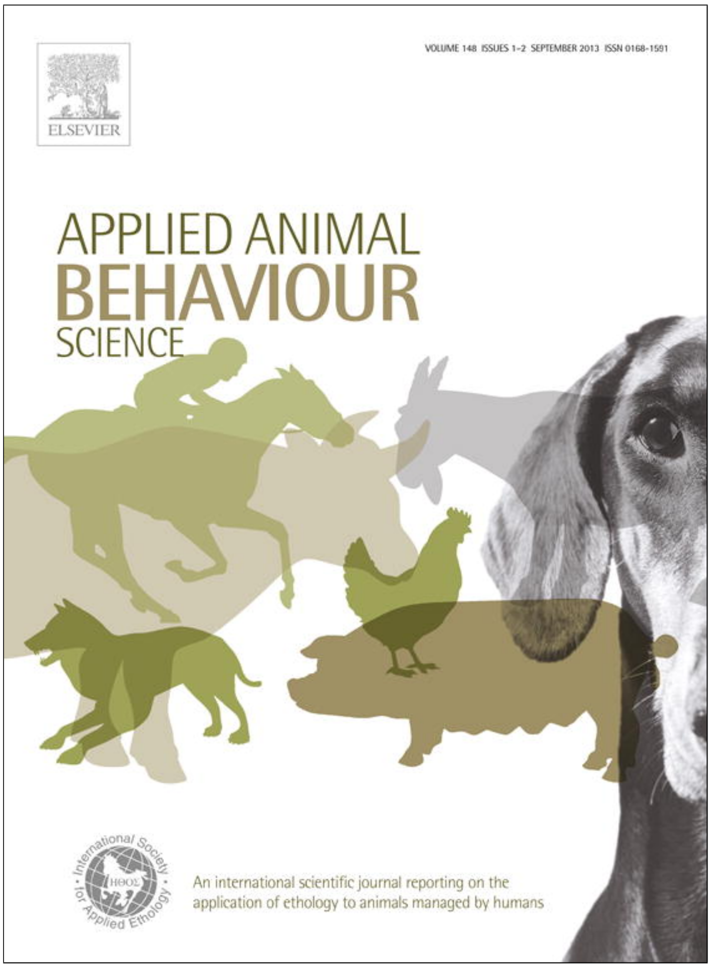
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The importance of diet choice on stress-related responses by lambs

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ABSTRACT

Farm animals are commonly restricted to a reduced array of foods, like total mixed rations or pastures with low species diversity. Under these conditions, animals are less likely to satisfy their specific and changing nutrient requirements. In addition, foods and flavors eaten too frequently or in excess induce sensory-specific satiety and can cause aversions. Thus, sensory and postingestive monotony may reduce animal welfare. We hypothesized that exposure to monotonous diets, even if they are considered to be nutritionally balanced, is stressful for sheep. Twenty-four 2-month-old male Corriedale lambs were randomly assigned to two experimental groups. One group (diversity treatment, DIV) received a free choice of four-way combinations of two foods with low and two foods with high protein/energy ratios from an array of seven foods (three foods high in protein/energy ratio: soybean meal, sunflower meal, and alfalfa pellets, and four foods low in protein/energy ratio: barley grain, oat grain, milo grain, and corn grain). The other group (monotony treatment, MON) was fed a balanced ration containing all foods offered to lambs in DIV. Foods were offered in four individual buckets and exposure lasted 55 days. During exposure, feeding behavior was assessed, and blood samples were taken for a complete blood cell count and to determine serum cortisol concentration. Lambs in MON showed greater cortisol levels (31.44 vs. 19.90 ± 3.30 nmol/L [means ± SEM]; $P = 0.025$) and a greater neutrophil to lymphocyte ratio (0.37 vs. 0.26 ± 0.05; $P = 0.044$) than lambs in DIV. Lambs in DIV spent a lower proportion of time eating (0.38 vs. 0.49 ± 0.02; $P < 0.001$) and showed a greater intake rate (17.73 vs. 14.09 ± 1.26 g/min, $P < 0.044$) than lambs in MON. They also showed a greater proportion of time lying (0.44 vs. 0.36 ± 0.03; $P = 0.049$) and greater activity (0.047 vs. 0.035 ± 0.003; $P = 0.003$) than lambs in MON. However, final body weight and the average daily weight gain were not affected by treatment ($P > 0.05$). Our results showed that restricting lambs' dietary breadth produced changes in blood and behavioral parameters previously shown to be indicative of stress in sheep. The importance of incorporating food choice as an alternative practice to overcome stress associated to the traditional livestock feeding management is discussed.

1. Introduction

Animal welfare has gained relevance as an essential component of livestock production systems (Rushen et al., 2011). Management practices involving procedures meant to minimize stressful events in different phases of animal

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production are being increasingly required (Fitzpatrick et al., 2006). Among the different stressors affecting ruminants, fear (e.g. Boissy et al., 2005), heat (e.g. Silanikove, 2000), and transportation (e.g. Broom, 2003) have received the most attention. Paradoxically, less importance has been given to the feeding practices that impact nutrition and welfare in ruminant production systems (but see Catanese et al., 2012; González et al., 2009; Manteca et al., 2008).

