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# Oestrogen receptor α and β mRNA expression in testis of ganders fed diets containing different levels of phytoestrogens*

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ABSTRACT

The aim of this study was to examine the effect of diets with different levels of phytoestrogens on testicular changes of oestrogen receptor (ER) α and β mRNA expression in Biłgoraj ganders during three different phases of reproductive activity. Birds were fed diets with low phytoestrogens levels (15.40-40.44 μg/g of total phytoestrogens) based on grass meal (control), or diets with higher levels of phytoestrogens (118.54-151.19 μg/g of total phytoestrogens) based on lucerne meal and soyabean meal. Testes were dissected from animals at the peak of reproductive activity (March), during the descending of reproductive activity (May) and at the beginning of photorefractoriness period (July). The expression of the ERα and β mRNA in testis was examined by semiquantitative RT-PCR. There were not differences in testicular expression of ERα mRNA between ganders fed both diets in March and May. An increasing of this expression in group fed diets with higher phytoestrogens levels in July in comparison to control group as well as to both groups in March and May was observed (281% as well as 441 and 446%, and 830 and 1388%, respectively). The testicular expression of ERβ mRNA between ganders fed both diets during all the experiment time was not significantly different. The greater ERβ mRNA expression in testis from ganders fed diets with higher phytoestrogens levels diets in July than from males fed both diets in March and May (153 and 197%, and 380 and 298%, respectively), and the increasing of this expression in control birds from July in comparison to animals from May (231 and 175%, respectively) were noted.

KEY WORDS: phytoestrogens, oestrogen receptors, gene expression, testis, goose

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## INTRODUCTION

Domestic geese are fed diets containing plant-derived components among which soya and lucerne are the source of phytoestrogens (e.g., genistein, daidzein) well recognized for their oestrogen-like activity. Although phytoestrogens exhibit nonsteroidal, diphenolic structure they can interact with oestrogen receptors and in consequence influence animal reproduction (Adams, 1995) including bird (Leopold et al., 1976; Lien et al., 1987). In our previous study, we noted that phytoestrogens inhibited *in vitro* secretion of testosterone by gander testicular cells (Opałka et al., 2006).

In mammals, phytoestrogens can act by a number of mechanisms (Benassayag et al., 2002): interaction with enzymes involved in steroidogenesis, inhibition of protein kinases, effect on steroid binding protein as well as binding to oestrogen receptors (ER), especially to ERβ because of their higher affinity for ERβ than ERα (Kuiper et al., 1998). Moreover, phytoestrogens may alter ER expression in tissues. Dietary genistein inhibited expression of ERα and β in the rat prostate (Dalu et al., 2002; Fritz et al., 2002). The treatment with genistein decreased ERα and β mRNA expression in the rat uterus (Diel et al., 2006). In mouse testis the injections of genistein inhibited mRNA expression of ERα (Adachi et al., 2004).

In birds, the mRNA expression of ER were detected in testis of chicken (Nakabayashi et al., 1998) and duck (Koba et al., 2008) embryos. In adult Japanese quail the testicular expression of ERα (Ichikawa et al., 2003) as well as ERβ (Lakaye et al., 1998; Foidart et al., 1999) were demonstrated. These facts indicate that oestrogens and also phytoestrogens can control of avian testicular functions. In mammal, the role of oestrogen in testis biology is well documented (Sierens et al., 2005). No data up to now are available concerning the effect of phytoestrogens on expression of ER in avian testis. In laying Shaoxing ducks dietary daidzein decreased ERβ mRNA expression in hypothalamus (Zhao et al., 2004). However, it remains to be elucidated whether and how dietary phytoestrogens may affect function of avian testis. Therefore, the aim of this study was to examine the effect of diets with different levels of phytoestrogens on testicular changes of oestrogen receptor α and β mRNA expression in Biłgoraj ganders during three different phases of reproductive activity.

## MATERIAL AND METHODS

All experiments were performed in accordance with the principles and procedures of the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn. Animals were kept and fed as previously described (Opałka et al., 2006).

