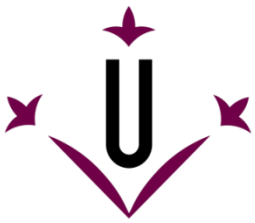

Maternal nutrient restriction in early pregnancy increases the risk of late embryo loss despite no effects on peri-implantation interferon-stimulated genes in suckler beef cattle

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Abstract

Reducing feeding costs in suckler beef herds to improve economic returns could have detrimental impacts on fertility. This study sought to determine whether maternal nutrient restriction during early pregnancy affects interferon-stimulated gene (ISG) expression in peripheral blood mononuclear cells during the peri-implantation period in two beef cattle breeds. Relationships were also examined between subnutrition and pregnancy failure defined according to ISG fold changes on Days 18 and 21 and to plasma pregnancy specific protein B (PSPB) concentrations on Day 28 post-artificial insemination (AI). Pirenaica or Parda de Montaña dams were assigned to a control (n = 23) or subnutrition (n = 30) group, receiving 100% or 65% of their estimated nutritional requirements from Day 1 to 82 post-AI, respectively. Treatment did not affect ISG expression or fertility. According to ISG fold changes (chi-square P=0.023) or PSPB levels (chi-square P=0.04) recorded in the subnutrition group, late embryo loss was more likely than in controls. Positive correlation was detected between Day 28 PSPB concentrations and both Day 18 *MX1*, *MX2* and *ISG15* expression, and Day 21 *OAS1* expression. *OAS1* and *MX1* fold changes were found to be the best variables to discriminate pregnancy status. Our findings indicate that maternal nutrient restriction during the first third of pregnancy does not impair embryo signalling yet may increase the risk of pregnancy failure.

Keywords: Beef cattle; Interferon tau; Undernutrition; Pregnancy loss.

1. Introduction

The productivity of livestock enterprises is determined as much by genetic factors as by management factors such as nutrition or environmental conditions (reviewed by Chavatte-Palmer et al., 2018). The economic feasibility of beef cattle herds relies on reduced feeding costs along with the good reproductive performance of dams and high growth rates of their offspring. However, feeding costs minimized through diet restriction or low quality grazing resources could have negative impacts on reproduction (Sanz et al., 2004). A negative energy balance during pregnancy has dramatic effects on postnatal development through its direct influence on foetal growth rate and calf endocrine regulation with detrimental consequences on carcass quality (Long et al., 2009; Funston et al., 2010a; Wang et al., 2015; Lemaster et al., 2017; Taylor et al., 2018; Noya et al., 2019).

During the peri-implantation period, the developing conceptus relies on histotroph secretion into the uterine lumen (Gray et al., 2002; Bazer et al., 2009). Such secretions consist of a complex mixture of nutrients, enzymes, growth factors, hormones, and transport proteins regulated by embryo-maternal crosstalk (Groebner et al., 2011; Bazer et al., 2012; Forde et al., 2014). Pregnancy establishment requires luteal progesterone, which stimulates uterine receptivity for conceptus implantation and development (Spencer et al., 2016), and the pregnancy recognition signal interferon tau (IFN-τ), which orchestrates luteotropic and immune mechanisms for successful embryo implantation (Roberts et al., 1992; Mann and Lamming 2001). Interferon tau is released by trophoblast cells and induces temporal changes perceptible in local and peripheral tissues (Binelli et al., 2001; Gifford et al., 2007; Pugliesi et al., 2014; Ruhmann et al., 2017). Accordingly, poor embryo signalling during this critical period may lead to

pregnancy loss (Garret et al., 1988; Matsuyama et al., 2012; Wijma et al., 2016). During this period of IFN-τ release, the antiviral genes, interferon-stimulated genes (ISGs), undergo short-lived activation (Roberts, 2007). In effect, maternal blood ISG profiles have proved to be an excellent tool to assess conceptus viability during the peri-implantation period in cattle (Green et al., 2010a; Matsuyama et al., 2012; Ahmad Sheikh et al., 2018).

