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Original article

Sex-dependent differences in the effect of early weaning on the chosen hormones secretion in sheep during the postnatal transition to puberty – Preliminary results

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Abstract

The influence of early weaning on the cortisol, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and growth hormone (GH) secretion in lambs of both sexes and testosterone (T4) level in male lambs during the postnatal transition to puberty was investigated by radioimmunoassay. It was hypothesized that this influence is long-term and sexually dimorphic. Hence, the effect of weaning at 5 weeks of age in comparison with the weaning at 9 weeks of age on hormone concentrations in peripheral blood plasma of 5-, 9-, 12-, and 16-week-old lambs of both sexes was investigated. The cortisol concentrations were greater ($P<0.05$) in control and early weaned female lambs than in male lambs at investigated stages. Weaning at 5 weeks of age resulted in the lover ($P<0.05$) cortisol secretion in male lambs in contrast to the greater ($P<0.05$) cortisol secretion in female lambs at 16 weeks of age. Weaning at 5 weeks of age stimulated ($P<0.001$) the FSH secretion, but reduced ($P<0.001$) the LH, GH and T4 secretion in 16-week-old male lambs. In female lambs early weaning inhibited ($P<0.05$) the FSH secretion at 9 weeks of age, LH secretion after 9 weeks of age and GH secretion after 12 weeks of age. Thus, early weaning results in the sexually dimorphic stress reaction that is more potent and long-lasting in female in contrast to male lambs. This maternal deprivation stress contributes to the inhibition of LH and GH secretion in lambs of both sexes and T4 secretion in male lambs during the postnatal transition to puberty.

Key words: stress, luteinizing hormone, follicle-stimulating hormone, growth hormone, testosterone, lambs

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Introduction

The early postnatal development is under maternal regulations in mammals. Weaning involves a rupture of the dam-lamb nutritive bond and, if abrupt, results in the stress of maternal deprivation, i.e. the frustration arising from maternal feeding deprivation resulting in greater cortisol secretion and, in consequence, physiological disturbances of homeostasis (for review, see Wańkowska and Polkowska 2010a,b). The effect of late weaning in 12-week-old lambs involves the changes in secretion of corticotropin-releasing hormone (CRH), somatostatin and gonadotropin-releasing hormone (GnRH) in the hypothalamus and adrenocorticotropin (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and growth hormone (GH) in the pituitary (Wańkowska et al. 2006, Wańkowska and Polkowska 2009, 2010b, Polkowska and Wańkowska 2010). These endocrine changes, as the effect of stress response, are important for maintenance of homeostasis (Wańkowska and Polkowska 2010a) during the full presentation of juvenile phenotype. The specific features of the sexual maturation onset include greater LH and GH secretion in female lambs (Wańkowska et al. 2010a, Wańkowska et al. 2012), greater T4 secretion in male lambs (Wańkowska et al. 2010b) and the inhibition of LH and FSH secretion by the gonadal hormones (Wańkowska et al. 2010a,b) associated with the stimulation of GH secretion by gonadal steroids in lambs of both sexes (Wańkowska 2012, Wańkowska et al. 2012). The endocrine effects of neonatal maternal deprivation in lambs are poorly described and related to the cortisol secretion (Napolitano et al. 2003). It was previously suggested, that neonatal handling can induce lasting effects on the reproductive function in rats (Gomes et al. 2005). However the endocrine mechanisms implicated in larger, particularly farm animals have yet to be determined. It was, therefore, hypothesized that maternal deprivation stress influences the FSH, LH and GH secretion in lambs during the postnatal transition to puberty, and that this influence is long-term and sexually differentiated.

Hence, the aim of this study was to investigate the effect of weaning at 5 weeks of age in comparison with the weaning at 9 weeks of age on the cortisol, LH, FSH and GH concentrations in peripheral blood plasma of 5-, 9-, 12-, and 16-week-old lambs of both sexes. Additionally, concentrations of T4 in male lambs were measured as an indicator of maturation of testes.

Materials and Methods

Animals, management and experimental design

All procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments as approved by the Local Ethics Committee affiliated with Warsaw University of Life Sciences (number of opinion 49/2009) according to the Polish Law for the Care and Use of Animals (2 August 1997).

