

## Article

# Effects of Late-Gestation Nutritional Restriction and Hydroxytyrosol Supplementation on Behavioural Responses and Neuroendocrine Blood Markers in Beef Cows and Their Calves

Nieves Escalera-Moreno <sup>1,\*</sup>, Beatriz Serrano-Pérez <sup>1,2,\*</sup>, Isabel Blanco-Penedo <sup>1,2,3</sup>, Leire López de Armentia <sup>4</sup>, Agustí Noya <sup>1,2</sup>, Albina Sanz <sup>4</sup> and Javier Álvarez-Rodríguez <sup>5</sup>

<sup>1</sup> Animal Science Department, University of Lleida, Av. Rovira Roure 191, 25198 Lleida, Spain; isabel.blancopenedo@udl.cat (I.B.-P.); agusti.noya@udl.cat (A.N.)

<sup>2</sup> Agrotecnio-CERCA Center, University of Lleida, 25198 Lleida, Spain

<sup>3</sup> Veterinary Epidemiology Unit, Department of Clinical Sciences, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden

<sup>4</sup> Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) (IA2-UNIZAR), Avda. Montañana 930, 50059 Zaragoza, Spain; llopezdearmentia@cita-aragon.es (L.L.d.A.); asanz@aragon.es (A.S.)

<sup>5</sup> Departamento de Producción Animal y Ciencia de los Alimentos, Escuela Politécnica Superior de Huesca, Universidad de Zaragoza-IA2, Carretera de Cuarte s/n, 22071 Huesca, Spain; javier.alvarezr@unizar.es

\* Correspondence: mariadelasnieves.escalera@udl.cat (N.E.-M.); beatriz.serrano@udl.cat (B.S.-P.)

## Abstract

Maternal nutrition during late gestation is critical for fetal development, neonatal resilience, and postnatal adaptation in beef cattle. This study aimed to evaluate the effects of nutritional restriction and supplementation of hydroxytyrosol (HT) in late pregnancy on behavioural, circadian, stress-related, and inflammatory responses in cows and their restricted nursed offspring. Pregnant cows were allocated to a 2 × 2 factorial experimental design (feeding level: T100% vs. T60% of nutrient requirements; HT: 0 vs. 180 mg/kg of diet). Cow behaviours were recorded during meals (from week –12 prepartum to term), and calf activities, body temperature, and mother–offspring interactions were assessed at 5 weeks postpartum. Nutritional restriction accelerated feed intake in cows and increased stress-related behaviours, while HT partially mitigated these effects. Molecular analyses in blood samples revealed dynamic prepartum upregulation of glucocorticoid-receptor *NR3C1* in week –6, and downregulation of circadian (*BMAL1*, *PER1*, *CRY1*) gene expression in week 5 after parturition, both in T60%-HT cows. In calves, maternal HT supplementation promoted active exploratory behaviour, and counteracted behavioural and circadian (*CRY1* and *PER1*) and inflammatory markers (*IL8*) gene expression resulting from prenatal nutrient restriction, leading to behavioural profiles and blood gene expression comparable to those observed in calves born to adequately fed dams.

**Keywords:** animal behaviour; clock genes; fetal programming; stress-related behaviour; time-budget; neonatal calves

Academic Editors: Sameer J. Mabweesh and József Rátky

Received: 16 February 2026

Revised: 24 March 2026

Accepted: 9 April 2026

Published: 12 April 2026

**Copyright:** © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\) license](https://creativecommons.org/licenses/by/4.0/).

## 1. Introduction

In recent years, beef cattle production systems have faced the challenge of maintaining productivity and animal welfare in a context of increasing nutritional uncertainty. Climate change has intensified the variability in forage availability in extensive livestock systems, directly impacting cattle feed and compromising the productive stability of these systems [1]. Late gestation constitutes a critical developmental window during which dams experience elevated metabolic demands to support fetal growth, mammary gland development, and the energy requirements of impending lactation. This homeorhetic shift includes increased lipolysis, intensified hepatic gluconeogenesis, and adaptive modulation of neuro-endocrine-immune pathways [2,3]. Concurrently, the maternal circadian system, driven by clock genes that control the circadian oscillation of physiological and molecular processes, orchestrates temporal patterns of catabolic and anabolic processes in cell systems, ensuring that energy mobilization aligns with environmental cues and physiological demands [4–7]. During the last third of gestation, cows may exhibit a reduction in relative appetite due to decreased abdominal cavity space as fetal growth progresses [8,9]. A slight decrease in activity may also be observed, accompanied by calmer social behaviour, occasional episodes of isolation, and increased resting periods [10].

Nutrient restriction during this period imposes metabolic stress, characterized by intensified catabolic activity, which is closely interconnected with immune-metabolic regulation [11,12]. Excessive energy mobilization, in addition to circadian fluctuations throughout the day [13], triggers hormonal and molecular signals that modulate glucocorticoid sensitivity, leading to disrupting oxidative homeostasis [14–16]. Nutritional restriction also perturbs the molecular circadian system, attenuating the amplitude or shifting the phase of clock gene expression in tissues such as immune cells. Collectively, these alterations can modify behavioural rhythms, providing a visible manifestation of these underlying physiological challenges, reflecting adaptive behavioural strategies to maintain homeostasis under limited resource availability [5,17–20]. Maternal nutritional restriction during late gestation has been shown to influence multiple aspects of neonatal development. Fetal exposure to an altered metabolic and inflammatory environment can modify organ development, endocrine regulation, energy allocation, and behavioural reactivity after birth [21,22]. In addition, programming of neonatal clock gene expression and functionality may affect circadian control of metabolism and thermoregulation, contributing to the variability observed in neonatal physiological states and coping strategies [6,11,22].

Among the strategies aimed at mitigating the consequences of gestational nutritional restriction, the use of natural antioxidants has garnered increasing interest [23,24]. Hydroxytyrosol (HT), a phenolic constituent commonly found in olive-derived products, exhibits a potent and rapid antioxidant capacity [25,26]. In mammals, HT has been shown to reduce oxidative damage, preserve lipid integrity, and influence energy metabolism [27–29]. Emerging evidence also suggests that polyphenols can influence circadian regulatory mechanisms by modulating clock gene expression and restoring rhythmicity in different tissues [30,31]. In ruminants, it has additionally been demonstrated that the inclusion of diet polyphenols influences feeding times and stress-related behaviours, showing manifestations consistent with an improvement in animal welfare [32,33].

Previous work by our group demonstrated that nutritional restriction during the last third of pregnancy reduced glucose oxidation and lipid synthesis in blood cells of pregnant cows, while HT supplementation improved glucose transport and increased the total antioxidant capacity of these cells after calving. In calves, maternal HT supplementation strengthened antioxidant defences and modulated genes related to the immune response, especially in those from mothers with nutritional restriction [29]. This study aimed at evaluating the impact of nutritional restriction and supplementation of HT during the last

third of pregnancy on activity and behavioural responses in gestating cows and cow–calf pairs during lactation. It also examined physiological and performance parameters in lactating calves, as well as the expression in blood samples of genes involved in glucocorticoid receptor signalling, clock-gene pathways, and inflammation regulation in late-pregnant cows and in cows and their offspring during early lactation.

## 2. Materials and Methods

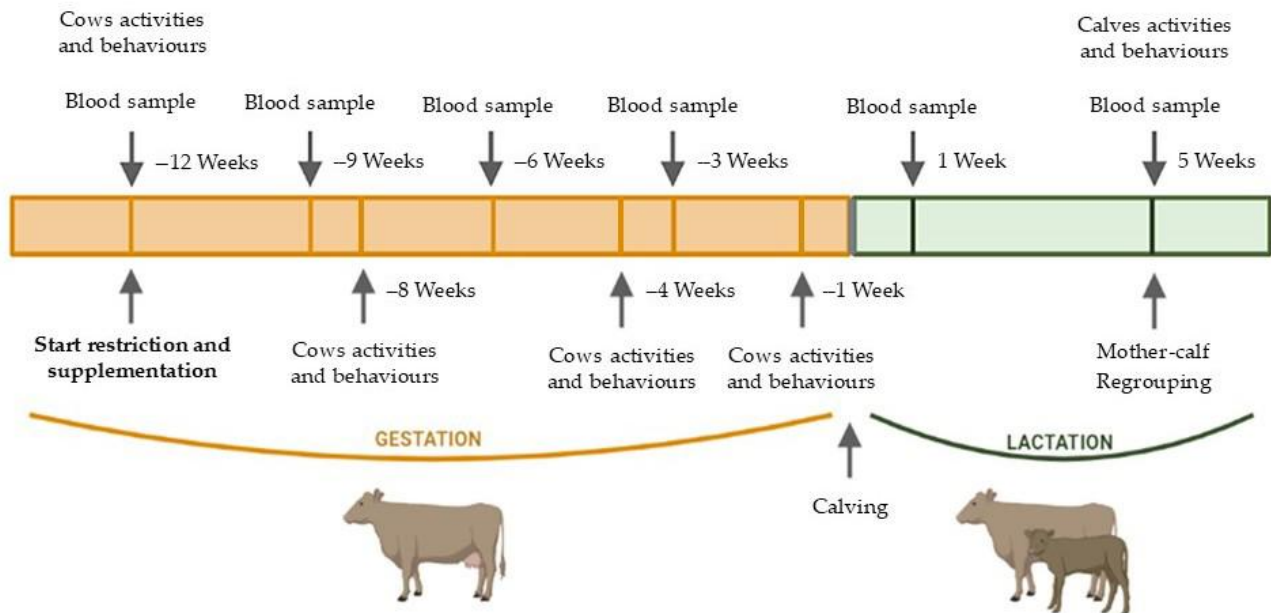
### 2.1. Animals, Diets and Management

The experiment was carried out at the experimental facilities of La Garcipollera Research Station (northeastern Spain, 945 m a.s.l., coordinates: 42.6° N, 0.54° W), from November 2021 to March 2022. During this period, the length of daylight hours decreases from 10 to 9 h between early November to late December before increasing again to approximately 12 h by late March. The involved cows were selected from a large experimental suckler cattle herd consisting solely of beef cattle breeds [34].

Concisely, at  $28 \pm 0.7$  weeks of gestation (12 weeks prior to parturition), 109 clinically healthy pregnant cows, without reproductive or clinical disorders, were stratified by expected calving date and age and randomly distributed according to one of four groups in a  $2 \times 2$  factorial experimental design which was implemented to evaluate the effects of feeding level (T100% vs. T60% of nutrient requirements) and HT supplementation during the last three months of gestation (0 vs. 180 mg HT/kg of total mixed ration, TMR, with initial body weight (BW) of  $662 \pm 51$  kg, means  $\pm$  standard deviations [29,34]). The TMR was formulated to satisfy gestational nutrient requirements, with an identical composition across treatments except for the inclusion of HT. Mineral blocks were available ad libitum for all groups. The reduced feeding level was consistent with standard commercial winter-feeding practices for late-gestation cows [35]. The HT was provided as a mash incorporated into the TMR, and the dose was selected based on previous studies showing health benefits without adverse effects in other species [36]. HT metabolites were assessed in maternal plasma and colostrum to confirm supplementation compliance [29]. Feed was offered once daily at 08:00 h, and cows were tied for 3 h until the assigned ration was consumed (10.5 kg/day for T100% or 7.0 kg/day for T60%, 1240 kcal of Net Energy for lactation and 114 g of crude protein/kg of TMR). This schedule was designed to implement a restricted feeding protocol, generating distinct nutritional levels between groups. No fixed feeding duration was set, allowing each group to consume its allocated ration fully. After calving, all cows received 100% of their postpartum requirements without HT supplementation. Detailed TMR composition and nutrient supply can be found in [29]. After birth, calves were nursed twice per day during 30 min sessions at 07:00 and 14:30 h. Housing paddocks were loose, with straw bedding and outdoor access. The BW, average daily gain (ADG), and body condition score of the cows during gestation can be found in [34]. The same data for the cows selected for the gene expression study in lactation are reported in [29]. Additionally, calves' weights were recorded at birth (<12 h) and at 5 weeks of age (one day after the behaviour recordings). ADG was estimated for this period as the difference between final and initial weight divided by the interval between recordings.

The analysis of cow behaviour during gestation included the whole pre-partum cows in the study (T100%-CTRL,  $n = 26$ ; T100%-HT,  $n = 25$ ; T60%-CTRL,  $n = 29$ ; T60%-HT,  $n = 29$ ). After calving, a representative sample of 48 calves ( $29 \pm 6$  days of age) were used for performance and body temperature recordings, as well as observation of their time-budget activity between suckling periods and cow-calf pair re-grouping behaviours. A visual description of samplings and recordings is shown in Figure 1. The animal sample for the analysis of gene expression consisted of a representative subset of 46 pre-partum cows (T100%-CTRL,  $n = 11$ ; T100%-HT,  $n = 10$ ; T60%-CTRL,  $n = 14$ ; T60%-HT,  $n = 11$ ) and 37 postpartum cows with their suckling calves (T100%-CTRL,  $n = 10$ ; T100%-HT,  $n$

= 9; T60%-CTRL,  $n = 9$ ; T60%-HT,  $n = 9$ ), selected following the conditions detailed in [29]. All treatment groups were managed simultaneously under the same housing and environmental conditions; therefore, all animals were equally exposed to the natural variations in day length occurring throughout the study period.



**Figure 1.** Samplings and recording dates for pre- and post-partum cows and their calves that were subjected to different feeding levels and supplementation of hydroxytyrosol during the last third of gestation.

