ADRENOCORTICAL AND THYROID FUNCTION, HORMONE AND METABOLITE PROFILES AND THE ONSET OF OVARIAN CYCLICITY IN DAIRY COWS SUFFERING FROM VARIOUS FORMS OF KETOSIS


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The involvement of adrenocortical and thyroid hormones in the pathogenesis of ketosis, as well as the ovarian consequences of this metabolic disorder, were studied in ≥2 parity cows (n=199) in 3 large scale dairy herds. To compare the plasma/serum concentrations of certain hormones [cortisol, thyroxin (T₄), triiodothyronine (T₃), insulin, insulin-like growth factor-1 (IGF-1)] and metabolites [glucose (G), acetoacetic acid (ACAC), β-OH-butyrate (BHB), non-esterified fatty acid (NEFA), trigliceride (TG), total cholesterol (TCh)], and the activity of aspartate aminotransferase (AST), blood samples were taken 1 to 3 days after calving and again 4 times 7 days apart. The ACTH-challenged cortisol responsiveness and the TRH-induced T₄/T₃ increase were determined between days 1 to 3 and again between days 28 to 35. The resumption of ovarian cyclicity was followed up by individual progesterone (P₄) profiles based on milk samples taken 3 times a week for about 80 to 85 days. BHB level of 1 mmol/L was estimated as a border line between hyper- (>1 mmol/L) and normoketonaemic (<1 mmol/L) conditions. Five different ketone patterns were distinguished: (1) non-ketotic (n=98; normoketonaemia in all samples), (2) early type ketosis (n=45; hyperketonemia was detected only in the first week after calving), (3) late type (lactational) ketosis (n=11; after a normoketonaemic period increasing hyperketonaemia was detected in the 5th, or in the 4th and 5th weeks), (4) temporary ketosis (n=11; hyperketonaemia was detected for 1-2 weeks in the 2nd and 3rd or in the 3rd and 4th weeks); (5) long-lasting ketosis (n=34; hyperketonaemia has been detected since calving for 4 to 5 weeks or until dying / emergency slaughtering). Simultaneously with the hyperketonaemic stage increased NEFA, ACAC, depressed TCh, glucose and decreased insulin, IGF-1, T₄ and T₃ concentrations were detected in almost all the cases. Obvious metabolic and endocrine alterations were found, however, only in long-lasting ketosis. The TRH-stimulated T₄ and T₃ responses remained almost unaffected proving intact thyroid function in early and late type as well as in temporary ketosis. Depressed thyroid
response and delayed onset of cyclic ovarian function were detected only in cases of long-lasting ketosis. The cows characterized by lower than normal (<mean-SD of non-ketotic cows) ACTH-stimulated cortisol response on days 1-3 after calving showed poorer chance for spontaneous recovery. There was a significant negative correlation between the IGF-1 level in the 1st week after calving and the duration of the postpartum acyclic period. In late type (lactational) ketosis the cessation of ovarian cyclicity was the most characteristic genital malfunction.