Briefly, 1-day-old (April/May hatched) Biłgoraj male goslings (*Anser anser*) were divided into a control group fed diets containing low levels of phytoestrogens (C), based on grass meal and a group fed diets containing with higher levels of phytoestrogens (P), based on lucerne meal and soyabean meal. During the first 12 weeks (to July) birds from C and P groups were fed diets containing 3.5-5% of grass meal and 3.5-5% of lucerne meal, respectively. Concentrations of total phytoestrogens in C and P diets during this period were 210.38 and 383.55 μg/g. Next, from August to July (including the reproductive season) diets for C group were enriched with 20% of grass meal while that for P group with 20% of lucerne meal. Levels of total phytoestrogens in C and P diets were 15.40-40.44 and 118.54-151.19 μg/g, respectively. Detailed diets compositions and concentrations of phytoestrogens were previously described (Opałka et al., 2006).

Testes were dissected from 30 ganders: at the peak of reproductive activity in March (11-month-old; n=5 for C and P groups), during the descending phase of reproductive activity in May (13-month-old; n=5 for C and P groups) and at the beginning of photorefractoriness period in July (15-month-old; n=5 for C and P groups). The removed glands were quickly frozen in liquid nitrogen and kept at −70°C.

Total RNA was isolated from frozen testis using RNeasy Mini Kit (Qiagen, USA). Complementary DNA (cDNA) was obtained, using 1 μg of total RNA, by the reverse transcription (at 37°C for 60 min), using Omniscript RT Kit (Qiagen, USA). The received cDNA was used in PCR reaction in presence of ERα primers (sense: 5'GTG CCT TAA GTC CAT CAT CCT 3'; antisense: 5'GCG TCC AGC ATC TCC AGT AAG 3') or ERβ primers (sense: 5'GGC TAC GGA AAT GCT ATG AA 3'; antisense: 5'CCA GTT TTG TCA GGG ACA TC 3') in co-amplification with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, as a reference gene (sense: 5'CAA CAT CAA ATG GGC AGA TG 3'; antisense: 5'AAC AGA GAC ATT GGG GGT TG 3'). The primers were constructed based on chicken ERα, ERβ and GAPDH mRNA sequences (Genbank Database accession numbers: X03805, NM_204794 and NM_204305, respectively). PCR was performed using HotStar Taq Master Mix Kit (Qiagen, USA). After an initial denaturation at 95°C for 15 min, the samples were coamplified for 28 cycles (ERα and GAPDH) or 36 cycles (ERβ) and 34 cycles (GAPDH) by denaturing at 94°C for 1 min, annealing at 61°C (ERα) or 63°C (ERβ) for 1 min and extension at 72°C for 1 min. The reaction was completed by final extension at 72°C for 10 min. The products of PCR (300, 282 and 478 bp for ERα, ERβ and GAPDH, respectively) were electrophoresed on 1.5% agarose, using pUC19 DNA/*Msp*I (Fermentas, Canada) as a molecular marker, visualized by ethidium bromide staining and pictures were saved by FOTO/Analist Archiver (Fotodyne, USA). For semi-quantity analysis of ERα or ERβ mRNA expression (in relation to

GAPDH), optical density was measured by densitometric scanning of archived pictures using GelScan for Windows ver. 1.45 software (Kucharczyk, Poland).

The data are presented as mean±SEM (standard error of mean). Differences between means were tested using the two-way analysis of variance following by LSD-test (Statistica, StatSoft Inc., USA). Significance is reported at $P<0.05$.

## RESULTS

The expression of ERα mRNA (Figure 1) in testis from ganders fed C and P diets at the peak (March) as well as during the descending phase of reproductive