## 2.2. Activities and Behavioural Recordings During the Meal in Gestating Cows

Behavioural patterns of pregnant cows during the daily meal were assessed using a predefined ethogram (Supplementary Materials Table S1 [37,38]), including maintenance activities, social behaviours (cohesive and agonistic), and stress-related behaviours. Behavioural recordings were conducted during the feeding period, as animals typically express dietary-motivated behaviours and social interactions most clearly around feeding events, allowing for a sensitive assessment of the effects of nutrient restriction and HT supplementation. Behavioural recordings were conducted by four trained observers once per month (starting at -12, -8, -4, and -1 weeks relative to calving) for 2 h, beginning 1 h after feed distribution. After cows were self-locked, the pen was virtually divided by observer into segments of 5 to 7 cows, and three segments per experimental group were observed for 10 min each. To minimize observer bias, all observers recorded all experimental groups on each recording day, and the order of treatments was randomized. Prior to data collection, all observers participated in joint training sessions and pilot recordings to harmonize the interpretation of behavioural categories and resolve ambiguities by consensus. The ethogram included clear operational definitions, and standardized recording procedures were used throughout the study to minimize subjectivity and maintain consistency among observers.

Main maintenance activity patterns were recorded on a segment level as the proportion of cows occupied in each activity, whereas social and stress-related behaviours were recorded individually using ear tag identification and expressed as events counts per 10 min. The individual eating time was recorded while daily feed intake was estimated by weighing the total feed offered to the group and dividing it by the number of animals. Intake rate was calculated as the ratio between the TMR supply and eating time [39]. No

feed refusals were recorded during the experiment. A single behaviour was counted as two or more events if the cow performing the behaviour stopped for more than 10 s and subsequently performed it again, regardless of if it was self-directed (e.g., head rub) or to alien counterparts (e.g., licking).

### 2.3. Calf Body Temperature and Time-Budget

After the morning suckling period, a total sample of 48 calves (5 weeks of age), consisting of four recording days with twelve calves each, balanced by maternal dietary treatments and calf sex, were housed in individual pens (1.5 m × 1.2 m) with straw bedding and had visual and olfactory contact with each other. Before entering the individual pens, their rectal and ear temperatures were measured with a digital probe (Inserbo SL, Lleida, Spain) and an infrared thermometer (Testo 830-T2, Testo SE & Co. KGaA, Titisee-Neustadt, Germany), respectively, according to [40].

Calves had been previously trained to teat buckets, which were placed two weeks earlier into the calves pens close to dams' area where they could rest between suckling periods. Therefore, two 5 L water ( $9.8 \pm 3.08$  °C) and warm milk replacer solution ( $46.5 \pm 6.63$  °C, with a final pH of 6.8) in buckets with teats (65 cm height) were provided from the moment they entered the individual pen. Time-budget-related activities were recorded individually by two trained observers using instantaneous observational sampling every 2 min, with each observer monitoring six calves simultaneously. Calves were habituated to human presence and routine handling throughout the experimental period. At the start of each observation session, the observers rested against an opposite fence at a convenience distance away from the individual pens (5 m) and waited 5 min before beginning the recordings to minimize any short-term disturbance caused by observer entry and to allow the calves to resume normal behaviour [41]. At the start of each observation session, the observer waited 5 min before beginning, allowing the calves to habituate to the observer's presence. Behaviour and posture (lying/standing) of each calf were then recorded every 2 min using the ethogram in Supplementary Table S2 [37,41]. Time spent in pluri-activity was also noted. Observations were conducted in three 1 h sessions per day, with a 30 min interval between sessions, starting ~1 h after morning suckling and ending 1 h before the afternoon suckling. Behavioural outcomes are expressed as minutes per hour, except vocalizations, which are reported as the number of events.

### 2.4. Cow-Calf Behaviour at the Afternoon Nursing Period

Mother-calf reunion behaviours were measured in straw-bedded group pens after calf behavioural recordings in individual pens. Each afternoon (14:30 h), after 7 h of separation without fence-contact (25 m away), the mothers' behaviour was recorded during the reunion with her calf. Observers remained at least 10 m away from the reunion area to avoid influencing behaviour. The time elapsed from the opening of the gate to the reunion was recorded, as well as the occurrence of maternal behaviours during the minute immediately after the reunion. Behaviours categories are described in Supplementary Table S3 [38] and grouped as maternal care, motivation-related vocalizations, and nursing behaviours.

### 2.5. Blood Sample Collection and Analysis

Blood samples were withdrawn at -9, -6, and -3 weeks relative to calving from the gestating cows, and at 1 and 5 weeks postpartum from both cows and their offspring, in different days of the week to behavioural recordings to avoid disturbances across measurements (Figure 1). Blood was collected into Tempus™ Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) subsequent whole-blood gene expression analysis,

according to the manufacturer's protocol. Immediately after collection these samples were stored at  $-20\text{ }^{\circ}\text{C}$  until use.

RNA extraction, DNase treatment, cDNA synthesis, and qPCR amplification were performed as detailed in [29]. Briefly, RNA quality and concentration were assessed spectrophotometrically, and reverse transcription was carried out with random hexamer primers. Messenger RNA expression was determined by qPCR using intron-spanning primers whenever possible. The genes selected for transcriptomic analysis were those associated with glucocorticoid receptor signalling (glucocorticoid receptor alpha (*NR3C1*, also named as *GRA*)), circadian rhythms (Aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*), Period circadian regulator 1 (*PER1*), Cryptochrome circadian regulator 1 (*CRY1*), Cryptochrome circadian regulator 2 (*CRY2*)), and inflammation regulation (Haptoglobin (*HP*), Interleukin 8 (*IL8*)). The target gene expression levels were normalized using the geometric mean of the two most stable reference genes (*ACTB* and *RPL19*), whose stability was confirmed with NormFinder v21 [42]. Primer sequences and their corresponding sources are provided in Table 1. Amplification and dissociation curves were evaluated to verify reaction quality, and relative gene expression levels were determined, including triplicate measurements and  $2^{-\Delta\Delta\text{Ct}}$  normalization to two reference genes.

**Table 1.** Primer sequences and sources for blood samples from cows.

Gene	General Gene Role	Forward and Reverse Primer (5'–3')	Base Pair	Access. No.	Efficiency (%)	nM	Source
<i>RPL19</i>	Housekeeping	F: GATCCGGAAGCTGATCAAAG R: ATTCGAGCATTGGCAGTACC	147	NM_001040516.1	97.5	200	[43]
<i>ACTB</i>	Housekeeping	F: CTGGACTTCGAGCAGGAGAT R: GATGTCGACGTCACACTTC	207	AY141970	105	200	[44]
<i>NR3C1</i>	Glucocorticoid receptor	F: GGCCAGATGTACCACTACGAC R: CCAGGGCTTGAATAGCCGTTAGAA	206	AY238475.2	100	400	[45]
<i>BMAL1</i>	Clock genes	F: AGGTCGAATGATTGCTGAGG R: TCTTCTTGCCCTCCTGGAGAA	134	XM_001251227	90	400	[46]
<i>PER1</i>	Clock genes	F: GCCAACCAGGAGTTCTACCA R: CCTGTCAGGAAGGAGACAGC	158	XM_594471	98	200	[46]
<i>CRY1</i>	Clock genes	F: CCATTGGGAGTGCAGAGTCTTAAC R: ACTTCTCTTGTCGCCAGAATTGATTTT	100	NM_001105415.1	92	400	[5]
<i>CRY2</i>	Clock genes	F: ATGCCCCAGAGTCCATTCAGAA R: TGAGGTCTTCCACACAGGAAGG	186	XM_585942	109	400	[46]
<i>HP</i>	Inflammation regulators	F: TGAGGCAGTGTGCGGGAAGCC R: AGCGTGGCTCCCGAGATGAGGTT	138	NM_001040470.1	103	400	[47]
<i>IL8</i>	Inflammation regulators	F: TGGGCCACACTGTGAAAATTC R: CCTTCTGCACCCACTTTTCC	92	ENSBTAG00000019716.3	93	400	[48]

## 2.6. Analysis of Data

The data were processed with JMP Pro v18 (SAS Institute Inc., Cary, NC, USA). Since several behavioural variables were zero-inflated or showed skewed distributions typical of behavioural datasets, they did not meet the assumptions of normality and homogeneity of variance required for parametric analyses. Therefore, during pregnancy, the cow activity (accounted as minutes/hour) and behaviours (as number of events per hour) during the eating meal were analyzed with nonparametric models, considering the independent effects of pre-partum maternal dietary treatment (T100%-CTRL, T100%-HT, T60%-

CTROL, T60%-HT), and gestation time (starting at -12, -8, -4, and -1 weeks before parturition). The count of focal behaviours per 10 min in pregnant cows were standardized to count of events per hour for statistical model comparisons. During lactation, calf time-budget (as minutes/hour) during the daily time between two restricted suckling periods at weeks 1 and 5 of lactation were analyzed with the same model than that used for gestating cows' behaviour, with additional consideration of calf sex effect. In both gestating cows and lactating calves' behaviour, means were separated using Wilcoxon and Kruskal–Wallis tests ( $p \leq 0.05$ ). Contingency tables and Pearson tests were used to evaluate the proportion of animals displaying each maternal behaviour recorded at the afternoon nursing meeting.

Calf temperature and performance data were analyzed using a standard least-squares means model that included dietary treatment and sex as fixed effects. The interaction between maternal dietary group and calf sex was excluded because it did not influence any behavioural variable ( $p > 0.05$ ). The relationship between rectal and ear temperatures at week 5 of age was assessed using a Pearson correlation test. Blood gene expression data collected at weeks -9, -6, and -3 pre-calving in pregnant cows, and at weeks 1 and 5 post-calving in dams and their calves, were analyzed in separate datasets using mixed models with repeated measures. Fixed effects included feeding level, HT supplementation, week relative to calving, and their interactions. Each cow was treated as the experimental unit and included as a random effect to account for individual variability. Starting body weight at week -12 pre-calving was included as a covariate in the gene expression model. Only significant interactions among fixed effects ( $p \leq 0.05$ ) are reported. Least-squares means and their standard errors are presented. Normalized gene expression values were analyzed statistically in log-transformed form to meet assumptions of normality and homoscedasticity, but are expressed as relative quantification (RQ). Tukey's test was used to compare means.

### 3. Results

#### 3.1. Activities and Behaviours During the Meal in Gestating Cows

During the meal at the feeder headlock, pregnant cows spent more time eating than those in the T60% groups ( $p < 0.001$ ). Conversely, T60% cows had higher feed intake per minute compared with the T100% groups ( $p < 0.01$ ). As expected, cows receiving less feed completed their meals more quickly than those in T100% group. No significant differences were observed for the other maintenance activities ( $p > 0.05$ ) (Table 2).

**Table 2.** Maintenance activities of cows during the last third of gestation at the feeder headlock during the meal period. Results are reported as mean  $\pm$  pooled standard error. Significant differences among maternal feeding level and supplementation of hydroxytyrosol (HT) groups were considered at  $p < 0.05$ . Different letters, a, b, within a row indicate statistically different groups.

	T100%-CTROL	T100%-HT	T60%-CTROL	T60%-HT	<i>p</i> -Values
Number of cows	26	25	29	29	
Age (years)	8.5 $\pm$ 0.4	8.8 $\pm$ 0.4	8.2 $\pm$ 0.4	8.5 $\pm$ 0.4	0.81
Eating (proportion of cows)	56.4 $\pm$ 6.5	58.0 $\pm$ 6.5	51.8 $\pm$ 5.9	56.6 $\pm$ 5.9	0.91
Idling (proportion of cows)	34.7 $\pm$ 5.7	35.6 $\pm$ 5.7	46.3 $\pm$ 5.2	41.4 $\pm$ 4	0.65
Ruminating (proportion of cows)	8.9 $\pm$ 1.9	6.4 $\pm$ 1.9	1.9 $\pm$ 1.7	2.0 $\pm$ 1.7	0.07
Duration of eating (hours)	3.0 $\pm$ 0.1 <sup>a</sup>	3.1 $\pm$ 0.1 <sup>a</sup>	1.5 $\pm$ 0.1 <sup>b</sup>	1.4 $\pm$ 0.1 <sup>b</sup>	<0.0001
Eating Rate (g of TMR/min)	62.2 $\pm$ 5.2 <sup>b</sup>	60.1 $\pm$ 5.2 <sup>b</sup>	84.8 $\pm$ 4.8 <sup>a</sup>	82.6 $\pm$ 4.8 <sup>a</sup>	0.002

Regarding the behaviour displayed by pregnant cows during meal, significant differences were observed in licking/sniffing their close counterparts, a behaviour indicative

