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Relationship of Ovine Glucose-6-phosphate Dehydrogenase and Cytosolic Isocitrate Dehydrogenase Gene Expression with Energy Balance Status During the Early Post Weaning Lactation Period

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Abstract

The aim of this study was to determine changes on gene expression of glucose-6-phosphate dehydrogenase (G6PD) and cytosolic isocitrate dehydrogenase (ICDH), during the early post weaning lactation period in ewes with a negative (<0 Mj; group NBS), positive (>0 Mj; group PBS), very low (< -1.74 Mj; group MinNBS) and very high (>2.56 Mj; group MaxPBS) energy balance status and to investigate their relationship. Adipose tissues samples from seven ewes at 1st, 2nd and 4th week post weaning were taken for RNA isolation and determination of genes expression using Real-Time PCR. Both genes showed a synchronized expression response. A higher (P<0.01) response of G6PD gene expression, also, observed in MinNBS or MaxPBS groups. ICDH showed a smoother response in regard to energy balance status, revealing the more crucial role of G6PD gene. The results provide valuable, novel information regarding lipogenesis during the early post weaning lactation period.

Keywords: Cytosolic Isocitrate Dehydrogenase, Energy Metabolism, Glucose-6-Phasphate Dehydrogenase, G6pd, Sheep, Ruminants

Introduction

The energy balance in a living organism is achieved by a sequence of biochemical reactions that are focused, mainly, on controlling the balance between lipogenesis and lipolysis (Elmquist *et al.*, 2006; Yadav *et al.*, 2013). Adipose tissue forms the predominant anatomic site for this control, as it provides the appropriate fuels by terms of fatty acids. The *de novo* biosynthesis of fatty acids, apart from acetylo-CoA (carbonic substrate) requires considerable amounts of reducing agents in the form of NADPH for the reduction of the carbon substrate to fatty acids. Several enzymes are linked to NADPH production of which the most important are the following: (i) cytosolic isocitrate dehydrogenase (ICDH); (ii) cytosolic NADP malic enzyme (ME1); (iii) glucose-6-phosphate dehydrogenase (G6PD) and (iv) 6-phosphogluconate dehydrogenase (6PGD) (Vernon, 1981).

Contrary to human and other mammalian species, in ruminants the major suppliers of NADPH equivalents are G6PD and ICDH, mainly due to differences observed in metabolic pathways of fatty acid synthesis and lipogenesis (Laliotis *et al.*, 2010). In sheep, the 50–80% of the essential NADPH compounds for fatty acid synthesis is produced by G6PD, while the rest is provided by ICDH. Unlike human and rodents, in ruminants ME1 is considered as a minor donor of reductive power (NADPH), because the metabolic sequence that catalyzes poses a minor activity and thus is rendered not significant. Many studies have been conducted revealing the enzymatic response of the two major ovine lipogenic dehydrogenases (G6PD, ICDH) in regard to various stimuli. Piperova and Pearce (1982) reported a significantly higher G6PD enzymatic activity in adipose tissue extracts from sheep fed concentrate diets compared to that of grass-fed animals. Antibiotics, like gentamicin sulfate and vancomycin hydrochloride, inhibited G6PD activity in vitro (Beydemir *et al.*, 2003). Yur *et al.* (2003) reported significant decrease of G6PD activity in fluorotic sheep. Babesiosis infection has been associated with decreased levels of blood G6PD activity in sheep (Esmaeilnejad *et al.*, 2014). The age as well as the anatomic site of adipose tissue influence, also, the enzymatic levels of ICDH (Rogdakis *et al.*, 199). According to Kelman *et al.* (2014) reduced levels of ICDH activity were observed during the selection for increased lean meat yield. Well-fed ewes in late pregnancy tended to possess a higher enzymatic activity than ewes that were underfed (Charismiadou *et al.*, 2008).

