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EFFECT OF MISSENSE MUTATION OF LEPTIN GENE ON SERUM LEPTIN CONCENTRATION AND SOME BLOOD METABOLIC PARAMETERS IN CZECH PIED BULLS

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Abstract

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The aim of present study was to examine the effect of leptin gene single nucleotide polymorphism (C/T) giving missense mutation (Arg25Cys) on concentration of some serum metabolic parameters in 58 Czech Pied bulls. Resulting genotypes in the exon 2 were CC (48.3%), CT (36.2%), and TT (15.5%). There were no differences in serum leptin, thyroxin (T_4), triiodothyronin (T_3), glucose, triacylglycerols and non-esterified fatty acid concentrations among the genotypes. However, concentration of serum insulin in bulls with TT genotype was significantly higher than those of TC and CC genotypes. Since it is shown that the T allele is associated with the trait of higher fat contents, higher insulin concentration in TT genotype might contribute to accumulation of triacylglycerols in the adipose tissue.

Key words: leptin gene, SNP, metabolic hormones, blood serum, bulls

Introduction

Leptin, a 167-amino acid hormone, is primarily secreted by white adipose tissue (Williams et al., 2002). Since its discovery in 1994 (Zhang et al., 1994), leptin has been implicated in several system such as regulation of energy, metabolism, and reproduction through endocrine, paracrine, and autocrine mechanisms (Williams et al., 2002). Leptin is sensitive to dietary manipulation and appears to play an important role in transmitting the status of energy reserves to the central nervous system to regulate feed intake and reproductive function in ruminants (Zieba et al., 2005). Intramuscular fat content, also known as marbling of meat, represents a valuable beef quality trait. Leptin is synthesized and released into the bloodstream in direct proportion to the amount of body fat, reflecting primarily the triacylglycerols content of lipid depots, but also functioning as a sensor of energy balance (Chilliard et al., 2005). The systemic leptin levels are strongly associated with mRNA levels in subcutaneous adipose tissue and cellularity [Delavaud et al. (2002)]. The leptin gene has been mapped to bovine chromosome 4 (Stone et al., 1996). Signle nucleotide polymorphisms (SNP) in the leptin gene have been associated with serum leptin concentration, feed intake, milk yield (Liefers et al., 2002) and body fatness (Buchanan et al., 2002; Nkrumah et al., 2004). Animals with TT genotype of a cytosine/thymine (C/T) substitution detected at position 528 in the bovine leptin promoter region (GenBank accession no AB070368) showed 13% and 9% increase in marbling score compared with CC and CT genotypes, respectively (Nkrumah et al., 2005). Some studies have reported the usefulness of using a single SNP in the leptin gene for sorting feedlot cattle (Engler*et al*. 2009). There is a clear correlation among leptin, IGF-I, insulin and BCS (León et al., 2004), but the metabolic activity of adipocytes in bovines is under the influence of several factors, e.g. breed (Hood, 1982). Some authors suppose, that changes in leptin and other metabolic hormones are depend on the animals fattening capacity and level of adiposity in some breeds (Ren et al., 2002; Marino et al., 2009)

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The aim of present study was to test hypothesis that there is existan effect of leptin gene single nucleotide polymorphism on serum leptin and other metabolites concentration in Czech Pied bulls.

Materials and Methods

Animals and breeding conditions

The experiment were performed in 58 Czech Pied bulls at 240 ± 9 days of age, Which were divided in three experimental groups depending on different leptin genotypes (CC, n=28; TC, n=21; TT, n=9). There were no differences in age and body weight among the groups. The feeding ration was based on corn silage. Components and the content of standard nutrients in the feeding ration are presented in Table 1.

