A negative energy balance during the peri-implantational period reduces dam IGF-1 but does not alter progesterone or pregnancy-specific protein B (PSPB) or fertility in suckled cows

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ABSTRACT

The aim of this study was to evaluate the effect of a negative energy balance during the first third of gestation on metabolic, endocrine and pregnancy recognition parameters in two beef cattle breeds adapted to semi-extensive conditions. Seventy-five lactating Parda de Montaña and 40 Pirenaica multiparous cows rearing calves were synchronized and timed artificial inseminated (TAI) on day 76 postpartum. Cows were assigned to one of two diets (CONTROL or SUBNUT; 100% or 65% of their requirements supplied) until day 82 of gestation. Pregnancy was diagnosed 37 days post-TAI using ultrasound. Blood samples were obtained to determine metabolic (glucose, NEFA, β-hydroxybutyrate, cholesterol and urea) and endocrine status (IGF-1) throughout the first third of gestation and to determine the concentrations of progesterone and pregnancy-specific protein B (PSPB) in the peri-implantational period. Undernutrition affected both cow and calf performance. The CONTROL cows maintained BCS and BW, whereas SUBNUT cows had negative daily gains. The CONTROL lactating calves had higher BW gains than SUBNUT. These negative effects were more evident in the Pirenaica breed, which was more sensitive to undernutrition. The negative energy balance was reflected in the cow metabolic profiles, with higher NEFA values and lower IGF-1 concentrations in SUBNUT cows.

However, undernutrition did not affect dam pregnancy/TAI or pregnancy recognition and maintenance, confirming that during periods of undernourishment, pregnant dams prioritize the allocation of dietary energy towards reproductive functions. Progesterone concentration on day 21 post-TAI (with a 4.8 ng/mL cutoff value) and PSPB on day 26 post-TAI (with a 0.57 ng/mL cutoff value) were determined as the earliest indicators to accurately establish dam pregnancy status, regardless of breed or nutrition treatment. In summary, early undernutrition affected cow performance and metabolic profiles and impaired lactating calf growth but did not affect progesterone or PSPB concentrations or the pregnancy/TAI rate in suckled cows.

Keywords: Malnutrition, Early-gestation, Pregnancy diagnosis, Metabolism, Calf performance

1. Introduction

Beef production systems are adapting to extensive conditions with the aim of reducing feed costs. This means that for long periods, cows will feed only on pastures or low-cost diets, which may compromise their nutritional status and reproductive performance. For instance, undernutrition during prepartum and/or postpartum periods negatively impacts pregnancy success and reproductive efficiency [1]. It is well established that when the nutrient requirements for maintenance and lactation exceed intake, fertility, embryo quality and viability rates are reduced. In fact, many metabolic and endocrine signals involved in reproductive processes are regulated by nutritional status [2]. A negative energy balance can impair the follicular development, the oocyte quality or the luteinizing hormone secretion, increasing the postpartum anestrus period [3]. Undernourishment following breeding can alter oviductal and uterine support for embryo growth, negatively impacting maternal embryo recognition and pregnancy maintenance. Similarly, alterations in hormone or metabolite concentrations, induced by changes in nutritional inputs, can also affect development of the early embryo and its ability to successfully trigger maternal recognition [4].

Embryo loss is a frequent occurrence that impairs dam efficiency, representing an important source of economic loss for livestock producers [5]. Early and accurate pregnancy detection is key to improve dam reproductive performance, since it allows the reduction of days open and thus the calving interval. Direct techniques such as transrectal palpation or ultrasonography are frequently used, providing an immediate diagnosis as early as day 35 and day 26 after breeding, respectively [6]; however, accuracy requires good technician skills. Indirect techniques, based on the detection of progesterone or pregnancy-specific proteins in cow plasma or milk, or the expression of interferon tau stimulated genes (ISGs) [7], are under development, but their precision and the earliest days when they can be applied remain unclear.

Furthermore, poor nutrition effects may elicit interbreed differences, since genetic background affects metabolic [8] and endocrine status. Parda de Montaña (PA) and Pirenaica (PI) are the two main beef cattle breeds adapted to the semi-extensive system in the Pyrenees mountain region (Northern Spain). Some interbreed differences have been reported in metabolic and hematologic profiles in response to differing managements, such as reduced granulocyte and mean corpuscular hemoglobin values in feed-restricted PI cows, but not in restricted PA cows [9], or reduced NEFA, total protein and urea plasma concentrations in PI, but not in PA cows, with restricted nursing periods [10].

We hypothesized that a negative energy balance during the peri-implantational period could be detrimental to dam pregnancy recognition and maintenance and, although interbreed differences have been reported, reproductive functions should not have been affected by the breed, provided they are crucial for the species survival. The aims of this study were to evaluate the effect of an energy-restricted diet during early gestation on performance, metabolic (glucose, NEFA, β-hydroxybutyrate, cholesterol and urea) and endocrine (IGF-1) status, pregnancy recognition and maintenance, and to establish the earliest day to use the pregnancy-specific protein B (PSPB) concentration as an accurate pregnancy test in PA and PI suckled cows.

2. Material and methods

All procedures were approved by the Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón. The care and use of animals were performed in accordance with the guidelines of the European Union on the protection of animals used for experimental and other scientific purposes [11].

2.1. Animals, management and diets

This study was conducted at CITA-La Garcipollera Research Station, in the mountain area of the central Pyrenees (Spain, 945 m a.s.l.). Seventy-five PA (560 ± 54.8 kg body weight (BW); 2.7 ± 0.03 body condition score (BCS) on a 5-point scale) and 40 PI (579 ± 54.9 kg BW; 2.9 ± 0.05 BCS) multiparous cows rearing a single calf were used for the study. The cows were synchronized to estrus at 65 ± 14 days postpartum with a protocol based on a progesterone-releasing intravaginal device (PRID Delta 1.55 g, CEVA, Loudéac, France) and a 10-µg injection of GnRH (Busol, INVESA, Barcelona, Spain), followed 7 days later by a 150-µg injection of prostaglandin $F_{2\alpha}$ (Galapán, INVESA, Barcelona, Spain). After 9 days, the PRID was removed and 500 IU of pregnant mare serum gonadotropin (Serigan, Laboratorios Ovejero, León, Spain) was administered, followed 48 h later by a second injection of GnRH (10 µg). Eight hours after the second GnRH injection, cows were randomly timed artificial inseminated (TAI) with sires of proven fertility (4 PA and 3 PI) by an expert technician. Pregnancy diagnosis to a single AI was performed by ultrasonography using a linear-array 7.5 MHz transducer (Aloka SSD-500V, Aloka, Madrid, Spain) on day 37 ± 2.5 post-TAI.