The embryonic period of gestation extends from conception to the end of the differentiation stage (about 45 days) and the foetal period extends from Day 45 to parturition (Committee on Bovine Reproductive Nomenclature, 1972). Pregnancy rates in beef cattle are 50-60%, slightly higher than for dairy cattle (Bridges et al., 2013). However, in both beef and dairy herds, most pregnancy losses occur during the embryonic period (Sreenan and Diskin, 1983). While intrinsic factors in the embryo can reduce fertility (Lonergan et al., 2016), a suboptimal uterine micro-environment has been strongly linked to pregnancy failure (Bazer et al., 2015). Effectively, a large body of literature exists describing the deleterious consequences of maternal undernutrition during the earlier stages of pregnancy on conceptus and placental development (Long et al., 2009, 2010; Wang et al., 2015; Kruse et al., 2017; McLean et al., 2018; Taylor et al., 2018).

The main factors reported to affect the reproductive performance of suckler beef cows are those related to feeding management, calving season, dam breed and calf suckling frequency (Sanz et al., 2004). The present study examines the effects of subnutrition during the first third of pregnancy in two local beef breeds widely distributed in the Spanish Pyrenees, Parda de Montaña (PA) and Pirenaica (PI). The former breed arises from the ancient Brown Swiss cow and its crosses with autochthonous breeds, while PI is a hardy autochthonous breed. To determine the

effects of subnutrition we examined: 1) ISG expression in peripheral blood mononuclear cells (PBMCs) 18 and 21 days after artificial insemination (AI) in cows of the two breeds subjected to nutrient restriction from Days 1-82 post-AI; and 2) relationships between subnutrition and pregnancy failure, which was confirmed by ISG fold changes recorded on post-AI Days 18 and 21 and by plasma pregnancy specific protein B (PSPB) concentrations recorded on post-AI Day 28.

2. Materials and methods

2.1. Cattle and herd management

This study was performed at La Garcipollera Research Station in the mountainous area of the central Pyrenees (northeastern Spain, 945 m a.s.l.) from September 2014 to March 2015. Cows were recruited from a large experimental suckler cattle herd including both PA and PI breeds. A full description of the animals included in this study can be found in Noya et al. (2019). The study population was comprised of 53 healthy multiparous cows (7.5 ± 3.5 years) with suckling calves, 34 PA and 19 PI. Exclusion criteria were: mastitis, lameness, digestive disorders and pathological abnormalities of the reproductive tract detectable by ultrasonography.

2.2. Experimental design

All procedures were approved by the Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón. Dams were handled in strict accordance with the guidelines of the European Union (Directive 2010/63/E.U.) on the protection of animals used for experimental and other scientific purposes (E.U., 2010).

Dams were artificially inseminated after a nine-day progesterone (P4)-based synchronization protocol at 65 ± 14 days post-partum. Briefly, cows were treated with a P4 releasing intravaginal device (PRID-Delta, containing 1.55 g of P4; CEVA, Loudéac, France) plus GnRH (10 µg i.m. Busol, INVESA, Barcelona, Spain). Seven days later, animals were also given PGF2α (150 µg Galapán, INVESA, Barcelona, Spain). After a further 48 h, the PRID was removed and animals were injected with equine chorionic gonadotropin (500 IU Serigan, Laboratorios Ovejero, León, Spain). Forty-eight hours after this first injection, the cows received a second GnRH dose. Cows were randomly inseminated by an expert technician eight hours later using semen from corresponding bulls of proven fertility (4 PA and 3 PI).

On the day of AI, dams were randomly allocated to two dietary treatments for the first 82 days of gestation. Groups were fed a diet that met 100% of their energy and protein requirements for maintenance, lactation and gestation (10.9 and 10.0 kg dry matter (DM)/cow/day for PA and PI, respectively) (CONTROL; n= 23), or was restricted (SUBNUT; n=30) to 65% of requirements of both protein and energy (7.0 and 6.4 kg DM/cow/day for PA and PI, respectively) (Table 1). Live weight (559 ± 11 kg and 564 ± 12 for SUBNUT and CONTROL, P=0.7912) and BCS (2.82 ± 0.05 and 2.75 ± 0.06 for SUBNUT and CONTROL, P=0.3529) were similar in both groups on the day of AI. Feed was provided at 08:00 a.m. and cows were tied up for maximum 2 h until they finished the restricted amount assigned to each one. During the experiment, all cows and calves were loose housed. From day 83 post-AI until parturition all cows were fed complete rations. Dams were weighed fortnightly. The average daily gain (ADG) was calculated by linear regression. Dam BCS was registered monthly by two expert technicians, based on the estimation of fat covering loin, ribs, and tailhead.