Leptin genotypes analysis

Blood samples (2 ml) were collected into tubes with EDTA stored at -20 °C. Genomic DNA was isolated from the samples using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA). The quality of DNA was verified by 1% agarose gel electrophoresis and sequential visualization with ethidium bromide. Genotypes were determined based on molecular genetic analysis of single-nucleotide polymorphism (SNP) in the exon 2 of the bovine leptin gene (transition C → T) (Buchanan et al., 2002). For testing, we used our own methodology. PCR primers were designed based on the nucleotide sequence of bovine leptin gene (GenBank U50365) (FW:5'TCGTTGTTATCCGCATCTGA3', REV: 5'TACCGTGTGTGAGATGTCATTG 3'). PCR was performed in 12.5 µl volumes containing 25 ng of bovine genomic DNA, 1x HotStarTaq Master Mix (Qiagen) and 0.2 µM of each forward and reverse primer. A PCR thermal profile consisted of pre-denaturation at 95°C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for30 s, elongation at 72°C for 30 s; and final extension at 72°C for 7 min. The PCR products of 278 bp in size were separated on 3% agarose gel and sequenced using the ABI PRISM 3100-Avant Genetic Analyzer. The polymorphic locus (C/T) is located at position 204 base of the fragment.

Table 1
Components and nutrients composition of the diets

Component/nutrient	Unit	Value
Corn silage	$kg.day^{-1}$	15
Clover haylage	$kg.day^{-1}$	5
CCM	$kg.day^{-1}$	1.5
Hay	$kg.day^{-1}$	0.5
Straw	$kg.day^{-1}$	0.5
Wheat meal	$kg.day^{-1}$	1.7
Maize meal	$kg.day^{-1}$	0.6
Rapeseed meal	$kg.day^{-1}$	0.5
Limestone powder	$kg.day^{-1}$	0.08
Feed salt	$kg.day^{-1}$	0.05
Mineral-vitamin feedstuffs for cattle (VVS, CzechRepublic)	$kg.day^{-1}$	0.18
Crudeprotein	$g.kg^{-1}$	125.5
Crudefiber	$g.kg^{-1}$	173.6
Net energy	$MJ.kg^{-1}$	6.1
PDIE	$g.kg^{-1}$	80.6
PDIN	$g.kg^{-1}$	82.3

Collection of blood samples

Blood for hormone and metabolite analyses was collected randomly from *vena jugularis externa* of bulls in three groups between 8.00 and 9.30 a.m., and sampled into the test tube with silicon gel separator and coagulation accelerator (Dispolab, Czech Republic). Serum was separated by centrifugation with 2 000 x g for 10 min at 4°C, and was stored at -20°C until analyzed.

Hormones and metabolite analyses

The triiodothyronine (T3) and thyroxine (T4) levels were determined using an IMMULITE automatic analyser (Siemens HealthcareDiagnostics, USA) and currently available commercial kits (Siemens s.r.o., Czech Republic).

Leptin concentration was analysed by ELISA (Bovine Leptin E90084Bo kit, USCN Life Science, China). Insulin concentration was analysed by Bovine Insulin ELISA 10-1201-01 kit (Mercodia, Sweden).

Triacylglycerols (TAG), glucoseand non-esterified fatty acids (NEFA) serum concentration were analysed on Konelab T20xt automatic analyser (Thermo Fisher Scientific, Finland) using currently available commercial kits (Biovendor-Laboratornimedicina, Czech Republic).

Statistical evaluation

Changes in serum hormones and metabolites were analyzed by one-way ANOVA for factors leptin genotype. ANOVA was followed by post-hoc Fischer LSD test. Correlations between leptin, hormones and blood metabolites were evaluated by means of the correlation coefficient at the level of probability ($P<0.05$). All statistical analyses were performed by Statistica 8.0 statistical software (StatSoft Inc., Tulsa, USA).Data represent mean ± SE. The overall level of statistical significance was defined as $p< 0.05$.