Advances on molecular biology level shed, also, light on the genes controlling ovine ICDH and G6PD enzymatic response (Kenoutis *et al.*, 2004; Laliotis *et al.*, 2007) as well as their structure (Laliotis *et al.*, 2007, 2018), promoting the research on ovine lipogenesis. However, sparse knowledge exists regarding the response of ovine G6PD and ICDH genes to external stimuli and especially under extreme fluctuations of these stimuli. A previous study showed that gradually changes on the dietary energy intake of ewes influence the transcript response of G6PD and ICDH genes (Laliotis *et al.*, 2012). However, response of these genes under extreme levels of energy balance status (either negative or positive) is not known with certainty. Our working hypothesis is that the energy balance status (negative or positive) in ewes influences G6PD and ICDH gene expression response. The aim of the present study was: (a) to determine the effect of negative or positive energy balance status on the expression levels of ICDH and G6PD gene; (b) to determine whether further gene expression fluctuations exist under extreme low or extreme high levels of energy balance and (c) to investigate if any relation between the response of ICDH and G6PD gene expression exist in regard to changes of extreme energy balance levels.

Materials and Methods

Animal Treatment and Measurements

A total of seven randomly selected lactating ewes (*Ovis aries*) of Chios breed housed within the premises of the experimental farm of the Agricultural University of Athens were used in the study. After weaning (45 days post-partum) ewes were fed individually twice per day (7:00 and 16:00 h) on an alfalfa hay (4.14 MJ/kg) and on a concentrated diet consisting of a basal (7.1 MJ/kg) and a lactation (7.3 MJ/kg) ration. In the two first experimental weeks the amount of diet was set to create a negative energy balance in animals as follows: 0.4 kg alfalfa hay, 0.5 kg basal ratio, 1 kg lactation ratio. In the next two weeks the lactation ration was increased 0.5 Kg to create in animals a positive energy balance. Water was freely available. Samples of the subcutaneous adipose tissue from the tail region from each ewe were taken by biopsy at the end of the first (1st), second (2nd) and fourth (4th) week. Prior to biopsy the ewes were fasted but with free access to water. Samples of adipose tissues were immediately frozen in liquid N_2 and stored at −80 °C for further RNA extraction. Body weight, milk yield and milk fat content were also recorded weakly in each ewe. Energy balance (Mj) was estimated weekly for each ewe as described previously (Mavrogenis *et al.*, 1988).

RNA Manipulation and Real-Time PCR

Total RNA from ovine adipose tissue was extracted using “Rneasy Lipid Tissue Kit” (Qiagen). A two- step RT-PCR procedure was followed for the synthesis of first-strand cDNA, using 1 μg of the eluted total RNA and Omniscript Reverse Transcriptase (Qiagen) in a total volume of 20 μl. Real-time quantitative RT-PCR was implemented to quantify the relative levels of mRNA. Two custom designed TaqMan® gene expression assays for ovine G6PD and ICDH transcripts using FAM as a reporter dye were used (Applied Biosystems). A TaqMan® Eukaryotic 18s rRNA Endogenous Control using VIC as a reporter dye (Applied Biosystems) was also used in multiplex reactions as a reference gene to normalize the amount of sample RNA. All probes had minor grooved binders (MGB) as quencher. Each multiplex reaction for the quantification of either G6PD or ICDH and 18S rRNA was conducted in triplicates (Table 1). The $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001) was used to determine the relative mRNA expression.

Table 1: Gene expression assays and thermal cycling protocol used for Real-Time PCR in the present study

Gene	Sequence	Position	Reporter Dye /Quencher	Accession Number	PCR Protocol
G6PD				DQ377363 / DQ377364	**1 x** {50 °C for 2’},
Forward	5’-AGAAGCCCTTCGGGAAGGA-3’	574-594			**1 x** {95 °C for 10’}
Reverse	5’-CTCGTGTAACAGGTAGTCGATGT-3’	624-646			**40x** {95 °C for 15’’& 60 °C for 1’}
Probe	5’-CCAACCAGCTGTCCAACC-3’	606-623	FAM/MGB		
ICDH				AY208679	
Forward	5’- TCACTACCGAATGTACCAGAAAGGA-3’	1121-1145			
Reverse	5’-CTCTGGTCCAGGCAAAAATGG-3’	1174-1195			
Probe	5’-ACGTCCACCAATCCCA-3’	1152-1167	FAM/MGB		

Statistical Analysis

The gene expression data (N=21) were divided into two groups according to ewes’ energy balance status; one group of ewes with negative energy balance status (< 0 Mj; namely hereafter: group NBS, N=9); and a second group of ewes with positive energy balance status (> 0 Mj; namely hereafter: group PBS, N= 12). An independent *t*-test was performed to compare the effect of energy balance status on the examined gene expressions. A P value of less than 0.05 ($P < 0.05$) was accepted as significant.

