

Changes in Estrogen Receptor ER α and ER β Expression in Chicken (*Gallus domesticus*) Adrenal Gland during Short-fasting and Refeeding*

Małgorzata BŁACHUTA and Danuta WROŃSKA-FORTUNA

Accepted May 22, 2012

BŁACHUTA M., WROŃSKA-FORTUNA D. 2012. Changes in estrogen receptor ER α and ER β expression in chicken (*Gallus domesticus*) adrenal gland during short-fasting and refeeding. *Folia Biologica* (Kraków) **60**: 199-203.

Estrogen receptors have been found in the adrenal gland of rodents, monkeys, mares and sheep, indicating a connection between sex steroids and the activity of the adrenal gland. In the present study, the expression of estrogen receptors alpha (ER α) and beta (ER β) in the chicken adrenal gland during stress induced by 24 h fasting and after refeeding was determined using reverse transcription and the polymerase chain reaction (RT-PCR). The presence of both ER mRNAs in the adrenal gland of all examined groups was found. The relative expression of ER α mRNA was higher than ER β mRNA. There were no significant differences in ER α mRNA expression among the examined groups. On the contrary, we observed changes in ER β expression during stress conditions. These findings indicate different pathways of estrogen action in the avian adrenal gland. Furthermore, changes in ER β level suggest that this form of estrogen receptor plays a predominant role for estrogen action in the chicken adrenal gland during stress.

Key words: ER α , ER β , adrenal gland, RT-PCR, chicken, stress.

Małgorzata BŁACHUTA, Danuta WROŃSKA-FORTUNA. Department of Animal Physiology and Endocrinology, University of Agriculture, Mickiewicza 24/28, 30-059 Kraków, Poland.
E-mail: malgorzata.blachuta@wp.pl

Estrogens play a key role in the regulation of reproductive functions in reptiles (LANCE & BOGART 1992), birds (ICHIKAWA *et al.* 2003) and mammals (HALL *et al.* 2001). In birds, estrogens are synthesized by the theca cells of the ovarian follicles (KATO *et al.* 1995) and they are involved in ovarian formation, differentiation of the oviduct (PALMITER & MULVIHILL 1978) and demasculinization of the brain during early life (BALTHAZART *et al.* 1996). Moreover, estrogens increase adrenal gland activity in rats (SARUHAN & OZDEMIR 2005). On the other hand, the release of corticoids from the adrenal gland affects the reproductive system by activation of the hypothalamic-pituitary-gonadal (HPG) axis (TILBROOK *et al.* 2000).

Biological effects of estrogens are mediated through two forms of receptors, alpha (ER α) and the more recently discovered beta (ER β) (KUIPER *et al.* 1996; PETTERSSON & GUSTAFSSON 2001). These receptors are encoded by two different genes and regulate the expression of E2 target genes by direct binding with a specific DNA do-

main – estrogen-responsive element (ERE) (KLINGE 2000). In birds, both forms of ER mRNAs have been found in the neuroendocrine system, the liver, ovary and oviduct (ICHIKAWA *et al.* 2003; HRABIA *et al.* 2008).

So far, research in molecular biology has been mainly based on mammalian organisms such as the rat, mouse and monkey. Therefore, expression of ERs has been shown in the adrenal gland of rats (KUIPER *et al.* 1997), monkeys (HIRST *et al.* 1992), mares (ALM *et al.* 2009) and sheep (VAN LIER *et al.* 2003). Even though the chicken (*Gallus domesticus*), as well as mammals, is an important organism for biomedical research, development and aging, there is no information about ER expression in the avian adrenal gland. The adrenal gland plays a role in the integration of metabolic activity and energy balance, implicating feeding as a major regulator of rhythms in the hypothalamic-pituitary-adrenal (HPA) axis (DALLMAN *et al.* 1999). Physiological stress, such as starvation, leads to substantially increased release of cortisol

*Supported by grant no. N N311 098834 and DS 3243/KFiEZ/10.

from the adrenal cortex (SEED *et al.* 2000). This pathway often has an inhibitory effect on the reproductive system (TILBROOK *et al.* 2000). Therefore, the aim of the present study was to determine mRNA ER α and ER β presence in the chicken adrenal gland and changes in the level of ERs during stress induced by feed withdrawal and after refeeding.