Results

Genotypes were determined based on the sequence of the leptin gene in 58 Czech Pied bulls, and resulting genotypes were CC (48.3%), CT (36.2%), and TT (15.5%). No obvious

difference was found in serum leptin concentrations among the genotypes, but the highest concentration of serum leptin was recorded in TT genotype (7.27 ± 1.892 ng.ml^{-1}) (Figure 1). When lipogenesis-related serum parameters were examined, serum insulin concentration in bulls of TT genotype (1.042 ± 0.231ng.ml^{-1}) was significantly higher than those of CC (0.544 ± 0.084 ng.ml^{-1}) and CT genotypes (0.365± 0.045 ng.ml^{-1}) (Figure 2). There was also some tendency to decrease in serum triacylglycerols concentration in TT genotype, while there were no differences in glucose concentration among the genotypes (Figure 3). On the other hand, when lipolysis-related serum parameters were examined, there were no apparent changes in serum T3, T4, glucose and NEFA concentrations among the genotypes (Figures 3, 4, 6 and 7).Contrariwise,

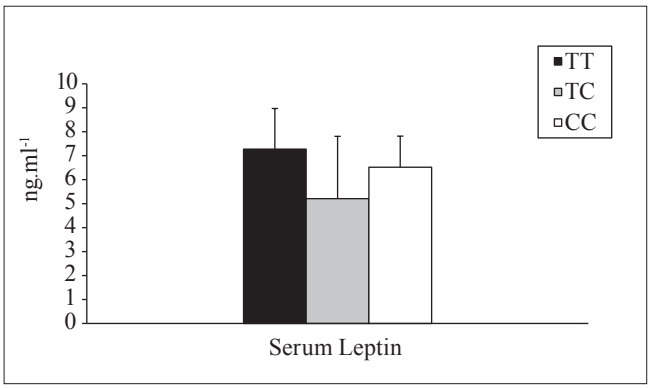

Fig. 1. Serum leptin concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

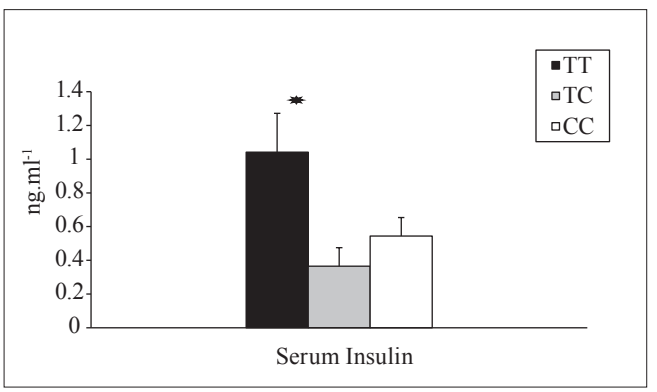

Fig. 2. Serum insulin concentration of 240 days old bulls divided into 3 groups depending on leptin genotype (*P<0.05)