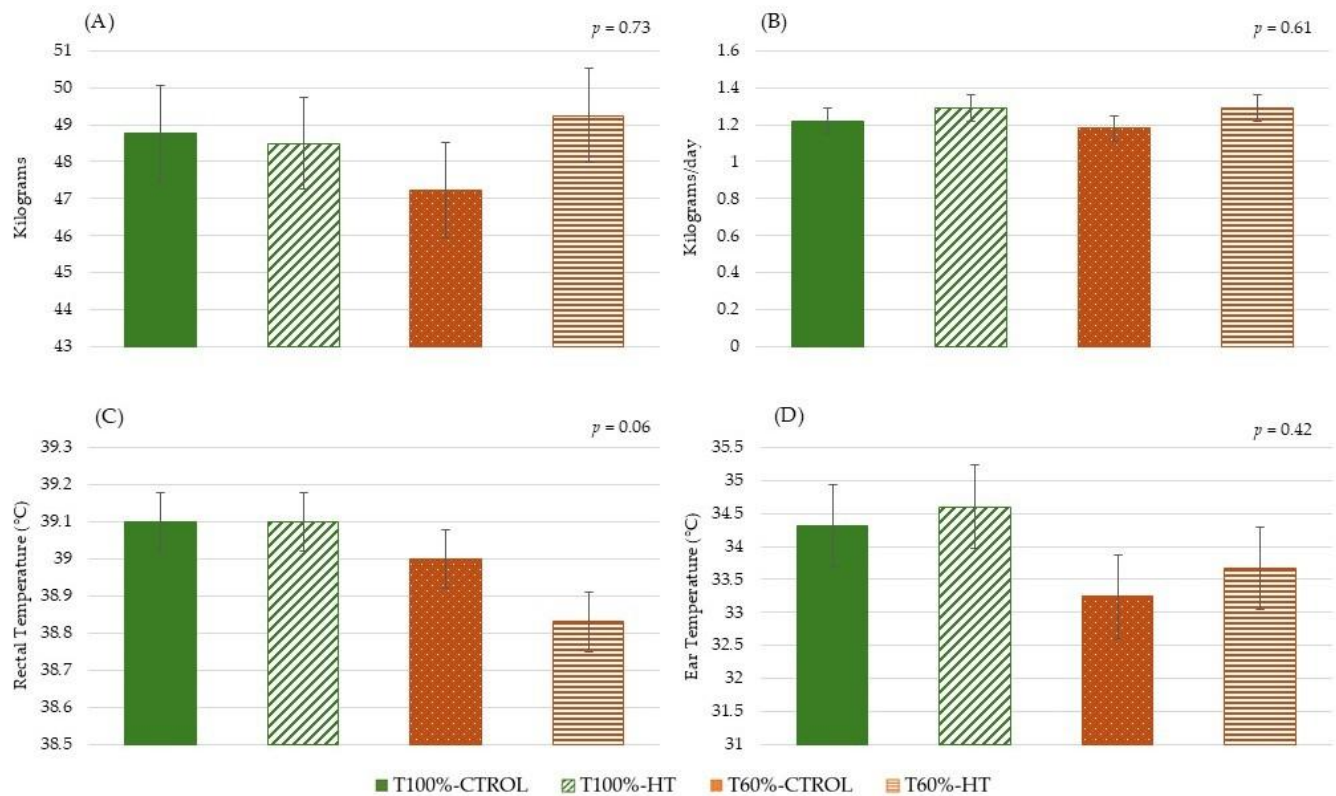
of social cohesion. This activity was performed for a longer duration by cows in the T60%-CTROL group, followed by those in the T60%-HT group, compared with both T100% groups, which engaged in this behaviour for fewer time ( $p < 0.001$ ). Respect to behaviours associated with stress, significant differences were found for head rubbing/licking against concrete, metal or ground, with both T60% groups exhibiting a greater number of these behavioural events compared with the T100% groups ( $p < 0.01$ ). In addition, tongue rolling was more frequently observed in cows from the T60%-CTROL group, followed by the T60%-HT group, whereas this behaviour was almost absent in both T100% groups ( $p < 0.01$ ). No significant differences were found for the remaining cohesive or stress-related behaviours (step back, head shake and vocalizations), nor for activities indicative of agonistic behaviour (head butts) ( $p > 0.05$ ) (Table 3).

**Table 3.** Behaviour of cows during the last third of gestation at the feeder headlock during the meal period. Results are reported as mean  $\pm$  pooled standard error number of events per hour for each behaviour. Significant differences among maternal feeding level (T100% vs. T60%) and supplementation of hydroxytyrosol (HT) (Control (CTROL) vs. HT) groups were considered at  $p < 0.05$ . Different letters, a, b, c, within a row indicate statistically different groups.

	T100%-CTROL	T100%-HT	T60%-CTROL	T60%-HT	<i>p</i> -Values
Numbers of cows	26	25	29	29	
<b>Cohesive</b>					
Licking/sniffing	0.06 $\pm$ 0.07 <sup>c</sup>	0.04 $\pm$ 0.07 <sup>c</sup>	0.45 $\pm$ 0.07 <sup>a</sup>	0.22 $\pm$ 0.07 <sup>b</sup>	0.0006
Vocalizations	0.02 $\pm$ 0.03	0.08 $\pm$ 0.03	0.09 $\pm$ 0.03	0.10 $\pm$ 0.03	0.15
<b>Agonistic</b>					
Head butt	0 $\pm$ 0.01	0.01 $\pm$ 0.01	0.03 $\pm$ 0.01	0.01 $\pm$ 0.01	0.12
<b>Stress-Related</b>					
Step back	0.3 $\pm$ 0.09	0.28 $\pm$ 0.09	0.2 $\pm$ 0.09	0.35 $\pm$ 0.09	0.85
Head shake	0.03 $\pm$ 0.02	0.05 $\pm$ 0.02	0.04 $\pm$ 0.02	0.11 $\pm$ 0.02	0.25
Head rub/lick concrete/metal/ground	0.13 $\pm$ 0.08 <sup>b</sup>	0.03 $\pm$ 0.08 <sup>b</sup>	0.35 $\pm$ 0.08 <sup>a</sup>	0.44 $\pm$ 0.08 <sup>a</sup>	0.007
Tongue rolling	0.03 $\pm$ 0.03 <sup>c</sup>	0.01 $\pm$ 0.03 <sup>c</sup>	0.19 $\pm$ 0.03 <sup>a</sup>	0.11 $\pm$ 0.03 <sup>b</sup>	0.005

### 3.2. Physiological and Performance Parameters in Lactating Calves

During the first month of life, the prepartum maternal feeding level and HT supplementation did not affect ( $p > 0.05$ ) the calves' birth BW and ADG (Figure 2). Regarding calf body temperatures, rectal temperature was, as a trend, higher in calves born to T100%-CTROL and T100%-HT dams compared with those born to T60%-HT dams, which tended to exhibit lower rectal temperatures ( $p = 0.06$ ). Conversely, no significant differences were detected in ear temperatures across treatments ( $p > 0.05$ ) (Figure 2). The correlation between rectal and ear temperature was positive and significant ( $r = 0.55$ ;  $p < 0.001$ ).



**Figure 2.** (A) Birth weight (kg), (B) average daily gain (kg/day) from birth to the fifth week of age; (C) rectal temperature (°C), and (D) ear temperature (°C) of calves at the fifth week of age. Results are reported as mean  $\pm$  pooled standard error. Significant differences among the maternal feeding (T100% vs. T60%) and supplementation of hydroxytyrosol (HT) (Control (CTRL) vs. HT) groups were considered at  $p < 0.05$ .

### 3.3. Activities and Behaviours in Lactating Calves

Prepartum maternal feeding level and HT supplementation significantly affected the posture activity most frequently performed by the calves. Calves in the T60%-HT group spent more time standing compared with those in the T100%-HT and T60%-CTRL groups, which spent more time lying down ( $p = 0.05$ ). Regarding maintenance activities, significant differences were observed for idling behaviours. Calves in the T60%-CTRL group showed longer periods of inactivity compared with calves in the T100%-CTRL and T60%-HT treatment groups, both of which were more active. Significant differences were also found for urination, as T100%-CTRL calves spent more minutes urinating than the rest of the groups ( $p < 0.01$ ) (Table 4).

Significant variations were also identified in some stress-related behaviours, such as sniffing/olfactory manipulation of pen fixtures. Calves from the T60%-HT group performed this behaviour for a longer than those in the T100%-HT and T60%-CTRL groups ( $p < 0.05$ ). Although no calves performed effective water or milk intake from buckets, it is noteworthy that T60%-HT calves showed a tendency toward a higher frequency of visiting the milk bucket without intake compared with the T100%-HT group, while T60%-CTRL calves tended to vocalize more frequently than the other treatments ( $p < 0.1$ ). No additional maintenance activities differed significantly among the groups or other stress-related behaviours, nor for any of the observed cohesive behaviours ( $p > 0.05$ ) (Table 4).

**Table 4.** Postures, maintenance activities, and behaviours of individually housed 5-week-old calves between morning and afternoon nursing periods. Results are reported as mean  $\pm$  pooled standard error. Activities are expressed as minutes per hour, except vocalizations (events per hour). Pluriactivity indicates simultaneous occurrence of two activities. Different letters (a, b) within a row indicate significant differences among maternal feeding (T100% vs. T60%) and supplementation of hydroxytyrosol (HT) (Control (CTROL) vs. HT) groups ( $p < 0.05$ ).

	T100%-CTROL	T100%-HT	T60%-CTROL	T60%-HT	<i>p</i> -Values
Number of calves	12	12	12	12	
<b>Posture</b>					
Standing (min/h)	24.0 $\pm$ 2.5 ab	17.6 $\pm$ 2.5 b	18.4 $\pm$ 2.5 b	27.0 $\pm$ 2.5 a	0.05
Lying (min/h)	36.0 $\pm$ 2.5 ab	42.4 $\pm$ 2.5 a	41.6 $\pm$ 2.5 a	33.0 $\pm$ 2.5 b	0.05
<b>Maintenance Activities</b>					
Idling (min/h)	29.6 $\pm$ 2.4 b	34.2 $\pm$ 2.4 ab	37.2 $\pm$ 2.4 a	29.5 $\pm$ 2.4 b	0.05
Ruminating (min/h)	11.9 $\pm$ 1.6	11.3 $\pm$ 1.6	7.7 $\pm$ 1.6	9.1 $\pm$ 1.6	0.27
Urination (min/h)	1.1 $\pm$ 0.2 a	0.4 $\pm$ 0.2 b	0.4 $\pm$ 0.2 b	0.3 $\pm$ 0.2 b	0.002
Defecation (min/h)	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	0.51
<b>Cohesive behaviours</b>					
Exploring bed or eating straw bed (min/h)	4.6 $\pm$ 0.7	2.9 $\pm$ 0.7	3.2 $\pm$ 0.7	4.7 $\pm$ 0.7	0.43
Socializing (min/h)	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2	0.74
Self-grooming (min/h)	1.7 $\pm$ 0.4	1.7 $\pm$ 0.4	1.3 $\pm$ 0.4	1.8 $\pm$ 0.4	0.96
<b>Stress-Related behaviours</b>					
Sniffing/olfactory manipulation of pen fixtures (min/h)	6.2 $\pm$ 1.0 ab	5.37 $\pm$ 1.0 b	5.2 $\pm$ 1.0 b	8.3 $\pm$ 1.0 a	0.04
Licking/biting persistently pen fixtures (min/h)	1.6 $\pm$ 0.5	1.6 $\pm$ 0.5	1.8 $\pm$ 0.5	1.2 $\pm$ 0.5	0.72
Visiting water bucket without intake (min/h)	1.9 $\pm$ 0.5	1.2 $\pm$ 0.5	1.6 $\pm$ 0.5	2.4 $\pm$ 0.5	0.52
Visiting milk bucket without intake (min/h)	1.0 $\pm$ 0.4	0.7 $\pm$ 0.4	1.2 $\pm$ 0.4	2.1 $\pm$ 0.4	0.08
Vocalization (number of events/h)	1.1 $\pm$ 0.5	1.0 $\pm$ 0.5	0.38 $\pm$ 0.5	1.7 $\pm$ 0.5	0.07
Time in pluriactivity (min/h)	1.4 $\pm$ 2.2	1.0 $\pm$ 1.5	0.8 $\pm$ 1.5	1.0 $\pm$ 1.7	0.65

### 3.4. Activities and Behaviours at Daily Mother–Calf Reunion

Concerning the behavioural results obtained during the mother–calf reunion period, significant differences were observed only for biological mother–calf licking. A higher number of cows from both T60% groups exhibited this maternal care behaviour toward their calves compared with cows from the T100% groups, which showed this cohesive behaviour only rarely ( $p < 0.05$ ). For the remaining behavioural parameters observed, no significant differences were found among groups ( $p > 0.05$ ) (Table 5).