Pregnancy diagnosis was performed by ultrasonography using a linear-array 7.5 MHz transducer (Aloka SSD-500V, Aloka, Madrid, Spain) on Day 37 post-AI and confirmed at 90 days post-AI. Late embryo mortality was assumed in dams classified as pregnant according to the ISG fold change produced from Day 18 to Day 21 that were diagnosed as non-pregnant by ultrasonography on Day 37 post-AI. This assumption was contrasted with embryo mortality predicted by PSPB concentrations on Day 28. Late embryo/foetal loss was recorded when the 90-day diagnosis proved negative.

2.3. Sample analysis

2.3.1. Blood sample collection

Blood samples were collected from each animal by tail vein puncture into EDTA vacuum tubes (BD Vacutainer™, Becton, Dickinson and Company, Plymouth, UK) on Days 18 and 21 post-AI for PBMC isolation (8 mL) and on Day 28 for plasma PSPB determination (4 mL). Samples were placed immediately on ice, and those for PSPB determination were centrifuged (3500 rpm for 20 min at 4ºC) within 30 min of collection and the plasma stored at −20°C until analysis.

2.3.2. Pregnancy specific protein B assay

Plasma PSPB concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit BioPRYN® (BioTracking Inc., Moscow, Russia) following the manufacturer's instructions. Assay sensitivity was 0.22 ng/mL. Intra- and inter-assay coefficients of variation respectively were: 6.3% and 10.4% for a plasma pool of 2.2 ng/mL, and 5.3% and 6.7% for a plasma pool of 1.3 ng/mL. Baseline levels calculated in 30 plasma samples from 10 non-pregnant cows were 0.34 ng/mL. Based on optical density (OD) standards for the assay, dams showing plasma PSPB

concentrations <0.6 ng/mL were recorded as non-pregnant, those with concentrations >1.1 ng/mL as pregnant and those with concentrations between 0.6 and 1.1 ng/mL as being at risk of pregnancy loss (Gabor et al., 2016).

2.3.3. PBMC isolation, RNA extraction and cDNA synthesis

Peripheral blood mononuclear cells were isolated by centrifugation on a Ficoll density gradient (Histopaque, Sigma, St Louis, MO) followed by repeated rinsing in phosphate buffered saline (PBS). Isolated mononuclear cells were lysed in Trizol™ (Invitrogen Corp., Carlsbad, CA, USA) and kept at −80ºC until RNA analysis. Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). RNA concentrations were determined spectophotometrically. Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Complementary DNA (cDNA) was synthesized in a total volume of 20 µL from 1 µg of total RNA in the presence of random primers and reverse transcriptase using the RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

2.3.4 Quantitative real-time PCR

Messenger RNA expression was determined by quantitative real-time PCR (qPCR) for four target genes—*interferon-stimulated gene 15 (ISG15), 20-50-oligoadenylate synthase 1 (OAS1)*, *myxovirus resistance 1 (MX1)*, and *myxovirus resistance 2 (MX2)*—and two reference genes—*β-actin (ACTB)* and *ribosomal protein L19 (RPL19)* (Table 2). To avoid gene contamination, all primers were selected to span an intron. For each gene, a standard curve was generated by amplifying serial dilutions of a control cDNA sample to check for linearity between initial template concentration

and cycle threshold (Ct) values. Amplification was conducted using the SYBR green method of the ABI PRISM^{TM} 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) under the conditions recommended by the manufacturer: an initial activation and denaturation step of 10 min at 95°C followed by 40 cycles consisting of 10 s at 95°C and 1 min at 60°C. PCR reactions were run using 3 μL of 30-fold diluted cDNA as template in a total volume of 8 μL containing 1× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA), and 200 nM of forward and reverse primers as reported elsewhere (Serrano-Pérez et al., 2016). Each PCR was run in triplicate and the average used to calculate the relative gene amount. Data were normalized and analysed by the $2^{-\Delta\Delta Ct}$ method using the mean Ct value obtained for the two reference genes and the Ct values for each ISG primer (Schmittgen and Livak, 2008).