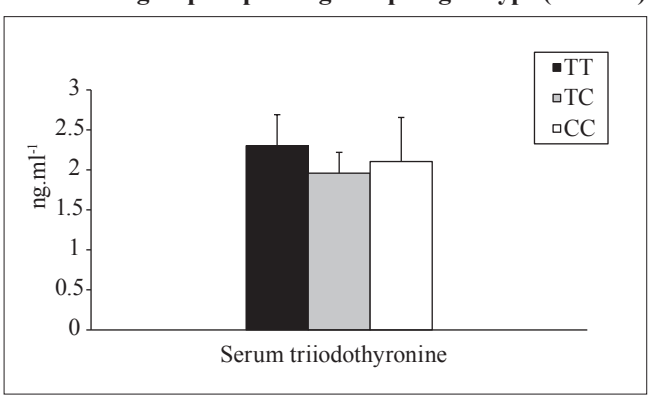

Fig. 3. Serum T3 concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

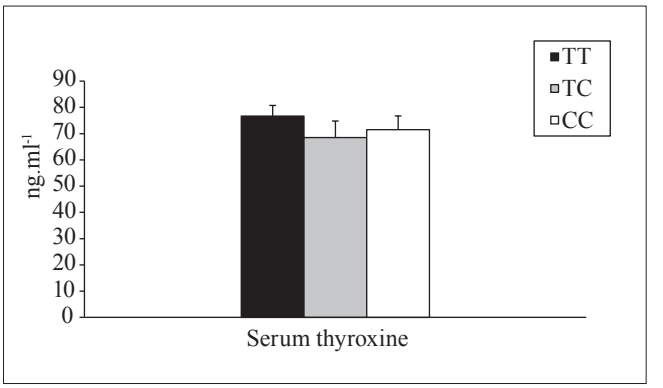

Fig. 4. Serum T4 concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

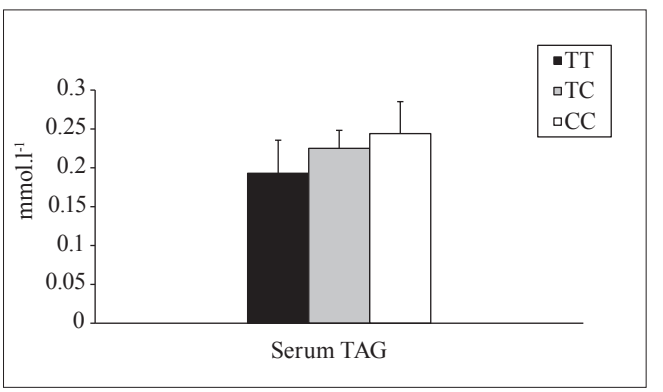

Fig. 5. Serum TAG concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

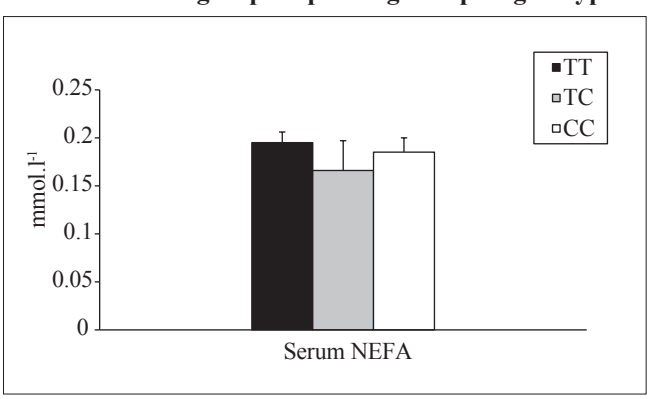

Fig. 6. Serum NEFA concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

Fig. 7. Serum glucose concentration of 240 days old bulls divided into 3 groups depending on leptin genotype

serum TAG was found as highest in bulls group TC (0.244 mmol.l^{-1}) as shows Figure 5.

Discussion

The mean value of plasma leptin from the present research (ranged between 5.21 and 7.27 ng.ml^{-1}) was greater than leptin concentration reported by Daix et al. (2008) in a different beef cattle breed, whereas it was comparable with values reported by Yamada et al. (2003). With regard to the other metabolite hormones, the plasma concentration of insulin was in agreement with those measured by Hornick et al. (1998) and results of plasma concentration in T3 and T4 were similar to those founded by Pagano-Toscano et al. (1993) and Pavlik et al. (2009, 2010).

Several experiments have indicated that plasma leptin concentrations are correlated with fat thickness in cattle (Geary et al., 2003). In relation to the physiological mechanism of leptin, it has been documented that leptin may regulate lipolysis by controlling both the levels of HSL mRNA (Sarmiento et al., 1997) and cellular cAMP concentration (Holm et al., 2000). Studies *in vitro* (Fruhbeck et al., 1997) and *in vivo* (Siegrist-Kaiser et al., 1997) have demonstrated that leptin increased lipolytic rates of the adipocytes. Also Chilliard et al. (2005) have shown, through effects on either the central nervous system or endocrine glands, that leptin decreases insulin and stimulates growth hormone, catecholamines and thyroid hormone secretions, thus increasing adipose tissue lipolysis. Simultaneously, in several studies (Buchanan et al., 2002, Liefers et al., 2002) the T allele was associated with higher level of leptin in the blood. Based on the information it would be expected, that intensity of lipolysis as well as serum lipids concentrations would be higher in TT bulls compared to other experimental groups. However, no differences in serum TAG and NEFA were found in this experiment, according to Ban-Tokuda et al. (2008), who not found any relationship between leptin, TAG and NEFA blood plasma concentration. Delavaud et al. (2002) recorded positive correlations between leptin and blood metabolites of young bulls and mentioned that leptin could be a useful indicator of nutritional status of the cattle, but in this study, any relationship between leptin and investigated blood metabolites was found.