The 3- to 4-year-old Polish Longwool ewes were mated naturally at a commercial sheep facility and were transported to the Institute of Animal Physiology and Nutrition in September. The ewes were fed a diet of commercial concentrates, with hay and water available *ad libitum*. Twelve male and 12 female lambs were born in February, weaned at 39 days of age (early weaning – maternally deprived female and male groups, n=6 each) or at 65 days of age (control female and male groups, n=6 each) and divided into four male and four female timing groups. Animals were assigned at 5 weeks (37 days of age) to be sampled again at either 9 (63 days of age), 12- (86 days of age) or 16 weeks (112 days of age) (week 5, n=24 lambs; week 9 early weaned and control groups, n=6 each; week 12 early weaned and control groups, n=6 each; and week 16 early weaned and control groups, n=6 each). This assignment was performed according to two phases of postnatal development, infantile (before 9 weeks of age) and the beginning of the juvenile period (after 9 weeks of age). The 5-week-old lambs suckled their mothers approximately 6 times per 24 hours. Suckling lambs were penned indoors with their dams in individual straw-bedded pens. After weaning, the male and female lambs were housed separately in big straw-bedded pens. Weaned lambs had the social contact with other lambs but were deprived of maternal stimulation. Early weaned lambs were fed 240 ml of commercially available milk replacer (containing 6% of fat, 5.3% total proteins, 5% of lactose; 4.5 MJ/kg), 3-6 times a day until 9 weeks of age. All lambs were fed hay *ad libitum* and a complete pelleted concentrate for calves and lambs supplemented with vitamins, minerals and containing 17% of protein twice daily.

A day before collecting blood plasma, a jugular venous catheter was inserted and filled with heparinized saline. Blood samples of 3 ml were collected every 10 min over a 5-h period (from 08:30 am to 01:20 pm) in 5- (37 days of age), 9- (63 days of age), 12- (86 days of age) and 16-week-old (112 days of age) control lambs and 9-, 12- and 16-week-old maternally deprived lambs of both sexes. During collection, the lambs were kept in their pens where they had unlimited access to hay, water and/or dams. The blood samples were centrifuged in heparinized tubes and the plasma was stored at -20°C until analyses.

Determination of hormones in peripheral blood plasma

The concentration of cortisol was determined in duplicate 100-μl aliquots by radioimmunoassay (RIA) procedure using rabbit anti-ovine cortisol antisera (R/75; Stupnicki 1985a) and HPLC grade cortisol standard (Sigma). The assay detection limit was 0.95 ng/ml of sample. The intra- and interassay coefficients of variations were 10 and 12%, respectively

Mean and basal concentrations and pulse characteristics of cortisol were calculated using the Pulsar Computer Program developed by Merriam and Wachter (1982). The cut-off parameters $G_{(n)}$ derived empirically for the analysis of ovine cortisol data sets sampled at 10-min intervals were G(1): 3.98, G(2): 2.40, G(3): 1.68, G(4): 1.24 and G(5): 0.93 standard deviations. The cut-off parameters $G_{(n)}$ were set as 5% error rate assuming a normal distribution of data. Analysis was performed individually for every lamb and included the entire sampling period. Basal cortisol secretion was determined by the compound measurements of interpulse (nadir) hormone concentrations. Pulse frequency was defined as the number of identified pulses per collecting period.

The concentration of LH was determined in duplicate 100-μl aliquots by double-antibody RIA procedure using anti-ovine LH, anti-rabbit-gammaglobulin antisera, and bovine LH standard NIH-LH-B6 (Stupnicki and Madej 1976). The assay detection limit was 0.312 ng/ml of sample. The intra- and interassay coefficients of variations calculated for control samples at concentrations of 5 ng/ml and 1 ng/ml of LH were 4 and 10%, respectively.

Plasma FSH concentration was estimated by double-antibody RIA, using anti-ovine-FSH and anti-rabbit gammaglobulin-antisera. The anti-FSH, as well as the FSH standard was kindly donated by Dr. L.E. Reichert Jr. (Tucker Endocrine Research Institute LLC, Atlanta, Georgia, USA). The assay sensitivity was 1.56 ng/ml and the intra- and inter-assay coefficients of variation were 3 and 11%, respectively.