Material and Methods

Animal experiment

The experiment was conducted according to a research protocol approved by the Local Animal Ethics Committee in Cracow (No. 49/OP/2004). Immature (15-week-old) Hy-Line Brown hens (n=18) were kept in individual cages under a light regimen of 14 h light and 10 h dark (lights-on at 0800 h and off at 2200 h) with free access to water and commercial food (DKMII).

The birds were divided into three equal groups: (i) with feed and water *ad libitum* (control), (ii) fasted for 24 h (short fasting) and (iii) fasted for 24 h and then allowed access to food for 24h (refeeding). All chickens were decapitated, and the liver (used as a positive control) and the adrenal gland tissues were isolated, quickly placed into RNAlater and stored in -20°C until total RNA extraction.

The chemicals were purchased from the following companies: TRI-reagent (MRC, Inc., Cincinnati, OH, USA), RevertAid M-MuLV Reverse Transcriptase, Ribonuclease inhibitor, dNTP mix, MgCl₂, Pol Taq DNA Polymerase, buffers, molecular weight marker – 100 bp DNA ladder (Fermentas, Vilnius, Lithuania), primers, oligo-dT₁₈ (IBB, Warszawa, Poland). All other reagents were obtained from ICN Biomedicals (Aurora, IL, USA) or Sigma (St. Louis, MO, USA).

RNA isolation and polymerase chain reaction

Total RNA was extracted from the liver and adrenal tissues using the TRI-reagent according to

the manufacturer's recommendations. 2 μ g of total RNAs isolated from each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200U) and oligo-dT₁₈ primers (0.5 μ g). As a negative control, untranscribed tissue RNA (reverse transcriptase omitted) was used. RT products (1 μ l) were amplified in a Thermocycler Gradient (Eppendorf, Germany) according to HRABIA *et al.* (2008) in a 12.5 μ l reaction mixture containing 1.25 μ l of buffer (100 mmol Tris-HCl, pH 8.8, 500 mmol KCl, 0.8% Nonidet P40), 0.312 unit pol Taq DNA polymerase, 0.2 μ mol sense and antisense primers, 0.2 mmol each dNTP, 1.5 mmol MgCl₂, and water. PCR conditions were as follows: the initial denaturation for 5 min at 95°C (ER α , GAPDH) or 4 min at 94°C (ER β), then 30 s at 95°C (ER α , GAPDH) or 30 s at 94°C (ER β), 30 s (ER α , ER β) or 15 s (GAPDH) at the annealing temperature, and 30 s at 72°C. Amplifications were completed with an additional extension at 72°C for 7 min. Primers, number of cycles and annealing temperatures for ER α , ER β and GAPDH are described in Table 1. Negative control (water) was included in all reactions. All PCR products were analysed using agarose gel electrophoresis with a 1.5% agarose gel in TBE buffer (0.5X TBE solution composed of 0.045M Tris-borate and 0.001M EDTA) and stained by ethidium bromide. The gel was examined under UV light and photographed with a digital camera. The net intensities of individual bands were measured using Scion Image for Windows. The ratios of net intensity of examined genes to GAPDH were used to represent the relative level of target gene expression. The average abundance of six repeats was used for statistical analysis.

Statistical analysis

Statistical procedures used for treating data obtained in this study were performed with two-way analysis of variance. Data were tested for significant differences (at the level of P<0.05) with the Tukey test. Results are expressed as the mean \pm SEM.

Table 1

Primer sequences and PCR conditions used for polymerase chain reactions

| Gene (GeneBank) | Primer sequence | Amplicon size | Annealing temperature | Number of cycles |
|-----------------------|--|-----------------------|-----------------------|------------------|
| GAPDH (K01458) | F: 5'-GTGGAGAGATGACAGAGGTG-3' R: 5'-AACAAGCTTGACGAAATGCT-3' | 349 bp (635-983) | 52°C | 28 |
| ER α (X03805) | F: 5'-GTGCCTTAAGTCCATCATCCT-3' R: 5'-GCGTCCAGCATCTCCAGTAAG-3' | 300 bp (1522-1821) | 58°C | 30 |
| ER β (AB036415) | F: 5'-TGATATGCTCCTGGCCATGAC-3' R: 5'-CTTCATGCTCAGCAGATGCTC-3' | 304 bp (1374-1677) | 55°C | 30 |