Key words: ketosis, dairy cows, adrenal, thyroid, ovary

INTRODUCTION

Ketosis is the known consequence of the unbalanced energy supply to high-producing postpartum dairy cows (Duffield et al., 1998). Several endocrine aspects of this metabolic disorder have already been reported (Nikolić et al., 1997). The lower than normal rate of insulin to glucagon and growth hormone is proved as its main endocrine predisposing factor (Sartin et al., 1988). The involvement of thyroid hormones in the postpartum adaptation of energy metabolism is a generally accepted idea, and glucocorticoid containing preparations have been widely used in the therapy of bovine ketosis for many years (Baird, 1982). The possible role of these hormones, as well as of the (mal)function of the thyroid gland and/or the adrenal cortex, in the pathogenesis of ketosis was supposed a long time ago (Hill et al., 1950; Robertson et al., 1957). These ideas, however, have remained poorly documented. In most of the trials only the circulating total thyroxine (T4), 3,3',5-triiodo-thyronine (T3), and cortisol levels were determined (Tiirats, 1997; Đurđević et al., 1980). The postpartum changes in blood level of the inactivated thyroid hormone formation (3,3',5-triiodothyronine or reverse-triiodothyronine, rT3) were followed-up only in cows with various degrees of energy imbalance (Tiirats, 1997; Kunz and Blum, 1985; Pethes et al., 1985), but not in those affected by ketosis. There are only limited data on the determination of standard low dose adrenocorticotrophin hormone (ACTH)-induced cortisol response (Kolk, 1991; Andriaens et al., 1995; Lay et al., 1996), as well as of the thyrotrophin releasing hormone (TRH) challenged T4/T3 increases (Lapierre et al., 1990; Tveit et al., 1990a; Tveit et al., 1990b; Andriaens et al., 1995), which can inform us on the functional reserve capacity of these endocrine glands. In addition, the degree of energy imbalance and/or the presence of hyperketonemia were identified in few of these studies (Nikolić et al., 1977). The actual levels of the biologically active, protein-unbound T4, T3, and cortisol levels remained unknown in healthy and different level of hyperketonaemic postpartum cows. The low concentration of insulin-like growth factor-1 (IGF-1) was proved to reflect the degree of energy imbalance in cows early in their lactation (Chelikani et al., 2004; Zulu et al., 2002), however, limited data are available on hyperketonaemic individuals. Recently a stimulatory role has been attributed to both insulin and IGF-1 in follicular growth and maturation.
(Diskin et al., 2003; Whates et al., 2003), directing the attention of gynaecologists to the postpartum changes of these hormones nowadays. Butler et al., (1981) and many others clearly demonstrated that the first postpartum ovulation takes place about 10 days after the nadir of the energy imbalance in the non-suckled dairy cows. Thereafter the cow becomes cyclic. The negative influence of the energy-imbalance on reproductive performance has become widely accepted (Lucy, 2003; Taylor et al., 2002). However, its detrimental effect on the resumption of cyclic ovarian function is only poorly documented and the possible involvement of insulin and IGF-1 levels in the supposed (Zulu et al., 2002), hyperketonaemia-induced delay of the onset of ovarian cyclicity has not been clearly demonstrated yet. This trial was conducted to study whether there are any interrelations of circulating ketone body (e.g. acetoacetic acid, ACAC and OH-butyrate, BHB) levels with (1) andrenocortical and/or thyroid gland functions, (2) circulating insulin and IGF-1 concentrations and (3) the onset of cyclic ovarian activity.

MATERIALS AND METHODS

Experimental animals

Interrelationships of circulating ketone body (e.g. acetoacetic acid, ACAC and OH-butyrate, BHB) levels with (1) andrenocortical and/or thyroid gland functions, (2) circulating insulin and IGF-1 concentrations and (3) the onset of cyclic ovarian activity were studied in ≥2 parity cows in 3 large-scale dairy herds with about 1850, 500 and 950 animals. All individuals calving within a pre-selected period in October-November (Farm A and B) or in May-June (Farm C) were involved in the study unless they needed veterinary intervention at calving, calved twins and/or showed clinical symptoms of parturient paresis just after calving. All cows on these farms including those enrolled in this study were kept and fed in groups of 80 to 100 individuals under free housing conditions with no possibility for individual feeding or pasturing. These groups of cows were formed in accordance with their monthly checked daily milk yield. Their daily ration was made up from ensiled maize and alfalfa products, alfalfa and grass hay and cereals completed with vitamins and minerals, in accordance with the NRC (1989) recommendations.