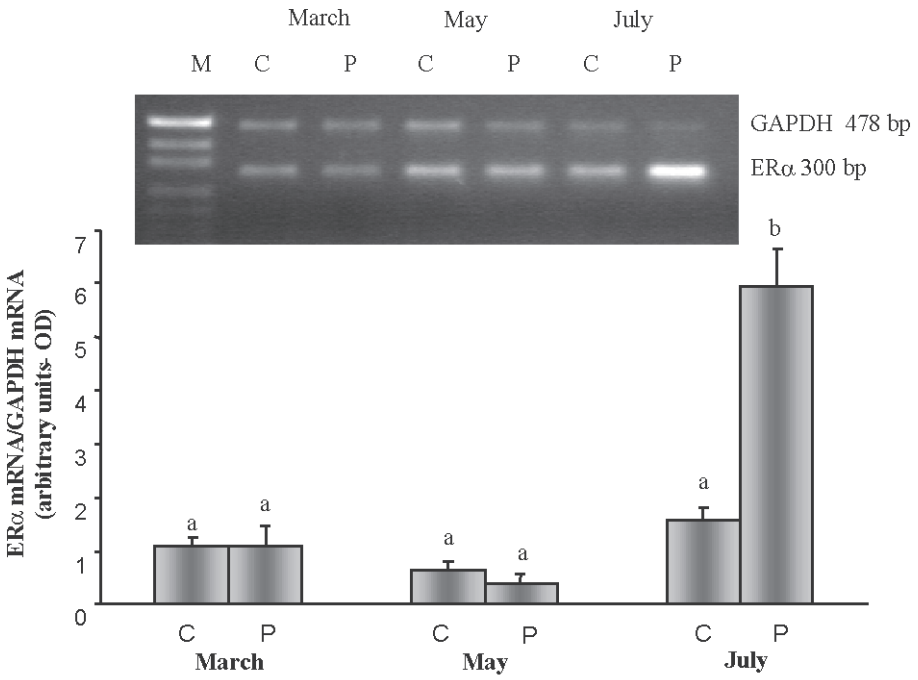


Figure 1. Semi-quantitative analysis of ERα mRNA expression in testis from ganders fed control diets (C) and diets with higher levels of phytoestrogens (P) at the peak of reproductive activity (March), during the descending phase of reproductive activity (May) and at the beginning of photorefractoriness period (July). GAPDH was used as a reference gene. M - molecular marker (pUC19 DNA/*Msp*I). Bars with the same superscripts are not significantly different ($P>0.05$). OD - optical density

activity (May) was not significantly different. A 281% increasing ($P<0.05$) in level of this expression in testis from P group than C group at the beginning of photorefractoriness period (July) was observed. Moreover, the testicular expression of ERα mRNA in ganders fed C diets in July was higher ($P<0.05$) than in males

from C and P groups in March and May (441 and 446%, and 830 and 1388%, respectively). There were not statistical differences between control groups at the peak of reproduction, during the descending phase of reproductive activity and at the beginning of photorefractoriness period.

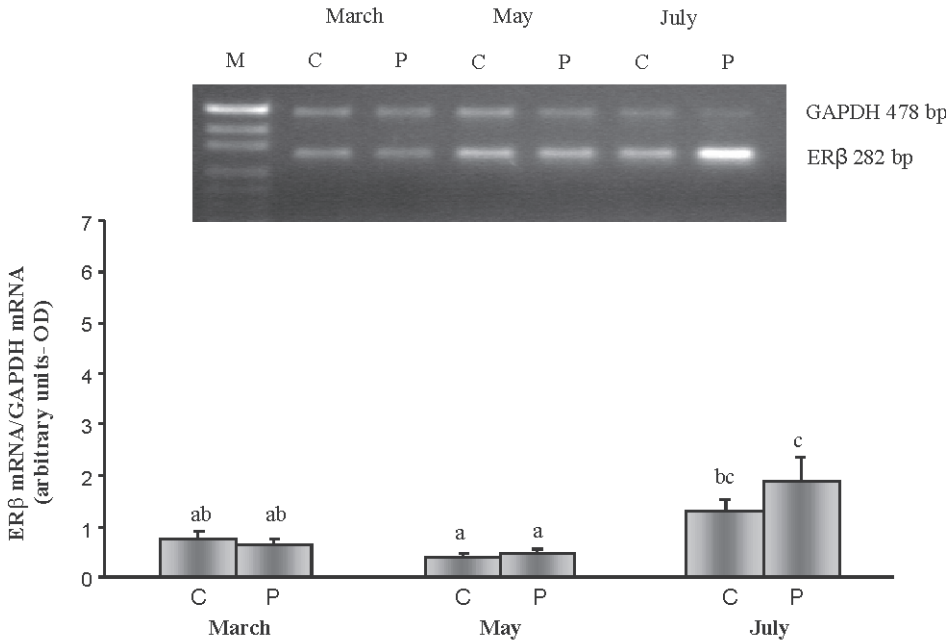


Figure 2. Semi-quantitative analysis of ERβ mRNA expression in testis from ganders fed control diets (C) and diets with higher levels of phytoestrogens (P) at the peak of reproductive activity (March), during the descending phase of reproductive activity (May) and at the beginning of photorefractoriness priod (July). GAPDH was used as a reference gene. M - molecular marker (pUC19 DNA/*Msp*I). Bars with the same superscripts are not significantly different (P>0.05). OD - optical density

The ERβ mRNA (Figure 2) expression in testis from ganders fed P diets in July was greater (P<0.05) than from males fed C and P diets in March and May (153 and 197%, and 380 and 298%, respectively). The increasing (P<0.05) of this expression in control birds from July in comparison to C and P groups from May (231 and 175%, respectively) was noted. The testicular expression of ERβ mRNA between C and P groups during all the experiment time was not significantly different.