In order to examine the effect of “extreme” levels of energy balance status, thus very low or very high energy balance status on the expression of the examined genes, two different groups were set considering the determined animal’s energy balance values. The two groups were set following a 25% and 75% quartile statistical approach, based on the energy balance mean, which allows to separate the distribution of the observations into extreme-marginal situations. Thus, two groups were created; one with very low energy balance status (namely hereafter MinNBS, N=5) or very high energy balance status (namely hereafter MaxPBS, N=6). The aforementioned percentages (25% and 75%) were chosen in order to obtain sufficiently large sample sizes of animal cases in each group. Gene expression data of the two groups (MinNBS, MaxPBS) were further analyzed applying again an independent t-test as previously performed. Linear regression analysis was also used to relate changes of ICDH and G6PD gene expression with very low or very high energy balance values.

Results

Mean values of energy balance in ewes with negative and positive energy balance status is shown in Fig. 1 A. The mean energy balance of group NBS was estimated at -1.89 ± 0.56 Mj, while the respective value for group PBS was 3.48 ± 0.48 Mj, indicating a significant difference ($P<0.01$). Milk production of ewes in respect to energy balance status is also shown in Fig.1B. Group NBS had significant ($P< 0.01$) higher milk production, compared to group PBS.

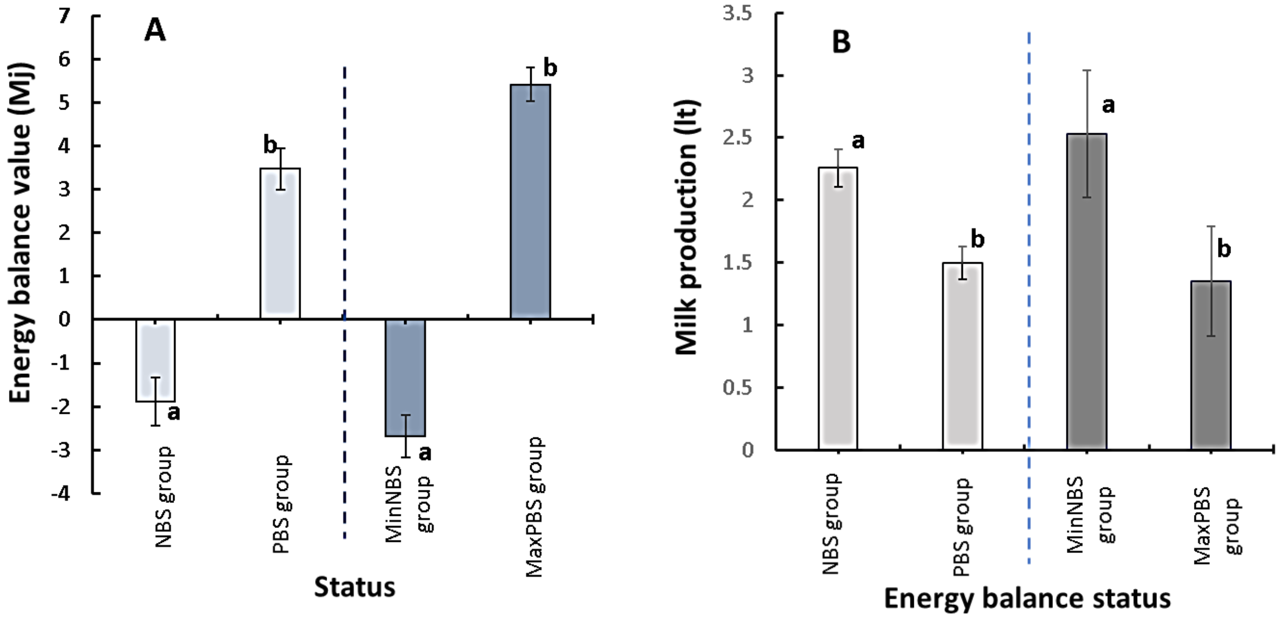

Figure 1: Mean energy balance values (Mj) and milk yield (lt) of Chios breed ewes at different energy balance status. Figure 1A presents the average energy balance values at NBS group, PBS group, MinNBS group and MaxPBS group. Figure 1B shows the respective average milk yield of the aforementioned animal groups. Values are presented as LSM ± SEM. Means with different superscripts (a, b) indicate statistically significant differences ($P<0.01$).