2.4. Statistical analysis

The following data were recorded for each animal: parturition and AI date; dam age (two categories: 5-10 years old *vs.* more than 10 years old); breed (PA *vs.* PI), treatment (CONTROL *vs.* SUBNUT), *ISG15*, *OAS1*, *MX1* and *MX2* gene expression in PBMCs on Days 18 and 21 post-AI, plasma PSPB concentrations on Day 28 post-AI and pregnancy status (pregnant *vs.* non-pregnant) on Day 90 post-AI.

Data were analysed using the SAS and JMPro package (SAS Institute Inc., Cary, NC). When necessary, data were logit transformed to meet the assumption of normality and homoscedasticity. The normality of data was confirmed by the UNIVARIATE procedure ($P > 0.05$). Gene expression of ISG on Days 18 and 21 was analysed through analysis of variance using a general linear model (GLM) and dam age, breed, pregnancy

status, treatment and their interactions as fixed effects. Multiple comparisons among treatments were performed using Tukey's test.

The Spearman's rho (sr) test was used to identify possible relationships between ISG expression on Days 18 and 21 and plasma PSPB concentrations on Day 28. Decision trees were constructed to explore the importance of the four ISG to explain the pregnancy status using the partition modelling option in JMPPro. The partition algorithm searched all possible splits of predictors to best predict the response (pregnancy status). These splits (or partitions) of the data were done recursively to form a tree of decision rules. The variables that explain better the response were selected from the four ISG according to G2 (likelihood-ratio chi-square) test of association and logworth (-log(p-value)) value. The logworth values are the logs of adjusted p-values for the chi-square test of independence. Goodness of prediction of the decision three was obtained by a 3-fold cross-validation. Chi-square tests were used to assess associations between pregnancy failure (according to the ISG fold change produced from Day 18 to Day 21 and PSPB concentrations on Day 28) and factors of interest (age, breed and treatment). Data are expressed as the least square (LS) means. Significance was set at $P < 0.05$.

3. Results

Treatment affected significantly cow average daily gain (ADG; $P < 0.001$) and body condition score change (BCS; $P < 0.05$) from AI to day 82 post-AI. CONTROL cows gained weight and maintained BCS (+0.214±0.10 kg/d and +0.083±0.073 points BCS/month) whereas SUBNUT cows loss weight and BCS (-0.650±0.09 kg/d and -0.107 ±0.062 points BCS/month). The ADG and BCS change were similar in both

breeds with no treatment x breed interaction ($P = 0.6254$ and $P = 0.9090$ for ADG and BCS respectively).

Over the study course, 35 of the 53 cows (66%) enrolled became pregnant (n=14 CONTROL, n=21 SUBNUT). Of the remaining 18 non-pregnant dams (n=9 CONTROL, n=9 SUBNUT), 17 were open at the time of pregnancy diagnosis on Day 37, and one dam was diagnosed as suffering late embryo/foetal loss on Day 90. Mean plasma PSPB concentrations in dams diagnosed as pregnant were 2.35±1 ng/mL. In contrast, PSPB concentrations were under 1.1 ng/mL in 12 of the non-pregnant dams and just over 1.1 ng/mL (1.3±0.45) in the remaining six.

No significant effects were observed for treatment ($P = 0.36$), breed ($P = 0.06$) or dam age ($P = 0.30$) on pregnancy status. Plausible interactions such as breed x treatment or dam age x treatment were not detected.