During the experiment, all cows and calves remained indoors in a loose housing system. After TAI (day 0), cows were group-fed and distributed into two maternal nutrition treatments with a total mixed ration (10.96 MJ ME/kg DM and 124 g CP/kg DM) (Table 1) during the first 82 days of pregnancy. The control group (CONTROL, n = 53) was fed a diet that supplied 100% of the estimated energy requirements for cow maintenance, lactation and gestation (10.9 and 10.0 kg DM/cow/d for PA and PI, respectively); and the nutrient-restricted group (SUBNUT, n = 62) received 65% of their requirements (7.0 and 6.4 kg DM/cow/d for PA and PI, respectively) for a 580-kg beef cow producing 9 kg (PA) or 8 kg (PI) of energy-corrected milk [12]. Groups were randomly balanced according to cow BW (565 ± 60.6 and 568 ± 50.9 kg for CONTROL and SUBNUT, respectively), BCS (2.8 ± 0.27 and 2.8 ± 0.29, respectively) and postpartum period (78 ± 12.0 and 74 ± 14.6 d, respectively) at TAI. Cows were supplied water and vitamin-mineral supplements (lick blocks) *ad libitum*. During the experiment, suckling calves had a restricted twice-daily nursing system and their diets consisted exclusively of milk.

2.2. Animal weight, BCS assessment and blood sample collection

Dams were weighed every two weeks and calves were weighed on day 0, 54 and 82 of the experimental period. The 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 (using a 1 – 5 scale). Blood samples were collected every two weeks for metabolic profiles; monthly for endocrine profiles; on days 14, 18, 21, 28, 42, 56, 69 and 82 post-TAI for plasma progesterone concentration; and on days 25, 26 and 28 post-TAI for PSPB concentration. Blood samples were collected before morning feeding by tail vessel puncture between the 6^{th} and 7^{th} coccygeal vertebrae. Samples to determine glucose, NEFA, β-hydroxybutyrate, cholesterol and PSPB concentration were collected into 10 mL tubes containing EDTA (BD Vacutainer, Becton-Dickenson and Company, Plymouth, UK). Samples to determine urea, IGF-1 and progesterone concentration were collected into 10 mL heparinized tubes (BD Vacutainer). After bleeding, samples were centrifuged at 1,500 x g for 20 min at 4ºC and plasma was stored at -20ºC until analysis.

2.3. Assays

An automatic analyzer (GernonStar, RAL/TRANSASIA, Dabhel, India) was used to measure blood concentrations of glucose (glucose oxidase/peroxidase method, sensitivity: 0.056 mmol/L); β-hydroxybutyrate (enzymatic colorimetric method, sensitivity: 0.03 mmol/L); cholesterol (enzymatic colorimetric method, sensitivity: 0.256 mmol/L); and urea (kinetic UV test, sensitivity: 0.170 mmol/L). The mean intra- and interassay coefficients of variation for these compounds were <5.4% and <5.8% respectively. Nonesterified fatty acids (NEFA, enzymatic method, sensitivity: 0.06 mmol/L) were analyzed using a commercial kit (Randox Laboratories Ltd., Crumlin Co., Antrim, UK). The mean intra- and interassay coefficients of variation were 5.1% and 7.4%, respectively. Insulin-like growth factor 1 (IGF-1, enzyme immunoassay, sensitivity: 20 ng/mL) was determined using a solid-phase enzyme-labeled chemiluminescent immunometric assay (Immulite, Siemens Medical Solutions Diagnostics Limited, Llanberis, Gwynedd, UK). The mean intra- and interassay coefficients of variation were 3.1% and 12.0%, respectively. Plasma progesterone concentration (ELISA test, sensitivity: 0.27 ng/mL) was measured using a specific kit for cattle (Ridgeway Science, Lydney, UK). The mean intra- and interassay coefficients of variation were 8.0% and 10.4%, respectively. Pregnancy-specific protein B (PSPB) (ELISA test, sensitivity: 0.25 ng/mL) was determined using a specific bovine kit (bioPRYN, Bio Tracking Inc., Moscow, Russia). The mean intra- and interassay coefficients of variation were <5%.