Nutrition is considered to influence animal welfare because animals must be provided with all the nutrients needed for proper growth and development (Kyriazakis and Savory, 1997). This is usually attempted by offering animals a high quality forage or a complete mixed ration formulated to satisfy the average nutrient requirements of a given group of animals. Nevertheless, individuals within a herd vary substantially in their intake and preferences for foods, and tolerance for excesses and deficits of nutrient in their diets (Manteca et al., 2008). Differences in food intake depend on an animal's past experiences with foods as well as on variations in how animals are built morphologically and how they function physiologically (Provenza et al., 2003). As suggested by Forbes (2007), nutrients ingested in excess or deficit could produce a nutritional discomfort which motivates animals to search for other foods that alleviate the imbalance. But if no alternatives are available, it is unlikely animals will achieve a state of nutritional homeostasis and well-being (Broom, 1991).

Domestic livestock are generalist herbivores that evolved to eat a wide array of vegetal species and typically select a diverse diet even when their nutritional requirements can be met by ingesting a single food (Provenza, 1996). Animals develop transient aversions to foods or flavors that are eaten too often or in excessive amounts, while they increase preference for alternative foods or flavors (Provenza, 1996). This phenomenon, known as sensory-specific satiety (Rolls, 1986), encourages animals to eat a diverse array of foods. Seeking for alternative foods, sampling those available, and experiencing novel flavors and textures, are probably "behavioral needs" (*sensu* Jensen and Toates, 1993) most likely to be satisfied with a wide array of options.

Following this theoretical background, we hypothesized that the exposure to monotonous diets, even if they are considered to be nutritionally balanced, is stressful for lambs. The objective of this study was to determine behavioral responses and blood parameters in lambs exposed to two different feeding regimes: (1) a free-choice between several nutritionally complementary foods or (2) exposure to a single nutritionally balanced diet.

2. Materials and methods

The study was conducted at the "Centro de Recursos Naturales Renovables de la Zona Semiárida" (CERZOS) located in Bahía Blanca (38° 44′ S; 62° 16′ W), Argentina, from September 2011 to February 2012. All experimental protocols fulfilled animal welfare regulations of the Universidad Nacional del Sur (Bahía Blanca, Argentina) and adhere to the ASAB/ABS (2006) guidelines for the use of animals in research. Throughout the study lambs had free access to water and trace mineral salt blocks.

2.1. Animals, housing, and treatments

We used 24 2-month-old male Corriedale lambs with an average initial body weight (BW) of 19.2 ± 2.6 kg (mean ± SD). Animals were weaned, placed in a protected enclosure (20 m × 20 m), and fed alfalfa pellets *ad libitum* and 300 g/d of barley grain per animal for 15 d to satisfy their average nutrient requirements for maintenance and growth (National Research Council, 1985). Then, lambs were weighed and penned outdoors under a protective roof in individual adjacent pens measuring 2.5 m × 2.5 m.

Lambs were randomly assigned to two treatments (12 lambs/treatment) with restrictions of randomization such that all treatments were balanced for body weight. One group of lambs (hereafter "DIV") was fed simultaneously an array of four foods taken from a group of seven; three foods high in protein/energy ratio: soybean meal, sunflower meal, and alfalfa pellets; and four foods low in protein/energy ratio: barley grain, oat grain, milo grain, and corn grain (Table 1). Lambs from this treatment received 11 four-way choice combinations of two foods high in protein/energy ratio and two foods low in protein/energy ratio selected at random from all possible combinations (Table 2). Different four-way combinations of foods were

Table 1
Nutritional composition of foods offered during the 55-d exposure period (DM basis).

Foods	Nutrient composition				
	ME[a] (MJ/kg)	CP (g/100 g)	DigP[b] (g/100 g)	DigP/ME	NDF (g/100 g)
Low protein/energy ratio					
Barley grain	13.01	13.00	10.69	0.82	21.21
Oat grain	11.63	11.32	8.85	0.76	35.21
Milo grain	13.01	8.55	6.32	0.49	20.86
Corn grain	13.18	9.81	6.31	0.48	12.11
High protein/energy ratio					
Soybean meal	12.84	46.75	40.09	3.12	15.62
Sunflower meal	6.82	32.12	26.04	3.82	30.47
Alfalfa pellets	9.08	17.75	11.93	1.31	44.67
Mixed diet offered to MON	11.49	17.19	13.19	1.17	25.58

ME, metabolizable energy; CP, crude protein; DigP, digestible protein; NDF, neutral detergent fiber; MON, treatment fed only with this mixed diet.
[a] Estimated values from National Research Council (1985).
[b] Crude protein digestibility coefficients were estimated from National Research Council (1985).

Table 2
Different combinations of four foods offered free-choice and at random to lambs exposed to a diverse food environment (DIV).

