

CHANGES IN THYROID HORMONES LEVELS AND METABOLISM IN DAIRY COWS AROUND CALVING

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The hormonal activity of the thyroid gland has an important role in ruminants for the modulation of metabolic variables. In this study changes in thyroid hormones and biochemical parameters in dairy cows around calving were evaluated and the critical thyroid hormones thresholds for predicting the risk of ketosis were estimated. Blood samples were collected from 82 dairy cows at 5 ± 3 days pre-partum and 5 ± 3 days post-partum. Serum values of triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), non-esterified fatty acids (NEFA), β -hydroxybutyrate (BHB), insulin, glucose, Ca, Cl, Mg, P, K, Na, aspartate transaminase (AST), alanine transaminase (ALT) and urea were evaluated. Significant decrease in the levels of T3, T4 and TSH was found in the postpartum period. The values of T3 and T4 were negatively correlated with NEFA and BHB levels, and TSH values were negatively correlated with NEFA. A critical T3 threshold was found by means of ROC analysis for predicting the risk of ketosis. T3 values < 1.23 nmol/L were associated with BHB > 1.10 mmol/L in postpartum, which represents the BHB threshold commonly used as the indicator of hyperketonaemia.

The results confirm that the peripartum period is accompanied by marked changes in circulating thyroid hormone profile that is correlated with lipomobilization predictors.

Key words: dairy cow, metabolism, thyroid hormone, transition period

INTRODUCTION

The time from late pregnancy to early lactation is known as the transition period and it is recognized as the period between 3 weeks before to 3 weeks after parturition in the ruminant [1-3].

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Cows in late gestation have higher metabolic demands and less feed intake compared to cows in the early dry period and enter a period of negative energy balance (NEB) intensified in early lactation when feed intake lags behind milk production [4,5].

Thyroid gland hormonal activity has an important role in the periparturient period for determining the cell metabolism intensity, the metabolism of lipids and carbohydrates, as well as the course of lactation. Thyroid hormones modulate metabolism in ruminants in which carbohydrates and lipids are the major constituents [6]. Transition dairy cows are often characterized by a decrease in serum glucose level and an increase of non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) serum concentrations, suggesting the difficulty of dairy cows to cope with the energy demand which characterizes the transition period [7].

Concentrations of blood metabolites including BHB, NEFA, glucose and insulin may provide some indication of postpartum disease risk and can be useful as a herd monitoring tool [7-9]. The positive correlation between circulating thyroid hormones concentrations and metabolic variables is well known in many animal species including cattle [10-14]. At the level of the hypothalamus, thyrotropin-releasing hormone is released which stimulates the pituitary to secrete the thyroid-stimulating hormone (thyrotropin or TSH). TSH in turn drives the thyroid gland to release the prohormone thyroxine T₄ into the circulation. Conversion of T₄ in peripheral tissues produces the active hormone 3,5,3'-tri-iodothyronine (T₃) and reverse T₃ (rT₃) which is thought to be metabolically inactive. Both T₃ and T₄ are present in the blood circulation, although the physiological effects are attributed almost only to T₃ [15-17]. In view of such considerations, the aim of the present study was to evaluate the changes of some biochemical parameters and of serum thyroid hormones including T₃, T₄ and TSH, as well as their relationship in dairy cows around calving. Moreover, the critical thyroid hormones thresholds based on the critical values of BHB and NEFA, previously identified as predictors of ketosis, were estimated in order to identify possibly diseased dairy cows.

MATERIALS AND METHODS

All treatments, housing and animal care were carried out in accordance with the standards recommended by EU Directive 2010/63/EU for animal experiments.