2.4. Statistical analysis

All statistics were calculated using SAS statistical package v 9.4 (SAS Institute Inc., USA). Normality of data was assessed with the Shapiro-Wilk test. Normality could not be confirmed for PSPB concentration, and therefore, it was expressed as a decimal logarithm for further analyses. The ADG of both dams and calves was analyzed using a general linear model (GLM procedure) with the breed (PA *vs.* PI) and nutritional treatment (CONTROL *vs.* SUBNUT) as fixed effects. In the case of cows, BW at TAI was added as a covariate, and in the case of calves, calf gender (male *vs.* female) was added as fixed effect. Pregnancy/TAI and embryo mortality rate were analyzed using a logistic regression model (LOGISTIC procedure) considering breed, nutritional treatment, ADG during the first month of subnutrition (from TAI to ultrasound scanning day), the cow BSC at TAI and the interval from the last calving to TAI as covariates. Embryo mortality was established in those dams that were diagnosed by ultrasonography as nonpregnant on day 37, but that presented one of these situations: (1) concentrations of progesterone on day 14 and PSPB on day 25 above the cutoff values proposed, (2) concentrations of progesterone on day 14 and PSPB on day 26 above the cutoff values, (3) concentrations of PSPB on days 25 and 28 above the cutoff values, or (4) concentrations of PSPB on days 26 and 28 above the cutoff values. Metabolites (glucose, NEFA, β-hydroxybutyrate, cholesterol and urea), IGF-1, progesterone and PSPB concentrations were analyzed using a mixed linear model (MIXED procedure) for repeated measures based on Kenward-Roger's adjusted degrees of freedom solution. The fixed factors were breed and nutritional treatment as the between-subject effects; sampling day as the within-subject effect; animal as the random effect (experimental unit) and the BCS at TAI as a covariate. In case of progesterone and PSPB concentrations, the pregnancy status (pregnant *vs.* nonpregnant) was considered as a fixed effect. Pregnancy/TAI and embryo mortality rate were analyzed using a logistic regression model (LOGISTIC procedure) considering metabolites (on days 0, 14 and 28) and IGF-1 (on days 0 and 28) as covariates. The least square (LS) means of the treatments were estimated per fixed effect, and pair-wise comparisons of the means were obtained by the probability of difference (PDIFF) option of the LS means procedure. Estimated cutoff values of progesterone and PSPB for diagnosing a dam as pregnant or nonpregnant were estimated using a linear logistic regression (LOGISTIC procedure), with breed and nutritional treatment as possible fixed effects. The Youden index was used to determine the sensitivity, specificity and the cutoff value of the proposed model. Relationships among the studied parameters were determined using Pearson's correlation coefficients. The level of significance for all tests was $P < 0.05$. The results are presented as LS means ± standard error.

3. Results

3.1. Animal performance

No breed effect was found for dam BW during the experiment ($P > 0.05$) but PI dams had higher mean BCS than PA dams (2.7 ± 0.03 *vs.* 2.9 ± 0.04 for PA and PI, respectively, $P < 0.001$). Cow BW and BCS were affected by an interaction between time and nutritional treatment ($P < 0.001$), BW and BCS from the second half of the experimental period being lower in the SUBNUT than in the CONTROL group, as shown in Figure 1. Throughout the experiment, cows in the CONTROL group maintained BW, whereas those in the SUBNUT group experienced a negative ADG (0.11 ± 0.031 *vs.* -0.37 ± 0.026 kg/d, respectively, $P < 0.001$). Regarding calf performance, an interaction effect of breed and nutritional treatment influenced ADG. Calves from PA-CONTROL and PI-CONTROL groups had greater weight gains than their counterparts (0.62 ± 0.020, 0.55 ± 0.020, 0.62 ± 0.034 and 0.44 ± 0.025 kg/d for PA-CONTROL, PA-SUBNUT, PI-CONTROL and PI-SUBNUT, respectively, $P < 0.05$). However, whereas no differences were found between CONTROL subgroups ($P > 0.05$), ADG was greater in PA-SUBNUT than in PI-SUBNUT calves ($P < 0.001$). No gender effect was found in the calf ADG (0.57 ± 0.017 *vs.* 0.55 ± 0.017 kg/d for male and female, respectively, $P > 0.05$).

3.2. Metabolic and endocrine profiles

Plasma concentrations of glucose, NEFA, β-hydroxybutyrate, cholesterol, urea and IGF-1, commonly associated with ruminant energy metabolism, were analyzed in order to characterize the nutritional status of suckled cows. Their profiles during the first third of gestation are displayed in Figure 2. Triple interaction effects of breed, nutritional treatment and sampling day affected both metabolite and IGF-1 concentrations ($P < 0.05$).

Glucose concentrations fluctuated over the course of the experiment. Glucose concentrations in PI-CONTROL cows were equal to or higher than those of their PI-SUBNUT counterparts, unlike PA-CONTROL cows, which had lower values than PA-SUBNUT cows at day 56.

In general, PI had higher NEFA concentration than PA throughout the experiment (0.24 ± 0.017 *vs.* 0.32 ± 0.024 mmol/L for PA and PI, respectively, $P < 0.05$). From the second half of the experiment, PI-SUBNUT had higher NEFA concentrations than PI-CONTROL on day 56 and 82 ($P < 0.05$), and PA-SUNBUT had higher NEFA values than PA-CONTROL from day 56 to the end of the experiment ($P < 0.05$). NEFA levels during the experiment were positively correlated with BCS at TAI, the highest correlations being observed on day 56 ($r = 0.39$, $P < 0.001$).

Few differences were found throughout the experimental period in β-hydroxybutyrate concentrations. PA-CONTROL on day 0 and 82 and PI-CONTROL on day 69 had higher values than their respective SUBNUT counterparts ($P < 0.05$).

Regarding cholesterol concentrations, no differences were found between PA-CONTROL and PA-SUBNUT cows throughout the experiment ($P > 0.05$), however, on days 28, 69 and 82 PI-SUBNUT had lower values than PI-CONTROL ($P < 0.05$). The evolution of cholesterol concentration during the experimental period was similar to that of glucose concentration, with a positive correlation on day 42 (r = 0.33, P < 0.001).

Similarly, no differences were found in urea concentrations between PA-CONTROL and PA-SUBNUT cows throughout the experimental period (P > 0.05), but PI-CONTROL cows had higher values than PI-SUBNUT cows on days 28, 56 (P < 0.05) and 69 (P < 0.001).

In general, CONTROL groups had higher IGF-1 concentrations than SUBNUT groups (82.7 ± 4.65 *vs.* 67.0 ± 3.99 ng/mL for CONTROL and SUBNUT, respectively, P < 0.05). Specifically, PA-CONTROL had higher values than PA-SUBNUT on day 82 (P < 0.01) and PI-CONTROL higher values than PI-SUBNUT on day 28, 56 and 82 (P < 0.05). A negative relationship between IGF-1 and NEFA concentration was found at AI time (r = -0.26, P < 0.01).