In total, data of 199 cows were evaluated (milk production in previous lactation: 5069 to 16 291 kg FCM). Using 1 mmol/L of BHB threshold as a cut off value 5 different ketone patterns could be distinguished: (1) Non-hyperketonemic (n=98): normoketonemia in all samples; (2) Early type hyperketonemia (n=45): hyperketonemia was detected only in the first week after calving. (3) Late type (lactational) hyperketonemia (n=11): after a normoketonemic period increasing hyperketonemia was detected in the 5th (or 4th and 5th) week(s); (4) Temporary hyperketonemia (n=11): (usually mild) hyperketonemia was detected for 1-2 weeks in the 2nd and 3rd or 3rd and 4th weeks; (5) Long-lasting hyperketonemia (n=34): (usually severe) hyperketonemia has been detected since calving for 4 to 5 weeks or until dying / emergency slaughtering.
Blood sampling, endocrine and metabolic parameters determination

To assay the concentrations of cortisol, $T_4$, $T_3$, insulin, IGF-1, and glucose, ACAC, BHB, non-esterified fatty acid (NEFA), trigliceride (TG) and total cholesterol (TCh), as well as the activity of aspartate aminotransferase (AST) enzyme, blood samples were taken 1 to 3 days after calving and again 4 times 7 days apart. All these samples were taken before the morning milking. We have used 1 mmol/L of BHB level as a border line between hyperketonemic ($>1$ mmol/l) and normoketonemic ($<1$ mmol/L) conditions.

The ACTH-challenged cortisol responsiveness and the TRH-induced $T_4/T_3$ increase were estimated on days 1 to 3 and again on days 28 to 35. For this reason 60 minutes after the morning milking blood samples were collected to determine the baseline cortisol, $T_4$ and $T_3$ levels. Just after sampling $60\, \text{g} \, \text{Cortrosin inj.®}$ (Organon, Oss, The Netherlands) and $400\, \text{g} \, \text{TRH (pGLU-HIS-PRO amide acetate salt}}$, Sigma, St. Louis, USA) dissolved in saline were administered in the jugular vein simultaneously. The endocrine response was determined 60 ($\text{cortisol}$), 240 and 360 ($T_4$ and $T_3$) minutes later.

The resumption of ovarian cyclicity was followed up by individual progesterone ($P_4$) profiles based on ELISA determination of $P_4$ from defatted milk samples taken 3 times a week for about 80 to 85 days after calving. The laboratory procedures used for all these determinations were validated for bovine samples, as described elsewhere (Huszenicza et al., 1998; Nikolić et al., 2003; Meikle et al., 2004). The milk production was checked every 7 days for 10 weeks and once a month thereafter. Body weight was measured on days 1-3, 28-35 and 63-70. Clinical events and data on reproduction were followed for 150 days after calving.

Statistical analysis

Data were analyzed using Students t-test, Chi-square test, Pearson correlation coefficients, regression analysis, or by one way analysis of variance. If the between-group differences were significant ($P<0.05$ or more), the least significant difference (LSD) was given on 5 % level for pair-wise comparison of group means (Kleinbaum and Kupper, 1978; Juvancz and Paksy, 1982; Snedecor and Cochran, 1982).

RESULTS AND DISCUSSION

Values of insulin, IGF-1, $T_4$ and $T_3$ concentrations in cows with different ketone bodies pattern are given in the Table 1. Simultaneously, with the hyperketonemic stage we have recorded increased NEFA and ACAC concentrations, depressed TCh and glucose levels, elevated AST activity (details are not given), as well as decreased insulin, IGF-1, $T_4$ and $T_3$ concentrations in almost all the cases. There is evidence that peripheral plasma concentrations of insulin, $T_4$ and $T_3$ are positively correlated with energy balance in dairy cows in the period from 5-70 days after parturition (Kunz et al., 1985). It also seemed possible to increase milk production by feeding a iodinated casein containing 1% of
Table 1. Base-line levels (mean ± SD) of insulin, IGF-1 and thyroid hormones in cows showing various ketone patterns