Low protein/energy ratio foods		High protein/energy ratio foods	
Food combinations[a]			
Corn grain	Oat grain	Sunflower meal	Alfalfa pellets
Corn grain	Barley grain	Soybean meal	Alfalfa pellets
Milo grain	Barley grain	Sunflower meal	Alfalfa pellets
Corn grain	Barley grain	Soybean meal	Sunflower meal
Oat grain	Barley grain	Sunflower meal	Alfalfa pellets
Corn grain	Oat grain	Soybean meal	Alfalfa pellets
Milo grain	Oat grain	Soybean meal	Alfalfa pellets
Milo grain	Barley grain	Soybean meal	Sunflower meal
Corn grain	Milo grain	Sunflower meal	Alfalfa pellets
Milo grain	Oat grain	Soybean meal	Sunflower meal
Corn grain	Barley grain	Sunflower meal	Alfalfa pellets

[a] Foods within a row represent a single food choice combination. Each food choice combination was provided at random for 5 days to each lamb; following this, a different food choice combination was offered until a total of 11 combinations were exhausted along a 55-day exposure period.

offered to avoid lambs to focus in the most palatable foods and to encourage them to mix and eat diverse (Provenza et al., 2003). Additionally, pairs of nutritionally complementary foods were used in each combination to ensure that lambs had choices available from which they could select a balanced diet. Each choice combination was offered for a period of 5 consecutive days, and all periods occurred in a concatenated sequence until all the selected combinations were offered. Exposure to each choice combination was randomly assigned among animals in such a way that the sequence of food combinations in the experimental design was counterbalanced. The other group of lambs (hereafter "MON") received a monotonous balanced diet throughout the whole period of exposure, i.e. 55 days. The balanced diet (Table 1) was formulated according to specific nutrient requirements of sheep (National Research Council, 1985) with a mix of the seven foods used to feed the lambs in DIV: 20% milo grain, 20% corn grain, 20% alfalfa pellets, 10% oat grain, 10% barley grain, 10% soybean meal, and 10% sunflower meal. The familiar foods, alfalfa pellets and barley, were fed to both MON and DIV lambs; although, for MON lambs these familiar foods were mixed with the rest of the foods. Sheep can recognize familiar flavors in novel foods (Villalba and Provenza, 2000).

All foods fed to lambs were ground to pass through a 4-mm screen. For DIV lambs, every day the four foods were randomly distributed in four individual plastic buckets, whereas for MON lambs the four buckets had the same food. All lambs had *ad libitum* access to their respective food treatment from 09:00 to 16:00 h for 55 d. Offered and refused foods were weighed (08:45 and 16:15 h, respectively) to determine daily intake. Lambs were weighed at the end of exposure (at d 56 of exposure).

2.2. Blood parameters

Blood samples were taken from 08:00 to 09:00 h at the beginning and end of the period of exposure. Blood sampling frequency was decided in order to have a baseline (beginning of the exposure period) and an endpoint, because the effect of chronic stress has commonly consequences in the long run (Dhabhar, 2009). Two 10-mL samples (with or without heparin added, Becton Dickinson Vacutainer System, New Jersey, United States) per animal were collected by puncture of the jugular vein. Samples with heparin were immediately submitted to the Veterinary Diagnostic Laboratory, Bahía Blanca, Argentina, for total blood cell count (ABX Micros 60 counter, ABX Diagnostics, Montpellier, France). Samples with no heparine were allowed to clot for 45 min; immediately after, serum was separated by centrifugation (2300 × *g* for 25 min; 4 °C) and stored at −20 °C until analysis. Serum cortisol concentration was determined using a commercial radioimmunoassay kit (Cortisol RIA CT, DIAsource Immuno Assays SA, Belgium). Minimum detectable concentration of cortisol in serum was 1 nmol/L. Inter- and intra-assay coefficient of variation was 8% and 3%, respectively.

2.3. Behavioral observations

The behavior of lambs was recorded with six video cameras (Foscam FI8904W, ShenZhen Foscam Intelligent Technology Co., Shenzhen, China) at 30 frames/s (i.e., real-time) from 09:00 to 16:00 h on 2 successive days, every 10 days, and after a month of exposure to ensure familiarization with the experimental conditions (d 30–31, 40–41, and 50–51 of exposure). Due to technical limitations video recordings were analyzed in two periods: from 09:00 to 11:00 h (morning observations) and from 14:00 to 16:00 h (afternoon observations). However, given the diurnal pattern of intake expressed by sheep and the time we offered the fresh food, we considered that peaks of intake and activity will be contained by these periods (Forbes, 1995; Baumont et al., 2000; González et al., 2008b). Activities (Table 3) were selected following the visual observation of the most common patterns of behavior observed inside the pens (see Wemelsfelder et al., 2000), and recorded by taking instantaneous scan samples on each animal at 1-min time intervals (Martin and Bateson, 1993). Average intake rate was calculated as total intake divided by total time spent eating, and was expressed as g/min.