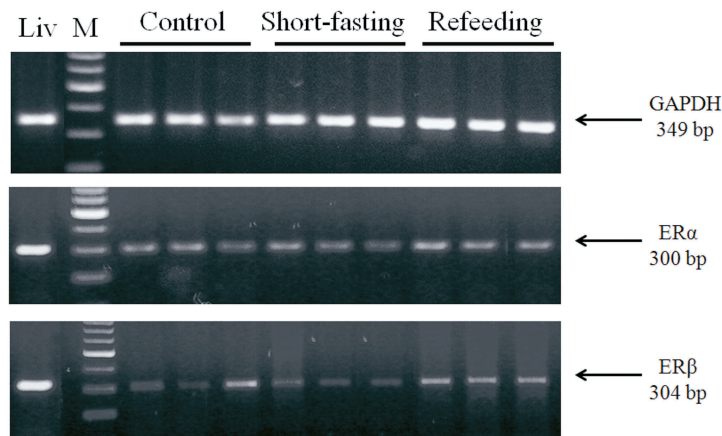


Fig. 1. Chicken adrenal gland distribution of ER α and ER β analyzed by RT-PCR. GAPDH was used as a control. Lane 1 shows the control expression in the liver (Liv); lane 2 shows a DNA size ladder (M).

Results

The presence of ER α and ER β mRNAs was found in the liver (a positive control) and in the adrenal tissue of all examined groups of chickens. The products were 300, 304 and 349 bp for ER α mRNA, ER β mRNA and GAPDH mRNA, respectively, and corresponded to the approximate size for each as predicted (Fig. 1).

The relative expression of ER α was significantly higher than ER β in the control (0.51 ± 0.066 vs. 0.24 ± 0.033) and in the fasted birds (0.44 ± 0.034 vs. 0.18 ± 0.044). In the case of the adrenal gland of

the chickens after refeeding, differences between expression of ER α mRNA and ER β mRNA were insignificant (0.55 ± 0.044 vs. 0.46 ± 0.076). There were no significant differences in ER α mRNA expression among the examined groups (0.51 ± 0.066 vs. 0.44 ± 0.034 vs. 0.55 ± 0.044 in control, short-fasting and refeeding group respectively) (Fig. 2).

With respect to ER β there was no difference in mRNA expression between the control and fasted birds (0.24 ± 0.033 vs. 0.18 ± 0.044), whereas in the chickens after refeeding the expression was significantly elevated by 162% and 94% compared to the fasted and control chickens, respectively (Fig. 2).

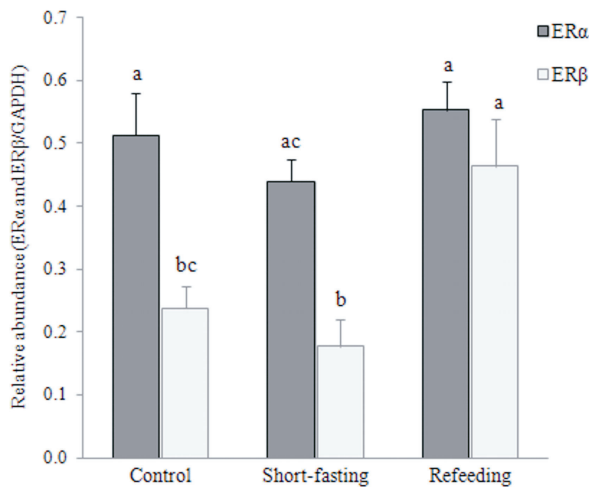


Fig. 2. ER α mRNA and ER β mRNA in chicken adrenal tissue: Control – chickens fed *ad libitum*; Starvation – chickens fasted for 24h; Refeeding – chickens fasted for 24h + refed for 24h. Each value represents the mean \pm SEM from six determinations that were measured as a relative density of RT-PCR products compared to GAPDH. Means with different letters are significantly different from each other ($P < 0.05$).