The concentration of GH was determined in duplicate 100 μl aliquots by double-antibody RIA procedure. The pituitary-derived bovine GH, purified by chromatography on SE-Sephadex C-50 in sodium acetate buffer for iodination and reference standards were used (Slaba et al. 1994). The first antibody was produced in rabbit against pituitary-derived bGH NIDDK-GH-B-1003 A. The assay sensitivity for GH was 0.6 ng/ml, and the intra- and inter-assay coefficients of variation averaged to 5.9% and 10.2%, respectively.

T4 was assayed in duplicate 100-μl aliquots by a direct RIA method, according to the procedure described by Stupnicki (1985b). The sensitivity of the assay was 0.6 ng/ml. The intra- and interassay coefficients of variations were 12 and 15%, respectively.

Statistical analyses

The 30 measurements taken from each peripheral blood collection were averaged to obtain a mean estimate of LH, FSH, GH or T4 concentrations for each animal. Then, the mean data were pooled to represent the individual groups. The mean concentrations of hormones and the characteristics of cortisol pulses were measured from the control 5-, 9-, 12- and 16-week-old lambs and maternally deprived 9-, 12- and 16-week-old lambs of both sexes, and compared between sexes within groups (between infancy and the juvenile period) at 5, 9, 12 and 16 weeks of age and between sexes across groups (control compared to maternally deprived) at 9, 12 and 16 weeks of age (n=6 per group). The effects of sex, age and early weaning on the mean plasma hormone concentrations were analyzed by ANOVA followed by Tukey's post-test using Statistica 9 software (StatSoft, Tulusa, USA). The significance of differences in cortisol pulse frequency across groups was assayed by the Mann-Whitney test. The levels of significance for differences between data for amplitude of pulses across the groups were calculated by the ANOVA rank Kruskal-Wallis test. All data are expressed as means ± SEM. Significance was defined as $P<0.05$

Results

Concentrations of cortisol were greater ($P<0.05$) in control female lambs than in male lambs at 5, 9 and 12 weeks of age and in early weaned female lambs than in male lambs at 9, 12 and 16 weeks of age (Fig. 1).

Following the weaning at 5 weeks of age, mean and basal concentrations of cortisol and amplitude of its pulses were greater ($P<0.001$) in 9-week-old male lambs, whereas in 12-week-old and 16-week-old male lambs they were lover ($P<0.05$) than in lambs weaned at 9 weeks of age (Fig. 1). The number of cortisol pulses did not differ ($P>0.05$) in early weaned and control male lambs in the studied age stages (Fig. 1). The mean concentrations of LH were lover ($P<0.05$) in the blood plasma of exposed to stress 12-week-old and 16-week-old male lambs compared to the control

