not only the present diet, but also on their previous diets. Future investigations in protein expression and translocation are needed to improve our overall understanding for beef cattle diets in water, urea and ammonia regulation.

Author contributions

Aghata E. Moreira da Silva contributed to facilities preparation, conducting the experiments, data collection and analysis, wrote the original manuscript, review and editing. **Arturo Macias Franco** contributed to facilities preparation, conducting the field experiment, data interpretation, review and editing of the manuscript. **Bradley S. Ferguson** contributed conceiving the laboratory study, data interpretation and review and editing of the manuscript. **Mozart A. Fonseca** contributed to conceptualization, methodology, resources, funding, project management, data interpretation and review and editing of the manuscript. All authors read and approved the final version of the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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