3.1. Factors affecting ISG mRNA expression

No effects of maternal nutrient restriction or breed on ISG expression levels were observed. The factors found to significantly affect ISG expression were sampling day, pregnancy status and dam age. Levels of *MX1*, *MX2* and *ISG15* were significantly increased on Day 21 compared to Day 18 ($P < 0.001$ for all genes). Higher *MX2* and *ISG15* expression were observed in pregnant than non-pregnant cows ($P = 0.001$, $P < 0.001$, respectively). Expression levels of *OAS1* were higher and increased between Day 18 and 21 in the pregnant versus non-pregnant cows ($P < 0.001$) (Fig. 1). Dams aged 5 to 10 years old showed significantly higher expression of *MX1* than dams >10 years ($P < 0.05$) (data not shown).

3.2. Predicting embryo loss

Significant positive correlations were observed between Day 28 PSPB concentrations and Day 18 *MX1*, *MX2* and *ISG15* expression levels (sr: 0.64, sr: 0.45, sr: 0.65, respectively; $P \leq 0.001$) or Day 21 *OAS1* expression (sr: 0.59; $P < 0.001$).

The decision tree developed to predict pregnancy status on Day 37 included the factors fold changes in *MX1, MX2, OAS1* and *ISG15* expression from Days 18 to 21 post-AI. The final decision tree contains 2 nodes (Fig. 2). The combined cutoffs *OAS1* fold change > 3.91 and *MX1* fold change < 0.42 served to define pregnancy status (R^2=0.30, Fig. 2). *OAS1* fold change was the first variable able to discriminate between pregnant and non-pregnant dams. Dams exhibiting an increase of *OAS1* fold change higher that 3.91 between Days 18 to 21 were classified as pregnant. In the remaining cows, a node with dams exhibiting a decrease of *MX1* fold change higher than 2.38 (1/0.42) were also classified as pregnant. Out of the 36 dams classified as pregnant, 32 were confirmed as positive on Days 37 and 90. Four dams that fulfilled these thresholds but were diagnosed as non-pregnant on Days 37 and 90 belonged to the SUBNUT group (one PI and three PA dams), and were assumed as suffering pregnancy loss. Two of these dams also showed PSPB concentrations higher than 1.1 ng/mL, and one dam between 0.6-1.1 ng/mL. The remaining non-pregnant dams (*n*=14) were classified as non-pregnant in the partition analysis, although three of these dams showed PSPB concentrations between 0.6-1.1 ng/mL (n=1) or higher than 1.1 ng/mL (n=2) on Day 28 (Fig 2).

A significant association was observed between nutrition level and embryo loss according to ISG fold change (chi-square $P = 0.028$) or PSPB concentration (chi-square $P = 0.04$). In the group of cows whose nutrition was restricted (SUBNUT), embryo loss was more likely than in the control group (Table 3). Plausible interactions such as breed x embryo loss or dam age x embryo loss were not detected ($P > 0.05$).

4. Discussion

This study was designed to examine embryo-maternal crosstalk during the peri-implantation period in beef cows subjected to restricted nutrient intake (from Days 1 to 82 post-AI) by determining the relative expression of ISG in PBMCs on Days 18 and 21 post-AI. Our main findings were that: 1) maternal undernutrition affected neither ISG expression nor fertility, and 2) that, based on ISG fold changes and PSPB concentrations, pregnancy failure was more likely to occur in dams subjected to nutrient restriction.

During early gestation, nutrient demands for embryo growth are lower than in later stages. However, the developing conceptus requires an adequate nutritional uterine microenvironment for normal growth (Groebner et al., 2011; Crouse et al., 2016). The short-term consequence here of 65% nutrient restriction was unimpaired embryo signalling, as neither ISG expression nor fertility were affected. Despite dramatic effects of subnutrition on embryo growth during early stages of pregnancy (Long et al., 2009; Micke et al., 2010; Taylor et al., 2018), our results suggest that most dams were able to develop adaptive responses to guaranty blastocyst formation and pregnancy maintenance (Velazquez, 2015). Similar observations were made in beef heifers subjected to 50 days of 40% nutrient restriction (McLean et al., 2018). However, Kruse et al. (2017) found that 50-80% nutrient restriction for as little as 6 days post-AI in beef heifers gave rise to poorer quality embryos. Similarly, Dunne et al. (1999) detected a significantly reduced embryo survival rate when beef heifers were shifted from high to low herbage allowance for a 10-day period after AI. Older cows seem more capable of supplying nutrients to the conceptus during early pregnancy compared with younger