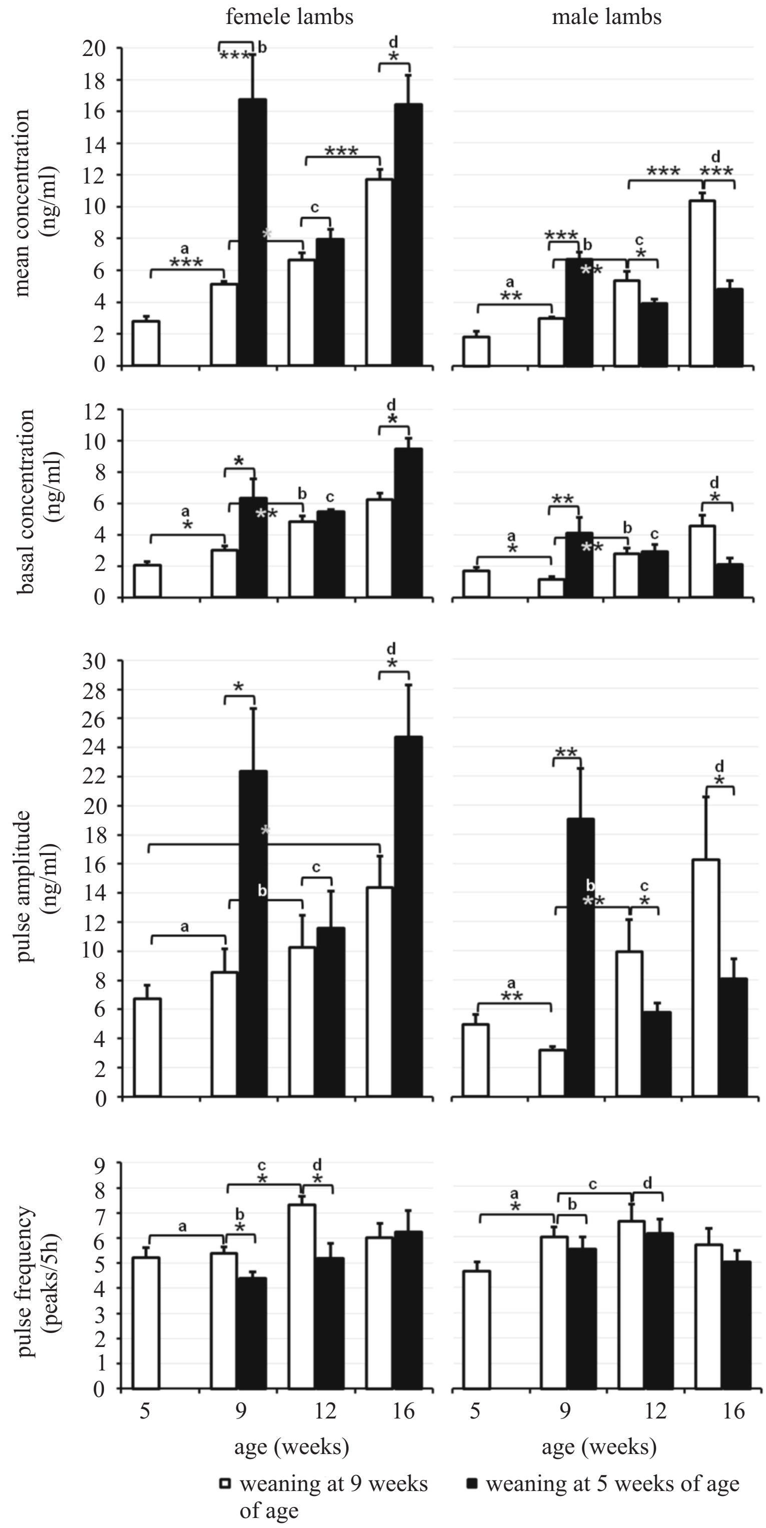

Fig. 1. Cortisol concentrations, basal concentrations, pulse amplitude and pulse frequency in peripheral blood plasma of female and male lambs after weaning at 9 (control) and 5 weeks of age (early weaning). Values are means ±SEM; *P<0.05, **P<0.01, and ***P<0.001. The same letters (a, b, c, d) above bars indicate statistical differences (P<0.01) for female versus male lambs.

lambs (Fig. 2). This difference was more prominent at 16 weeks of age than at 12 weeks of age (P<0.001 vs. P<0.01), whereas the mean concentrations of FSH were greater (P<0.001) in maternally-deprived 16-week-old male lambs compared to the control lambs (Fig. 2). The mean concentrations of GH were greater (P<0.01) in maternally-deprived 9-week-old male lambs, whereas in 12-week-old lambs they were relatively similar (P>0.05) and in 16-week-old male lambs were less (P<0.001) than in the control lambs