Farm and animals

Eighty-two multiparous Holstein Frisian cows (*Bos taurus*) were enrolled from a high production dairy farm in Northeast Italy: 29 cows in the second lactation, 27 in the third lactation and 24 in the fourth lactation. Farm management included a dry period of 60 days and a period of 15 days before calving in which the cows are fed a diet with increased energy density. The chemical composition of the diets used during steaming-up and subsequent early lactation is reported in Table 1. The cows produced an average of 9490.58 ± 2461.39 kg milk per lactation, with an average of 3.64%

of milk-fat and 3.25% of milk-protein. All the animals were clinically healthy and free from internal and external parasites. Their health status was evaluated based on rectal temperature, heart rate, respiratory profile, appetite and fecal consistency. Body condition score (BCS) of each cow was evaluated on a scale from 0 to 5 [18] at 5 ± 3 d before and 5 ± 3 d after calving.

Table 1. Feed ingredients and chemical composition of the total mixed ratio (TMR) administered in pre-partum and post-partum periods

Total mixed ratio (TMR)	Pre-partum	Post-partum
Feed basis (kg/animal)	20.40	25.10
Dry Matter (DM) (kg/animal)	11.74	19.86
Dry Matter Intake (DMI) (kg/animal)	11.14	19.60
Chemical composition (%)	Pre-partum	Post-partum
Crude protein	13.42	16.83
Ethereal extract	4.27	5.89
NDF	41.12	30.10
ADF	25.96	20.35
NFC	34.28	38.06
Starch	14.87	27.99

NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; NFC: Non Fiber Carbohydrates

Blood sampling and biochemical analysis

Blood sampling was performed from each animal at 5 ± 3 d pre-partum and 5 ± 3 d post-partum. Polyethylene catheters were aseptically inserted into the external jugular vein. Blood samples were collected into 8 mL serum vacuum tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). The samples were allowed to clot for 30 minutes at room temperature, then the tubes were centrifuged (Labfuge 400, Heraeus) at 1750 g for 10 minutes and the obtained serum was stored at -18°C . Hormonal profile analyses included: triiodothyronine (T3; ng/dL), thyroxine (T4; $\mu\text{g}/\text{dL}$) and thyroid-stimulating hormone (TSH; $\mu\text{IU}/\text{mL}$). T3, T4, TSH concentration in each sample was quantified by means of IMMULITE[®] 2000 (Siemens) instrument and commercial kits (Immunolite 2000 Total T3/L2KT36; Immunolite 2000 Total T4/L2KT46; Immunolite 2000 Third Generation TSH/L2KTS6; Siemens, Italy). Biochemical analysis included the measurement of NEFA, BHB, glucose, Ca, Cl, Mg, P, K, Na, aspartate transaminase (AST), alanine transaminase (ALT) and urea concentrations. Biochemical parameters were measured using available commercial kits by means of the automated clinical chemistry analyzer Konelab 60I (Thermo Electron Corporation). Free-insulin concentration in each sample was quantified with a commercial 125I-IRMA kit developed for human samples (BI-Ins IRMA kit; CIS Bio International Ltd.) and previously validated for bovine plasma samples [19]. NEFA concentration was determined by using a colorimetric method, NEFA RX Monza test

(kit no. FA 115, Randox, Crumlin, UK) while BHB concentration was determined with the RANBUT RX Monza test (kit no. RB 1007, Randox, Crumlin, UK).

Statistical analysis

One-way analysis of variance (ANOVA) was performed in order to assess statistically significant effects of period (pre-partum vs post-partum) on the investigated parameters. $P < 0.05$ was considered statistically significant.

Pearson's correlation test was applied in order to investigate the relationship among thyroid hormones and biochemical parameters.

Critical values of BHB and NEFA previously identified as predictors of ketosis [8] were used to dichotomize data in 4 models: pre-partum BHB; post-partum BHB; pre-partum NEFA; post-partum NEFA.

Data were dichotomized: at pre-partum BHB values < 0.6 mmol/L (CL_BHB < 0.6) or ≥ 0.6 mmol/L (CL_BHB ≥ 0.6); at post-partum BHB values < 1.1 mmol/L (CL_BHB < 1.1) or ≥ 1.1 mmol/L (CL_BHB ≥ 1.1); at pre-partum NEFA values < 0.3 mmol/L (CL_NEFA < 0.3) or ≥ 0.3 mmol/L (CL_NEFA ≥ 0.3); at post-partum NEFA values < 0.6 mmol/L (CL_NEFA < 0.6) or ≥ 0.6 mmol/L (CL_NEFA ≥ 0.6).