Insulin's effects on leptin secretion have been demonstrated in dairy (Leury et al., 2003; Bradford et al., 2006) and beef cattle (Lents et al., 2005). These authors showed, that plasma leptin concentrations were significantly related to insulin. In our study, significant effect of leptin single nucleotide polymorphism in Czech Pied bulls on serum insulin concentration was found. The highest insulin concentration was found in the group of TT bulls. However, no significant relationship between serum leptin and insulin concentration was recorded.

The thyroid hormones maintain the homeostasis of energy and protein metabolism, thermoregulation, growth and productivity parameters (Huszenicza et al., 2002). Thyroxin (T4) and triiodothyronin (T3) are factors that regulate leptin expression or its plasma concentration to some extent (Ahima et al, 1996; Chilliard et al., 2005), thyroid hormone status then influences leptin, but the relationship between leptin and thyroid hormones is controversial. Thyroid dysfunction causes changes in appetite and body weight, which might involve the regulation of leptin (Medina-Gomez et al., 2003). It has been shown that thyroid hormones exert a negative influence on serum leptin levels in rodents (Escobar-Morreale et al., 1997). On the other hand, leptin is an important neuroendocrine regulator of the hypothalamicpituitary-thyroid axis (Feldt-Rasmussen, 2007) by regulation of TRH gene expression in the paraventricular nucleus, and TSH in turn will stimulate leptin secretion by human adipose tissue (Oge et al., 2005). Leptin also affects thyroid deiodinase activities with activation of T4 to T3 conversion (Reinehr, 2010). All the foregoing data support the concept of an inverse relationship between thyroid hormone and leptin. On the contrary, there were no significant differences in thyroid hormones between experimental groups of bulls in our study. We, as well as Delavaud et al. (2002), have found not significant correlation between thyroid hormones, leptin, insulin and other monitored parameters.

Conclusion

In this study, there were investigated the effects of single nucleotide polymorphism of leptin gene. We found significant effect of leptin SNP on serum insulin concentration, but any effect of leptin SNP on other monitored parameters was recorded. No significant correlations between monitored meta-

bolic hormones in serum of Czech Pied bulls were found. We supposed that differences in comparison with results found in other studies are given by differences in fattening capacities particular breeds.

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References

Ahima, R. S., D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier and J. S. Flier, 1996. Role ofleptin in theneuroendocrine response to fasting. *Nature,* **382:** 250-252.

Ban-Tokuda, T., C. Delavaud, Y. Chilliard and T. Fujihara, 2008. Plasma leptin, feedintake and body fat accumulation in fatteningcastrated male and femalelambs. *Anim Sci.,* **79:** 58-67.

Bradford, B. J., M. Oba, R. A. Ehrhardt, Y. R. Boisclair and M. S. Allen, 2006. Propionateis not an important regulator of plasma leptin concentration in dairy cattle. *Domest. Anim. Endocrinol.,* **30:** 65-75.

Buchanan, F .C., C. J. Fitzsimmons, A. G. Van Kessel, T. D. Thue, D. C. Winkelman-Sim and S. M. Schmutz, 2002. Association of a missensemutationin the bovine leptin gene with carcass fat content and leptin mRNA levels. *Genet. Sel. Evol.,* **34:** 105-116.

Chilliard, Y., C. Delavaud and M. Bonnet, 2005. Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism. *Domest. Anim. Endocrinol.,* **29:** 3-22.