2.4. Chemical analyses of foods

All foods used during the study were sampled each time before feeding (08:45 h), pooled for periods of 5 consecutive days corresponding to each food choice combination

Table 3
Ethogram of recorded activities.

Behavior	Description
Eating	Eating from one of the food bunks
Idling on food bunks	Placing the head over the food bunks, smelling or searching for food but not eating
Stereotypes	Licking or biting elements of the pen
Standing	Standing inactive or ruminating
Moving	Changing position while standing except those activities included in stereotypes
Lying	Lying inactive or ruminating
Drinking	Drinking water from automatic nipple drinker

offered to DIV, and then prepared for chemical analyses. Samples were dried for 48 h at 60 °C, ground using a Wiley Mill (1-mm mesh), and analyzed for crude protein (Method 990.03; Association of Official Agricultural Chemists, 1990) and neutral detergent fiber (Goering and Van Soest, 1970).

2.5. Statistical analyses

Statistical analyses were performed using the R environment (R Development Core Team, 2012). Mixed effects models were evaluated during the selection process according to the procedure detailed in Zuur et al. (2009). Model diagnostics also included testing for normal distribution, homogeneity of variance, and linearity. Least square means and standard errors were obtained with the “lsmeans” package (Lenth, 2012). All data are reported as means ± standard error of the mean (SEM).

Total dry matter (DM) intake, metabolizable energy intake, digestible protein intake, intake rate, average daily weight gain (ADG), final body weight (BW), and blood parameters of lambs in MON and DIV were compared using a one-way ANOVA, with the initial estimates used as covariates for final BW and blood parameters.

Behavioral data were averaged across days (d 30–31, 40–41, and 50–51 of exposure) because preliminary analyses did not show a differential evolution of behavior over time. Behavioral data were analyzed using a mixed effects model (Pinheiro et al., 2012) which included treatment (MON and DIV), hour (scans separated at 1-min intervals were averaged over 10 min to improve visual representation of results; e.g., Villalba et al., 2011), and treatment × hour interaction as fixed effects, and lamb as random effect that was nested within treatment levels. The model was fitted with an autoregressive order-1 covariance structure ($\chi^2{}_1 > 15.01$, $P < 0.001$; for all analyses). The probability of a given animal to alternate between three different states: eating, not-eating but active, or lying, was analyzed using a time-inhomogeneous Markov model with treatment as a covariate factor. Eating and lying states account for the activities depicted in Table 3, whereas not-eating but active state represented the sum of the rest of the activities listed in Table 3 but “Standing”. This model was run using the “msm” package (Jackson, 2011). Separate analyses were conducted for morning observations (09:00–11:00 h) and afternoon observations (from 14:00 to 16:00 h).

3. Results

3.1. Total and macronutrient intake, BW, and ADG

Mean daily total DM intake during exposure did not differ between lambs in DIV and MON treatments (31.75 vs. 32.31 ± 0.86 g/kg BW, respectively; $F_{1,22} = 0.22$, $P = 0.644$). Mean daily digestible protein intake was greater by lambs in DIV that by those in MON (6.76 vs. 5.32 ± 0.30 g/kg BW, respectively; $F_{1,22} = 11.13$, $P = 0.003$), whereas mean daily metabolizable energy intake was similar between groups (466.72 vs. 465.88 ± 12.73 kJ/kg BW; for DIV and MON, respectively; $F_{1,22} < 0.01$, $P = 0.962$).

Final BW was similar between lambs in DIV and those in MON (33.8 vs. 34.9 ± 0.9 kg, respectively; $F_{1,22} = 0.74$, $P = 0.399$), as well as ADG (234.6 vs. 250.1 ± 14.1 g/d, respectively; $F_{1,22} = 0.63$, $P = 0.437$).

3.2. Blood parameters

Data on blood parameters measured at the end of the exposure period are presented in Table 4. Red blood cell morphology and composition showed no differences between experimental groups ($P > 0.05$). Lambs in MON had a greater amount and percentage of segmented neutrophils relative to lambs in DIV ($P = 0.048$). Lymphocytes count was not affected by treatment ($P = 0.897$), but the percentage of lymphocytes relative to the total count of leukocytes was lower in lambs exposed to MON than in lambs exposed to DIV ($P = 0.013$). The ratio of segmented neutrophils to lymphocytes count was reduced when alimentary diversity of lambs was increased ($P = 0.044$). Platelet count was lower in lambs exposed to MON than in those exposed to DIV ($P = 0.011$). Lambs fed a monotonous diet (MON) showed greater serum cortisol levels than lambs fed free-choice different foods (DIV) ($P = 0.025$).