3.3. Progesterone and PSPB concentrations, pregnancy diagnosis and embryo mortality

Progesterone concentrations were affected by a triple interaction among nutritional treatment, pregnancy status and sampling day (Figure 3), but not by breed (P > 0.05). No differences were found in progesterone concentration between pregnant-CONTROL and pregnant-SUBNUT dams, or between nonpregnant-CONTROL and nonpregnant-SUBNUT throughout the experiment (P > 0.05). Pregnancy status affected progesterone concentration, pregnant cows having statistically higher values than their nonpregnant counterparts from day 21 to the end of the assay (P < 0.001). The estimated cutoff value of progesterone concentration to determine pregnancy status at each sampling day, as well as the sensitivity and the specificity each model, are presented in Figure 3. The earliest accurate cutoff value to diagnose gestation was 4.8 ng/mL on day 21 post-TAI, with an area under the curve (AUC) value of 0.93. On earlier days (14 and 18), the specificity was lower since the difference was not enough to discriminate the progesterone values from a gestational corpus luteum in a pregnant cow from a corpus luteum in the luteal phase in a nonpregnant cow (AUC = 0.66 and 0.77 for days 14 and 18, respectively). On day 28, the accuracy had slightly diminished (AUC = 0.91). Progesterone concentration from pregnant dams was quite constant from day 28 to the end of the experiment regardless of the breed and the nutritional treatment (7.1 ± 2.1 ng/mL).

A triple interaction effect of nutritional treatment, pregnancy status and sampling day affected the PSPB concentration (Figure 4). No differences were found between breeds ($P > 0.05$) neither between pregnant-CONTROL and pregnant-SUBNUT dams, or between nonpregnant-CONTROL and nonpregnant-SUBNUT dams throughout the experiment ($P > 0.05$). Pregnancy status affected PSPB concentration on days 26 and 28, with higher values in pregnant than in nonpregnant dams ($P < 0.001$). No statistical differences were found on day 25 between pregnant-CONTROL and nonpregnant-SUBNUT dams ($P > 0.05$). The estimated cutoff value to diagnose pregnancy status according to PSPB concentration, its sensitivity and its specificity are displayed in Figure 4. For pregnancy diagnosis at day 25, a 0.76 AUC value was obtained, but no cutoff value was proposed because of the overlap between pregnant and nonpregnant PSPB values. On days 26 and 28, the AUC values were 0.88 and 0.93, respectively, but no significant differences were found between these logistic models ($P > 0.05$). Thus, the first cutoff value obtained to diagnose pregnancy was 0.57 ng/mL on day 26 post-TAI. Concerning only pregnant dams, PSPB concentration increased over time ($P < 0.001$), with no breed or nutritional treatment effect ($P > 0.05$). A negative relationship was found between PSPB and progesterone concentrations in pregnant dams throughout the experiment. Specifically, the PSPB concentrations on day 26 were negatively related to progesterone concentrations on days 14 ($r = -0.41$, $P < 0.01$), 21 ($r = -0.29$, $P < 0.05$), 28 ($r = -0.37$, $P < 0.01$), 56 ($r = -0.45$, $P < 0.001$) and 82 ($r = -0.29$, $P < 0.05$), among others. Concentration of PSPB was also negatively correlated with IGF-1 on day 28 post-TAI ($r = -0.40$, $P < 0.001$).

The pregnancy rate obtained by ultrasonography 37 days post-TAI was 77% (89/115), with no breed (73 *vs.* 85%, for PA and PI) or nutritional treatment (71 *vs.* 82%, for CONTROL and SUBNUT) effect ($P > 0.05$). The ADG during the first month of the experiment, the cow BSC at TAI, the calving to TAI interval or the metabolite concentrations had not a significant effect on pregnancy rate ($P > 0.05$). Neither IGF-1 on day 0 was related with the fertility rate ($P > 0.05$), however, IGF-1 concentration on day 28 had a negative relationship with fertility rate ($P < 0.01$), the probability to be pregnant decreasing by 2.2% for each extra point of IGF-1.

Embryo mortality rate, diagnosed in 8 dams (8/97 possibly pregnant cows, according to their progesterone and PSPB concentrations), was not related to breed (5/60 PA and 3/37 PI) or nutritional treatment (2/40 CONTROL and 6/57 SUBNUT, $P > 0.05$). The ADG during the first month of the experiment, the cow BSC at TAI, the calving to TAI interval, metabolite and IGF-1 concentrations had not a significant effect on embryo mortality rate ($P > 0.05$).

4. Discussion

4.1. Animal performance

Nutritional restriction at 65% of cows' requirements over 82 days reduced BCS and BW throughout the study with no difference between breeds, indicating that the estimated requirements, specifically calculated for each breed, were well adjusted. The lower calf gains observed in SUBNUT groups resulted from the negative effects of feed restriction on dam milk yield and its protein concentration [13]. However, whereas no differences were found between CONTROL subgroups, PA-SUBNUT calves had higher gains than PI-SUBNUT calves, suggesting that nutritional restriction in PI dams may more severely impair milk yield and/or composition.

4.2. Metabolic and endocrine profiles

Circulating glucose is an indicator of energy balance that shows a strong dependence on the current energy and protein intake at a given time [14]. In our study, CONTROL groups had higher or equal values than SUBNUT groups in most of cases. Similarly, Richards et al. [15] found lower glucose concentrations in restricted cows compared to cows fed at maintenance after 30 weeks.

A negative energy balance increases plasma NEFA concentration as a consequence of fatty acid release from adipose tissue. In the current study, SUBNUT cows had higher NEFA concentrations than CONTROL cows from the second half of the experiment. Pirenaica cows had higher NEFA concentrations than PA, which was related with their higher BCS during the experiment.

Ketogenesis increases blood glucose concentrations when glucose becomes scarce and glycolysis falls to very low levels [16]. In the current study, despite the greater fat tissue mobilization in SUBNUT cows, few differences were found in β-hydroxybutyrate concentration between CONTROL and SUBNUT groups. This implies that β-hydroxybutyrate, which is the predominant circulating ketone body, was not the main energy source used by SUBNUT groups. The mobilization of NEFA from adipose tissue is not associated with concomitant increases in their oxidative metabolite (β-hydroxybutyrate) [10].