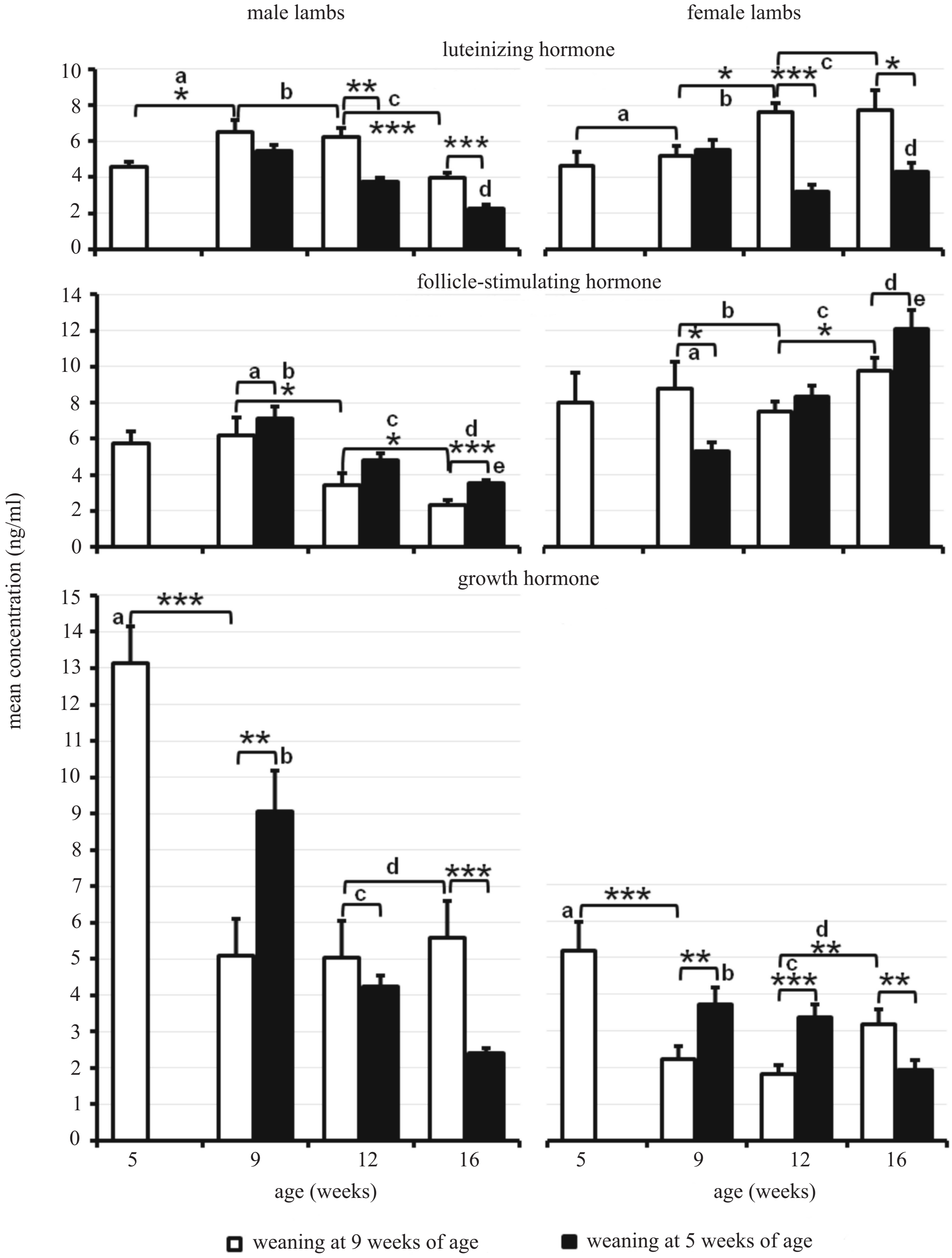

Fig. 2. The luteinizing hormone, follicle-stimulating hormone and growth hormone mean concentrations in peripheral blood plasma of male and female lambs after weaning at 9 (control) and 5 weeks of age (early weaning – maternal deprivation stress). Values are means ±SEM; **P*<0.05, ***P*<0.01, and ****P*<0.001. The same letters (a, b, c, d) above bars indicate statistical differences (*P*<0.01) for male versus female lambs.

(Fig. 2). The mean concentrations of T4 were greater (*P*<0.01) in maternally-deprived 9-week-old and 12-week-old lambs, and then lover (*P*<0.01) in 16-week-old lambs compared to the control group (Fig. 3).

In contrast to male lambs, mean and basal cortisol concentrations and cortisol pulse amplitude were greater (*P*<0.05) in 9-week-old and 16-week-old female lambs weaned at 5 weeks of age compared to lambs weaned at 9 weeks of age (Fig. 1). The number of cortisol pulses was lover (*P*<0.05) in early weaned 9-week-old and 12-week-old female lambs than in control lambs. In exposed to stress female lambs the mean concentrations of LH were relatively similar