3.3. Behavioral observations

Lambs in DIV spent a lower proportion of time eating during the morning observations (from 09:00 to 11:00 h) than lambs in MON (0.38 vs. 0.49 ± 0.02, respectively; $F_{1,22} = 15.55$, $P < 0.001$; Fig. 1a). During afternoon observations (from 14:00 to 16:00 h) there was a significant treatment × hour interaction ($F_{1,236} = 7.12$, $P = 0.008$), explained by a lower proportion of time eating by lambs in DIV than by lambs in MON during the two peaks of food intake (at 14:10 and 15:40 h). Intake rate was greater for lambs in DIV than for lambs in MON (17.73 vs. 14.09 ± 1.26 g/min, respectively; $F_{1,22} = 4.55$, $P < 0.044$).

A treatment × hour interaction in both morning and afternoon observations was detected for the proportion of time that lambs spent idling on food buckets ($F_{1,236} = 5.78$, $P = 0.017$ and $F_{1,236} = 6.89$, $P = 0.009$; respectively; Fig. 1b). During the first 30 min of the morning observation period (from 09:00 to 09:30 h) the proportion of time engaged in the latter activity was greater for lambs in DIV than for lambs in MON, whereas a similar response was observed during the first peak of intake (14:10 h) and following the last peak of intake (16:00 h) in the afternoon observation period.

Table 4
Complete blood cell count and cortisol level of lambs ($n = 12$) fed a single diet (MON) or fed free-choice different combinations of four foods (DIV) at the end of an exposure period of 55 days.

Item	Treatment		SEM	*P*	$F_{1,21}$
	MON	DIV			
Red blood cells ($\times 10^6/\mu L$)	9.77	9.09	0.33	0.111	2.76
Hemoglobin (g/dL)	12.72	12.73	0.23	0.985	0.00
Packed cell volume (%)	47.30	45.60	1.28	0.360	0.87
Mean cell volume (fL)	33.50	33.40	0.26	0.876	0.02
MCH (pg)	13.73	13.60	0.31	0.570	0.33
MCHC (g/dL)	40.96	40.72	1.05	0.874	0.03
RDW	9.00	8.96	0.11	0.775	0.08
Leukocytes ($\times 10^3/\mu L$)	7.05	6.48	0.50	0.956	0.00
Lymphocytes ($\times 10^3/\mu L$)	4.96	4.91	0.36	0.897	0.02
Lymphocytes (%)	71.42	78.95	2.35	0.013	7.30
Neutrophils ($\times 10^3/\mu L$)	1.71	1.18	0.17	0.048	4.38
Neutrophils (%)	24.32	15.55	2.47	0.017	6.67
Monocytes ($\times 10^3/\mu L$)	0.19	0.24	0.03	0.259	1.34
Monocytes (%)	3.10	3.36	0.45	0.680	0.17
Eosinophils ($\times 10^3/\mu L$)	0.63	0.62	0.01	0.938	0.01
Eosinophils (%)	8.95	9.50	0.22	0.766	0.09
Platelets ($\times 10^3/\mu L$)	190.89	219.45	6.50	0.011	7.71
N/L	0.37	0.26	0.05	0.044	4.57
Cortisol (nmol/L)	31.44	19.90	3.30	0.025	5.79

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; Neutrophils, segmented neutrophils; N/L, segmented neutrophils to lymphocytes ratio.

The average proportion of time lying during morning observations was greater for lambs in DIV than for those in MON (0.44 vs. 0.36 ± 0.03, respectively; $F_{1,22} = 4.35$, $P = 0.049$; Fig. 1c). The pattern of lying was similar between groups during afternoon observations ($F_{1,22} = 0.01$, $P = 0.916$).

During morning observations, lambs in DIV showed a greater proportion of time moving than lambs in MON (0.047 vs. 0.035 ± 0.003, respectively; $F_{1,22} = 11.86$, $P = 0.003$; Fig. 1d). However, during afternoon observations a significant treatment × hour effect was observed ($F_{1,236} = 6.25$, $P = 0.013$). This effect was explained by a