Cholesterol is related to glucose concentration [17], with both metabolites indicating a positive energy balance. Accordingly, a positive relationship between them was found in our study. Furthermore, PI-CONTROL had greater cholesterol concentrations than PI-SUBNUT, whereas no differences were found between PA subgroups, highlighting the greater sensitivity to undernutrition of the PI breed.

Blood urea is a good indicator of the protein status of the animal, directly related to degradable protein intake, but also to the catabolism of body protein in periods of energy shortfall [18]. Blood urea concentrations have long been known to reflect inefficient utilization of dietary CP by ruminants [19]; i.e., blood urea concentration increases in a cow fed excess dietary protein. In our experiment, CONTROL groups had equal or higher urea concentrations than SUBNUT groups, mostly in PI breed, reflecting their greater CP intake.

In the current study, PI-CONTROL dams had the highest IGF-1 concentrations, whereas PI-SUBNUT concentrations were similar than that obtained in PA groups. The differences between PA-CONTROL and PI-CONTROL cows contrasts with other experiments where IGF-1 differences between these breeds were not found [10,18]. Nutrient intake is positively related to IGF-1 concentration [20], and accordingly IGF-1 concentration was higher in CONTROL than in SUBNUT cows, with negative correlation between NEFA and IGF-1 concentration. At parturition, six months after the nutrient treatment was finished, calves born from CONTROL cows had also higher IGF-1 blood concentration than those from SUBNUT cows [9] , highlighting the maternal-embryo cross-talk and its role in embryonic and fetal development.

4.3. Progesterone and PSPB concentrations, pregnancy diagnosis and embryo mortality

Progesterone plays a central role in the establishment of uterine receptivity to the embryo and drives conceptus elongation through molecular changes induced in the endometrium [21]. A negative energy balance is detrimental for the early growth of ovarian follicles and after ovulation, progesterone secretion of the corpus luteum can be reduced [22]. In the current study, nutritional treatment did not affect progesterone concentration between pregnant dams, allowing for the maintenance of pregnancy in both CONTROL and SUBNUT cows. On the contrary, other studies have described an inverse relationship between energy intake and systemic progesterone concentration. High energy intake increases metabolic rate and the blood flow through the liver, resulting in an increased clearance rate of progesterone [23,24]. Accordingly, Nolan et al. [25] found 25% lower progesterone concentrations in heifers fed a high *vs.* a low-energy diet.

In our experiment, day 21 was determined to be the earliest accurate day to diagnose pregnancy status based on progesterone concentration, with a 4.8 ng/mL cutoff value and both high sensitivity and specificity values. In agreement with our results, Otavă et al. [26] found that in pregnant cows the progesterone levels increase continuously up to day 21 postfertilization and established the progesterone levels between days 18 and 24 as an indirect method for pregnancy diagnosis. Similarly, Humblot [27] established a combination of <3.5 ng/mL on day 0 and >5 ng/mL on days 21 – 24 as criteria to diagnose a dam as pregnant.

Pregnancy-specific protein B, formerly known as pregnancy-associated glycoprotein 1 [28], is a glycoprotein synthesized by the binucleate trophoblastic cells of the bovine placenta [29]. Unlike progesterone, PSPB is a specific pregnancy signal induced as a result of the presence of a conceptus [30]. According to Humblot [27], PSPB concentrations rise from days 15 to 35 to reach 2 to 3 ng/mL at this stage, the critical period for maternal recognition of pregnancy taking place between days 15 and 18 of gestation [28]. The earliest day when the PSPB pregnancy test can yield accurate and consistent results remains unclear, with estimates ranging from day 24 postconception [30], 25 [31], 28 [32] to day 30 [33]. Nevertheless, PSPB clearance from circulation during the postpartum period is extremely slow [34], involving the persistence of high peripheral PSPB concentrations in postpartum cattle. In the current study, the day 25 blood sample was taken on day 100.8 ± 13.5 after parturition, consistent with the manufacturer’s instructions (more than 73 days since last calf). However, residual PSPB concentrations in nonpregnant dams on day 25 did not permit the determination of an accurate cutoff value to diagnose pregnancy. The low metabolic rates of beef compared to dairy cattle might have delayed the clearance of the residual PSPB from the last gestation. The PSPB concentration on day 26 yielded a 0.57 ng/mL cutoff value, with both high sensitivity and specificity and similar accuracy to that from day 28. This suggests that in our conditions, day 26 was the earliest day to diagnose pregnancy, regardless of the nutritional treatment or breed.

Surprisingly, the PSPB concentration was negatively correlated with progesterone values from the critical period of days 15 – 18 until day 82 post-TAI. We hypothesized that higher PSBP concentration may compensate for lower progesterone production, due to the response by the trophoblastic cells to establish a stronger maternal-embryo cross-talk to permit maternal recognition and ensure the maintenance of gestation. Humblot et al. [35] found negative but nonsignificant correlations between circulating progesterone on day 24 and PSPB on days 24, 26 and 30-35 and therefore concluded that there was no relationship between them in pregnant animals. Similarly, López-Gatius et al. [36] discounted any potential involvement of progesterone with pregnancy-associated glycoproteins from the placenta or *vice versa*. However, Ayad et al. [37] observed that pregnancy-associated glycoproteins tended to be higher in pregnant females with higher progesterone concentrations. Additional research is needed to determine the role of PSPB in the maternal recognition of a viable conceptus and in pregnancy maintenance, which is not yet fully understood.