For each model thyroid hormones were evaluated as predictors of ketosis. Thyroid hormones were evaluated with receiver operator characteristic (ROC) analysis in order to determine critical thresholds for predicting the risk of disease. Data from the pre- and post-partum cohorts were analyzed separately.

The ROC curves analyze sensitivity (Se) versus 100-specificity (Sp). The point on the ROC curve that has the highest combined Se and Sp was considered the critical threshold. Interpretation of this critical threshold depends on the area under the curve (AUC), such that if $AUC > 0.7$ the test is considered accurate [20]. On the basis of ROC analysis we dichotomized data at $<$ or $>$ of critical thresholds and we investigated the effect of hormones levels and time period on the biochemical parameters by means of two-way ANOVA.

Statistical analyses were performed by means of SAS software (version 9.3; SAS Institute, Inc., Cary, NC, USA, 2009).

RESULTS

Mean values \pm SD of metabolic parameters obtained at the pre-partum and post-partum time points are reported in Table 2. A significant decrease in the levels of thyroid hormones and TSH were found in post-partum cows. Statistical analysis showed lower values for T3 ($P < 0.01$), TSH ($P < 0.05$), T4 ($P < 0.001$), insulin ($P < 0.01$), glucose ($P < 0.01$), ALT ($P < 0.001$) and urea ($P < 0.05$) in post-partum than in pre-partum; whereas, higher BHB and NEFA values were found at post-partum than at pre-partum ($P < 0.01$). No statistically significant effect of the peripartum period was found on the values of blood minerals, with the exception of K that showed significantly decreased values at post-partum ($P < 0.01$). Pearson's correlation test results obtained between thyroid hormones and biochemical parameters are shown in Table 3.

Table 2. Mean values \pm SD of metabolic parameters measured in cows 5 ± 3 days pre-partum and 5 ± 3 days post-partum periods

Parameters	Pre-partum	Post-partum	P
BCS	3.34 \pm 0.23	2.98 \pm 0.46	***
T3 (nmol/L)	1.75 \pm 0.27	1.39 \pm 0.34	**
TSH (nIU/mL)	0.032 \pm 0.003	0.012 \pm 0.002	*
T4 (nmol/L)	40.93 \pm 11.20	23.38 \pm 6.69	***
BHB (mmol/L)	0.59 \pm 0.12	1.16 \pm 0.36	**
NEFA (mmol/L)	0.24 \pm 0.08	0.86 \pm 0.47	**
Insulin (pmol/L)	28.30 \pm 5.90	11.85 \pm 2.49	**
Glucose (mmol/L)	3.98 \pm 0.91	3.46 \pm 0.95	**
ALT (U/L)	18.93 \pm 5.70	16.46 \pm 4.50	***
AST (U/L)	72.17 \pm 34.10	107.41 \pm 24.40	**
Urea (mmol/L)	9.42 \pm 2.32	7.79 \pm 2.81	*
K (mmol/L)	4.36 \pm 0.95	3.96 \pm 1.10	**

BCS = Body Condition Score; NEFA = Non-Esterified Fatty Acids; BHB = β -hydroxybutyrate; ALT = Alanine transaminase; AST = Aspartate transaminase
Significances: *P < 0.05; **P < 0.01; ***P < 0.001

Table 3. Significant Pearson's correlation results found among thyroidhormones and biochemical parameters