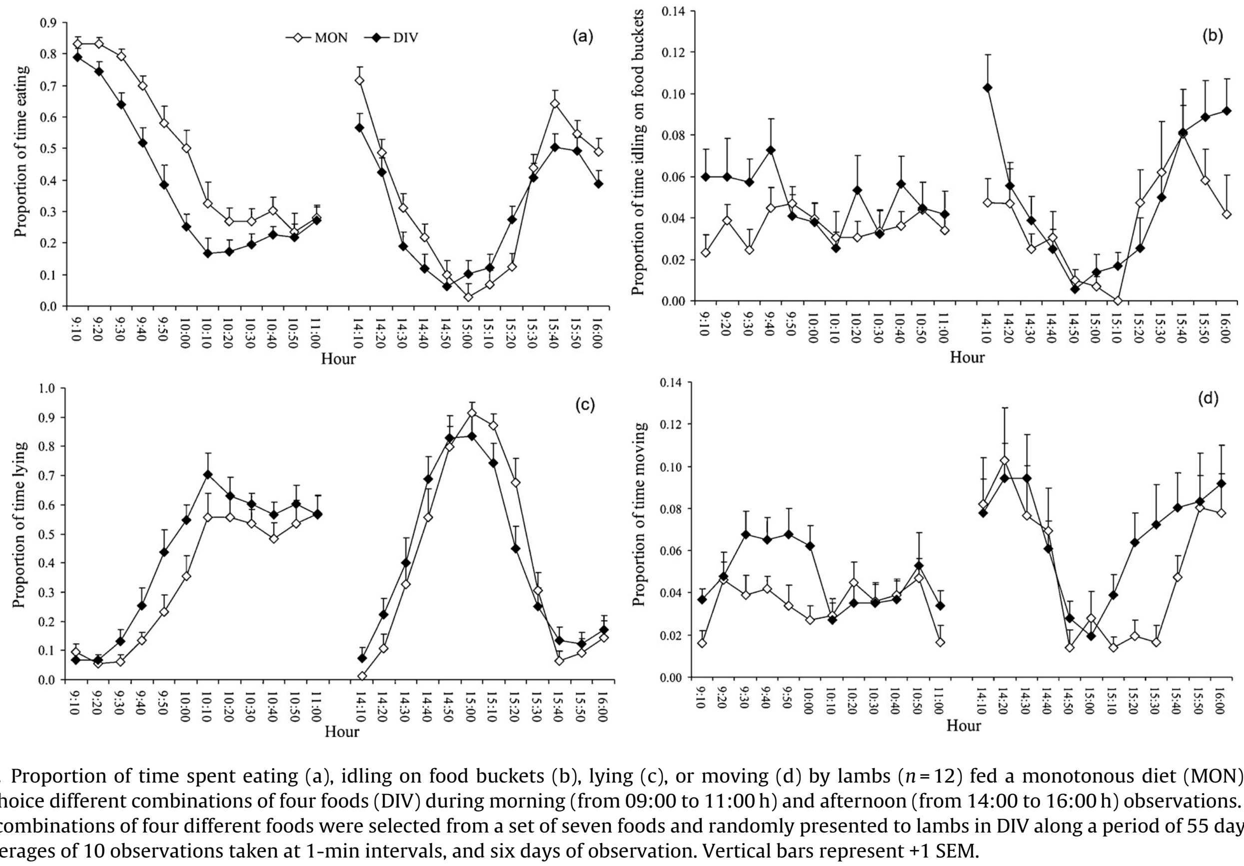

Fig. 1. Proportion of time spent eating (a), idling on food buckets (b), lying (c), or moving (d) by lambs ($n = 12$) fed a monotonous diet (MON) or fed free-choice different combinations of four foods (DIV) during morning (from 09:00 to 11:00 h) and afternoon (from 14:00 to 16:00 h) observations. A total of 11 combinations of four different foods were selected from a set of seven foods and randomly presented to lambs in DIV along a period of 55 days. Data are averages of 10 observations taken at 1-min intervals, and six days of observation. Vertical bars represent +1 SEM.

greater proportion of time moving for lambs in DIV than for lambs in MON only from 15:00 to 15:30 h.

No differences between DIV and MON were observed during morning and afternoon observations for the proportion of time spent standing ($F_{1,22}$ = 2.51, P = 0.127 and $F_{1,22}$ = 0.21, P = 0.654; respectively), engaged in stereotyped activities ($F_{1,22}$ < 0.01, P = 0.990 and $F_{1,22}$ < 0.01, P = 0.925; respectively), or drinking ($F_{1,22}$ = 1.36, P = 0.256 and $F_{1,22}$ < 0.01, P = 0.987; respectively).

The inclusion of treatment as a covariate improved the Markov model for morning observations ($\chi^2{}_4$ = 17.26, P = 0.002). Lambs in DIV were more likely to switch from eating to displaying other activities than lambs in MON (0.22 [0.26–0.19] vs. 0.14 [0.16–0.12], respectively [upper and lower 95% confidence intervals]), whereas lambs in MON were more likely to keep on eating than lambs in DIV (0.84 [0.87–0.81] vs. 0.75 [0.79–0.72]), respectively [upper and lower 95% confidence intervals]). In contrast, during afternoon observations the inclusion of treatment as a covariate did not improve the model ($\chi^2{}_4$ = 5.25, P = 0.263).

4. Discussion

Our results were consistent with the hypothesis that dietary monotony, even for nutritionally balanced diets, is stressful for lambs. We observed that restricting lambs' dietary breadth produced changes in blood and behavioral parameters indicative of stress in sheep (as discussed below). Because there is a well supported relationship between stress and animal well-being (Broom and Johnson, 2000), it can be inferred that restricting dietary choice has the potential to compromise animal welfare (Manteca et al., 2008; Villalba et al., 2010).