Discussion

Estrogen receptors ER α and ER β were found in the adrenal gland of several species such as rodents and primates (WEISS & RUO-JUN XU 1990; HIRST *et al.* 1992). Furthermore, data from many experiments indicate that gonadal hormones have a direct effect on the physiology of the adrenal tissue. For instance, female sheep secreted more cortisol after exogenous ACTH treatment than male sheep. Gonadectomy in these animals reduced the sex differences, suggesting a role for circulating gonadal steroids in the regulation of cortisol secretion at the adrenal gland level (VAN LIER *et al.* 2003). SARUHAN and OZDEMIR (2005) received similar results in rats with bilateral ovariectomy, i.e. a decrease in the activity of the adrenal cortex. In contrast, estrogen supplementation causes a significant increase in the activity of the adrenal cortex and medulla. LO *et al.* (2000) indicate that estrogens may enhance corticosterone feedback by stimulating corticosteroid

terone production at the adrenal gland or by reducing corticosterone metabolism.

We reveal the presence of two transcripts (α and β) of ER in the chicken adrenal gland. The presence of both ERs indicates different pathways of estrogen action in the avian adrenal gland. These findings correspond to previous observations implying distinct pathways of estrogen action (LINDNER *et al.* 1998; PRINS *et al.* 1998). GUSTAFSSON (1999) assumed that ER α and ER β differentially expressed in several tissues have varied or even opposite biological actions.

Furthermore, we demonstrated a significant difference between ER α and ER β levels. A markedly higher expression of ER α mRNA suggests that this type of estrogen receptor is mainly involved in the modulation of adrenal functions in chickens. This is in accordance with a study in the rat that showed higher expression of ER α mRNA than ER β mRNA in the adrenal gland (KUIPER *et al.* 1997). On the other hand, we observed changes in ER β expression level after stress conditions while there were no significant differences in ER α among control, short-fasting and refeeding group. These data indicate that ER β is predominantly involved in the modulation of adrenal activity by estrogens in response to stress conditions. This conclusion is consistent with previous studies which have shown that estrogens take part in limiting the responses to physiological stress. For example, estrogen administration to postmenopausal women attenuates cortisol responses to mental stress (LINDHEIM *et al.* 1992). These data are also consistent with those of KOMESAROFF *et al.* (1998) who showed that in ovariectomized ewes administration of estrogen at physiological levels decreases glucocorticoid responses to stressors.

The HPA system is the main neuroendocrine pathway in the mediation of physiological responses to stressors. Activation of this axis leads to higher endogenous glucocorticoid levels and further exacerbates the pathologies associated with stressors (McEWEN 1998; BAO *et al.* 2008). Stress suppresses the reproductive system at various levels: through inhibiting the luteinizing hormone-releasing hormone (LHRH) secretion or repressing LH-induced ovulation and sperm release. In addition, glucocorticoids inhibit the testes and ovaries directly, hindering production of the male and female sex hormones (SWAAB 2003). The interaction between the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis may act in both ways with reproductive hormones also influencing adrenal function (YOUNG 1995). In rodents basal and stress-induced activity of the HPA axis is higher in females than in males (VIAU *et al.* 2005). This discrepancy suggests that gonadal steroid hormones might be in part responsible for

these sex differences. BEUVING *et al.* (1989) showed corticosterone depletion during chronic stress. Our suggestion is that estrogens, in the case of corticosterone deficiency, may control the adrenal gland activity through ER β . The specific structure of avian adrenal gland with cortical tissue intermingled with the medullary tissue also seems to be significant.

It is known that estrogens are well-established mood modulators in both males and females (ARPELS 1996). This may be a consequence of two main receptor systems of estradiol ER α and ER β . Animal studies support an anxiogenic and depressant effect of ER α activation and anxiolytic and antidepressant effect of ER β activation (LUND *et al.* 2005; WAIF & FRYE 2005; HUGHES *et al.* 2008). This is in agreement with our studies that showed higher expression of ER β during the time when an organism returns to "normal" conditions. Estradiol's overall effect on HPA axis activity may be in part due to impairment in glucocorticoid receptor function thereby impairing glucocorticoid negative feedback (TURNER 1990). ISGOR *et al.* (2003) showed that in rats, ER β has an important role in HPA axis activation at the hypothalamus level, and is regulated by circulating corticosterone. Adrenalectomy reduced ER β mRNA expression in the paraventricular nucleus (PVN), whereas corticosterone replacement fully reversed this effect in a dose-dependent fashion. On the other hand, local delivery of estradiol or ER α agonist to the PVN increases stress-induced plasma corticosterone. These findings correspond with our suggestion that ER β may be an important mediator between the HPA and HPG axis during recovery.