In regard to the examined lipogenic genes' expression, G6PD gene expression was 72.5 % lower ($P<0.01$) in group NBS compared to its expression levels in group PBS (Table 2). The same trend but with slight lower fluctuations was noticed for ICDH gene expression. ICDH gene expression levels were 69 % lower ($P<0.01$) in ewes with NBS in respect to group PBS. Differences were also observed in the expression levels between the two lipogenic genes. G6PD gene expression was significant higher ($P<0.01$) compared to ICDH expression in both examined energy balance statuses (negative or positive). Investigating further the effect of energy balance status on G6PD and ICDH genes expression, we statistically separate our dataset into two "extreme" energy groups; one group with very low negative energy balance status, that of values ≤ - 1.74 Mj (group MinNBS), and a second group with very high energy balance status, that of values > 2.56 Mj (group MaxPBS). The two groups included all values that corresponded to the 25% and the 75% quintile of the whole dataset, respectively. According to our results (Table 2), an 82.1 % diminish ($P<0.01$) of G6PD expression was determined in ewes being in group MinNBS compared to ewes being in group MaxPBS. ICDH gene expression followed a same trend with G6PD response, but with lower fluctuations. Ewes group MinNBS showed a 69.1 % lower ($P<0.01$) gene expression levels compared to group MaxPBS, retaining the same levels of its expression as observed in group NBS (Table 2).

Table 2: Effect of energy balance status on the glucose-6phosphate dehydrogenase (G6PD) and cytosolic isocitrate dehydrogenase (ICDH) gene expression during the early post weaning lactation period of Chios breed ewes. Values are presented as LSM ± SEM

Parameter	Energy Balance groups	
	NBS group (<0 Mj)	PBS group (> 0 Mj)
G6PD expression	$1.57^{a,1} \pm 0.31$	$5.71^{b,1} \pm 0.51$
ICDH expression	$0.65^{c,2} \pm 0.14$	$2.10^{d,2} \pm 0.49$
	MinNBS group	MaxPBS group
	(<1.74Mj)	(>2.56 Mj)
G6PD expression	$1.34^{a,3} \pm 0.42$	$7.47^{b,3} \pm 0.74$
ICDH expression	$0.75^{a,4} \pm 0.22$	$2.43^{b,4} \pm 0.72$

^{a,b} Different superscripts within the same row indicate significant difference (a,b: $P<0.01$; c,d: $P<0.05$) between the examined energy balance status. ^{1,2,3,4} Different superscripts within the same column indicate significant difference ($P<0.01$) for the examined gene expression.

The relationship of G6PD and ICDH gene expression in respect to extreme high or low values of energy balance is shown in Figure 2. G6PD and ICDH gene expression is increased with the increase of energy balance, in a co-synchronized trend. In addition, a same transcript accumulation, thus a same gene expression response is noted at E= - = -3.5 Mj (cross point of the two graphs). Based on intercept values (b), ICDH showed a lower response under extreme changes of energy balance status compared to the respective reaction of G6PD gene expression ($b_{ICDH} < b_{G6PD}$). According to the ratio of the intercepts (b_{G6PD} / b_{ICDH}) G6PD expression appears to have a 3.54-fold higher response, revealing a more triggered response towards ICDH as well as smoother fluctuations of ICDH gene expression towards energy balance changes.