cows (Long et al., 2009; McLean et al., 2018; Taylor et al., 2018). Some authors argue that a more extended subnutrition period is needed for any perceivable deleterious effects on embryo development (McLean et al., 2018). However, changes in embryo metabolism under these circumstances may result in re-programming of foetal and post-natal development (Funston et al., 2010b). Recently, we observed long-term effects on hormone profiles in calves born from dams that were undernourished in early pregnancy (Noya et al., 2019).

Maternal undernutrition may influence fertility via direct effects on the reproductive tract (Hill et al., 1970; Kruse et al., 2017). However, it is unknown to what extent the embryo's metabolism can be downregulated before it becomes incompatible with life preservation (Leese, 2002). In the present study, as indicated by ISG expression and plasma PSPB levels, an increased risk of pregnancy failure was observed in the subnutrition group especially in PA dams. In dairy cattle, a negative energy balance has been shown to compromise oocyte quality (Snijders et al., 2000). In our experiment, as subnutrition commenced at AI, pregnancy failure in undernourished dams was unlikely due to poor oocyte quality. According to Wiltbank et al. (2016), varying body condition scores in dairy cows from calving to AI play a major role among numerous other factors in increasing the risk of pregnancy loss in the second month of gestation. However, the cows in our trial were fed according to their needs before AI such that no negative energy balance or dramatic change in BCS would be expected (similar mean body condition score changes close to 0 were observed in control and subnutrition group dams; data not shown). Despite the long-term carryover effect of a negative energy balance on reproductive performance (Diskin et al., 2016), post-AI maternal subnutrition seemed here to produce negative effects mainly at later stages of embryo development.

Our findings suggest that PI dams were more able to develop adaptive mechanisms that guaranteed embryo survival during dietary restriction than PA dams. Differences might be attributed to genetic background differences between these two cattle breeds (Villalba et al., 2000; Casasús et al., 2002; Sanz et al., 2003). These differences lead to a higher milk production potential and intake capacity of PA dams during the post-partum period (Casasús et al., 2002), but in consequence, to a higher susceptibility to calf suckling (Sanz et al., 2003; Álvarez-Rodríguez and Sanz, 2009). Interbreed differences have been also observed in the haematological profile response to undernutrition (Noya et al., 2019).

Lastly, as anticipated, the detection of ISGs in maternal blood emerged here as an excellent measure of embryo viability in cattle (Sheikh et al., 2018; Yoshino et al., 2018). Given the trophoblast origin of both IFN-τ and pregnancy associated glycoproteins, positive correlation was observed between PSPB concentrations on Day 28 and both *MX1*, *MX2* and *ISG15* expression on Days 18 and *OAS1* expression on Day 21. Probably, dams classified as non-pregnant with PSPB levels higher than 0.6 ng/mL suffered failure of pregnancy establishment. Decision trees are a reliable and effective decision making technique in medicine (Podgorelec et al., 2002). In our study, dams were classified as pregnant or non-pregnant according to a partition tree threshold based on ISG fold changes and differentiated according to various decision classes (treatment, breed and PSPB concentration). Since older cows seem to show a lesser response to implantation signals received from the conceptus (Green et al., 2010a), *OAS1* and *MX1* fold changes were found to be the best variables to discriminate pregnancy status in multiparous beef cattle. Previous reports have described the efficiency of OAS1 as biomarker to assess the presence of a viable conceptus in beef (Pugliesi et al., 2014) or dairy cattle (Green et al., 2010b).

5. Conclusions

As an overall conclusion, our findings indicate that maternal nutrient restriction in suckler beef cattle does not influence conceptus signalling during the peri-implantation period. However, pregnancy failure was more likely in dams subjected to nutrient restriction in early pregnancy.