## DISCUSSION

This is the first study in geese regarding the effects of dietary phytoestrogens on the expression of ER mRNA in testis. Briefly, the ERα mRNA expression increased in testis from ganders fed diets with higher levels of phytoestrogens at the beginning of photorefractoriness period (in July) and the expression of ERβ mRNA in gander testis changed seasonally and was the highest in July, but no differences in this expression between both experimental groups were noted.

The effect of phytoestrogens on the ER expression in avian tissues has not been extensively examined. The trend of decrease in ERα mRNA expression in shell glands of hens fed diet with daidzein (10 mg/kg diet for 9 weeks) were observed (Ni et al., 2007). In female Shaoxing ducks dietary daidzein (5 mg/kg for weeks) decreased ERβ mRNA expression in hypothalamus (Zhao et al., 2004). The effect of phytoestrogens on the testicular mRNA expression of ER were observed in mouse (Adachi et al., 2004). The injections for 5 days of genistein (1000 μg/mouse/day) inhibited expression of ERα mRNA in testis, and had no effect on ERβ. In this study, diets with higher levels of phytoestrogens altered ERα expression in gander testis, but the increasing of this expression was observed. These facts suggest that phytoestrogens may differentially act on the expression of ERα or ERβ. Moreover, it may be occurred in the species-dependent manner.

In our study the elevated ERβ mRNA expression was noted after breeding period. The investigations in songbirds showed that the expression of ER in canary brain was increased when plasma levels of androgens and oestrogens were decreased (Fusani et al., 2000). Low concentrations of plasma steroids are characterized for post-breeding period in goose (Peczely et al., 1993; Hischenhauser et al., 1999; Opałka et al., 2008). The rise of tissue sensitivity to sex steroids during nonbreeding season by increasing of steroids receptors mRNA expression in male birds were observed (Canoine et al., 2006).

Moreover, the lack of effects of phytoestrogens on ER mRNA expression during reproductive activity in our study may be caused by relatively low exposure of experimental ganders to phytoestrogen. In this investigation the only sources of phytoestrogens in the diet were soya and lucerne, thus, doses of phytoestrogens had to be limited in order to balance of diets (used in practical goose breeding) for crude protein, metabolizable energy and exogenous amino acids. Moreover, the microbiological metabolism of phytoestrogens in intestines of geese is not well known, yet. For example, genistein and daidzein may be transformed by microorganisms in the rumen to para-ethyl phenol and equol, respectively (Lund, 1995). These metabolites demonstrate weaker biological activity than their substrates.

In conclusion, the obtained results suggest that the phytoestrogens may differentially affect the mRNA expression of ERα or ERβ in gander testis and

this effect depends on seasonal stage. Further studies are required to elucidate the mechanism of phytoestrogen action on ER expression in gander gonads.

REFERENCES

Adachi T., Ono Y., Koh K.B., Takashima K., Tainaka H., Matsuno Y., Nakagawa S., Todaka E., Sakurai K., Fukata H., Iguchi T., Komiyama M., Mori C., 2004. Long-term alternation of gene expression without morphological change in testis after neonatal exposure to genistein in mice: toxicogenomic analysis using cDNA microarray. Food Chem. Toxicol. 42, 445-452

Adams N.R., 1995. Detection of the effects of phytoestrogens on sheep and cattle. J. Anim. Sci. 73, 1509-1515

Benassayag C., Perrot-Applanat M., Ferre F., 2002. Phytoestrogens as modulators of steroid action in target cells. J. Chromatogr. B 777, 233-248

Canoine V., Fusani L., Schlinger B., Hau M., 2006. Low sex steroids, high steroid receptors: Increasing the sensitivity of the nonreproductive brain. J. Neurobiol. 67, 57-67