In the current study, 77% of cows were pregnant at the TAI, a higher pregnancy rate than other studies using similar synchronization protocols [18,38,39], regardless of the breed or the nutritional treatment, probably because of their optimal BCS at TAI. Nutrition determines cow BW and BCS, which underpin fertility rate in postpartum cows [40]. In the current study, despite the SUBNUT group being in a negative energy balance after TAI, these cows' optimal BCS at TAI allowed the conception and maintenance of gestation. Keady et al. [41] found similar fertility between a control group fed with ad libitum grass silage as the sole diet and a group supplemented with 5 kg/d of concentrate during late gestation. Contrastingly, Perry et al. [42] found that post-AI supplementation improved pregnancy success and Fontes et al. [43] reported an increased pregnancy failure rate associated to a nutrient restriction during early gestation. Metabolite concentration during the first month of the experiment and IGF-1 concentration on day 0 were not related with the pregnancy/TAI rate. Surprisingly, lower IGF-1 concentrations on day 28 were associated with higher pregnancy success. High plasma IGF-1 concentration at TAI has been described as a useful predictor of reproductive success in cattle [23]. Taylor et al. [44] reported that cows with plasma IGF-1 values greater than 50 ng/mL at first service exhibited a five-fold increase in likelihood of conception and Moyes et al. [45] found that plasma IGF-1 concentrations in pregnant cows were numerically higher than those of nonpregnant cows after conception; however, these differences were not significant until 15 weeks postconception. On the other hand, Falkenberg et al. [46] found no significant differences in IGF-1 concentration between cows that conceived at the first AI, in later services, or in cows that did not become pregnant. In the current study, no IGF-1 effect on pregnancy/TAI rate was found at day 0. At that moment, all cows had an optimal BCS and the IGF-1 concentration of all groups was above the threshold before reproduction is adversely affected [47], which implies that IGF-1 concentration did not determine the reproductive performance. From day 0 onwards, due to the nutritional treatment, IGF-1 concentration in SUBNUT cows started to decrease, specially in PI breed. Although the differences in pregnancy/TAI rate between CONTROL and SUBNUT cows were not significant, 57% of pregnant dams belonged to SUBNUT group, while 58% of nonpregnant dams belonged to CONTROL group. That could be the reason why on day 28 pregnant dams (most from SUBNUT group) had lower IGF-1 concentration than nonpregnant dams (most from CONTROL group). Our hypothesis is that despite these lower values at the onset of pregnancy, according to Moyes et al. [45], IGF-1 concentration of pregnant dams increases above that of nonpregnant dams as gestation proceeds.

In our study, an 8% embryo mortality rate was reported. Although fertility rates are usually high in beef cattle, pregnancy outcome may decrease due to embryo losses, which can account for up to 29 - 39% of pregnancies after fertilization, most of them between days 8 and 16 after insemination [48]. Nutritional and metabolic status of the cow can affect embryonic development and survival [2]. In beef heifers Dunne et al. [24] found that a short-term (2 weeks) reduction in energy intake after AI severely reduced embryo survival rates by 41%, but Doyle et al. [23] reported no effect of postinsemination plane of nutrition. In the current study, SUBNUT cows had higher embryo loss rates than their CONTROL counterparts, but the difference was not significant, probably due to the low incidence of embryo losses. Therefore, more studies are needed to assess the impact of the negative energy balance on embryo mortality in adult beef cows.

Therefore, in our study undernutrition during the first third of pregnancy did not impair the cow reproductive performance and allowed to establish and maintain the gestation. A 65% energy restricted diet was a severe feed restriction, reflected in most of the metabolites and IGF-1 SUBNUT cow profiles. Nevertheless, at the beginning of the experiment all cows had an optimal BCS to face this nutritional challenge. Animals with lower BCS and a worse metabolic status at the beginning of the study would possibly have obtained a worse reproductive performance. Fernández-Foren et al. [49] affirmed that initial body reserves determine the endocrine response to undernutrition. As in our experiment, undernourished animals with optimal initial BCS developed compensatory mechanisms against adverse environmental factors, counteracting the negative effects caused by a food restriction on reproduction. However, it is interesting to highlight that in our study, although the reproductive performance was not initially affected by undernutrition, an altered maternal environment compromised the fetal programming with long-term consequences in the newborns [9].

4.4. Conclusions

A restrictive diet during the first 82 days after TAI induced a negative energy balance in suckled cows, reflected in higher NEFA and lower IGF-1 concentrations, which affected dam performance and impaired calf growth. These negative effects were more evident in the PI breed, which was more sensitive to feed restriction.

Undernutrition did not affect dam pregnancy recognition, maintenance of gestation or pregnancy/TAI rate, confirming that pregnant dams cope with undernourishment by prioritizing the allocation of dietary energy towards reproductive functions.

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

Ingredients	
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
Chemical composition	
DM (g/kg)	908 ± 5.8
CP (g/kg DM)	124 ± 10.2
NDF (g/kg DM)	466 ± 34.8
ADF (g/kg DM)	253 ± 25.1
ADL (g/kg DM)	40 ± 4.7
Ash (g/kg DM)	113 ± 15.3
ME (MJ/kg DM)	11 ± 0.4

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid-detergent lignin; ME, metabolizable energy.