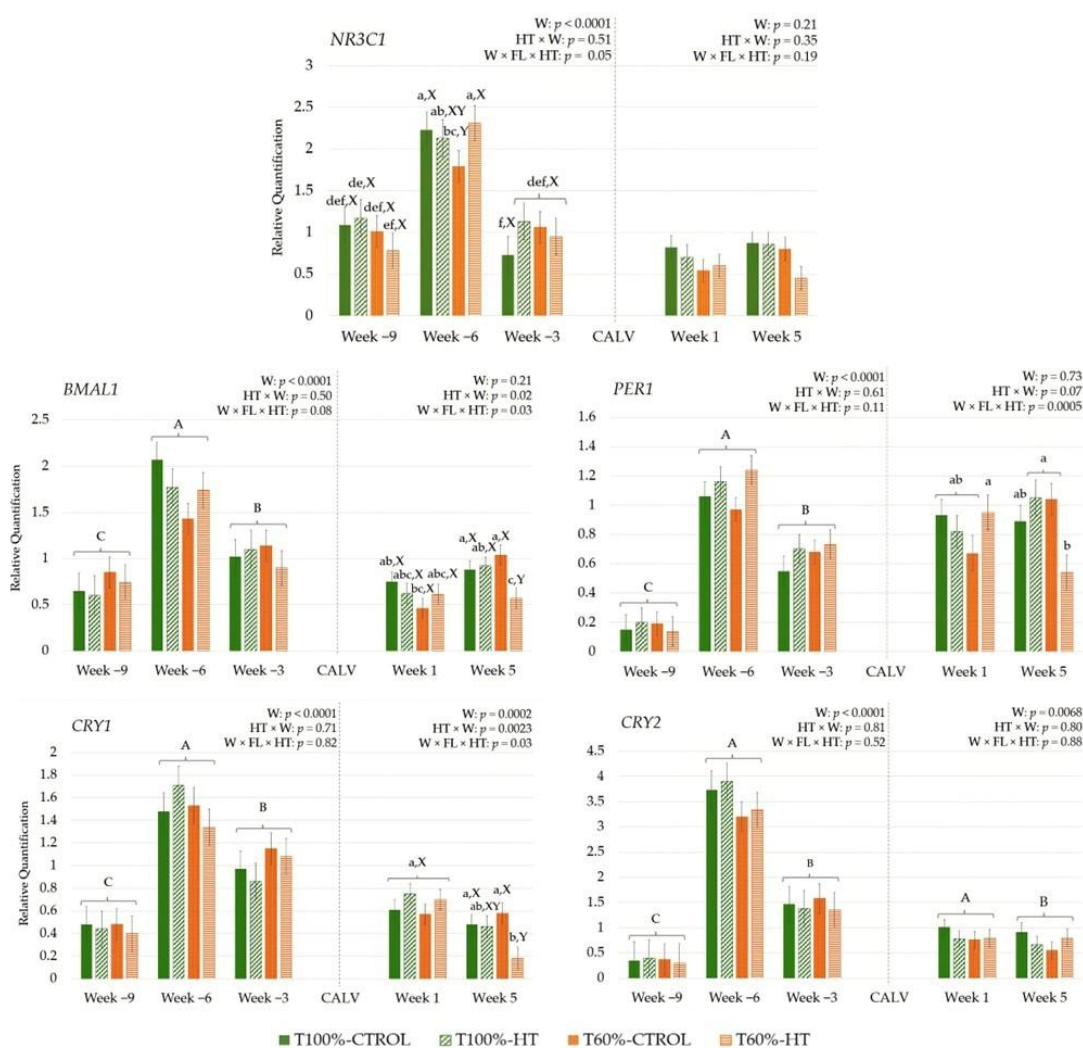
**Table 5.** Activities and behaviours observed during daily mother–calf reunion at the afternoon nursing period. Values are expressed as the proportion of animals displaying each behaviour, except reunion time, which is presented as mean  $\pm$  SEM (min). Different letters (a, b) within a row indicate significant differences among maternal feeding (T100% vs. T60%) and supplementation of hydroxytyrosol (HT) (Control (CTROL) vs. HT) groups ( $p < 0.05$ ).

	T100%-CTROL	T100%-HT	T60%-CTROL	T60%-HT	<i>p</i> -Values
<b>Number of mother-calf pairs</b>					
Reunion time > 1 min	5/12	4/12	7/12	7/12	0.52
Reunion time in min	1.36 $\pm$ 0.56	1.4 $\pm$ 0.56	1.56 $\pm$ 0.56	2.71 $\pm$ 0.56	0.75
<b>Maternal care</b>					
Biological mother–calf licking	2/12 b	4/12 b	9/12 a	7/12 a	0.02

Nasal Care-Contact/Play	5/12	6/12	7/12	5/12	0.82
<b>Motivation-related</b>					
Maternal vocalization	4/12	4/12	3/12	4/12	0.96
Calf vocalization	0/12	3/12	1/12	1/12	0.24
<b>Nursing-related</b>					
Attempted nursing from another mother	4/12	4/12	4/12	5/12	0.96
Nursing from biological mother	11/12	12/12	10/12	9/12	0.28
Calf tail movement during nursing	9/12	9/12	10/12	8/12	0.83

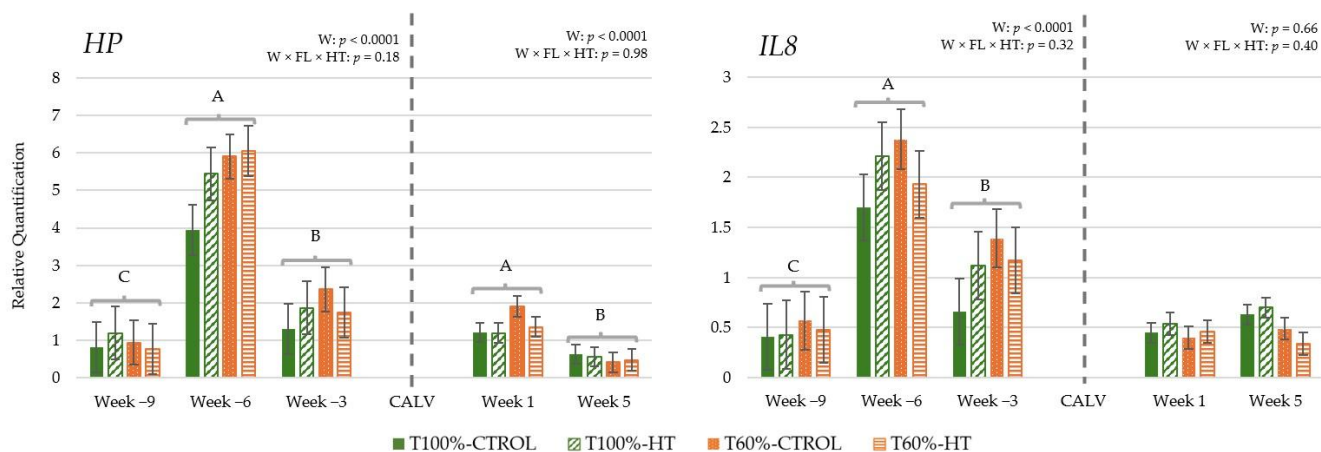
### 3.5. Genes Expression Markers in Blood

During the last third of gestation, a significant effect of feeding level, HT supplementation, and week affected the expression of the target stress hormone signalling marker *NR3C1*, with higher levels observed in the T60%-HT and T100%-CTROL groups compared to the T60%-CTROL group during week -6 (Figure 3). Overall, the week of gestation affected all gene expressions (Figures 3 and 4). The expression of *NR3C1* was upregulated from week -9 to a peak at week -6 and subsequently decreased at week -3 ( $p < 0.001$ ). On the other hand, genes involved in circadian rhythms, *BMAL1*, *PER1*, *CRY1* and *CRY2*, as well as genes involved in inflammatory response, *HP* and *IL8*, showed significantly the lowest levels of expression at week -9 but the highest expression levels at week -6 compared with the rest of the weeks ( $p < 0.001$ ) (Figure 4).



**Figure 3.** Peripheral gene expression according to week (W) relative to calving (CALV), maternal feeding level (FL), and supplementation of hydroxytyrosol (HT) in cows during gestation (T100%-

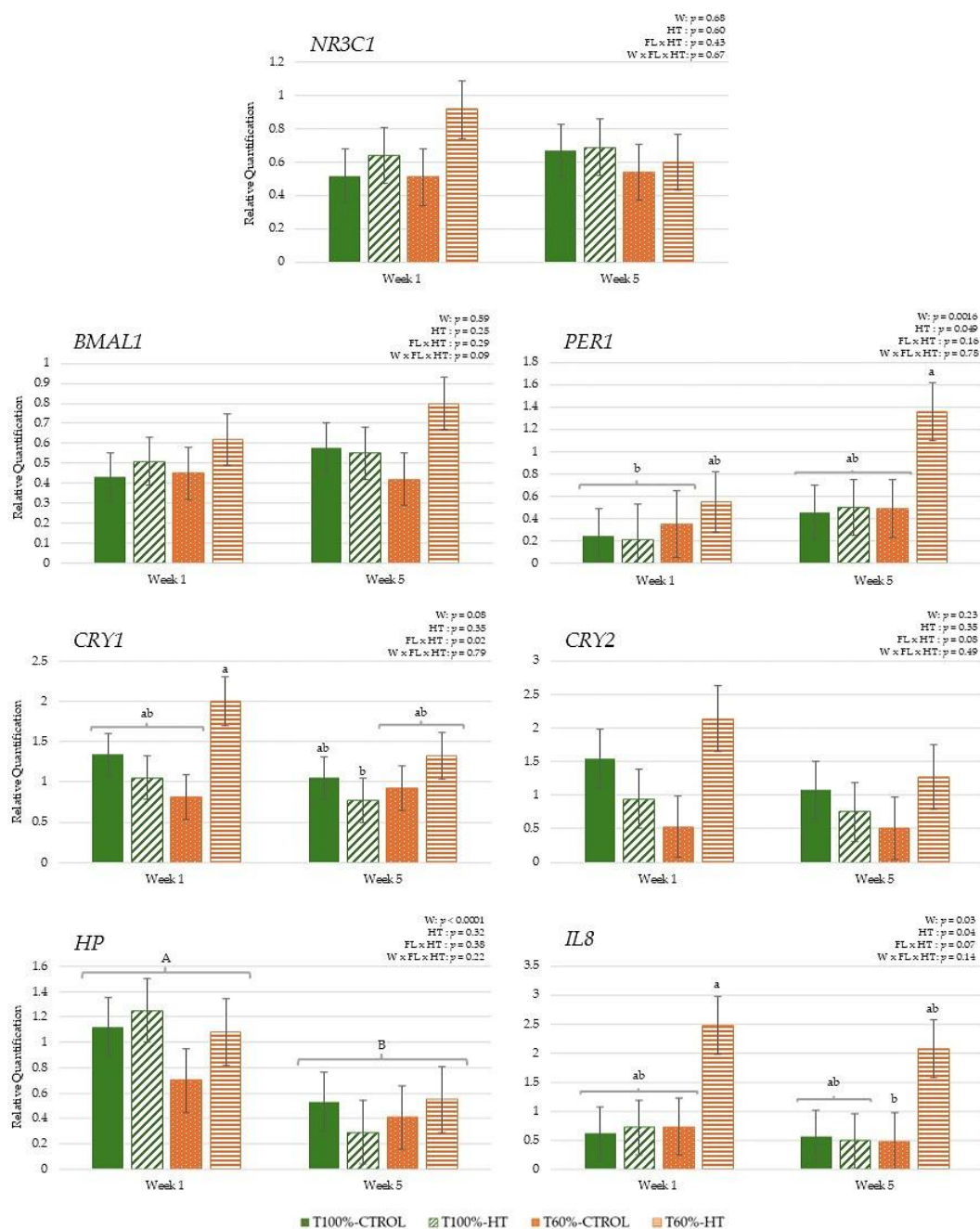
CTROL,  $n = 11$ ; T100%-HT,  $n = 10$ ; T60%-CTROL,  $n = 14$ ; T60%-HT,  $n = 11$ ) and lactation (T100%-CTROL,  $n = 10$ ; T100%-HT,  $n = 9$ ; T60%-CTROL,  $n = 9$ ; T60%-HT,  $n = 9$ ) for the following genes: nuclear receptor subfamily 3 group C member 1 (*NR3C1*), basic helix-loop-helix ARNT-like 1 (*BMAL1*), period circadian regulator 1 (*PER1*), cryptochrome circadian regulator 1 (*CRY1*), and cryptochrome circadian regulator 2 (*CRY2*). Means with different letters differ at  $p \leq 0.05$ . When only week has a significant effect, letters A, B, C are shown. When other significant effects occur, letters from the three-way interaction (a, b, c) are used; if interactions between feeding level or HT supplementation and week are significant, differences within the same week are indicated by X, Y. If no significant effects are detected, no letters are displayed. Letter groupings for gestation and lactation are independent and not comparable across periods.



**Figure 4.** Peripheral gene expression according to week (W) relative to calving (CALV), maternal feeding level (FL), and supplementation of hydroxytyrosol (HT) in cows during gestation (T100%-CTROL,  $n = 11$ ; T100%-HT,  $n = 10$ ; T60%-CTROL,  $n = 14$ ; T60%-HT,  $n = 11$ ) and lactation (T100%-CTROL,  $n = 10$ ; T100%-HT,  $n = 9$ ; T60%-CTROL,  $n = 9$ ; T60%-HT,  $n = 9$ ) for haptoglobin (*HP*) and interleukin-8 (*IL8*). Means with different letters differ at  $p \leq 0.05$ . When only week has a significant effect, letters A, B, C are shown. If no significant effects are detected, no letters are displayed. Letter groupings for gestation and lactation are independent and not comparable across periods.

In early postpartum (week 5), the interaction between week, prepartum maternal feeding level, and HT supplementation affected clock genes in dams. At week 5, *BMAL1*, *PER1*, and *CRY1* gene expression levels were lower in the T60%-HT group compared to the T60%-CTROL group ( $p < 0.05$ ). Regarding the effect of lactation week, the expression of *CRY2* and decreased significantly at week 5 compared to week 1 ( $p < 0.05$ ) (Figure 3).

In calves, prepartum maternal feeding level and HT supplementation affected the regulation of circadian rhythms and the inflammatory regulation (Figure 5). The interaction between maternal feeding level and HT supplementation showed higher *CRY1* expression in the T60%-HT compared with the other groups ( $p < 0.05$ ). Furthermore, prepartum HT supplementation significantly increased *PER1* and *IL8* gene expression in the two groups of calves from HT dams ( $p < 0.05$ ). Regarding the effect of the week, the expression of the clock gene *PER1* increased at week 5 compared to week 1, while the expression of *HP* and *IL8* decreased at week 5 ( $p < 0.05$ ).



**Figure 5.** Interaction between maternal feeding level (FL) and supplementation of hydroxytyrosol (HT) on peripheral gene expression in calves at specific weeks (W) relative to birth (T100%-CTROL,  $n = 9$ ; T100%-HT,  $n = 9$ ; T60%-CTROL,  $n = 9$ ; T60%-HT,  $n = 9$ ) for the following genes: nuclear receptor subfamily 3 group C member 1 (*NR3C1*), basic helix-loop-helix ARNT-like 1 (*BMAL1*), period circadian regulator 1 (*PER1*), cryptochrome circadian regulator 1 (*CRY1*), cryptochrome circadian regulator 2 (*CRY2*), haptoglobin (*HP*), and interleukin-8 (*IL8*). Means with different letters differ at  $p \leq 0.05$ . When only week has a significant effect, letters A and B are shown. When other significant effects occur, letters from the three-way interaction (a, b) among week, maternal FL, and HT supplementation are used. If no significant effects are detected, no letters are displayed.