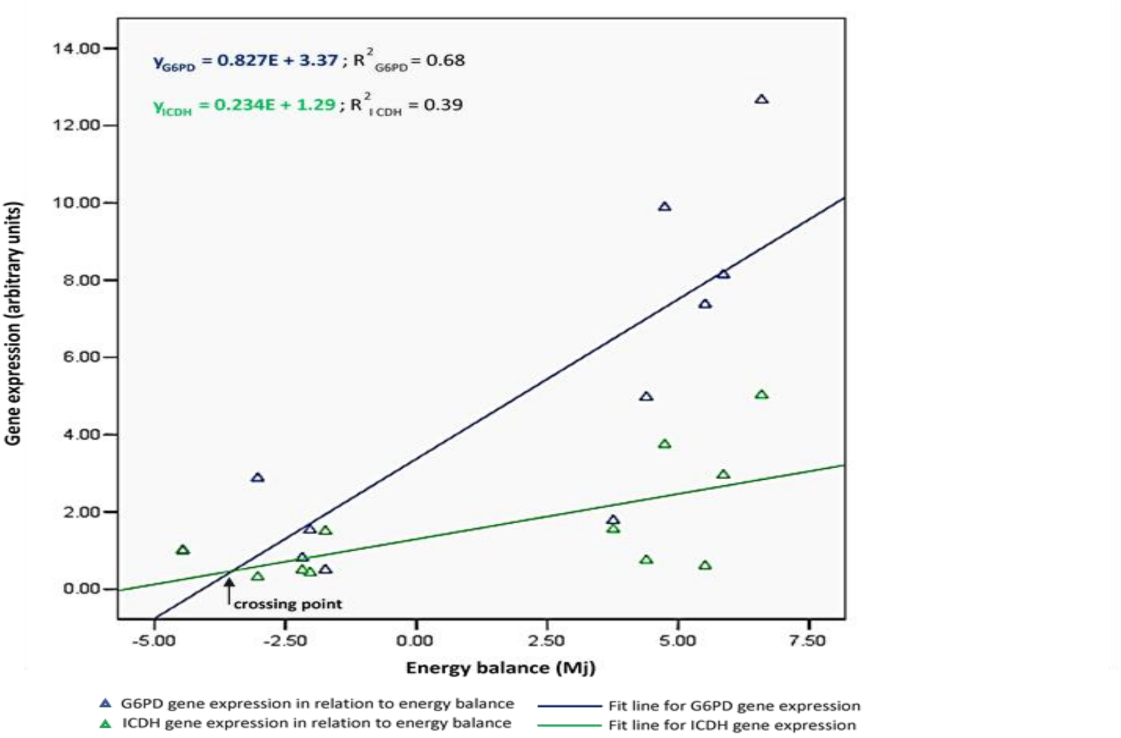

Figure 2: Linear regression analysis of glucose-6phosphate dehydrogenase (G6PD) and cytosolic isocitrate dehydrogenase (ICDH) gene expression in ovine adipose tissue as a function of extreme energy balance values. Blue and green triangles represent relative G6PD and ICDH gene expression (in arbitrary units) in regard to energy balance values (Mj) of MinNBS and MaxPBS animal groups. Blue and green line represent the fit line of linear equation of G6PD and ICH gene expression in respect to the respective energy balance values (Mj). The black arrow shows the crossing point of the two fitted lines, where both studied genes appears to have the same levels of transcript accumulation.

Discussion

Energy metabolism, defined as the processes underlined by food intake, food burning to release energy as well as storage of energy excess for use in time of energy shortage, plays a pivotal role in organism's sustain to life (Yadav, 2013). These processes include a variety of complex metabolic reactions, with lipogenesis being one of them. Lipogenesis, through the lipogenic pathways, assists organisms to avoid metabolic diseases and to maintaining their homeostasis (Solinas *et al.*, 2015). In addition, in ruminants influences also the productive traits and the economic return of a production system (Laliotis *et al.*, 2007; 2010). Herein the association of ewes' energy balance status, thus negative (< 0 Mj; group NBS) or positive (> 0 Mj; group PBS), with the expression of two major ovine lipogenic genes, the G6PD and ICDH gene, is examined during the early post weaning lactation period. Response of the aforementioned genes' expression under very low (group MinNBS) or very high (group MaxPBS) energy balance status, is also reported.