<table>
<thead>
<tr>
<th>Day 1-3</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Day 1-3</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
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<tr>
<td>Insulin (IU/l)</td>
<td>Insulin-like growth factor (g/l)</td>
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<tr>
<td>Non-hyperketonemic (n=98)</td>
<td>mean</td>
<td>4.67</td>
<td>4.77</td>
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<td>SD</td>
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<tr>
<td>Early type hyperketonemia (n=45)</td>
<td>mean</td>
<td>1.32</td>
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<tr>
<td>SD</td>
<td>0.74</td>
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<td>Late type hyperketonemia (n=11)</td>
<td>mean</td>
<td>3.23</td>
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<td>SD</td>
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<tr>
<td>Temporary hyperketonemia (n=11)</td>
<td>mean</td>
<td>3.59</td>
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<td>SD</td>
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<tr>
<td>Long-lasting hyperketonemia (n=34)</td>
<td>mean</td>
<td>2.28</td>
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<td>SD</td>
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LSD(_P<0.05_) = 1.29 0.78 0.94 1.24 1.63 6.34 5.41 7.14 8.22 10.05

Thyroxine (nmol/l) | Triiodothyronine (nmol/l)
<table>
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<tr>
<td>Non-hyperketonemic (n=98)</td>
<td>mean</td>
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<tr>
<td>SD</td>
<td>10.21</td>
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<td>Early type hyperketonemia (n=45)</td>
<td>mean</td>
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<tr>
<td>SD</td>
<td>7.03</td>
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<tr>
<td>Late type hyperketonemia (n=11)</td>
<td>mean</td>
</tr>
<tr>
<td>SD</td>
<td>6.20</td>
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<tr>
<td>Temporary hyperketonemia (n=11)</td>
<td>mean</td>
</tr>
<tr>
<td>SD</td>
<td>7.73</td>
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<tr>
<td>Long-lasting hyperketonemia (n=34)</td>
<td>mean</td>
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</table>

F = 6.94 9.53 19.46 19.52 14.00 20.11 25.53 20.68 22.05 20.03
LSD(_P<0.05_) = 4.82 4.30 4.18 4.23 5.92 0.13 0.13 0.17 0.17 0.21
thyroxine to lactating cows (Shaw et al., 1975), but other authors did not confirm an association between milk yield and thyroid hormone concentrations (Hoshino et al., 1991). Investigating thyroid hormones in the blood of ketotic cows Đurđević et al., (1980) found significantly lower levels of T₃ and T₄ in comparison to healthy cows. More recent investigation confirmed those previous results (Nikolić et al., 1997), even though the authors remarked the possibility that some of the experimental animals may have been suffering from a functional iodine deficiency. Decrease in the serum insulin and IGF-I levels in almost all ketotic cows in our experiment probably reflects the fact that the balance between anabolic and catabolic hormones due to the NEB is set to a lower point.

Functional tests of adrenal and thyroid gland in ruminants have been used in clinical endocrinology during early 1980ies (Hurley et al., 1981; Alan et al., 1981). Since that time they have been widely accepted (Tveit et al., 1990a; Tveit et al., 1990b; Kolk, 1991; Rumsey et al., 1999; Bage et al., 2000).

Obvious metabolic and endocrine alterations were found, however, only in cows with long-lasting hyperketonemia. Compared with those of non-hyperketonemic animals, in cows with early and late type, as well as with temporary hyperketonemia, the TRH-stimulated T₄ and T₃ responses were only slightly lower, proving an almost intact thyroid function. A more depressed response was detected, however, in cases of long-lasting ketosis. The peak level of T₃ seen at 240 min. after the TRH remained almost unchanged in normoketonemic condition, but decreased significantly if hyperketonemia was detected simultaneously (Fig. 1).

In dairy cows at the beginning of lactation, during the period of NEB, plasma concentrations of T₄ and T₃ decrease, while the rT₃ level increases (Pethes et al., 1985; Ronge et al., 1989; Rudas, 1990; Rudas, 1994; Tiirats, 1997; Huszenicza et al., 2002; Stojić et al., 2001). Kapp et al., (1979) noticed significant changes in thyroid gland histology, in cows that died because of the fatty liver syndrome. Potentially decreased T₄-response to TRH in cases of long-lasting hyperketonemia could be induced by the functional damage of thyroid epithelial cells. Plasma levels of T₃ in cows with hyperketonemia lasting 240. and 360. minutes was significantly decreased compared to the normoketonemic animals. This could be explained by the increased activity of 5D enzyme system that inactivates T₄ and T₃ (Pethes et al., 1985; Rudas, 1990; Rudas, 1994), which is in accordance with significantly higher plasma level of rT₃ in cows with hyperketonemia.