#### 4. Discussion

This study examined the effects of feeding levels and supplementation of HT during the final third of pregnancy on maintenance activities and behaviours in both cows and their calves. All animals included in this study were free of reproductive or clinical

diseases, indicating that the observed molecular and behavioural changes should be interpreted in a context of metabolic or physiological adaptation and not as part of pathological processes. An increase in grooming behaviours was observed in cows toward calves in the T60% groups, as well as changes in blood cell expression of genes involved in stress response pathways involving the glucocorticoid receptor. These genes showed higher expression during gestation in the T60%-HT group at week -6, and decreased expression of circadian rhythms in this same group at week 5 of lactation. Furthermore, fluctuations in the expression of genes involved in inflammatory regulation were observed throughout the peripartum weeks in all groups. The results obtained in the calves demonstrate that maternal feeding level and dietary supplementation of HT during the final third of pregnancy exert significant effects on early behavioural patterns and the regulation of circadian and inflammatory pathways.

Notably, our results indicate that nutritional restriction during the final third of gestation profoundly influenced the eating pattern of pregnant cows. Had the T100% cows been fed ad libitum, they would have consumed more feed than the actual supply. Likewise, the feeding restriction protocol allowed to observe expected differences in feeding behaviour, which was consistent with the intended nutritional levels and provided meaningful insights into animal behaviour and gene expression in blood cells. T60%-cows (T60%) exhibited a need to maximize nutrient intake, consuming feed at an accelerated rate and spending less time eating, which may reflect either anxious behaviour or a strategy to optimize nutrient acquisition [49]. In contrast, T100% cows displayed prolonged feeding rate, without behavioural indications of food-related anxiety, in line with previous studies in cattle and swine [39,50]. This pattern of accelerated feed intake was accompanied by stress-related behaviours such as head rubbing, licking surfaces, and oral stereotypes (tongue rolling), reflecting animal discomfort and further highlighting the stress induced by nutritional restriction. It is worth-mentioning that cows that received HT exhibited fewer occurrences of tongue rolling than those that did not, indicating that stress-related behavioural alterations were mitigated by the polyphenol extract. Consequently, the social and maintenance behaviours of T60%-HT cows approached those of T100% cows, which showed no visible signs of stress, in agreement with other reports working on cows [51–53]. In parallel, T60%-cows exhibited more frequent affiliative behaviours toward conspecifics, such as licking and sniffing, likely reflecting attempts at stress regulation through mechanisms that may involve oxytocin associated with positive social interactions in gregarious animals [54,55]. In agreement with the afore-mentioned results, T60%-HT cows did not exhibit this behaviour as prominently as T60%-CONTROL cows, suggesting a reduced expression of stress-related social behaviours. These findings suggest that, in beef cows, dietary HT supplementation may have partially attenuated behavioural indicators associated with undernutrition-induced stress in beef cows. In the lactation period, a greater number of cows in both T60% groups exhibited licking behaviour toward their calves compared to cows in the T100% groups during reunion for nursing. This finding suggests the presence of compensatory behavioural adjustments during lactation, as maternal licking constitutes one of the most critical care behaviours in ruminants, stimulating circulation and thermoregulation in the neonate, as well as reinforcing the dam-offspring bond, thereby contributing to the reduction in postnatal stress in both. The fact that cows subjected to nutritional restriction prioritized this behaviour, increasing its frequency, indicates that prenatal metabolic experience may modulate the expression of maternal behaviours, enhancing responses that promote appropriate development, environmental adaptation, and calf survival, which can be interpreted as a form of biological compensation [54,56–58]. Previous studies have demonstrated, across various species, that gestational stress can intensify certain maternal behaviours postpartum, likely mediated

by alterations in the expression of neuroendocrine hormones (e.g., oxytocin, prolactin) or via epigenetic mechanisms affecting maternal care circuits [59–61].

In calves, the current maternal diet restriction did not affect prenatal or early postnatal growth, since no differences were observed in calf birth weight or ADG during the first month of life. The activation of maternal compensatory mechanisms may have helped in maintaining homogeneity in early somatic development and may have contributed to maintain early somatic development [62]. However, changes were detected in behaviour, thermoregulation, and the expression of circadian genes and inflammatory regulation markers, indicating functional adaptations that were not immediately reflected in calf growth but may influence neonatal robustness and the quality of early-life development, as found in other experiments [6,63,64].

During the first month of life, the ability of calves to maintain body temperature depends on the interplay between metabolic thermogenesis—namely, the capacity to generate brown adipose tissue—behavioural mechanisms, such as increased mobility or social huddling, and physiological adjustments and maturation processes, including peripheral vasoconstriction [65,66]. T60%-HT calves exhibited, as a trend ( $p < 0.06$ ), lower rectal temperatures than T100% calves. This trend, which should be interpreted with caution, could suggest potential metabolic and energetic adjustments in thermoregulation, and reduced mean temperature may represent an adaptation to conserve calories, potentially allowing energy to be allocated to other processes [67,68]. This was likely facilitated by the transfer of HT-derived metabolites through colostrum to the offspring [69]. In our previous study, HT metabolites were detected in maternal plasma and colostrum, confirming supplementation compliance and transfer to the calves, providing early-life exposure to HT-derived compounds [29,69]. Complementarily, the stability of ear surface temperature suggests that thermoregulatory adaptations are selective, focusing on central regulation without compromising peripheral homeostasis [27,70,71]. On the other hand, the greater urination observed in T100%-CTRL calves, which spent more time on this activity, is likely due to a higher water and energy availability resulting from adequate maternal nutrition, which favours an increased renal filtration volume and metabolically generated heat production, thereby increasing the need to eliminate fluids [72]. The behavioural profile of T60%-HT calves reflects this adaptation, as they spent more time standing, exploring, and manipulating objects in the pen, reaching activity levels like T100%-CTRL calves. This active and exploratory behaviour indicates that HT supplementation allows these calves to achieve an optimal functional phenotype, sustaining behaviours that facilitate resource acquisition and adaptation to the postnatal environment [73], along with circadian synchronization observed in the present study.

Circadian clock genes play a central role in coordinating metabolic dynamics in both pregnant cows and their neonatal offspring [6]. The expression of these genes is consistent with cellular catabolic processes, facilitating energy mobilization to support the substantial metabolic demands of fetal development and maternal maintenance [74]. As pregnancy progresses towards term, leukocyte function must transition from predominant catabolic pathways, linked to the anti-inflammatory state of gestation, to primarily anabolic pathways that underpin the pro-inflammatory responses required for parturition [75,76]. Accordingly, all the studied circadian rhythm, stress response or inflammatory regulation gene expression markers in whole blood of cows peaked at week  $-6$  relative to parturition, defined by hormonal adaptations that facilitate the upcoming parturition [29]. The inherent stress of parturition was supported by *CRY1*, *CRY2*, and *HP*, that showed a marked decrease between weeks 1 and 5 postpartum in all cow groups reflection of a progressive adjustment of physiological postpartum inflammation and a concomitant re-entrainment of the circadian clock in peripheral blood cells [77]. In an earlier study, maternal nutrient restriction in pregnant dams shifted leukocyte metabolism towards catabolic pathways

and reduced the expression of genes involved in lipid synthesis. Following parturition, when both lactation and immune activation increase glucose requirements, T60% dams continued to exhibit a predominantly catabolic metabolic profile in their blood cells [29].

An increased *NR3C1* gene expression was observed in T60%-HT dams compared T60%-CTRL at week -6 prepartum. Under optimal conditions, when cortisol concentrations remain within the physiological range, *NR3C1* expression typically stays high, reflecting an enhanced sensitivity and a greater capacity to regulate processes such as glucose homeostasis and other metabolic cellular pathways [78]. Conversely, when animals are exposed to elevated stress levels—as in this study, due to feed restriction—the resulting potential rise in cortisol may downregulate *NR3C1* expression, probably to prevent excessive glucocorticoid signalling. Thus, *NR3C1* can be considered a reliable marker of cellular stress [79,80]. The decrease in *NR3C1*, circadian genes, and inflammatory response markers in all cows at week -3 suggests enhanced blood cellular sensitivity to fetal glucocorticoid [77,78]. The similar *NR3C1* gene expression in this week across groups is interpreted as a more catabolic cell state in conjunction with a promotion of fatty acid utilization and inhibition of glucose oxidation for energy production, favouring gluconeogenesis [81]. This pattern is consistent with previous results and reflects a dynamic immunometabolism characterized by pronounced endocrine changes inherent to gestational progression [29]. Collectively, this utilization of fatty acids for energy and the enhanced conservation of glucose is proposed to reduce the metabolic stress signal resulting from nutrient deficiency, which, via the HPA axis, could induce increased cortisol production and maintain low *NR3C1* levels, thereby stabilizing this cellular metabolic pathway [78]. Consequently, T60%-CTRL cows exhibited lower *NR3C1* levels (week -6) and higher cortisol concentrations, consistent with a stress response, which may manifest as stress-related behaviours that could potentially indicate a heightened state of agitation, likely resulting from a less optimized metabolic regulatory capacity [82]. It is noteworthy that the T60%-HT group tended to exhibit *NR3C1* expression levels comparable to those observed in the T100%-CTRL group. In line with the trends observed in [34], where both HT cow groups exhibited slightly lower cortisol levels than CTRL cows, regardless of feeding level, and consistent with previous findings in the same group reported by [29], which showed higher *PDK4* expression levels, a key regulator of glucose conservation and indirect support of gluconeogenesis.

In early postpartum (week 5), peripheral gene expression markers related with circadian rhythm (*BMAL1*, *PER1*, *CRY1*) were downregulated in T60%-HT dams in comparison with the other treatments. The T60%-HT group exhibited a downregulation of *BMAL1*, a core clock regulator that controls numerous downstream genes, including those involved in antioxidant defence such as *NRF2*, a key transcription factor that activates the antioxidant enzyme cascade [83,84], as well as *NF-κB*, another key transcription factor in inflammatory responses and cellular stress, and genes involved in energy metabolism. The T60%-HT group also showed reduced expression of *PER1* and *CRY1*, which participate in the negative feedback loop of the circadian clock, modulating the expression of inflammation-responsive genes such as leukocyte sensitivity to hormonal signals [84], reaching levels like those observed in the T100%-CTRL group. *BMAL1* acts as the primary activator of the other clock genes, typically peaking around the beginning of the day and gradually decreasing as *Per* (midday/afternoon) and *Cry* (afternoon/night) levels rise, thereby enabling synchronization with the light-feeding cycle [4,5,85]. Under conditions of dietary restriction, *BMAL1* exhibits an increase in expression, along with its regulatory genes *PER1* and *CRY1*, in response to cellular and endocrine signals that reflect the physiological stress status of the blood cell [86–88].

These findings indicate that dietary HT may contribute to the decreasing of *BMAL1*, *CRY1*, and *PER1*, potentially reducing the amplitude of the circadian feedback loop,

possibly via antioxidant-mediated catalysis of *PER1* mRNA degradation, although the underlying mechanism has not yet been elucidated [89]. This modulation may enhance energy cellular efficiency and prioritize energetic resources for maintaining homeostasis in blood cells during lactation period. In an earlier study, T60%-HT cows displayed reduced *ACADVL*, IGF signalling, and immune-related antioxidant responses during early postpartum, although their overall antioxidant capacity was improved [29]. The higher levels observed in the T60%-CTROL compared with T60%-HT group at week 5 postpartum could reflect a pattern characteristic of periods of high metabolic demand, coordinating critical cellular processes, inflammation, and glucocorticoid sensitivity [31,90,91].

In neonatal calves, the same molecular clocks facilitate cellular anabolic pathways, promoting tissue growth, thermoregulation, and the maturation of metabolic and immune cell functions during the early postnatal period [92,93]. This temporal orchestration ensures that energy utilization and physiological priorities are optimally synchronized with the specific requirements of each developmental stage.

A state of metabolic activation with precise biological synchronization is required to optimize energy utilization during the post-natal period [94]. Accordingly, a functional similarity between T60%-HT and T100%-CTROL was observed in blood cellular circadian regulation, leading to an upregulation in T60%-HT calves for the genes *CRY1* and *PER1*. Furthermore, both *Per1* and *Cry1* indirectly activate antioxidant enzymes via the *Nrf2* pathway [95] providing antioxidant protection to these blood cells. This is consistent with previously observed expression levels in T60%-HT calves for *NRF2*, *SOD1*, *CAT*, and *GPX1* [29].

These results are corroborated by the upregulation of the clock genes *CRY1* and *PER1* in blood cells, which are involved in the coordination of energy metabolism and exploratory behaviour, allowing the anticipation of environmental changes, synchronization of biological processes, and more precise utilization of available energy was described in small ruminants [93]. Its elevated expression of *CRY1* and *PER1* indicates that HT preserving glucose for dependent tissues while allowing efficient utilization of lipids as an energy source. This aligns with the capacity to maintain exploratory activity without compromising central homeostasis, integrating metabolism, and behaviour into a unified adaptive pattern. In contrast, T60%-CTROL calves, with lower expressions of *CRY1* and *PER1* in blood cells, did not appear able to distribute energy effectively, showing lower activity spent more time lying down reflecting a diminished capacity to maintain an active and adaptive behavioural pattern, likely associated with constraints in blood cellular circadian regulation [96].