In conclusion, the data presented here clearly show the involvement of estrogens in reaction to stress by changes of ER α and ER β mRNAs in the chicken adrenal gland. The obtained results indicate that this gland seems to be also a target tissue for estrogen auto-, para-, and endocrine actions.

References

- ALM Y. H., SUKJUMLONG S., KINDAHL H., DALIN A. M. 2009. Steroid hormone receptors ER α and PR characterised by immunohistochemistry in the mare adrenal gland. *Acta Vet. Scand.* **51**: 31.
- ARPELS J. C. 1996. The female brain hypoestrogenic continuum from the premenstrual syndrome to menopause. A hypothesis and review of supporting data. *J. Reprod. Med.* **41**: 633-639.
- BALTHAZART J., TLEMCANI O., BALL G. F. 1996. Do sex differences in the brain explain sex differences in the hormonal induction of reproductive behavior? What 25 years of research on the Japanese quail tells us. *Horm. Behav.* **30**: 627-661.
- BAO A. M., MEYNEN G., SWAAB D. F. 2008. The stress system in depression and neurodegeneration: Focus on the human hypothalamus. *Brain Res. Rev.* **57**: 531-553.

- BEUVING G., JONES R. B., BLOKHUIS H. J. 1989. Adrenocortical and heterophil/lymphocyte responses to challenge in hens showing short or long tonic immobility reactions. *Br. Poult. Sci.* **30**: 175-184.
- DALLMAN M. F., AKANA S. F., BHATNAGAR S., BELL M. E., CHOI S., CHU A., HORSLEY C., LEVIN N., MEIJER O., SORIANO L. R., STRACK A. M., VIAU V. 1999. Starvation: early signals, sensors and sequelae. *Endocrinology* **140**: 4015-4023.
- GUSTAFSSON J. A. 1999. Estrogen receptor β – a new dimension in estrogen mechanism of action. *J. Endocrinol.* **163**: 379-383.
- HALL J. M., COUSE J. F., KORACH K. S. 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J. Biol. Chem.* **276**: 36869-36872.
- HIRST J. J., WEST N. B., BRENNER R. M., NOVY M. J. 1992. Steroid hormone receptors in the adrenal glands of fetal and adult rhesus monkeys. *J. Clin. Endocrinol. Metab.* **75**: 308-314.
- HRABIA A., WILK M., RZASA J. 2008. Expression of α and β estrogen receptors in the chicken ovary. *Folia Biol. (Kraków)* **56**: 187-191.
- HUGHES Z. A., LIU F., PLATT B. J., DWYER J. M., PULICICCHIO C. M., ZHANG G., SCHECHTER L. E., ROSENZWEIG-LIPSON S., DAY M. 2008. WAY-200070, a selective agonist of estrogen receptor β as a potential novel anxiolytic/antidepressant agent. *Neuropharmacology* **54**: 1136-1142.
- ICHIKAWA K., YAMAMOTO I., TSUKADA A., SAITO N., SHIMADA K. 2003. cDNA cloning and mRNA expression of estrogen receptor α in Japanese quail. *J. Poult. Sci.* **40**: 121-129.
- ISGOR C., CECCHI M., KABBAJ M., AKIL H., WATSON S. J. 2003. Estrogen receptor β in the paraventricular nucleus of hypothalamus regulates the neuroendocrine response to stress and is regulated by corticosterone. *Neuroscience* **121**: 837-845.
- KATO M., SHIMADA K., SAITO N., NODA K., OHTA M. 1995. Expression of P45017 α -hydroxylase and P450aromatase genes in isolated granulosa, theca interna, and theca externa layers of chicken ovarian follicles during follicular growth. *Biol. Reprod.* **52**: 405-410.
- KLINGE C. M. 2000. Estrogen receptor interaction with coactivators and co-repressors. *Steroids* **65**: 227-251.
- KOMESAROFF P. A., ESLER M., CLARKE I. J., FULLERTON M. J., FUNDER J. W. 1998. Effects of estrogen and estrous cycle on glucocorticoid and catecholamine responses to stress in sheep. *Am. J. Physiol. Endocrinol. Metab.* **275**: E671-E678.