Our results indicate that, on day 1-3 after calving, cows showing lower than normal ACTH-stimulated cortisol response (hypocorticism: <mean-SD of non-hyperketonemic cows; e.g. <44 nmol/L) could hardly tolerate the simultaneous hyperketonemia and had poorer chance for recovery. Altogether 79 cows had elevated BHB levels at that time, and 14 of them were characterized also with hypocorticism. Eight of them were lost due to ketosis, whereas the rate of dying was only five out of 65 (P<0.001) among the hyperketonemic individuals with normocorticism. Application of ACTH in a dose of 25 IU/100 kg BW could effectively increase serum cortisol and glucose levels in ketotic cows (Đurđević et al., 1985). It seems that in the case of reduced cortisol response ketotic cows are
unable to sustain metabolic stress induced by hyperketonemia. Similar situation could be found in the *E.coli* mastitis, where endotoxin initiates delayed cortisol response due to the release of TNF (Jánosi, 2002).

Only the cows with long-lasting hyperketonemia were characterized with delayed onset of cyclic ovarian function (Table 2).

There was a significant negative correlation between the insulin and IGF-1 levels in the 1st week after calving and the duration of the postpartum acyclic period. In late type (lactational) ketosis the cessation of ovarian cyclicity was the most characteristic ovarian malfunction. The first visible estrus, the first insemination and the day of re-conception was delayed by both the late type and

![Figure 1. Changes in T4 and T3 levels (mean±SD) after TRH challenge in cows with normoketonemic (BHB level: <1.00 mmol/l) vs. hyperketonemic (BHB level: >1.00 mmol/l) conditions (ns: not significant difference; *P<0.05; **P<0.01; ***P<0.001)
long-lasting hyperketonemia. The within-150-days pregnancy rate was lowered, however, only by the most severe, long-lasting form (Table 2). Blood concentrations of insulin, IGF-I, and leptin decrease shortly after calving (Butler, 2000; Lucy, 2000; Block et al., 2001; Meikle et al., 2004). Insulin and IGF-I concentrations gradually increase postpartum, while leptin concentration remains low in lactating cows. Cows with negative energy balance and cows selected for milk production have lower blood concentrations of insulin and IGF-I (Snijders et al., 2001; Gong, 2002). Those metabolic hormones could influence GnRH neurons in the hypothalamus or on the pituitary gonadotroph (Adam et al., 2000; Williams et al., 2002). On the other side, ovarian cells treated with insulin or IGF-I have greater numbers of gonadotrophin receptors and greater activation of second messenger pathways in response to gonadotrophins (Lucy, 2000).

Table 2. Reproductive performance of cows showing various ketone pattern (mean ± SD)

<table>
<thead>
<tr>
<th>Period of ketone pattern</th>
<th>Acyclic period</th>
<th>Postpartum day of AI/pregnancies</th>
<th>Rate of pregnant</th>
<th>F=</th>
<th>LSD(P&lt;0.05)</th>
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<tr>
<td></td>
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<td>1st estrus</td>
<td>1st insemination</td>
<td>re-conception</td>
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</tr>
<tr>
<td>Non-hyperketonemic (n=98)</td>
<td>31.2±16.7</td>
<td>81.9±29.4</td>
<td>83.6±29.2</td>
<td>101.4±27.2</td>
<td>1.86±0.77</td>
</tr>
<tr>
<td>Early type hyperketonemia (n=45)</td>
<td>36.95±14.72</td>
<td>88.9±25.1</td>
<td>92.7±24.3</td>
<td>113.3±30.3</td>
<td>1.95±0.74</td>
</tr>
<tr>
<td>Late type hyperketonemia (n=11)</td>
<td>25.36±11.56</td>
<td>116.2±21.8</td>
<td>116.2±21.8</td>
<td>132.7±21.7</td>
<td>1.67±0.71</td>
</tr>
<tr>
<td>Temporary hyperketonemia (n=11)</td>
<td>31.27±9.32</td>
<td>90.3±29.1</td>
<td>90.3±29.1</td>
<td>114.5±28.9</td>
<td>2.00±0.76</td>
</tr>
<tr>
<td>Long-lasting hyperketonemia (n=34)</td>
<td>59.0±17.7</td>
<td>112.3±28.1</td>
<td>114.4±24.6</td>
<td>129.5±22.7</td>
<td>1.62±0.65</td>
</tr>
<tr>
<td>F=</td>
<td>15.32</td>
<td>7.13</td>
<td>7.20</td>
<td>5.15</td>
<td>0.50</td>
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</table>