Finally, the early immune response in neonatal calves was also modulated. Both *PER1* and *CRY1* are involved in the expression of transcription factors such as *NF-κB*, which in turn regulates the expression of *IL8*, *TLR4*, and *TNFA* [97] and indirectly affects *HP* through these pathways. Although *IL8* and *HP* levels decreased by the end of the first month of life, T60%-HT calves seemed to maintain a relatively more active immune response, exhibiting higher *IL8* expression than T60%-CTROL calves, yet comparable to T100%-CTROL calves. *IL-8* is critical for the recruitment of immune cells and the activation of effective innate defence mechanisms [98], and tissue repair and development [99] indicating a functional immune response in T60%-HT calves. This is consistent with the previously observed enhanced activation of immune-metabolic pathways in T60%-HT calves, characterized by elevated *TNFA*, *TLR4*, and *ALOX5* expression, genes that play central roles in the regulation of inflammatory processes, contributing to both the initiation and resolution phases of inflammation [29].

Taken together, these findings suggest that maternal supplementation with HT compensates for the effects of nutritional restriction, restoring behavioural patterns such as licking/sniffing, metabolic and circadian patterns such as *CRY1*, *PER1* and *IL8* in T60%-

HT calves comparable to those of calves born to well-nourished mothers. However, functional analyses including neuroendocrine regulators would be necessary to confirm these results. Maternal nutritional restriction during gestation, particularly when combined with dietary supplementation of olive-derived polyphenols, can modulate early-life behavioural phenotypes in beef calves. While nutritional restriction alone is known to alter fetal programming by improving caregiving, resource-seeking behaviour and alterations in circadian regulation, the concurrent supply of polyphenols may partially buffer oxidative and endocrine stress, probably thereby supporting a more balanced neurodevelopment. This interaction may result in calves that display increased exploratory activity during their first month of life, reflecting an adaptive response to perceived prenatal resource scarcity.

## 5. Conclusions

Late-pregnancy nutritional restriction altered the feeding patterns, social behaviours, and stress-related behaviours of healthy pregnant beef cows, as well as the regulation of glucocorticoid-receptor pathways (*NR3C1*). After parturition, it also altered the circadian (*BMAL1*, *PER1*, and *CRY1*) blood pathways, with associated effects such as more non-nutritive resource-seeking motivation and grooming to their calves during the early stages of life. Dietary HT supplementation partially counteracted these alterations by modulating behavioural responses in dams subjected to nutritional restriction, and up-regulating circadian (*PER1* and *CRY1*) and immunoregulatory (*IL-8*) gene expression in blood cells in their offspring, comparable to those observed in animals born to adequately fed dams. Whether these adaptations observed in the calves are beneficial or detrimental in the long term will need to be elucidated in future studies.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16080859/s1>, Table S1: Definitions of activities and behaviours observed during instantaneous observations of all cows during the gestation period. Prepared based on the methodology described in [37,38]; Table S2: Definitions of observed activities and behaviours in calves during the lactation period. Expressed as minutes per hour except vocalization. Prepared based on the methodology described in [37,41]; Table S3: Definitions of behaviours observed during the lactation period at the time of mother-calf reunion. Quantified as the proportion of animals showing each behaviour. Prepared based on the methodology described in [38].

**Author Contributions:** Conceptualization, J.Á.-R., A.S., and B.S.-P.; methodology, N.E.-M., L.L.d.A., A.N., I.B.-P., A.S., J.Á.-R., and B.S.-P.; validation, N.E.-M., J.Á.-R., and B.S.-P.; formal analysis, N.E.-M. and J.Á.-R.; investigation, N.E.-M., J.Á.-R., L.L.d.A., A.S., and B.S.-P.; data curation, N.E.-M., J.Á.-R., and B.S.-P.; writing—original draft preparation, N.E.-M., J.Á.-R., and B.S.-P.; writing—review and editing, N.E.-M., J.Á.-R., L.L.d.A., I.B.-P., A.N., A.S., and B.S.-P.; supervision, J.Á.-R. and B.S.-P.; project administration, A.S. and B.S.-P.; funding acquisition, A.S. and B.S.-P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was part of the project PID2020-113617RR-C22, funded by MICIU/AEI/10.13039/501100011033 (FETALNUT, the Spanish Ministry of Science, Innovation and Universities, Government of Spain), the Government of Aragon (grant research group INPASS A25\_23R), and the Government of Catalunya (2021 SGR 01361). N. Escalera-Moreno received a PhD grant from University of Lleida, and L. López de Armentia received a FPI-AEI grant.

**Institutional Review Board Statement:** The animal study protocol was approved by the Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Spain (protocol number CEEA-04 2021-09; approved on 18 October 2021). The care and use of animals were conducted in accordance with the European Parliament and Council of the European

Union on the protection of animals used for experimental and other scientific purposes (Directive 2010/63/EU).

**Data Availability Statement:** The data that support the study findings will be available in public Repositori Obert UdL at [repositori.udl.cat](https://repositori.udl.cat). Until then, data are available from the authors upon reasonable request.

**Acknowledgments:** We greatly appreciate the technical assistance of Teresa Giró, Anna Ñaco, Joan Carles Melo and La Garcipollera staff during sample collection and processing.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

- Rubio, A.; Roig, S. *Impactos, Vulnerabilidad y Adaptación al Cambio Climático en los Sistemas Extensivos de Producción Ganadera en España*; Oficina Española de Cambio Climático, Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente: Madrid, Spain, 2017. Available online: [https://www.miteco.gob.es/es/cambio-climatico/publicaciones/publicaciones/informe\\_ganaderia\\_extensiva\\_tcm30-435573.pdf](https://www.miteco.gob.es/es/cambio-climatico/publicaciones/publicaciones/informe_ganaderia_extensiva_tcm30-435573.pdf) (accessed on 9 February 2026).
- Sordillo, L.M.; Raphael, W. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. *Vet. Clin. N. Am. Food Anim. Pract.* **2013**, *29*, 267–278. <https://doi.org/10.1016/j.cvfa.2013.03.002>.
- Celi, P.; Gabai, G. Oxidant/antioxidant balance in animal nutrition and health: The role of protein oxidation. *Front. Vet. Sci.* **2015**, *2*, 48. <https://doi.org/10.3389/fvets.2015.00048>.
- Piccione, G.; Cannella, V.; Monteverde, V.; Bertolucci, C.; Frigato, E.; Congiu, F.; Guercio, A. Circadian gene expression in peripheral blood of *Bos taurus* under different experimental conditions. *J. Appl. Biomed.* **2014**, *12*, 271–275. <https://doi.org/10.1016/j.jab.2014.07.002>.
- Wang, M.; Zhou, Z.; Khan, M.J.; Gao, J.; Loor, J.J. Circadian gene network expression in bovine adipose, liver, and mammary gland during the transition from pregnancy to lactation. *J. Dairy Sci.* **2015**, *98*, 4601–4612. <https://doi.org/10.3168/jds.2015-9430>.
- Li, H.; Li, K.; Zhang, K.; Li, Y.; Gu, H.; Liu, H.; Yang, Z.; Cai, D. The circadian physiology: Implications in livestock health. *Int. J. Mol. Sci.* **2021**, *22*, 2111. <https://doi.org/10.3390/ijms22042111>.
- Suarez-Trujillo, A.; Hoang, N.; Robinson, L.; McCabe, C.J.; Conklin, D.; Minor, R.C.; Townsend, J.; Plaut, K.; George, U.Z.; Boerman, J.; et al. Effect of circadian system disruption on the concentration and daily oscillations of cortisol, progesterone, melatonin, serotonin, growth hormone, and core body temperature in periparturient dairy cattle. *J. Dairy Sci.* **2022**, *105*, 2651–2668. <https://doi.org/10.3168/jds.2021-20691>.
- Braun, U.; Tschoner, T.; Hässig, M. Evaluation of eating and rumination behaviour using a noseband pressure sensor in cows during the peripartum period. *BMC Vet. Res.* **2014**, *10*, 195. <https://doi.org/10.1186/s12917-014-0195-6>.
- Miranda-Moreira, G.; Aguiar, G.L.; Moreno-Meneses, J.A.; Nascimento, K.B.; Ramírez-Zamudio, G.D.; Costa, T.C.; Duarte, M.S.; Casagrande, D.R.; Gionbelli, M.P. Pregnancy affects maternal performance, feed intake, and digestion kinetics parameters in beef heifers. *J. Anim. Sci.* **2025**, *103*, skae328. <https://doi.org/10.1093/jas/skae328>.
- Hendriks, S.J.; Phyn, C.V.C.; Turner, S.-A.; Mueller, K.M.; Kuhn-Sherlock, B.; Donaghy, D.J.; Huzzey, J.M.; Roche, J.R. Lying behavior and activity during the transition period of clinically healthy grazing dairy cows. *J. Dairy Sci.* **2019**, *102*, 7371–7384. <https://doi.org/10.3168/jds.2018-16045>.
- Sanglard, L.P.; Nascimento, M.; Moriel, P.; Sommer, J.; Ashwell, M.; Poore, M.H.; Duarte, M.d.S.; Serão, N.V.L. Impact of energy restriction during late gestation on the muscle and blood transcriptome of beef calves after preconditioning. *BMC Genom.* **2018**, *19*, 702. <https://doi.org/10.1186/s12864-018-5089-8>.
- Redifer, C.A.; Wichman, L.G.; Davies-Jenkins, S.L.; Rathert-Williams, A.R.; Freetly, H.C.; Meyer, A.M. Late gestational nutrient restriction in primiparous beef females: Performance and metabolic status of lactating dams and pre-weaning calves. *J. Anim. Sci.* **2024**, *102*, skae015. <https://doi.org/10.1093/jas/skae015>.
- Hudson, S.; Mullard, M.; Whittlestone, W.G.; Payne, E. Diurnal variations in blood cortisol in the dairy cow. *J. Dairy Sci.* **1975**, *58*, 30–33. [https://doi.org/10.3168/jds.S0022-0302\(75\)84513-9](https://doi.org/10.3168/jds.S0022-0302(75)84513-9).
- McFadden, J.W. Review: Lipid biology in the periparturient dairy cow: Contemporary perspectives. *Animal* **2020**, *14*, s165–s175. <https://doi.org/10.1017/S1751731119003185>.
- Abuelo, A.; Hernández, J.; Benedito, J.L.; Castillo, C. Redox biology in transition periods of dairy cattle: Role in the health of periparturient and neonatal animals. *Antioxidants* **2019**, *8*, 20. <https://doi.org/10.3390/antiox8010020>.