Dalu A., Blaydes B.S., Bryat C.W., Latendresse J.R., Weis C.C., Delclos K.B., 2002. Estrogen receptor expression in the prostate of rats treated with dietary genistein. J. Chromatogr. 777, 249-260

Diel P., Hertrampf T., Seibel J., Laudenbach-Lschowsky U., Kolba S., Vollmer G., 2006. Combinatorial effects of the phytoestrogen genistein and of estradiol in uterus and liver of female Wistar rats. J. Steroid Biochem. Mol. Biol. 102, 60-70

Foidart A., Lakaye B., Grisar T., Ball G.F., Balthazart J., 1999. Estrogen receptor-β in quail: cloning, tissue expression and neuroanatomical distribution. J. Neurobiol. 40, 327-342

Fritz W.A., Wang J., Eltoum I.E., Lamartiniere C.A., 2002. Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate. Mol. Cell. Endocrinol. 186, 89-99

Fusani L., Van't Hof T., Hutchison J.B., Gahr M., 2000. Seasonal expression of androgenreceptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. J. Neurobiol. 43, 254-268

Hischenhauser K., Mostl E., Kortrschal K., 1999. Seasonal patterns of sex steroids determined from feces in different social categories of greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 114, 67-79

Ichikawa K., Yamamoto I., Tsukada A., Saito N., Shimada K., 2003. cDNA cloning and mRNA expression of estrogen receptor α in Japanese quail. J. Poultry Sci. 40, 121-129

Koba N., Ohfuji T., Ha Y., Mizushima S., Tsukada A., Saito N., Shimada K., 2008. Profiles of mRNA expression of FOXL2, P450arom, DMRT1, AMH, $P450_{c17}$, SF1, ERα and AR, in relation to gonadal sex differentiation in duck embryo. J. Poultry Sci. 45, 132-138

Kuiper G.G., Lemnen J.G., Carsson B., Corton J.C., Safe S.H., van der Saag P.T., van der Burg B., Gustafsson J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252-4263

Lakaye B., Foidart A., Grisar T., Balthazart J., 1998. Partial cloning and distribution of estrogen receptor beta in the avian brain. Neuroreport 9, 2743-2748

Leopold A.S., Erwin M., Oh J., Browning B., 1976. Phytoestrogens: adverse effects on reproduction in California quail. Science 191, 98-100

Lien R.J., Cain J.R., Beasom S.L., 1987. Effects of dietary and parenteral estrogens on bobwhites reproduction. Poultry Sci. 66, 154-161

Lund T.,1995. Metabolism of estrogenic isoflavones in domestical animals. Proc. Soc. Exp. Biol. Med. 208, 33-39

Nakabayashi O., Kikuchi H., Kikuchi T., Mizuno S., 1998. Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. J. Molecular Endocrinol. 20, 193-202

Ni Y., Zhu Q., Zhou Z., Grossmann R., Chen J., Zhao R., 2007. Effect of dietary daidzein on egg production, shell quality, and gene expression of ER-alpha, GH-R and IGF-IR in shell glands of laying hens. J. Agr. Food Chem. 55, 6997-7001

Opałka M., Kaminska B., Piskula M.K., Puchajda-Skowronska H., Dusza L., 2006. Effects of phytoestrogens on testosterone secretion by Leydig cells from Bilgoraj ganders (*Anser anser*). Brit. Poultry Sci. 47, 237-245

Opałka M., Kugla-Owczarska J., Kaminska B., Puchajda-Skowronska H., Hryniewicka W., Dusza L., 2008. Effects of dietary meals containing different levels of phytoestrogens on reproductive function in Bilgoraj ganders. Acta Vet. Hung. 56, 379-391

Peczely P., El Halawani M.E., Hargitai C., Mezes M., Forgo V., Janosi S., 1993. The photorefractoriness in domestic goose: effect of gonads and thyroid on the development of postbreeding prolactinemia. Acta Biol. Hung. 44, 329-352

Sierens J.E., Sneddon S.F., Collins F., Millar M.R., Saunders P.T.K., 2005. Estrogens in testis biology. Ann. N.Y. Acad. Sci. 1061, 65-76

Zhao R., Wang Y., Zhou Y., Ni Y., Lu L., Grossman R., Chen J., 2004. Dietary daidzein influences laying performance of ducks (*Anas platyrhynchos*) and early post-hatch growth of their hatchlings by modulating gene expression. Comp. Biochem. Physiol. Pt A 138, 459-466