Conflict of interest

None.

Acknowledgements

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Figure 1. Relative ISG expression levels recorded in peripheral blood mononuclear cells (PBMC) on Days 18 (1) and 21 (2) post-AI in cows diagnosed as pregnant or non-pregnant on Day 90 post-AI. Bars indicate the mean RQ value ± SEM. [a,b] Statistically significant differences when different letters among groups.

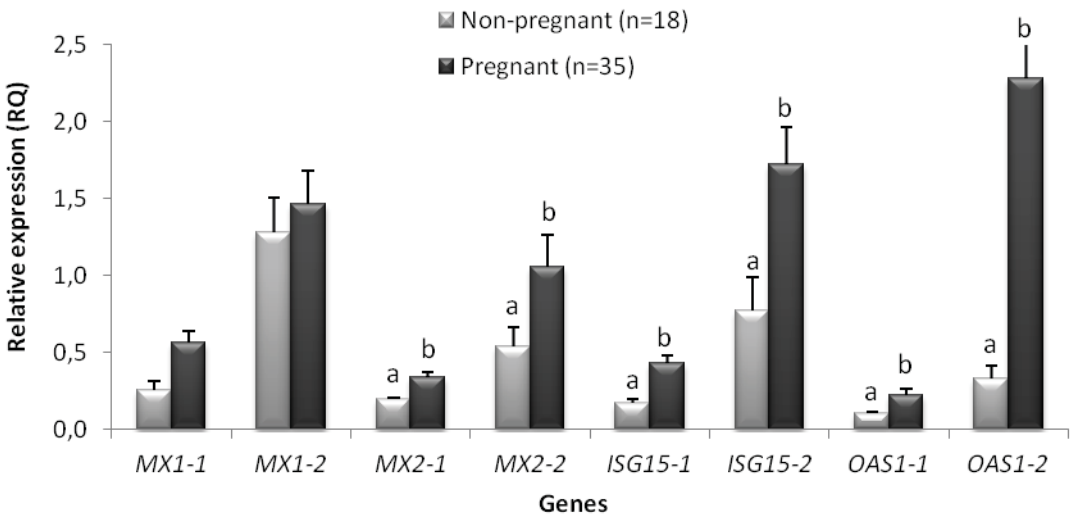
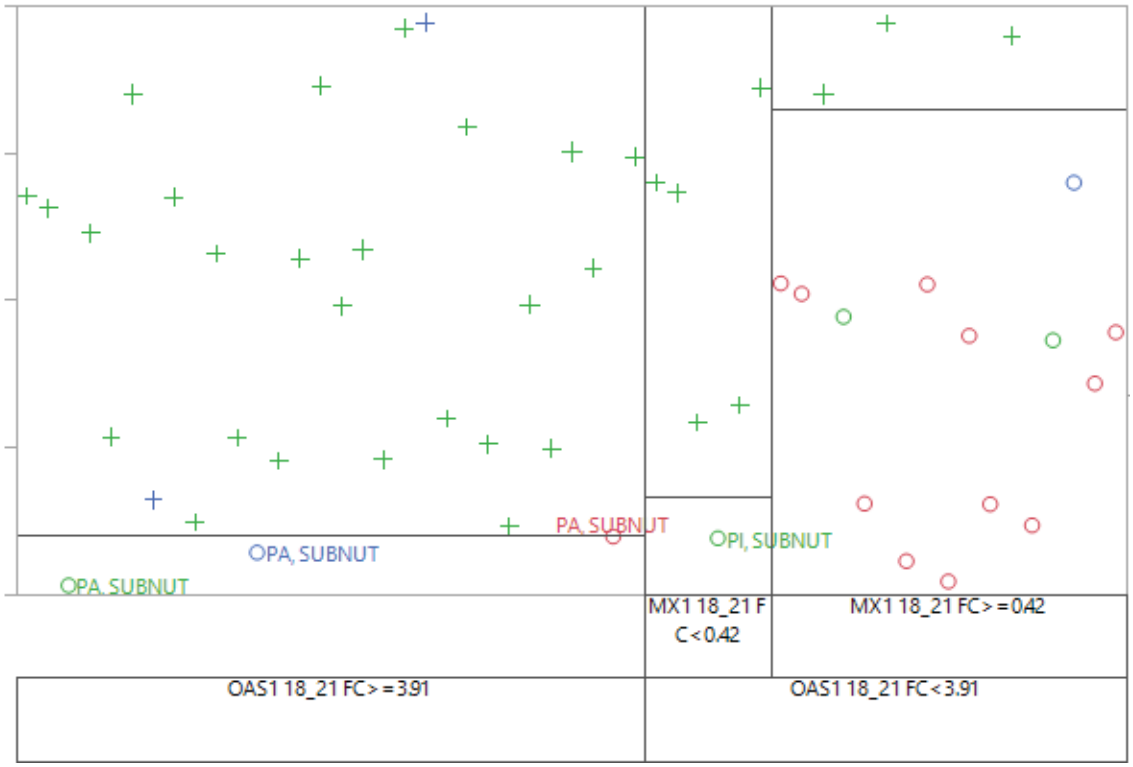