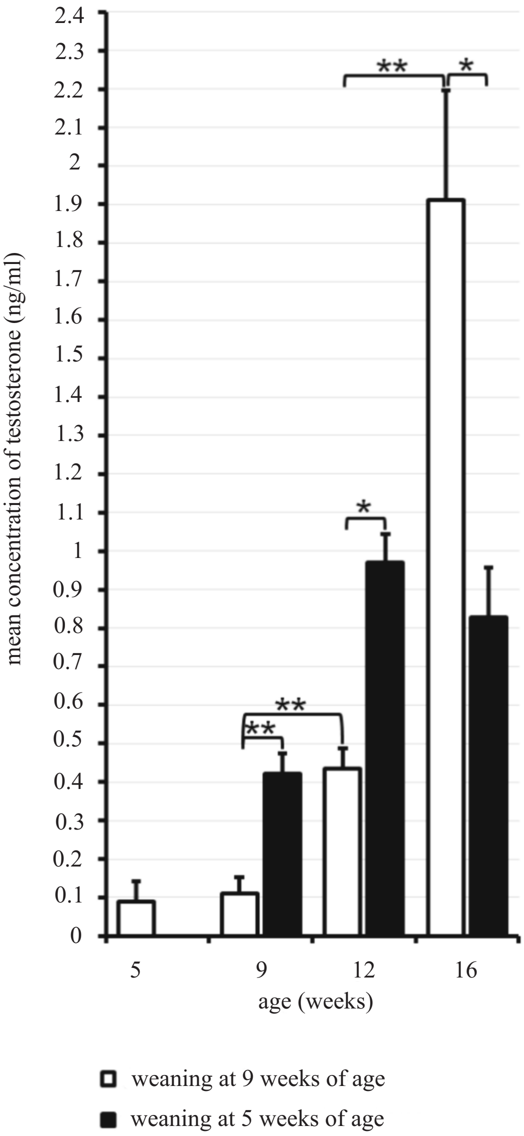

Fig. 3. The mean T4 (±SEM) concentrations in peripheral blood plasma of male lambs after weaning at 9 (control) and 5 weeks of age (early weaning – maternal deprivation stress). **P*<0.01, and ***P*<0.001.

(*P*>0.05) at 9 weeks of age and then lover (*P*<0.05) compared to the control group (Fig. 2). This difference was more prominent at 12 weeks of age than at 16 weeks of age (*P*<0.001 vs. *P*<0.05), while the mean concentrations of FSH were lover (*P*<0.05) at 9 weeks of age and then relatively similar (*P*>0.05) compared to the control lambs (Fig. 2). In maternally deprived female lambs, the mean concentrations of GH were greater (*P*<0.01) at 9 and 12 weeks of age and less (*P*<0.01) at 16 weeks of age in comparison with the control group (Fig. 2).

In control male lambs, mean concentrations of LH and FSH decreased (*P*<0.05) from 12 to 16 weeks of age (Fig. 2), whereas mean concentrations of T4 increased (*P*<0.0001) from 9 to 16 weeks of age (Fig. 3) and mean concentrations of GH were relatively similar (*P*>0.05) at 9, 12 and 16 weeks of age (Fig. 2). In contrast to male lambs, mean concentrations of LH were relatively similar (*P*>0.05) in control 12- and 16-week-old female lambs, whereas mean concentrations of FSH and GH were higher (*P*<0.05) in 16-week-old female lambs (Fig. 2).