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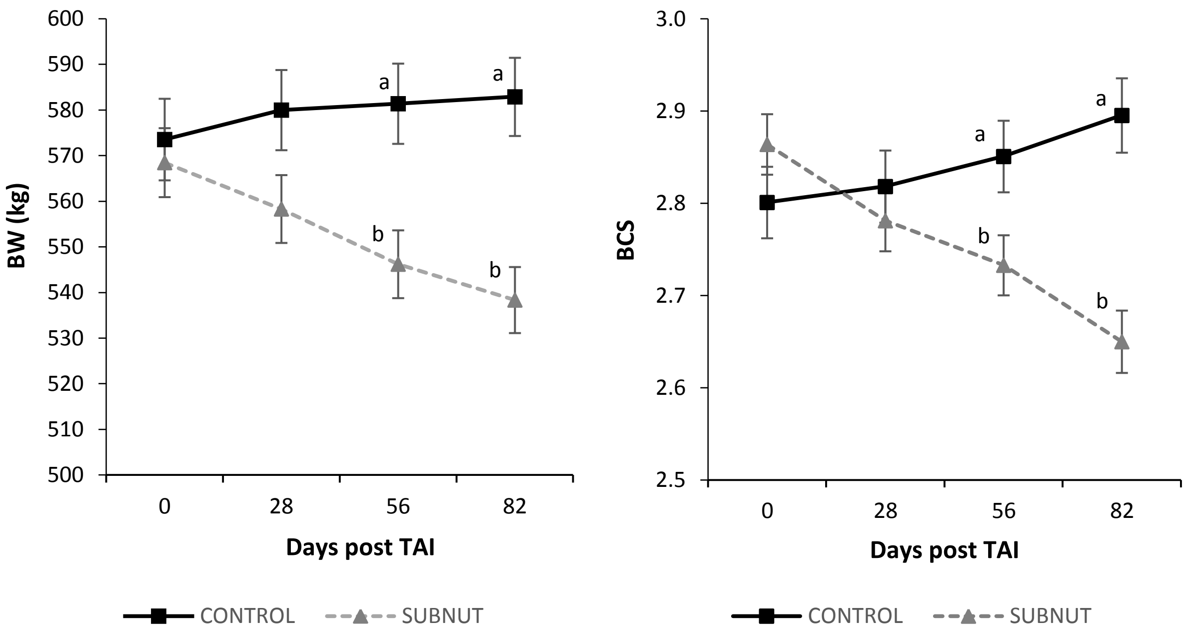
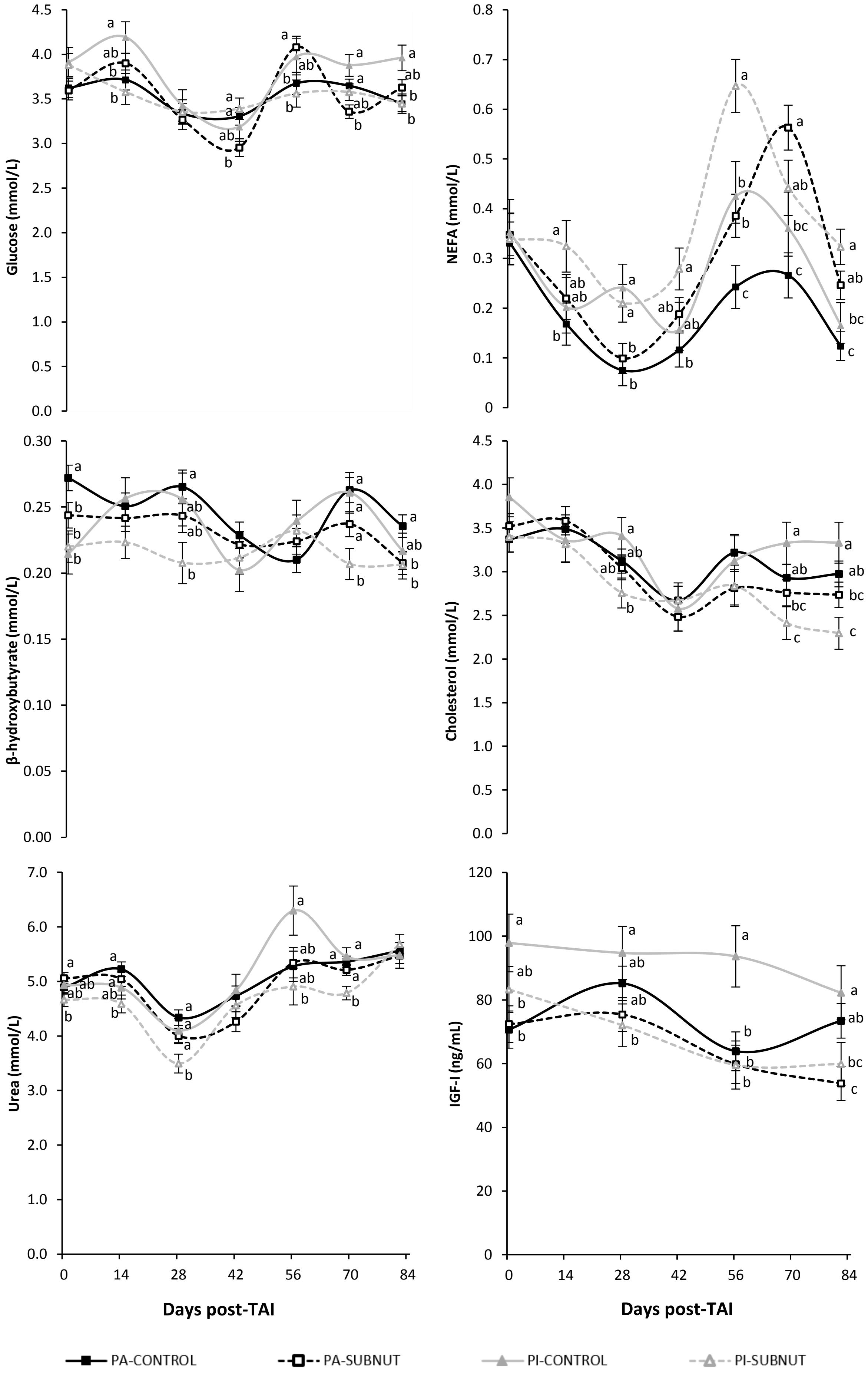
Figure 1. Body weight (BW) and body condition score (BCS) after TAI of suckled cows according to the nutritional treatment. [a-b], means at a given time with different superscripts differ significantly (P < 0.05); CONTROL, dams fed 100% of their nutritional requirements from day 0 to day 82 of pregnancy; SUBNUT, dams fed 65% of their nutritional requirements from day 0 to day 82 of pregnancy.