16. Zachut, M.; Contreras, G.A. Mechanistic insights into adipose tissue inflammation and oxidative stress in periparturient dairy cows. *J. Dairy Sci.* **2022**, *105*, 3670–3686. <https://doi.org/10.3168/jds.2021-21225>.
17. John, A.J.; Garcia, S.C.; Kerrisk, K.L.; Freeman, M.J.; Islam, M.R.; Clark, C.E.F. The diurnal intake and behavior of dairy cows when access to a feed of consistent nutritive value is restricted. *J. Dairy Sci.* **2017**, *100*, 9279–9284. <https://doi.org/10.3168/jds.2016-12245>.
18. Salfer, I.J.; Harvatine, K.J. Night-restricted feeding of dairy cows modifies daily rhythms of feed intake, milk synthesis and plasma metabolites compared with day-restricted feeding. *Br. J. Nutr.* **2020**, *123*, 849–858. <https://doi.org/10.1017/S0007114520000057>.
19. Casey, T.M.; Plaut, K. Circadian clocks and their integration with metabolic and reproductive systems: Our current understanding and its application to the management of dairy cows. *J. Anim. Sci.* **2022**, *100*, skac233. <https://doi.org/10.1093/jas/skac233>.
20. DeVries, T.J.; von Keyserlingk, M.A.G.; Weary, D.M. Effect of feeding space on the inter-cow distance, aggression, and feeding behavior of free-stall housed lactating dairy cows. *J. Dairy Sci.* **2004**, *87*, 1432–1438. [https://doi.org/10.3168/jds.S0022-0302\(04\)73293-2](https://doi.org/10.3168/jds.S0022-0302(04)73293-2).
21. Abuelo, A. Symposium review: Late-gestation maternal factors affecting the health and development of dairy calves. *J. Dairy Sci.* **2020**, *103*, 3882–3893. <https://doi.org/10.3168/jds.2019-17278>.
22. Long, J.M.; Trubenbach, L.A.; Hobbs, K.C.; Poletti, A.E.; Steinhäuser, C.B.; Pryor, J.H.; Long, C.R.; Wickersham, T.A.; Sawyer, J.E.; Miller, R.K.; et al. Maternal nutrient restriction in late pregnancy programs postnatal metabolism and pituitary development in beef heifers. *PLoS ONE* **2021**, *16*, e0249924. <https://doi.org/10.1371/journal.pone.0249924>.
23. Parraguez, V.H.; Sales, F.; Peralta, O.; De los Reyes, M.; Gonzalez-Bulnes, A. Oxidative stress and fetal growth restriction set up earlier in undernourished sheep twin pregnancies: Prevention with antioxidant and nutritional supplementation. *Antioxidants* **2022**, *11*, 1287. <https://doi.org/10.3390/antiox11071287>.
24. Vazquez-Gomez, M.; Garcia-Contreras, C.; Torres-Rovira, L.; Pesantez, J.L.; Gonzalez-Añoover, P.; Gomez-Fidalgo, E.; Sanchez-Sanchez, R.; Ovilo, C.; Isabel, B.; Astiz, S.; et al. Polyphenols and IUGR pregnancies: Maternal hydroxytyrosol supplementation improves prenatal and early-postnatal growth and metabolism of the offspring. *PLoS ONE* **2017**, *12*, e0177593. <https://doi.org/10.1371/journal.pone.0177593>.
25. Rigacci, S.; Stefani, M. Nutraceutical properties of olive oil polyphenols. An itinerary from cultured cells through animal models to humans. *Int. J. Mol. Sci.* **2016**, *17*, 843. <https://doi.org/10.3390/ijms17060843>.
26. Britton, J.; Davis, R.; O'Connor, K.E. Chemical, physical and biotechnological approaches to the production of the potent antioxidant hydroxytyrosol. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 5957–5974. <https://doi.org/10.1007/s00253-019-09914-9>.
27. Garcia-Contreras, C.; Vazquez-Gomez, M.; Pardo, Z.; Heras-Molina, A.; Pesantez, J.L.; Encinas, T.; Torres-Rovira, L.; Astiz, S.; Nieto, R.; Ovilo, C.; et al. Polyphenols and IUGR pregnancies: Effects of maternal hydroxytyrosol supplementation on hepatic fat accretion and energy and fatty acids profile of fetal tissues. *Nutrients* **2019**, *11*, 1534. <https://doi.org/10.3390/nu11071534>.
28. Gómez, G.; Laviano, H.D.; García-Casco, J.M.; Escudero, R.; Muñoz, M.; Heras-Molina, A.; González-Bulnes, A.; Óvilo, C.; López-Bote, C.; Rey, A.I. Different Effect of Vitamin E or Hydroxytyrosol Supplementation to Sow's Diet on Oxidative Status and Performances of Weaned Piglets. *Antioxidants* **2023**, *12*, 1504. <https://doi.org/10.3390/antiox12081504>.
29. Escalera-Moreno, N.; Álvarez-Rodríguez, J.; López de Armentia, L.; Macià, A.; Martín-Alonso, M.J.; Molina, E.; Villalba, D.; Sanz, A.; Serrano-Pérez, B. Maternal hydroxytyrosol supplementation enhances antioxidant capacity and immunometabolic adaptations in nutrient-restricted beef cows and their offspring. *Antioxidants* **2025**, *14*, 1097. <https://doi.org/10.3390/antiox14091097>.
30. Sulaimani, N.; Houghton, M.J.; Bonham, M.P.; Williamson, G. Effects of (poly)phenols on circadian clock gene-mediated metabolic homeostasis in cultured mammalian cells: A scoping review. *Adv. Nutr.* **2024**, *15*, 100232. <https://doi.org/10.1016/j.advnut.2024.100232>.
31. Man, K.; Loudon, A.; Chawla, A. Immunity around the clock. *Science* **2016**, *354*, 999–1003. <https://doi.org/10.1126/science.aah4966>.
32. Bottegal, D.N.; Lobón, S.; Serrano-Pérez, B.; Martín-Alonso, M.J.; Latorre, M.Á.; Álvarez-Rodríguez, J. Mild synergistic effects of a dietary source of polyphenols (*Ceratonia siliqua* L.) and vitamin E on light lambs' rumination activity, nutritional status, and gastrointestinal redox-immune markers. *Livest. Sci.* **2025**, *291*, 105628. <https://doi.org/10.1016/j.livsci.2024.105628>.
33. Amato, A.; Cavallo, C.; Minuti, A.; Trevisi, E.; Engler, P.; Bui, H.; Gugliandolo, E.; Llobat, L.; Laporta, J.; Emmanuele, G.; et al. Rumen-protected dry grape extract supplementation enhances milk production, behavior traits, and immunometabolism of mid-lactating Fleckvieh cows under naturally occurring heat stress. *J. Dairy Sci.* **2025**, *108*, 11586–11605. <https://doi.org/10.3168/jds.2025-26830>.

34. López de Armentia, L.; Noya, A.; Álvarez-Rodríguez, J.; Villalba, D.; Serrano-Pérez, B.; Casasús, I.; Alabart, J.L.; Sanz, A. Effects of undernutrition and hydroxytyrosol supplementation in late pregnancy on cow-calf performance, metabolic and immune status, and newborn vitality in beef herds. *Animal* **2026**, *20*, 101793. <https://doi.org/10.1016/j.animal.2026.101793>.
35. Sanz, A.; Bernués, A.; Villalba, D.; Casasús, I.; Revilla, R. Influence of management and nutrition on postpartum interval in Brown Swiss and Pirenaica cows. *Livest. Prod. Sci.* **2004**, *86*, 179–191. [https://doi.org/10.1016/S0301-6226\(03\)00165-9](https://doi.org/10.1016/S0301-6226(03)00165-9).
36. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Turck, D.; Bresson, J.-L.; Burlingame, B.; Dean, T.; Fairweather-Tait, S.; Heinonen, M.; Hirsch-Ernst, K.I.; Mangelsdorf, I.; McArdle, H.J.; et al. Scientific opinion on the safety of hydroxytyrosol as a novel food pursuant to Regulation (EC) No 258/97. *EFSA J.* **2017**, *15*, e04728. <https://doi.org/10.2903/j.efsa.2017.4728>.
37. Leruste, H.; Bokkers, E.A.M.; Sergent, O.; Wolthuis-Fillerup, M.; van Reenen, C.G.; Lensink, B.J. Effects of the observation method (direct vs. from video) and of the presence of an observer on behavioural results in veal calves. *Animal* **2013**, *7*, 1858–1864. <https://doi.org/10.1017/S1751731113001456>.
38. Roadknight, N.; Wales, W.; Jongman, E.; Mansell, P.; Hepworth, G.; Fisher, A. Does the duration of repeated temporary separation affect welfare in dairy cow-calf contact systems. *Appl. Anim. Behav. Sci.* **2022**, *249*, 105592. <https://doi.org/10.1016/j.applanim.2022.105592>.
39. Álvarez-Rodríguez, J.; Casasús, I.; Blanco-Penedo, I.; Sanz, A. Effect of feeding level and breed on the daily activity budget of lactating beef cows fed total mixed ration. *Agriculture* **2020**, *10*, 195. <https://doi.org/10.3390/agriculture10060195>.
40. Kovács, L.; Kézér, F.L.; Póti, P.; Boros, N.; Nagy, K. Short communication: Upper critical temperature-humidity index for dairy calves based on physiological stress variables. *J. Dairy Sci.* **2019**, *102*, 2707–2710. <https://doi.org/10.3168/jds.2019-17459>.
41. Martin, P.; Bateson, P. *Measuring Behaviour: An Introductory Guide*, 3rd ed.; Cambridge University Press: Cambridge, UK, 2007.
42. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **2004**, *64*, 5245–5250. <https://doi.org/10.1158/0008-5472.CAN-04-0496>.
43. Serrano-Pérez, B.; Hansen, P.J.; Mur-Novales, R.; García-Ispuerto, I.; de Sousa, N.M.; Beckers, J.F.; Almería, S.; López-Gatius, F. Crosstalk between uterine serpin (SERPINA14) and pregnancy-associated glycoproteins at the fetal-maternal interface in pregnant dairy heifers experimentally infected with *Neospora caninum*. *Theriogenology* **2016**, *86*, 824–830. <https://doi.org/10.1016/j.theriogenology.2016.03.003>.
44. Monteiro, P.L.J., Jr.; Ribeiro, E.S.; Maciel, R.P.; Dias, A.L.G.; Solé, E., Jr.; Lima, F.S.; Bisinotto, R.S.; Thatcher, W.W.; Sartori, R.; Santos, J.E.P. Effects of supplemental progesterone after artificial insemination on expression of interferon-stimulated genes and fertility in dairy cows. *J. Dairy Sci.* **2014**, *97*, 4907–4921. <https://doi.org/10.3168/jds.2013-7802>.
45. Alhussien, M.N.; Dang, A.K. Integrated effect of seasons and lactation stages on the plasma inflammatory cytokines, function and receptor expression of milk neutrophils in Sahiwal (*Bos indicus*) cows. *Vet. Immunol. Immunopathol.* **2017**, *191*, 14–21. <https://doi.org/10.1016/j.vetimm.2017.07.010>.
46. Nebzydoski, S.J.; Pozzo, S.; Nemeč, L.; Rankin, M.K.; Gressley, T.F. The effect of dexamethasone on clock gene mRNA levels in bovine neutrophils and lymphocytes. *Vet. Immunol. Immunopathol.* **2010**, *138*, 189–196. <https://doi.org/10.1016/j.vetimm.2010.07.017>.
47. Drong, C.; Bühler, S.; Frahm, J.; Hüther, L.; Meyer, U.; von Soosten, D.; Gessner, D.K.; Eder, K.; Sauerwein, H.; Dänicke, S. Effects of body condition, monensin, and essential oils on ruminal lipopolysaccharide concentration, inflammatory markers, and endoplasmic reticulum stress of transition dairy cows. *J. Dairy Sci.* **2017**, *100*, 2751–2764. <https://doi.org/10.3168/jds.2016-11819>.
48. Beltman, M.E.; Lewis, J.; McCabe, M.; Keogh, K.; Kenny, D.A. The effect of natural and induced calving of beef heifers on stress-related gene expression and maternal health and immunity. *Animal* **2022**, *16*, 100550. <https://doi.org/10.1016/j.animal.2022.100550>.
49. Lindström, T.; Redbo, I. Effect of feeding duration and rumen fill on behaviour in dairy cows. *Appl. Anim. Behav. Sci.* **2000**, *70*, 83–97. [https://doi.org/10.1016/S0168-1591\(00\)00148-9](https://doi.org/10.1016/S0168-1591(00)00148-9).
50. Bottegal, D.N.; Latorre, M.Á.; Lobón, S.; Verdú, M.; Álvarez-Rodríguez, J. Fattening pigs with tannin-rich source (*Ceratonia siliqua* L.) and high doses of vitamin E: Effects on growth performance, economics, digestibility, physiology, and behaviour. *Animals* **2024**, *14*, 1855. <https://doi.org/10.3390/ani14131855>.
51. Daddam, J.R.; Daniel, D.; Kra, G.; Pelech, I.; Portnick, Y.; Moallem, U.; Lavon, Y.; Zachut, M. Plant polyphenol extract supplementation affects performance, welfare, and the Nrf2-oxidative stress response in adipose tissue of heat-stressed dairy cows. *J. Dairy Sci.* **2023**, *106*, 9807–9821. <https://doi.org/10.3168/jds.2023-23549>.