It was concluded that adrenocortical and thyroid gland functions are involved in the pathogenesis of ketosis. However, only the long-lasting form of hyperketonemia is a real risk factor for subsequent reproduction. Besides the high risk of mortality it may usually result in a serious delay in the time of the first ovulation and first visible estrus. In the first few days after calving the decreased insulin and IGF-1 levels may be involved in the pathogenesis of this ovarian disorder.
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Holesterol (TCh), i aktivnosti aspartat aminotransferaze (AST) u krvnoj plazmi/se-rumu, uzmali su uzorci krvi od prvog do trećeg dana posle teljenja i ponovo 4 puta u razmacima od po 7 dana. Povećanje koncentracije kortizola, posle aplikacije ACTH, i povećanje koncentracije T4/T3 nakon aplikacije TRH je utvrđeno između 1. do 3. dana i ponovo između 28. do 35-og dana. Ponovo uspostavljanje cikličnosti funkcije jajnika bilo je praćeno individualnim profilima progesterona (P₄) na osnovu kontrole uzoraka mleka uzetih 3 puta nedeljno tokom perioda od 80 do 85 dana. Nivo BHB od 1 mmol/l je bio utvrđen kao granica između hiperke-tonemičnih (>1 mmol/l) i normoketonemičnih (<1 mmol/l) stanja. Na osnovu toga krave su razvrstane u pet jasno definisanih grupa:

1. krave koje nisu ketozne (n=98; normoketonemija u svim uzorcima),
2. rani tip ketoze (n=45; hiperketonemija utvrđena samo u prvoj nedelji posle teljenja),
3. kasni (laktacioni) tip ketoze (n=11; posle perioda normoketonemije detektovana je hiperketonemija u 4-oj i 5-oj nedelji, ili u 5-oj nedelji),
4. povremena ketoza (n=11; hiperketonemija je detektovana u trajanju od 1-2 nedelje u drugoj i trećoj ili u trećoj i četvrtoj nedelji),
5. dugotrajna ketoza (n=34; hiperketonemija je bila dijagnostikovana od teljenja tokom 4 do 5 nedelja ili posle uginuća/prinudnog klanja). Istovremeno sa stanjem hiperketonemije bilo je ustanovljeno povećanje koncentracija NEFA, ACAC, i smanjenje TCh, glikoze, IGF-1, T4 i T3 u skoro svim slučajevima. Ipak, očigledne metaboličke i endokrine promene su pronađene, samo kod dugotrajne ketoze. T₄ i T₃ odgovor stimulisan TRH-om ostao je skoro nepromenjen što dokazuje nepromenjenu funkciju tiroide u ranom i kasnom kao i u prolaznom tipu ketoze. Hipoefikuca tiroide je i usporeno uspostavljanje ciklične funkcije jajnika bilo je utvrđeno samo u slučajevima dugotrajne ketoze. Krave koje su imale nižu koncentraciju kortizola, kao odgovor na aplikovan ACTH 1-3 dana posle teljenja, imale su manje šanse za spontani oporavak. Postoji značajna negativna korelacija između nivoa IGF-1 u prvoj nedelji posle teljenja i dužine trajanja postpartalnog perioda aciklje.

U kasnom (laktacionom) tipu ketoze prekid ciklične funkcije jajnika bio je najkarakterističniji genitalni poremećaj.