Parameters	R	P	
T3	T4	+0.537	***
	TSH	+0.332	***
	Glucose	+0.367	***
	Insulin	+0.226	*
	NEFA	-0.383	***
	BHB	-0.339	***
	NEFA	-0.238	*
	Insulin	+0.368	***
TSH	Glucose	+0.364	***
	Cl	+0.371	***
	Mg	+0.344	***
	Na	+0.412	***
	K	-0.351	***
T4	Insulin	+0.513	***
	Glucose	+0.247	**
	Mg	+0.330	***
	BHB	-0.280	***
	NEFA	-0.402	***
	AST	-0.304	***

NEFA: Non-Esterified Fatty Acids; BHB: β -hydroxybutyrate; AST: Aspartate transaminase
Significances: * P < 0.05; ** P < 0.01; ***P < 0.001

Table 4. Statistical data of ROC analysis for thyroid hormones obtained from cows at 5±3 days pre-partum and 5±3 days post-partum period

Threshold	Thyroid Hormone		ROC						
	Parameters	Cut-off	AUC	SE	95% CI for SE	SP	95% CI for SP	LR+	P-value
Pre-partum (5±3 Days)	T3 (ng/dl)	>1.53	0.55±0.06	79.41	56.3 - 92.5	42.37	29.6 - 55.9	1.43	<0.0001
	T4 (ug/dl)	≤ 40.62	0.54±0.06	58.82	34.5 - 76.8	52.54	39.1 - 65.7	1.65	0.05
	TSH (uIU/ml)	>0.015	0.55±0.07	61.76	42.7 - 83.6	59.32	45.7 - 71.9	1.92	<0.0001
	T3 (ng/dl)	≤ 1.53	0.57±0.08	50.00	30.6 - 73.2	69.49	56.1 - 80.8	2.28	<0.0001
	T4 (ug/dl)	≤ 9.08	0.58±0.08	68.75	47.1 - 86.8	54.24	40.8 - 67.3	1.71	0.05
	TSH (uIU/ml)	≤ 0.004	0.63±0.08	43.75	23.2 - 65.5	76.27	63.4 - 86.4	2.57	<0.0001
Post-partum (5±3 Days)	T3 (ng/dl)	≤ 1.23	0.86±0.06	81.87	71.2 - 95.0	93.05	83.5 - 98.1	4.49	<0.0001
	T4 (ug/dl)	≤ 20.6	0.61±0.07	60.87	38.5 - 80.3	55.93	42.4 - 68.8	1.78	0.05
	TSH (uIU/ml)	≤ 0.004	0.93±0.06	78.26	56.3 - 92.5	61.02	49.1 - 75.0	2.10	<0.0001
	T3 (ng/dl)	≤ 1.12	0.60±0.06	30.43	13.2 - 52.9	94.44	83.5 - 98.1	4.49	<0.0001
	T4 (ug/dl)	≤ 14.87	0.57±0.06	32.61	16.4 - 57.3	86.11	75.0 - 94.0	3.53	0.05
	TSH (uIU/ml)	≤ 0.004	0.53±0.06	56.52	34.5 - 76.8	58.33	44.1 - 70.4	1.85	<0.0001

AUC: area under the curve; SE: Epidemiologic sensitivity; SP: Epidemiologic specificity; LP: Likelihood ratio positive.

T3 values results positively correlated with T4, TSH, glucose and insulin, and negatively correlated with NEFA and BHB. A significant negative correlation was found between TSH and NEFA values. Moreover, TSH positively correlated with insulin, glucose, Cl, Mg and Na. However, a negative correlation between TSH and K values was found. T4 positively correlated with insulin, glucose and Mg, and negatively correlated with BHB, NEFA and AST. The optimal cut-off points of thyroid hormones were considered based on ROC curves. As reported in Table 4, ROC analysis suggests that T3 concentration was a predictive variable at post-partum in the BHB model. The ROC