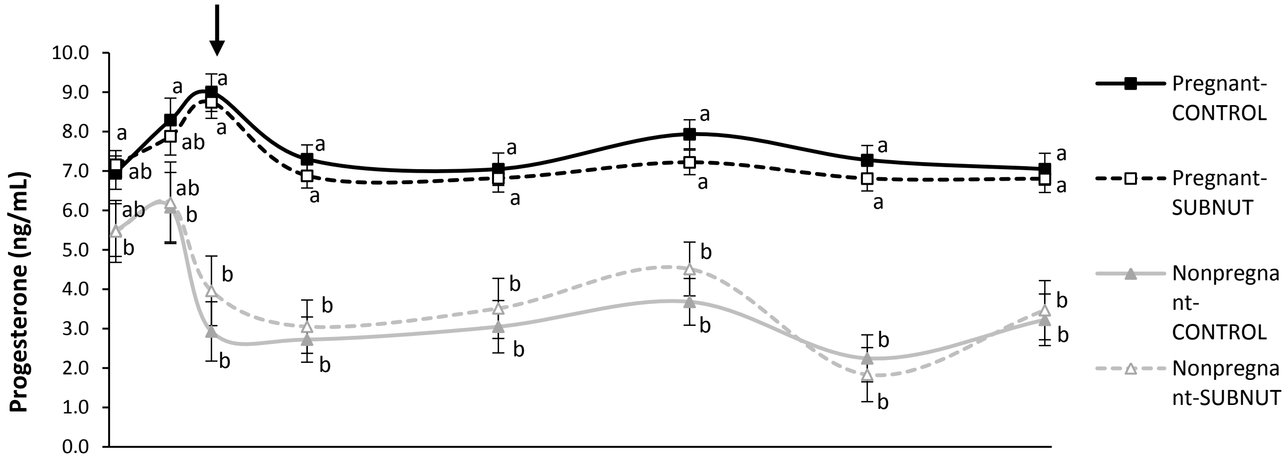
Figure 2. Plasma concentrations of glucose, NEFA, β-hydroxybutyrate, cholesterol, urea and IGF-1 after TAI of suckled cows according to the breed and the nutritional treatment. [a-c], means at a given time with different superscripts differ significantly (P < 0.05); PA, Parda de Montaña; PI, Pirenaica; CONTROL, dams fed 100% of their nutritional requirements from day 0 to day 82 of pregnancy; SUBNUT, dams fed 65% of their nutritional requirements from day 0 to day 82 of pregnancy.

Figure 3. Progesterone concentrations after TAI of suckled cows according to nutritional treatment and pregnancy status. [a-b], means at a given time with different superscripts differ significantly (P < 0.05); CONTROL, dams fed 100% of their nutritional requirements from day 0 to day 82 of pregnancy; SUBNUT, dams fed 65% of their nutritional requirements from day 0 to day 82 of pregnancy; the arrow marks the earliest day for an accurate diagnosis based on progesterone concentration.

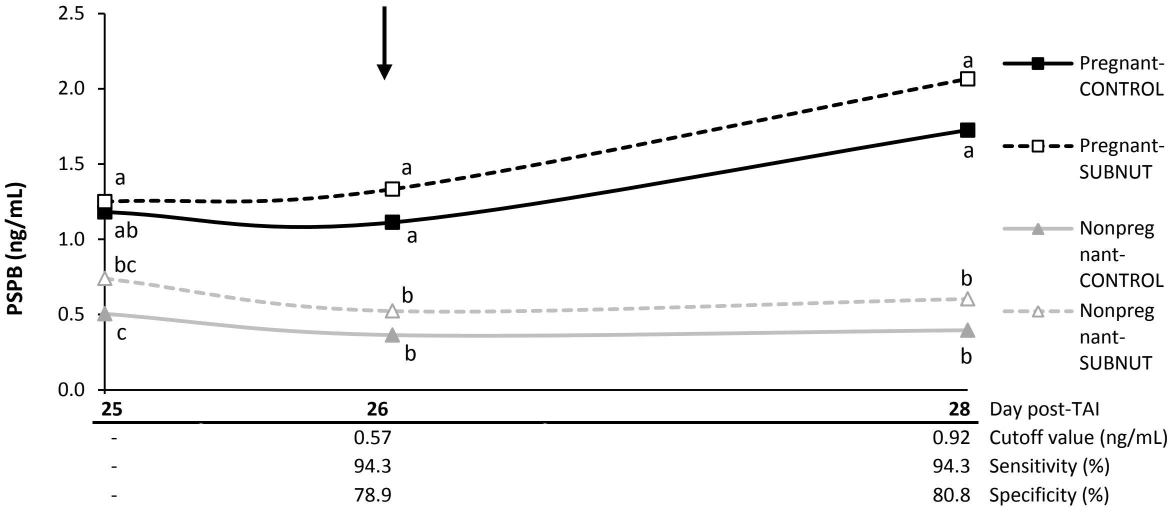
Figure 4. Pregnancy-specific protein B (PSPB) concentrations after TAI of suckled cows according to nutritional treatment and pregnancy status. [a-c], means at a given time with different superscripts differ significantly (P < 0.05); CONTROL, dams fed 100% of their nutritional requirements from day 0 to day 82 of pregnancy; SUBNUT, dams fed 65% of their nutritional requirements from day 0 to day 82 of pregnancy; the arrow marks the earliest day for an accurate diagnosis based on PSPB concentration.


600
590
580
570
560
550
540
530
520
510
500
BW (kg)
a
a
b
b
0
28
56
82
Days post TAI
CONTROL
SUBNUT
3.0
2.9
2.8
2.7
2.6
2.5
BCS
a
a
b
b
0
28
56
82
Days post TAI
CONTROL
SUBNUT


Glucose (mmol/L)
NEFA (mmol/L)
β-hydroxybutyrate (mmol/L)
Cholesterol (mmol/L)
Urea (mmol/L)
IGF-I (ng/mL)
Days post-TAI
Days post-TAI
PA-CONTROL
PA-SUBNUT
PI-CONTROL
PI-SUBNUT



14	**18**	**21**	**28**	**42**	**56**	**69**	**82**	Day post-TAI
3.1	7.1	4.8	4.1	4.1	6.1	3.6	4.8	Cutoff value (ng/mL)
100	65	98	93	97	79	98	87	Sensitivity (%)
19	73	81	77	64	84	84	68	Specificity (%)


2.5
2.0
1.5
1.0
0.5
0.0
PSPB (ng/mL)
a
a
a
a
ab
a
bc
b
b
c
b
b
Pregnant-CONTROL
Pregnant-SUBNUT
Nonpregnant-CONTROL
Nonpregnant-SUBNUT
25
26
28
Day post-TAI
- 0.57 0.92 Cutoff value (ng/mL)
- 94.3 94.3 Sensitivity (%)
- 78.9 80.8 Specificity (%)

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