4.1. Dietary monotony and blood parameters

Chronic stress in cattle activates the hypothalamic–pituitary–adrenal axis increasing peripheral levels of glucocorticoids (Mench et al., 1990; Napolitano et al., 2008). In this study we observed that lambs fed a monotonous diet had greater levels of serum cortisol than lambs fed a wider array of foods. Ruminants are sensitive to feeding practices and even small changes in the feeding schedule can generate stressful conditions followed by increased concentrations of blood cortisol (González et al., 2009). Bourguet et al. (2011) concluded that food deprivation is likely to cause psychological stress and frustration. In agreement with this, food deprived dairy cows showed greater blood cortisol levels than cows with a lower degree of deprivation (Samuelsson et al., 1996). In this study, lambs in MON were restricted in the amount of options but not in the amounts of food they had available to eat; however, the first type of restriction seems to have had similar effects on welfare as those observed for food deprived animals.

The immunosuppressive effects of chronic stress are well documented (Dhabhar, 2009). This phenomenon is explained by the adverse action of high and sustained levels of glucocorticoids on multiple components of the immune system (DeVries et al., 1997; Salak-Johnson and McGlone, 2007). A reduced number of lymphocytes (lymphopaenia) and an increased number of neutrophils (neutrophilia) are typical signs of elevated plasmatic levels of glucocorticoids (Broom, 2006). In accordance to this evidence, we observed a greater number of neutrophils in lambs fed a monotonous diet than in those exposed to a choice of foods, whereas no differences were evident for lymphocytes count. Nevertheless, the percentage of lymphocytes and neutrophils relative to the total amount of leukocytes were lower and higher, respectively, in lambs in MON than those in DIV. Hickey et al. (2003) showed a similar trend in lymphocytes and neutrophils percentages for lambs separated from their mothers compared to lambs that were not weaned. Moreover, lambs restricted to a monotonous diet showed a greater ratio of neutrophils to lymphocytes, which is indicative of distress and high glucocorticoids levels (Davis et al., 2008). In addition, lambs in MON showed lower number of platelets than those in DIV. Barbucci et al. (2002) showed that platelet adhesion to some materials commonly used during blood collection is increased in stressed animals relative to non-stressed individuals. Thus, it is likely that lower amounts of platelets were harvested during the blood sampling done on the animals under greater levels of stress (i.e., those in the MON treatment). Overall, these results on blood parameters suggest that lambs exposed to a monotonous diet experienced greater stress than lambs offered food choices.

4.2. Dietary monotony and animal behavior

Lambs exposed to a diverse alimentary environment spent less time eating than lambs restricted to a monotonous diet, which was consistent with (1) the greater proportion of time spent by lambs in DIV with the face over the food buckets but not eating at hours when food intake was relatively high, (2) the greater likelihood of lambs in DIV to switch between eating and doing other activities, and (3) the lower likelihood of lambs in DIV to remain eating if the immediate prior activity was also eating. Nevertheless, these differences in time spent eating did not translate into differences in total food intake between treatments. A similar amount of food eaten in less time by lambs in DIV was explained by a greater rate of food intake, when compared to lambs in MON. High intake rate can be a consequence of a higher motivation to eat by lambs in DIV (Baumont et al., 2000), because animals habituate and decrease their responses to foods eaten to often (like the monotonous diet; Rolls, 1986). On the other hand, a lower intake rate by lambs in MON can be explained by a greater amount of time invested in sorting for preferred dietary ingredients. Cows fed total mixed rations can spend considerable amounts of time sorting in order to achieve ruminal stability (DeVries et al., 2008). Contrary to our results, Keskin et al. (2004) observed that choice-fed lambs spent more time eating than animals restricted to a monotonous diet; however, in this work total food intake was greater in the former group. Differences in the composition and formulation of the monotonous diet (which commonly represents the control treatment in free-choice feeding experiments) may help to explain differences in total intake between studies. For instance, total intake of calves exposed to a choice of foods was observed to be higher (Boga et al., 2009), lower (Atwood et al., 2001), or similar (Montoro and Bach, 2012) than in calves exposed to a monotonous diet.

Stereotypic behaviors are repetitive and seemingly non-functional activities indicative of poor welfare (Broom, 1991; Bergeron et al., 2006). Oral manipulation of objects is the most common abnormal behavior in ungulates facing stressful situations (Mason et al., 2007). In the present study, all lambs showed sporadic licking and biting of different elements of the pen, but no differences between treatments were observed in the frequency of these behaviors. In previous studies, restrictions in the availability of food (Redbo et al., 1996) or roughage (Redbo and Nordblad, 1997) were shown to increase the frequency of stereotypic behaviors, as well as the number of animals involved in these actions. In contrast with the latter studies, we did not intentionally manipulate the duration of oral manipulation of foods and oral stereotypes in sheep can be reduced by the oral stimulation produced by rumination (Dwyer and Lawrence, 2008), which may have leaded to the lack of differences between groups.