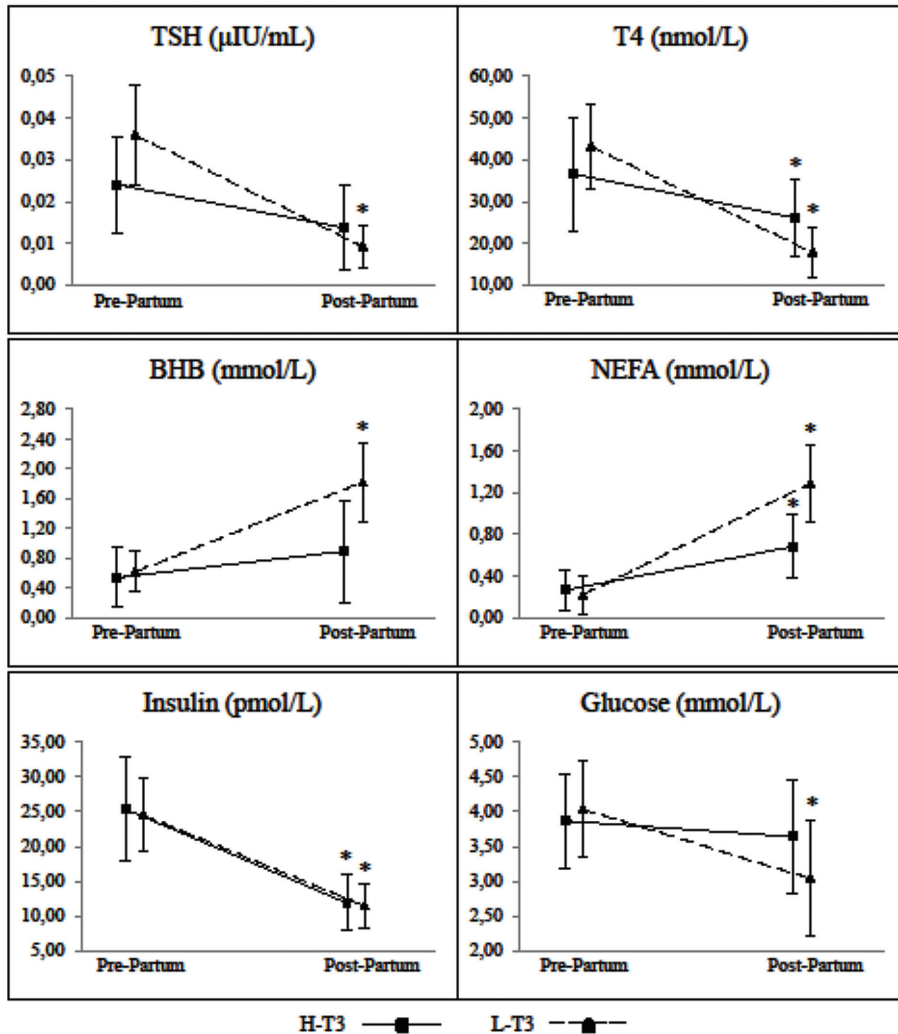


Figure 1. Significant relationships of T3 levels with other metabolic parameters in cows during the monitoring period (5±3 days pre-partum and 5±3 days post-partum). Animals with T3 > 1.23 nmol/L are represented as high T3 (H-T3), animals with T3 < 1.23 nmol/L are represented as low T3 (L-T3)
Significances: * vs Pre-Partum (P < 0.001)

curve for postpartum T3 showed that AUC was 0.86 ± 0.06 and the cut-off value was 1.23 nmol/L (Se = 0.81; Sp = 0.93). Animals with T3 > 1.23 nmol/L are presented as high T3 (H-T3), animals with T3 < 1.23 nmol/L are presented as low T3 (L-T3). TSH significantly varied ($P < 0.01$) in L-T3 but not H-T3 animals during post-partum. T4 differed between periods in both groups ($P < 0.001$). L-T3 was significantly associated with higher values of BHB ($P < 0.001$) and NEFA ($P < 0.001$) in dairy cows during post-partum. Moreover, BHB and NEFA levels in L-T3 animals were higher than in the H-T3 group during post-partum. H-T3 dairy cows showed significantly lower glucose levels at post-partum ($P < 0.001$); whereas, no effect of the peripartal period was found on glucose values in L-T3 cows (Figure 1).