Figure 2. Binary partition tree. Pregnancy status on Day 90 post-AI represented in the x-direction (+: pregnant, o: non-pregnant) according to partition tree thresholds represented in the y-direction. Colour of point is related to cow's PSPB (ng/mL) on Day 28 post-AI: Red for ≤ 0.6; Blue for 0.6-1.1; Green for >1.1. FC: Fold Change: PA: Parda de Montaña, PI: Pirenaica. SUBNUT: diet restricted to 65% of protein and energy requirements during the first 82 days post AI.



1 Table 1. Ingredients of feedstuffs used in the experiment (on an as-fed basis)

Ingredients	% of dietary dry matter
Alfalfa hay	25.0
Cereal straw	25.0
Crushed barley	25.0
Dehydrated alfalfa	10.0
Rapeseed meal	6.5
Citrus pulp	4.5
Soybean meal	2.5
Correctors (calcium carbonate, dicalcium phosphate, sodium chloride, vitamins, and trace elements)	1.5

2

Table 2. Sequence, NCBI sequence and reference of the primers used for quantitative PCR.

Gene		Sequence (5' – 3')	NCBI sequence	Reference
ISG15	Fwd	CGCAGCCAACCAGTGTCT	NM_174366.1	Paradis et al. 2015
	Rev	CGTCATGGAGTCCCTCAGA		
OAS1	Fwd	TCATCCGCCTGGTGAAGCACTGG	NM_174366.1	Manjari et al. 2016
	Rev	TTGCTCCCAGGCATAGACCGTCAG		
MX1	Fwd	GTACGAGCCGAGTTCTCCAA	AF047692	Ribeiro et al. 2014
	Rev	ATGTCCACAGCAGGCTCTTC		
MX2	Fwd	CTTCAGAGACGCCTCAGTCG	NM_173941	Ribeiro et al. 2014
	Rev	TGAAGCAGCCAGGAATAGTG		
ACTB	Fwd	CTGGACTTCGAGCAGGAGAT	AY141970	Monteiro et al. 2014
	Rev	GATGTCGACGTCACACTTC		
RPL19	Fwd	GATCCGGAAGCTGATCAAAG	NM_001040516.1	Serrano-Pérez et al. 2016
	Rev	ATTCGAGCATTGGCAGTACC		

Table 3. Proportions of non-pregnant cows and cows suffering embryo loss as defined by fold changes in ISG (A) or concentrations of PSPB (B) according to whether nutrition was restricted (SUBNUT) or complete (CONTROL) within 82 days of AI.

A)	Nutrition	
ISG-defined status	CONTROL	SUBNUT
Non-pregnant	9 (100%)	5 (55.6%)
Embryo loss	0 (0%)	4 (44.4%)

B)	Nutrition	
PSPB-defined status	CONTROL	SUBNUT
Non-pregnant	8 (88.9%)	4 (44.4%)
Embryo loss	1 (11.1%)	5 (55.5%)