The first finding emerging from the present study is a strong association of G6PD and ICDH gene expression with ewe's energy balance status, even in extreme situations of very high or very low values of energy balance. A synchronized response in the changes of energy balance either by decreasing or increasing their expression levels under negative or positive energy balance status was observed, respectively. Obviously, this association could be attributed to a "cause effect" relationship due to the need of covering ewes' metabolic requirements during the early post weaning lactating period. Excessive energy balance usually leads to fat accumulation to be used as energy source at a later point in time. Thus, lipogenesisis is stimulated and excess fat is further stored in reservoir anatomic sites. In such a case, metabolic homeostasis is achieved through the activation of genes participating in fatty acids synthesis and the increase of their gene expression (Collier *et al.*, 1984). Contrary, the presence of negative energy balance, as a result of fasting, increased energy demands i.e. during the initiation of lactation in the early post-partum period in lactating animals or even as a result of limited feed retrieval, leads to a mobilization of fatty acids from storage anatomic sites and as a consequence in the decrease of lipogenesis (McNamara and Hillers, 1989; Luo and Liu, 2016). Our results share similarities to previous reported studies. Very early reports on enzymatic level showed a synchronized enzymatic activity of the NADPH producing enzymes in ovine adipose tissue (Panopoulou *et al.*, 1989; Rogdakis *et al.*, 1997) implying an involved mechanism of gene expression control at molecular level. Changes on ewes' dietary-energy intake revealed a synchronized response of G6PD and ICDH gene expression (Laliotis *et al.*, 2012). Finally, fasting ewes for a two-week period has been, also, reported to lead to a synchronized decrease of G6PD and ICDH gene expression levels by 1.2-fold and 1.0-fold, respectively compared to well-fed animals (Laliotis and Bizelis, 2011).

Another finding revealed from this study is that G6PD response at transcriptional level was higher triggered compared to ICDH gene. An almost 3.5% higher levels of G6PD gene expression in respect to ICDH gene expression was observed in group NBS compared to group PBS. This percentage was higher under "extreme" energy balance status. G6PD gene expression was 13% higher in respect to ICDH gene response, in group MinNBS compared to group MaxPBS. In addition, the observed ICDH transcript response showed similar changes between the compared energy balance statuses, thus between groups NBS and PBS as well as between groups MinNBS and MaxPBS (69 % and 69.1 %, respectively). These data, taking together, suggest the higher importance of G6PD gene in controlling ewes' metabolic homeostasis during the presence of "extreme" energy status at the early post weaning lactating period.

The last finding emerging from the present study is that a pivotal point in energy balance values was observe (E= -3.5 Mj), where G6PD gene starts to response with more sharply fluctuations in respect to ICDH gene. At this point, the two studied genes reached the same expression level, indicating that both contribute equally to ewes' homeostasis response towards "extreme" energy balance statuses, thus very low or very high energy balance values. After this point, G6PD gene is triggered to respond more sharply ($b_{G6PD}/b_{ICDH}=3.53$) either by declining or increasing its expression level depending on energy balance level. ICDH gene appears to retain a more stable transcript response. The fact that in ruminants G6PD contributes in a higher proportion (over 50%) regarding the production of NADPH molecules that participate in the *de novo* synthesis of fatty acids (Vernon, 1981), could partially explain the observed sharply response of G6PD gene. G6PD is, also, associated with cell protection from oxidative stress (Hung-Yao *et al.*, 2007; Nobrega-Pereira *et al.*, 2016) by controlling the biochemical pathway of glutathione through the production of NADPH. Under high oxidation levels, mammalian cells usually respond with a diminish of G6PD activity (Nobrega-Pereira *et al.*, 2016; Zachut *et al.*, 2016). From very early studies it is well established that lactating mammals undergo high oxidative stress during the first stage of lactation period (Collier *et al.*, 1984; McNamara and Hillers, 1989; McNamara, 2004). Moreover, lipogenesis under negative energy balance is minimized in favor of lipid mobilization leading to an induce of oxidative stress in central and peripheral tissues (Zachut *et al.*, 2016). Thus, according to the aforementioned, decrease of lipogenesis and high oxidation stress could be responsible for the observed sharply response of G6PD gene towards very low energy balance status, during the early post weaning lactating period. However, we could not exclude the involvement of any pre-transcription mechanisms that may contribute to the observed response of the two examined genes in regard to extreme levels of energy balance; but this assumption needs further investigation.

Conclusion

In conclusion, a synchronized but higher response of G6PD gene expression compared to the respective ICDH gene expression was observed to be associated with ewes' energy balance status at the early post weaning lactation period. ICDH transcript fluctuations observed to be smoother in regard to energy balance status, revealing the more crucial role of G6PD gene, especially under a very low or very high energy balance status. The results of the present study provide valuable, novel information regarding ovine lipogenesis in respect to energy metabolism and assists the further investigation of ruminant lipogenesis in respect to external stimuli.

Ethics Statement

All procedures involving animals were approved by the bioethical committee of the Agricultural University of Athens under the guidelines of “Council Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes”.

Conflict of Interests

There is no conflict of interest.

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