The proportion of time spent lying during mornings was greater for lambs in DIV than for lambs in MON. Similarly, Keskin et al. (2004) showed that lambs offered food choices spent more time resting than lambs fed a monotonous diet. Moreover, dairy cows exposed to stressful conditions, like restricted availability of feeding places, spent less time lying and more time standing (González et al., 2008a,b). Increased resting by lambs in DIV could have been a consequence of the high activity produced by the periods of greater moving and searching for different foods between buckets (i.e., idling on food buckets), when compared to lambs in MON. Alternatively, increased resting by lambs in DIV may have been related to rumination. Ruminants prefer to ruminate while lying (Kilgour, 2012) and rumination can be taken as an indicator of low stress (Redbo and Nordblad, 1997; Cockram, 2004). However, due to technical limitations in this study we could not discriminate between the time spent lying inactive and ruminating. Finally, because abnormal inactivity is common in confined animals (Morgan and Tromborg, 2007), increased activity by lambs in DIV may reflect a reduced stress condition.

Behavioral responses cannot be readily associated to stressful processes, like it could be the case for some physiological responses that can be more closely related to the animal's biological functioning (e.g., immunology). Different behavioral responses are specific for the type of stressor involved (Cockram, 2004) and sometimes they can have an ambiguous interpretation. For instance, reduced activity in sheep can be taken as a sign of fear or docility (Romeyer and Bouissou, 1992). Therefore, the behavioral responses exposed in this work should be taken as probable descriptors of stress in the diet choice paradigm and further research is needed to provide validation.

4.3. *Diet choice and welfare: a probable link*

Ruminants evolved in complex environments in which food resources have not only diverse and changing nutritional properties but also variable spatial distribution (Day et al., 1998). Sheep seek for diversity in their diets; for instance, they preferred to eat a varied array of plant species and feeds (Provenza, 1996; Villalba et al., 2011) or feeds offered in different flavors (Scott and Provenza, 1998; Distel et al., 2007). Restricting dietary choices may reduce welfare by altering nutritional, sensory and cognitive processes.

Animals exposed to a monotonous diet cannot avoid nutrient imbalances by choosing complementary foods as they would typically do in a natural environment (Provenza et al., 2003). This may be true even for diets designed to satisfy average nutrient requirements (like the diet for lambs in MON in this study), because individual differences in morphological built and physiological function can lead to strikingly different requirements among animals (Provenza et al., 2003). Lambs in DIV showed a greater intake of protein than lambs restricted to a monotonous diet, which may have contributed to a better nutrition in the former. This assumption is supported by results observed in choice-fed lambs (Keskin et al., 2004; Rodríguez et al., 2007); although, it seems inconsistent with the lack of differences in final BW and ADG between treatments in present study. However, ADG and BW do not necessarily reflect animal's nutritional and physiological homeostasis. This is because animals can handle moderately unbalanced diets by overingesting some nutrients that are in excess in the diet, to ingest adequate amounts of others that are required but deficient in the diet (Simpson et al., 2004). This behavior may lead to a high ADG and BW but through the abnormal accumulation of fat, if animals are overingesting energy in order to meet protein requirements (Simpson and Raubenheimer, 2005). It has been proposed that both over- and under-ingestion of nutrients cause discomfort and stress in animals (Forbes and Provenza, 2000). Because of this, productive performance has been criticized as a reliable indicator of welfare (Dwyer and Lawrence, 2008). For instance, feedlot cattle show high performance on high-grain diets that cause them sporadic metabolic disorders known to reduce welfare (Russell and Rychlik, 2001).

Not only nutritional but also sensory processes are probably affected by the continuous exposure to the same food. Following the ingestion of a given food its palatability decline because an aversion develops toward its specific sensory properties (Rolls, 1986). Sheep show greater intake of the same food offered in different flavors than of that food offered in a single flavor (Distel et al., 2007; Villalba et al., 2011). In the present study, feeding animals a diverse diet did not improve total food intake but intake rate, which may more accurately represent animals' motivation to eat (Baumont et al., 2000). Moreover, the nutritional composition of the diet differed between experimental groups, which make meaningless the comparison of total food intake between them (Forbes, 2003).

Meehan and Mench (2007) argued that animals need to be faced with "appropriate challenges" (problems that can be solved by the animal's skills) because this enriches the interaction of the animal with its environment, favoring motivation and learning, while reducing frustration and distress. Dietary diversity challenges animals to learn about the postingestive consequences of foods, and then, to select the appropriate types and amounts of these foods relative to their needs. Complex alimentary environments are likely to provide multiple experiences with the potential to enhance motivation and cognitive development. For instance, lambs exposed to a diverse alimentary environment showed later in life an improved ability to learn about the nutritional properties of novel foods relative to lambs exposed to a monotonous diet (Catanese et al., 2012).

Theoretical and experimental evidence points out a strong link between dietary choice and welfare that is characterized by the interplay between an animal's sensory experiences and the need to achieve homeostasis.

5. Conclusions

Farm animals are commonly restricted to a reduced array of foods, like total mixed rations or pastures with a low diversity of vegetal species. In the present study, we found that feeding lambs a monotonous diet produces changes in blood and behavioral parameters indicative of stress. Our results emphasize the importance of incorporating food choice and diversity into feeding practices to reduce stress and enhance animal welfare.

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