DISCUSSION

The hormonal activity of the thyroid gland has an important role for peripartal cows and their offspring in order to determine cell metabolism intensity, metabolism of lipids and carbohydrates, and lactation course. Previous findings reported that cows in postpartum NEB show a decrease in the concentration of T3 and T4 and an increase in the concentration of reverse T3 [21,22]. Our results confirm that the act of parturition in cows is accompanied by marked changes in circulating thyroid hormones with a significant decrease in T3, T4 and TSH levels. The reduction of serum thyroid hormones levels found at post-partum, probably is a reflection of the decreased hormone secretion rate due to energy deficiency, as well as to the large demand for these hormones by the mammary gland [23]. Moreover, the decreased serum concentration of thyroid hormones has been described as an adaptation to NEB [24]. BHB and NEFA are the main blood indicators of ketosis and lipomobilization in ruminants [25,26]. According to Djoković *et al.* [16], we found negative correlations between serum NEFA concentrations and TSH and thyroid hormones and negative correlations between BHB and T3 and T4.

Serum glucose levels in this study significantly decreased in the post-partum period. However, the mean value remained within the reference range reported for cows (1.9-3.8 mmol/L) [27].

The decrease of glycemia in cows during early lactation may be due to the sudden activity of the mammary gland and increased lactose synthesis. Furthermore, the NEB and lipomobilization in cows affected by ketosis induce a reduction in gluconeogenesis in the liver [16]. Serum insulin values showed to be decreased in the post-partum period, as well. Insulin plays a role in the adaptation of metabolism in dairy cows during the transition period, particularly in terms of nutrient redistribution and partitioning towards the mammary gland during early lactation (insulin resistance) [28]. The decrease in insulin levels found during post-partum is consistent with previous studies which reported high insulin levels before parturition, followed by a gradual decrease from 10 days before parturition remaining relatively low throughout lactation [28]. Lower glucose and insulin levels found during the post-partum period relative to

the pre-partum period may be due to a decreased responsiveness of pancreatic β -cells to a state of hyperglycemia, caused by factors which inhibit the release of insulin [7].

Previous studies suggested that thyroid hormones may increase the level of glucose since they increase intestinal absorption of glucose, glycolysis and gluconeogenesis, facilitate glucose uptake by muscles and fat tissues, and increase insulin secretion [30,31]. The post-partum decrease of TSH and thyroid hormones could then be related to insulin resistance leading to a decrease in insulin and glucose levels, as confirmed by the positive correlations found among the three hormones and both insulin and glucose.

Thyroid hormones regulate body hemodynamics, thermoregulation and metabolism. Therefore, these hormones have an influence on renal hemodynamics, glomerular filtration and electrolyte handling. The influence of thyroid hormones on electrolytes seems to be confirmed by the correlations found in the current study among TSH and/or T4 and electrolytes values. In particular, the TSH values resulted positively correlated with Cl, Mg and Na values, and negatively correlated with K. In addition, a positive correlation between T4 and Mg values was found. The effect of thyroid hormones on electrolytes and minerals has not been well established and also the underlying mechanisms are not well understood [32]. The relationships found among these parameters are probably dependent on the action performed by thyroid hormones on the kidney and other tissues. It has been established that the increase of thyroid hormones leads to serum Na increase because of the rise of Na-H exchanger and Na-Pico-transporter activity first in the proximal tubules then almost in all segments of the nephron [33]. Sodium and potassium are important components of the enzyme Na-K ATPase, which is a cell membrane enzyme that helps in the transport of water and nutrients across the cell membrane. Thyroid hormones regulate the activity of sodium potassium pumps in most of the tissues [34].