52. Redbo, I.; Nordblad, A. Stereotypies in heifers are affected by feeding regime. *Appl. Anim. Behav. Sci.* **1997**, *53*, 193–202. [https://doi.org/10.1016/S0168-1591\(96\)01145-8](https://doi.org/10.1016/S0168-1591(96)01145-8).
53. Downey, B.C.; Tucker, C.B. Breed differences in oral behaviors in feed-restricted dairy heifers. *J. Dairy Sci.* **2023**, *106*, 9440–9450. <https://doi.org/10.3168/jds.2022-23208>.
54. Papageorgiou, M.; Simitzis, P.E. Positive welfare indicators in dairy animals. *Dairy* **2022**, *3*, 814–841. <https://doi.org/10.3390/dairy3040056>.
55. Fielding, H.R.; Silk, M.J.; McKinley, T.J.; Delahay, R.J.; Wilson-Aggarwal, J.K.; Gauvin, L.; Ozella, L.; Cattuto, C.; McDonald, R.A. Social interactions of dairy cows and their association with milk yield and somatic cell count. *Appl. Anim. Behav. Sci.* **2024**, *279*, 106385. <https://doi.org/10.1016/j.applanim.2024.106385>.
56. Michenet, A.; Saintilan, R.; Venot, E.; Phocas, F. Insights into the genetic variation of maternal behavior and suckling performance of continental beef cows. *Genet. Sel. Evol.* **2016**, *48*, 45. <https://doi.org/10.1186/s12711-016-0223-z>.
57. Nevard, R.P.; Pant, S.D.; Broster, J.C.; Norman, S.T.; Stephen, C.P. Maternal behavior in beef cattle: The physiology, assessment and future directions—A review. *Vet. Sci.* **2022**, *10*, 10. <https://doi.org/10.3390/vetsci10010010>.
58. Ospina-Rios, S.L.; Lee, C.; Andrewartha, S.J.; Verdon, M. Temperament of the dairy cow relates to her maternal behaviour in a pasture-based extended suckling system. *Appl. Anim. Behav. Sci.* **2024**, *279*, 106400. <https://doi.org/10.1016/j.applanim.2024.106400>.
59. Meek, L.R.; Dittel, P.L.; Sheehan, M.C.; Chan, J.Y.; Kjolhaug, S.R. Effects of stress during pregnancy on maternal behavior in mice. *Physiol. Behav.* **2001**, *72*, 473–479. [https://doi.org/10.1016/S0031-9384\(00\)00431-5](https://doi.org/10.1016/S0031-9384(00)00431-5).
60. Coulon, M.; Lévy, F.; Ravel, C.; Nowak, R.; Boissy, A. Mild effects of gestational stress and social reactivity on the onset of mother–young interactions and bonding in sheep. *Stress* **2014**, *17*, 460–470. <https://doi.org/10.3109/10253890.2014.969238>.
61. Schmidt, M.; Braun, K.; Brandwein, C.; Rossetti, A.C.; Guara Ciurana, S.; Riva, M.A.; Deuschle, M.; Bock, J.; Gass, P.; Gröger, N. Maternal stress during pregnancy induces depressive-like behavior only in female offspring and correlates to their hippocampal Avp and Oxt receptor expression. *Behav. Brain Res.* **2018**, *349*, 46–54. <https://doi.org/10.1016/j.bbr.2018.06.027>.
62. Caton, J.S.; Crouse, M.S.; Reynolds, L.P.; Neville, T.L.; Dahlen, C.R.; Ward, A.K.; Swanson, K.C. Maternal nutrition and programming of offspring energy requirements. *Transl. Anim. Sci.* **2019**, *3*, 976–990. <https://doi.org/10.1093/tas/txy127>.
63. Hicks, Z.M.; Yates, D.T. Going Up Inflammation: Reviewing the underexplored role of inflammatory programming in stress-induced intrauterine growth restricted livestock. *Front. Anim. Sci.* **2021**, *2*, 761421. <https://doi.org/10.3389/fanim.2021.761421>.
64. Merlot, E.; Quesnel, H.; Prunier, A. Prenatal stress, immunity and neonatal health in farm animal species. *Animal* **2013**, *7*, 2016–2025. <https://doi.org/10.1017/S175173111300147X>.
65. Carstens, G.E. Cold thermoregulation in the newborn calf. *Vet. Clin. N. Am. Food Anim. Pract.* **1994**, *10*, 69–106. [https://doi.org/10.1016/S0749-0720\(15\)30590-9](https://doi.org/10.1016/S0749-0720(15)30590-9).
66. Mota-Rojas, D.; Wang, D.; Titto, C.G.; Martínez-Burnes, J.; Villanueva-García, D.; Lezama, K.; Domínguez, A.; Hernández-Avalos, I.; Mora-Medina, P.; Verduzco, A.; et al. Neonatal infrared thermography images in the hypothermic ruminant model: Anatomical-morphological-physiological aspects and mechanisms for thermoregulation. *Front. Vet. Sci.* **2022**, *9*, 963205. <https://doi.org/10.3389/fvets.2022.963205>.
67. Brooke, O.G. Influence of malnutrition on the body temperature of children. *Br. Med. J.* **1972**, *1*, 331–333.
68. Zhou, X.; Yan, Q.; Yang, H.; Ren, A.; He, Z.; Tan, Z. Maternal intake restriction programs the energy metabolism, clock circadian regulator and mTOR signals in the skeletal muscles of goat offspring probably via the protein kinase A–cAMP-responsive element-binding proteins pathway. *Anim. Nutr.* **2022**, *8*, 1303–1314. <https://doi.org/10.1016/j.aninu.2021.09.006>.
69. Silva, F.L.M.; Miqueo, E.; da Silva, M.D.; Torrezan, T.M.; Rocha, N.B.; Vieira Salles, M.S.; Bittar, C.M.M. Thermoregulatory responses and performance of dairy calves fed different amounts of colostrum. *Animals* **2021**, *11*, 703. <https://doi.org/10.3390/ani11030703>.
70. Xue, Y.; Guo, C.; Hu, F.; Zhu, W.; Mao, S. Maternal undernutrition induces fetal hepatic lipid metabolism disorder and affects the development of fetal liver in a sheep model. *FASEB J.* **2019**, *33*, 9990–10004. <https://doi.org/10.1096/fj.201900406R>.
71. Laviano, H.D.; Gómez, G.; Núñez, Y.; García-Casco, J.M.; Benítez, R.M.; de las Heras-Molina, A.; Gómez, F.; Sánchez-Esquiliche, F.; Martínez-Fernández, B.; González-Bulnes, A.; et al. Maternal dietary antioxidant supplementation regulates weaned piglets' adipose tissue transcriptome and morphology. *PLoS ONE* **2024**, *19*, e0310399. <https://doi.org/10.1371/journal.pone.0310399>.
72. Vorndran, A.M.; Kehraus, S.; Kleigrewe, K.; Steinhoff-Wagner, J. Urinary dynamics and analytical composition of neonatal Brown Swiss calves' urine during the first week postpartum, fed either dam's milk or milk replacer. *J. Dairy Sci.* **2025**, *108*, 10956–10972. <https://doi.org/10.3168/jds.2025-26651>.

73. Kladt, L.V.; Jiang, M.; Li, Z.; Veloso, C.M.; Hare, K.S.; Silva, W.; Wood, K.M.; Serão, N.V.L.; Gionbelli, M.P.; Steele, M.A.; et al. Maternal metabolizable energy intake during late gestation affects energy metabolism of the skeletal muscle of beef offspring. *J. Anim. Sci.* **2025**, *103*, skaf203. <https://doi.org/10.1093/jas/skaf203>.
74. Wharfe, M.D.; Wyrwoll, C.S.; Waddell, B.J.; Mark, P.J. Pregnancy-induced changes in the circadian expression of hepatic clock genes: Implications for maternal glucose homeostasis. *Am. J. Physiol. Endocrinol. Metab.* **2016**, *311*, E575–E586. <https://doi.org/10.1152/ajpendo.00060.2016>.
75. Liu, S.; Zhang, S.; Hong, L.; Diao, L.; Cai, S.; Yin, T.; Zeng, Y. Characterization of progesterone-induced dendritic cells in metabolic and immunologic reprogramming. *J. Reprod. Immunol.* **2023**, *159*, 104128. <https://doi.org/10.1016/j.jri.2023.104128>.
76. Minuti, A.; Jahan, N.; Lopreiato, V.; Piccioli-Cappelli, F.; Bomba, L.; Capomaccio, S.; Loor, J.J.; Ajmone-Marsan, P.; Trevisi, E. Evaluation of circulating leukocyte transcriptome and its relationship with immune function and blood markers in dairy cows during the transition period. *Funct. Integr. Genom.* **2019**, *20*, 293–305. <https://doi.org/10.1007/s10142-019-00720-0>.
77. Trevisi, E.; Minuti, A. Assessment of the innate immune response in the periparturient cow. *Res. Vet. Sci.* **2018**, *116*, 47–54. <https://doi.org/10.1016/j.rvsc.2017.12.001>.
78. Preisler, M.T.; Weber, P.S.D.; Tempelman, R.J.; Erskine, R.J.; Hunt, H.; Burton, J.L. Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient Holstein cows. *J. Dairy Sci.* **2000**, *83*, 38–47. [https://doi.org/10.3168/jds.S0022-0302\(00\)74852-1](https://doi.org/10.3168/jds.S0022-0302(00)74852-1).
79. Palma-Gudiel, H.; Córdova-Palomera, A.; Leza, J.C.; Fañanás, L. Glucocorticoid receptor gene (*NR3C1*) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review. *Neurosci. Biobehav. Rev.* **2015**, *55*, 520–535. <https://doi.org/10.1016/j.neubiorev.2015.05.016>.
80. Zheng, G.; Von Furstenberg, G.; Meixner, W.; Creekmore, A.; Zong, Y.; Dame, M.K.; Colacino, J.; Dedhia, P.H.; Hong, S.; Wiley, J.W. Chronic stress and intestinal barrier dysfunction: Glucocorticoid receptor and transcription repressor HES1 regulate tight junction protein Claudin-1 promoter. *Sci. Rep.* **2017**, *7*, 4502. <https://doi.org/10.1038/s41598-017-04755-w>.
81. Wathes, D.C.; Cheng, Z.; Salavati, M.; Buggiotti, L.; Takeda, H.; Tang, L.; Becker, F.; Ingvarsen, K.I.; Ferris, C.; Hostens, M.; et al. Relationships between metabolic profiles and gene expression in liver and leukocytes of dairy cows in early lactation. *J. Dairy Sci.* **2021**, *104*, 3596–3616. <https://doi.org/10.3168/jds.2020-19165>. Corrigendum in *J. Dairy Sci.* **2021**, *104*, 6327. <https://doi.org/10.3168/jds.2021-104-5-6327>.
82. Boyle, M.P.; Kolber, B.J.; Vogt, S.K.; Wozniak, D.F.; Muglia, L.J. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. *J. Neurosci.* **2006**, *26*, 1971–1978. <https://doi.org/10.1523/JNEUROSCI.2173-05.2006>.
83. Pekovic-Vaughan, V.; Gibbs, J.; Yoshitane, H.; Yang, N.; Pathiranage, D.; Guo, B.; Sagami, A.; Taguchi, K.; Bechtold, D.; Loudon, A.; et al. The circadian clock regulates rhythmic activation of the NRF2/glutathione-mediated antioxidant defense pathway to modulate pulmonary fibrosis. *Genes Dev.* **2014**, *28*, 548–560. <https://doi.org/10.1101/gad.237081.113>.
84. Early, J.O.; Menon, D.; Wyse, C.A.; Cervantes-Silva, M.P.; Zaslona, Z.; Carroll, R.G.; Palsson-McDermott, E.M.; Angiari, S.; Ryan, D.G.; Corcoran, S.E.; et al. Circadian clock protein *BMAL1* regulates IL-1 $\beta$  in macrophages via NRF2. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E8460–E8468. <https://doi.org/10.1073/pnas.1800431115>.
85. Cox, K.H.; Takahashi, J.S. Circadian clock genes and the transcriptional architecture of the clock mechanism. *J. Mol. Endocrinol.* **2019**, *63*, R93–R102. <https://doi.org/10.1530/JME-19-0153>.
86. Kobayashi, H.; Oishi, K.; Hanai, S.; Ishida, N. Effect of feeding on peripheral circadian rhythms and behaviour in mammals. *Genes Cells* **2004**, *9*, 857–864. <https://doi.org/10.1111/j.1365-2443.2004.00769.x>.
87. Kawamoto, T.; Noshiro, M.; Furukawa, M.; Honda, K.K.; Nakashima, A.; Ueshima, T.; Usui, E.; Katsura, Y.; Fujimoto, K.; Honma, S.; et al. Effects of fasting and re-feeding on the expression of *Dec1*, *PER1*, and other clock-related genes. *J. Biochem.* **2006**, *140*, 401–408. <https://doi.org/10.1093/jb/mvj165>.
88. Patel, S.A.; Velingkaar, N.; Makwana, K.; Chaudhari, A.; Kondratov, R. Calorie restriction regulates circadian clock gene expression through *BMAL1* dependent and independent mechanisms. *Sci. Rep.* **2016**, *6*, 25970. <https://doi.org/10.1038/srep25970>.
89. Oishi, K.; Yamamoto, S.; Oike, H.; Ohkura, N.; Taniguchi, M. Cinnamic acid shortens the period of the circadian clock in mice. *Biochem. Biophys. Rep.* **2017**, *9*, 232–237. <https://doi.org/10.1016/j.bbrep.2016.12.008>.
90. Eckel-Mahan, K.; Sassone-Corsi, P. Metabolism and the circadian clock converge. *Physiol. Rev.* **2013**, *93*, 107–135. <https://doi.org/10.1152/physrev.00016.2012>.
91. Curtis, A.M.; Bellet, M.M.; Sassone-Corsi, P.; O'Neill, L.A.J. Circadian clock proteins and immunity. *Immunity* **2014**, *40*, 178–186. <https://doi.org/10.1016/j.immuni.2014.02.002>.

92. Sládek, M.; Jindráková, Z.; Bendová, Z.; Sumová, A. Postnatal ontogenesis of the circadian clock within the rat liver. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2007**, *292*, R1224–R1229. <https://doi.org/10.1152/ajpregu.00184.2006>.
93. Serón-Ferré, M.; Torres-Farfán, C.; Valenzuela, F.J.; Castillo-Galán, S.; Rojas, A.; Méndez, N.; Reynolds, H.; Valenzuela, G.J.; Llanos, A.J. Deciphering the function of the blunt circadian rhythm of melatonin in the newborn lamb: Impact on adrenal and heart function. *Endocrinology* **2017**, *158*, 2895–2905. <https://doi.org/10.1210/en.2017-00254>.
94. Furman-Fratczak, K.; Rzasa, A.; Stefaniak, T. The influence of colostral immunoglobulin concentration in heifer calves' serum on their health and growth. *J. Dairy Sci.* **2011**, *94*, 5536–5543. <https://doi.org/10.3168/jds.2010-3253>.
95. Xiong, L.; Lin, T.; Yue, X.; Zhang, S.; Liu, X.; Chen, F.; Guan, W. Maternal Selenium-Enriched Yeast Supplementation in Sows Enhances Offspring Growth and Antioxidant Status through the Nrf2/Keap1 Pathway. *Antioxidants* **2023**, *12*, 2064. <https://doi.org/10.3390/antiox12122064>.
96. Zhang, W.; Wang, Y.; Guo, L.; Falzon, G.; Kwan, P.; Jin, Z.; Li, Y.; Wang, W. Analysis and comparison of new-born calf standing and lying time based on deep learning. *Animals* **2024**, *14*, 1324. <https://doi.org/10.3390/ani14091324>.
97. Silver, A.C.; Arjona, A.; Walker, W.E.; Fikrig, E. The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity* **2012**, *36*, 251–261. <https://doi.org/10.1016/j.immuni.2011.12.017>.
98. Baggiolini, M.; Clark-Lewis, I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* **1992**, *307*, 97–101. [https://doi.org/10.1016/0014-5793\(92\)80909-Z](https://doi.org/10.1016/0014-5793(92)80909-Z).
99. Gao, L.; Gao, R.; Mao, W.; Liu, B.; Zhang, S.; Tahala, D.; Fu, C.; Shen, Y.; Wu, J.; Deng, Y.; et al. PTGFR activation promotes the expression of PTGS-2 and growth factors via activation of the PKC signaling pathway in bovine endometrial epithelial cells. *Anim. Reprod. Sci.* **2018**, *199*, 30–39. <https://doi.org/10.1016/j.anireprosci.2018.10.003>.

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.