Discussion

The present data clearly demonstrate the strong positive influence of early weaning on the cortisol secretion at 9 weeks of age in sheep of both sexes and the strong negative influence of early weaning on cortisol secretion in male lambs after 9 weeks of age. In contrast to male lambs, the positive influence of early weaning on the cortisol secretion in juvenile female lambs is reported. This study proves the sexual dimorphism of the stress response at the beginning of reproductive maturation. Moreover, the changes in the concentrations of cortisol in infantile and juvenile lambs of both sexes are associated with the effect of early weaning on pulse amplitude of this hormone. However, the decrease in the influence of early weaning in 12-week-old female lambs is associated with the effect of maternal deprivation on cortisol pulse amplitude and the number of pulses until 12 weeks of age. It was previously suggested, that the maternal stimulation actively inhibits adrenocorticotropic responses in postnatally developing offspring (Levine 2000, Hennessy et al. 2002). Feeding appears to regulate an adrenal sensitivity whereas tactile stimuli inhibit activation of centrally-controlled components of the hypothalamo-pituitary-adrenal (HPA) system in the developing mammal (Suchecki et al. 1993, McCormick et al. 1998). It was previously suggested, that the mother has rewarding properties for her young, associated with nutritive suckling, and maternal deprivation increases reward value of mother for the pup (Agmo et al. 1997). In female lambs maternally-deprived at 12 weeks (late weaning), the increase in CRH release from the nerve terminals in the median eminence to the pituitary portal circulation is followed by the increase in the storage of ACTH manifested by hypertrophy and hyperplasia of corticotropic cells (Wańkowska et al. 2006). Similar secretory changes are observed in the hypothalamus and pituitary of the adult ewes after prolonged stressful stimulation (Wańkowska and Polkowska 2010a). Thus, the attenuated release of ACTH causes the hypocortisolemia during longer exposure to stressful stimuli in lambs beyond the time of weaning and adult sheep. The restricted secretion in the HPA system similar in the stressed juvenile lambs and mature ewes, in contrast to the quite different cortisol secretion in stressed suckling lambs, suggests the development of this system during the weaning period. The sexual differentiation of the stress response after 9 weeks of age suggests the influence of hormones produced by the developing gonads on the adrenocortical functions.

The study demonstrates the strong positive influence of early maternal deprivation stress on the T4 secretion at 9 weeks of age and the decrease in this positive influence until 12 weeks of age in contrast to the following negative influence at puberty in male lambs. It should be noted, that the effect of maternal deprivation stress experienced at 5 weeks of age on the T4 secretion at puberty, i.e. the inhibitory effect, is similar to the recently described strong inhibitory effect of orchidectomy at 12 weeks of age, the stage of timing puberty in male lambs (Wańkowska et al. 2010b). In this regard, the maternal deprivation stress chronically affects the gonadotropic hormones secretion in lambs. The stress response in male lambs contributes to stimulating at puberty the secretion of FSH, a hormone essential for testes development (Wańkowska et al. 2010b), and the intensification of the negative influence on the LH secretion after 9 weeks of age, which results in the reduced T4 secretion during sexual maturation. In the female lambs, the stress response leads to the short-term and reversible inhibition of the FSH secretion and long-term inhibition of the LH secretion during the reproductive development. Luteinizing hormone is pivotal for ovaries maturation because the most important feature of juvenile phenotype is increase in the secretion of GnRH-LH which is determined by the reduction in sensitivity of the hypothalamus and pituitary to inhibitory feedback of the gonadal steroid hormones (Veldhuis et al. 2006, Wańkowska and Polkowska 2009, Wańkowska et al. 2010a). Thus, the long-term changes in the LH secretion after an early stressful stimulation suggest the endocrine modifications in the timing of testes and ovaries maturation in lambs.

In this study, maternal deprivation stress stimulated the GH secretion in infantile lambs but inhibited the GH secretion in juvenile lambs of both sexes. There is a close association between growth and reproductive development. Steroids produced by the developing gonads have weak effect on the GH secretion in suckling lambs but enhance GH release in juvenile lambs of both sexes. The gonadal factors modulate mechanisms within the somatotropic system in infantile and juvenile lambs to synchronize the somatic growth with gonadal development (Wańkowska 2012, Wańkowska et al. 2012). It is, therefore, assumed that endocrine modifications in the timing of testes and ovaries maturation in maternally deprived lambs involved decrease in the GH secretion.

In conclusion, early weaning in lambs is stressful and sexually dimorphic experience. In female lambs, the stress reaction is strong and long-lasting, whereas in male lambs this reaction is strong at first but reversible, i.e. resulted in the low cortisol secretion during puberty. It is concluded that the influence of hormones produced by the developing gonads on the cortisol secretion begins at the start of sexual maturation beyond the time of weaning. The influence of maternal deprivation stress on the LH secretion, increasing in male lambs and decreasing in female lambs, is sexually differentiated as late as the cortisol secretion in the maternally deprived lambs, in contrast to the early sexual differentiation of the FSH secretion under stressful conditions. Maternal deprivation stress inhibits the prepubertal increase in the LH and GH secretion in female lambs and pubertal LH, GH and T4 secretion in male lambs.

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