Daix, C., J. L. Pirotte, F. Bister, C. Wergifosse, J. F. Cuvelier, N. Cabaraux, L. Kirschvink, R. Istasse and Paquay, 2008. Relationship between leptin content, metabolic hormones and fat deposition in three beef cattle breeds. *Vet. Journal,* **177:** 273-278.

Delavaud, C., A. Ferlay, Y. Faulconnier, F. Bocquier, G. Kann and Y. Chilliard, 2002. Plasma leptin concentration in adult cattle: effects of breed, adiposity, feeding level and mealintake. *J. Anim Sci.,* **80:**1317-1328.

Engler, M., Defoor, P. andMarquess, L. 2009.Impactof a leptin SNP and zilpaterolhydrochloride on growth and carcass characteristics of finishing steers. In'Beef Improvement Federation 41st annual research symposium'. (Online). Available: http://www.bifconference.com/bif2009/ab_c2_5_engler.

Escobar-Morreale, H. F., del R. F. Escobar and G. Morreale de Escobar, 1997. Thyroid hormones influence serum leptin concentrations in the rat. *Endocrinology,* **138:** 4485- 4488.

Feldt-Rasmussen, U., 2007. Thyroid and leptin. *Thyroid.,* **17:** 413-419.

Fruhbeck, G., S. A. Jebb and A. M. Prentice, 1998. Leptin: Physiology and Pathophysiology. *J. Clin. Physiol.,* **18:** 399-419.

Geary, T. W., E. L. McFadin, M. D. MacNeil, E. F. Grings, R. R. Short, R. N. Funston and D. H. Keisler, 2003. Leptin as a predictor of carcass composition in beef cattle. *J. Anim. Sci.,* **81:** 1-8.

Holm, C., T. Osterlund, H. Laurell and J. A. Contreras, 2000. Molecularmechanismsregulating hormone-sensitive lipase and lipolysis. *Annu. Rev. Nutr.,* **20:** 365-393.

Hood, R. L., 1982. Relationships among growth, adipose cell size, and lipid metabolism in ruminant adipose tissue. *Fed. Proc.,* **41:** 2555-2561.

Huszenicza, G., M. Kulscsar and P. Rudas, 2002. Clinical endocrinology o fthyroid gland function in ruminants. *Vet. Med. Czech.,* **47:**199-210.

Lents, C. A., R. P. Wettemann, F. J. White, I. Rubio, N. H. Ciccioli, L. J. Spicer, D. H. Keisler and M. E. Payton, 2005. Influence of nutriet intake and body fat on concentrations of insulin-like growth factor-I, insulin, thyroxine and leptin in plasma of gestating beef cows. *J. Anim. Sci.,* **83:** 586-596.

Leon, H. V., J. Hernandez-Ceron, D. H. Keislert and C. G. Gutierrez, 2004. Plasma concentrations o fleptin, insulin-like growth factor-I, and insulin in relation to changes in body condition score in heifers. *J. Anim. Sci.,* **82:** 445-451.

Leury, B. J., L. H. Baumgard, S. S. Block, N. Segoale, R. A. Ehrhardt, R. P. Rhoads, D. E. Bauman, A. W. Bell and Y. R. Boisclair, 2003. Effect of insulin and growth hormone on plasma leptin in periparturient dairy cows. *Am. J. Physiol.,* **285:** R1107-1115.

Liefers, S. C., M. F. W. de Pas, R. F. Veerkamp and T. van der Lende, 2002. Associations between leptin gene polymorphism and production, liveweight, energy balance, feed intake and fertility in Holstein heifers. *J. Dairy Sci.,* **85:** 1633-1638.

Marino, R., A. Braghieri, M. Albenzio, M. Caroprese, A. Girolami, A. Santillo, and A. Sevi, 2009. Effect of rearing system and ofdietary protein level on leptin, growth, and carcass composition in young Podolian bulls. *J. Anim. Sci.,* **87:** 3097-3104.