Critical thresholds of thyroid hormones were determined using the ROC test. Our results indicate that serum T3 values <1.23 nmol/L were associated to hyperketonemic cows (BHB >1.1 mmol/L) at post-partum. Subsequent analysis of the differences between H-T3 and L-T3 animals highlighted a significant effect of time and T3 levels on BHB, NEFA and glucose values. In animals with low T3 serum concentrations correspond to high levels of BHB and NEFA and a low concentration of glucose. An opposite trend was detected in H-T3 cows. These results could confirm the effect of T3 on liver metabolism as previously described.

The significant variations found in the serum levels of AST, ALT, urea and K, confirm that the transition period represents a critical point in the cow's life since important metabolic changes occur in response to physiological modifications. A similar decrease of urea and K and the increase of AST levels found at post-partum in this study have been previously described by Fiore et al. [9]. The AST increase could be associated to disorders involving the liver, including ketosis, even at subclinical levels [35]. However, in this study mean values of AST remained within the physiological range reported

for the cow (78-132 IU/L) [27]. Animals during pre-partum showed higher levels of urea than the reference range of 2.0-7.5 mmol/L provided by Radostis *et al.* [27]. Urea decrease in post-partum may be due to low protein intake of periparturient cows [36].

CONCLUSION

The results obtained in the present study highlight the effect of the transition period on serum thyroid hormones, BHB, NEFA, glucose, insulin, AST, ALT, urea and K concentration in dairy cows. Moreover, a close correlation among studied hormones and biochemical parameters was detected suggesting a relationship between thyroid hormones and predictors of lipomobilization. Monitoring of thyroid hormones, especially T3, could represent an important tool to evaluate the metabolism adaptation in response to NEB in dairy cows during the peripartum period and in order to understand when regulatory mechanisms break through the physiological limits predisposing the cows to metabolic problems.

Authors' contributions

All Authors have made substantial contribution to each step of experimental procedure and manuscript preparation. In particular: the idea for the paper was conceived by EF and MG; the experiments were designed by SG and MM; the experiments were performed by GP and IV; the data were analyzed by BC, TO and FA; the paper was written by EF; the manuscript was critically revised for important intellectual content by MG. All authors have read and approved the final version of manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PROMENE U NIVOIMA TIREOIDNIH HORMONA I METABOLIZMU KOD MUZNIH KRAVA U PERIODU TELENJA

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Hormonska aktivnost tireoideje igra veoma važnu ulogu kod preživara a u cilju modulacije metaboličkih parametara. U studiji obavljena je evaluacija tireoidnih hormona i biohemijskih parametara kod muznih krava u periodu telenja pri čemu je ustanovljen kritičan prag koncentracije tireoidnih hormona u cilju procene rizika nastanka ketoze. Uzorci krvi su sakupljeni od 82 muzne krave u periodu od 5 ± 3 dana pre telenja kao i 5 ± 3 dana posle partusa. Posle izdvajanja seruma, ispitivane su koncentracije trijodotironina (T3), tiroksina (T4), tiroid-stimulirajućeg hormona (TSH), neesterifikovanih masnih kiselina (NEFA), β -hidroksibutirata (BHB), insulina, glukoze, Ca, Cl, Mg, P, K, Na, aspartat transaminase (AST), alanin transaminaze (ALT) kao i uree. U postpartalnom periodu uočen je značajan pad koncentracije T3, T4 i TSH. Vrednosti T3 i T4 su bile u negativnoj korelaciji sa NEFA i BHB koncentracijama a TSH vrednosti su bile u negativnoj korelaciji sa DEFA. Kritični nivoi tj. prag T3 bio je ustanovljen primenom ROC analize a sa ciljem predviđanja rizika od ketoze. Koncentracije T3 ispod 1,23 nmol/L bile su povezane sa BHB $>1,10$ nmol/L posle partusa, koje predstavljaju granične BHB vrednosti koje se uobičajeno koriste kao indikator hiperketonemije. Rezultati ukazuju da je peripartalni period povezan sa značajnim promenama u profilu i koncentracijama tireoidinih hormona u cirkulaciji što može da bude u korelaciji sa predviđanjem mobilizacije masti.