- KUIPER G. G., ENMARK E., PELTO-HUIKKO M., NILSSON S., GUSTAFSSON J. A. 1996. Cloning of the novel estrogen receptor expression in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93**: 5925-5930.
- KUIPER G. G., CARLSSON B., GRANDIEN K., ENMARK E., HAGGBLAD J., NILSSON S., GUSTAFSSON J. A. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* **138**: 863-870.
- LANCE V. A., BOGART M. H. 1992. Disruption of ovarian development in alligator embryos treated with aromatase inhibitor. *Gen. Comp. Endocrinol.* **86**: 59-71.
- LINDHEIM S. R., LEGRO R. S., BERNSTEIN L., STANCZYK F. Z., VIJOD M. A., PRESSER S. C., LOBO R. A. 1992. Behavioral stress responses in premenopausal and postmenopausal women and effects of estrogen. *Am. J. Obstet. Gynecol.* **167**: 1831-1836.
- LINDNER V., KIM S. K., KARAS R. H., KUIPER G. G., GUSTAFSSON J. A., MENDELSON M. E. 1998. Increased expression of estrogen receptor- β mRNA in male blood vessels after vascular injury. *Circ. Res.* **83**: 224-229.
- LO M. J., CHANG L. L., WANG P. S. 2000. Effects of estradiol on corticosterone secretion in ovariectomized rats. *J. Cell. Biochem.* **77**: 560-568.
- LUND T. D., ROVIS T., CHUNG W. C., HANDA R. J. 2005. Novel actions of estrogen receptor- β on anxiety related behaviors. *Endocrinology* **146**: 797-807.
- McEWEN B. S. 1998. Stress, adaptation and disease. Allostasis and allostatic load. *Ann. N. Y. Acad. Sci.* **840**: 33-44.
- PALMITER R. D., MULVIHILL E. R. 1978. Estrogenic activity of the insecticide kepone on the chicken oviduct. *Science* **201**: 356-358.
- PETERSSON K., GUSTAFSSON J. A. 2001. Role of estrogen receptor β in estrogen action. *Annu. Rev. Physiol.* **63**: 165-192.
- PRINS G. S., MARMER M., WOODHAM C., CHANG W., KUIPER G., GUSTAFSSON J. A., BIRCH L. 1998. Estrogen receptor β mRNA ontogeny in the rat prostate and affects of neonatal estrogen exposure on the expression pattern. *Endocrinology* **139**: 874-883.
- SARUHAN B. G., OZDEMIR N. 2005. Effect of ovariectomy and of estrogen treatment on the adrenal gland and body weight in rats. *Saudi. Med. J.* **26**: 1705-1709.
- SEED J. A., DIXON R. A., McCLUSKEY S. E., YOUNG A. H. 2000. Basal activity of the hypothalamic-pituitary-adrenal axis and cognitive function in anorexia nervosa. *Eur. Arch. Psych. Clin. Neurosci.* **250**: 11-15.
- SWAAB D. F. 2003. The human hypothalamus, basic and clinical aspects. Part I: Nuclei of the hypothalamus. In: Aminoff M. J., Boller F., Swaab D. F. (Eds) *Hand Book of Clinical Neurology 3rd series vol. 79*, Elsevier, Amsterdam, NE, pp. 1-297.
- TILBROOK A. J., TURNER A. I., CLARKE I. J. 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Rev. Reprod.* **5**: 105-113.
- TURNER B. B. 1990. Sex difference in glucocorticoid binding in rat pituitary in estrogen dependent. *Life Sci.* **46**: 1399-1406.
- VAN LIER E., MEIKLE A., BIELLI A., AKERBERG S., FORSBERG M., SAHLIN L. 2003. Sex differences in oestrogen receptor levels in adrenal glands of sheep during the breeding season. *Domest. Anim. Endocrinol.* **25**: 373-387.
- VIAU V., BINGHAM B., DAVIS J., LEE P., WONG M. 2005. Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat. *Neuroendocrinology* **146**: 137-146.
- WAIF A. A., FRYE C. A. 2005. Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity. *Neuropsychopharmacology* **30**: 1288-1301.
- WEISS M., RUO-JUN XU 1990. Estrogen receptors in the adrenal cortex of the possum (*Trichosurus vulpecula*). *Comp. Biochem. Physiol.* **96B**: 375-380.
- YOUNG E. A. 1995. The role of gonadal steroids in hypothalamic-pituitary-adrenal axis regulation. *Crit. Rev. Neurobiol.* **9**: 371-378.