Medina-Gomez, G., R. M. Calvo and M. J. Obregon, 2004. T3 and Triacinhibitleptin secretion and expression in brown and white rat adipocytes. *Biochim. Biophys. Acta,* **1682:** 38-47.

Nkrumah, J. D., C. Li, J. Yu, J. A. Basarab, S. Guercio, Y. Meng, B. Murdoch, C. Hansen and S. S. Moore, 2004. Association of a single nukleotide polymorphism in the bovine leptin gene with feed intake, growth, feed efficiency, feeding behavior and carcass merit. *J. Anim. Sci.,* **84:** 211-219.

Nkrumah, J. D., C. Li, J. Yu, C. Hansen, D. H. Keisler and S. S. Moore, 2005. Polymorphism in the bovine leptin promoter associated with serum leptin concentration, growth, feed intake, feeding behavior and measures of carcass merit. *J. Anim. Sci.,* **83:** 20-28.

Oge, A., F. Bayraktar, F. Saygili, E. Guney and S. Demir, 2005. TSH influences serum leptin levels independent of thyroid hormones in hypothyroid and hyperthyroid patients. *Endocr. J.,* **52:** 213-217.

Pagano Toscano, G., C. Lazzaroni, S. Gallo and D. Galleazzi, 1993. Study on muscle hypertrophy in beef cattle: thyroid hormones. In: Proceedings of the Atti 10th Congr.Naz.ASPA, Avenue Media,Bologna, pp. 311–315.

Pavlik, A., P. Jelinek, M. Matejicek and J. Illek, 2010. Blood

Plasma Metabolic Profile of Aberdeen Angus Bulls during Postnatal Ontogenesis. *Acta Vet. Brno,* **79**: 419-429**.**

Pavlik, A., R. Zahradkova, D. Bures, P. Jelinek and Z. Havlicek, 2009. Indicators of the Internal Environment of Gasconne Calves during Rearing. *Acta Vet. Brno,***78**: 37-45.

Reinehr, T., 2010. Obesity and thyroidfunction. *Mol. Cell Endocrinol.,* **316:**165-171.

Ren, M. Q., J. Wegner, O. Bellmann, G. A. Brockmann, F. Schneider, F. Teuscher and K. Ender, 2002. Comparing mRNA levels of genes encoding leptin, leptin receptor, and lipoprotein lipase between dairy and beef cattle. *Domest. Anim. Endocrinol*., **23:** 371-381.

Sarmiento, U., B. Benson, S. Kaufman, L. Ross, M. Qi, S. Scully and C. DiPalma, 1997. Morphologic and molecular changes induced by recombinant human leptin in the white and brown adipose tissues of C57BL/6 mice. *Lab Invest.,* **77:** 243-255.

Siegrist-Kaiser, C. A., V. Pauli, C. E. Juge-Aubry, O. Boss, A. Pernin, W. W. Chin, I. Cusin, F. Rohner-Jeanrenaud, A. G. Burger, J. Zapf and C. A. Meier, 1997. Direct effects of leptin on brown and white adipose tissue. *J. Clin. Invest.,* **100:** 2858-2864.

Stone, R. T., S. M. Kappes and C. W. Beattie, 1996. The bovine homologue of the obesegene maps to chromosome 4. *Mamm Genome,* **7:** 399-400.

Williams, G. L., M. Amstalden, M. R. Garcia, R. L. Stanko, S. E. Nizielski, C. D. Morrison and D. H. Keisler, 2002. Leptin and its role in the central regulation of reproduction in cattle. *Domest. Anim. Endocrinol.,* **23:** 339-349.

Yamada, T., S. I. Kawakami and N. Nakanishi, 2003. The relationship between plasma leptin concentrations and the distribution of body fat in crossbred steers. *Anim. Sci. J.,* **74:**95-100.

Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold and J. M. Freidman, 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* **372:** 425-432.

Zieba, D. A., M. Amstalden and G. L. Williams, 2005. Regulatory roles of leptin in reproduction and metabolism: A comparative review. *Domest. Anim. Endocrinol.,* **29:** 166-185.

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