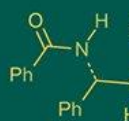


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 IJABR 2026; 10(9): 361-365
www.biochemjournal.com
 Received: 14-07-2026
 Accepted: 17-08-2026

A Aswathy
 College of Veterinary and
 Animal Sciences, Mannuthy,
 Kerala Veterinary and Animal
 Sciences University, Kerala,
 India

TR Sreekumar
 College of Veterinary and
 Animal Sciences, Mannuthy,
 Kerala Veterinary and Animal
 Sciences University, Kerala,
 India

R Uma
 College of Veterinary and
 Animal Sciences, Mannuthy,
 Kerala Veterinary and Animal
 Sciences University, Kerala,
 India

PS Megha
 College of Veterinary and
 Animal Sciences, Mannuthy,
 Kerala Veterinary and Animal
 Sciences University, Kerala,
 India

Corresponding Author:
TR Sreekumar
 College of Veterinary and
 Animal Sciences, Mannuthy,
 Kerala Veterinary and Animal
 Sciences University, Kerala,
 India

Soybean meal modulates estrogen receptor expression in non gonadal tissues of layer chicken

A Aswathy, TR Sreekumar, R Uma and PS Megha

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9e.9983>

Abstract

The current study investigates the effects of soyabean meal rich in phytoestrogen on estrogen receptor (ER) expression in layer chickens. ER α and ER β , in the liver, shell gland, and oviduct, are compared between diets with or without soybean meal. Key findings reveal that soybean meal significantly increases ER α expression in the liver and shell gland, enhancing estrogenic activity important for egg production. In contrast, ER β expression decreases in the oviduct, indicating tissue-specific effects of phytoestrogens. These results suggest that dietary soybean meal could improve reproductive performance and egg quality in poultry. Further research is needed to assess its long-term impact on health and productivity.

Keywords: Estrogen receptors, Phytoestrogens, non gonadal tissues, soyabean meal, expression

Introduction

In recent years, the poultry industry has witnessed a remarkable shift towards exploring alternative dietary ingredients to optimise the health and productivity of birds. Soybean is one of the most popular ingredient of poultry feed in both conventional and alternative poultry production systems. Soybean meal has high protein content and is an excellent source of amino acids for poultry diets. They also abundantly contain hormone-like substances called phytoestrogens.

Phytoestrogens are a phenolic non-steroidal heterogeneous group of herbal substances produced by legumes such as soya. There are three main classes of phytoestrogens - isoflavones, lignans, and coumestans, out of which isoflavones are predominantly found in soybeans [1]. Genistein and daidzein, are the two most prominent isoflavones found in soybeans which have a structural and functional similarity to that of 17 β -oestradiol and their concentration increase in the blood following diet containing soyabean meal [2]. This special feature enables them to mimic the actions of endogenous oestrogens, so they are also called oestrogen agonist [3].

In avian species, oestrogen is an important hormone, produced primarily in the theca cells of the ovarian follicles and a small amount in non-gonadal tissues, such as the brain and adrenal gland. The hormone plays a critical role in controlling reproductive processes like gonadal differentiation, development and sexual behavior. In addition, it also has various functions on non-gonadal tissues such as the production of egg yolk and egg white proteins in the liver and the mobilisation of calcium for the creation of the eggshell in the shell gland. In the oviduct, oestrogen triggers the final maturation of the reproductive tract and coordinates the egg formation processes. Moreover, oestrogen is crucial for bone conservation, health of the central nervous system and cardiovascular system [4].

The long-term physiological functions of oestrogen are mediated by its nuclear oestrogen receptor (ER) subtypes known as oestrogen receptor alpha (ER α) and oestrogen receptor beta (ER β) [5]. The distribution and expression of these receptors are found different in various tissues of avian species. The receptor ER α is found in the brain, hypothalamus, ovary, shell gland (uterus), kidney, liver and bones of mature laying chicken. Avian ER β expression is less common because it was only found in the embryonic liver, ovary and brain. The ER α subtype remains primarily responsible for triggering follicular hiring, maturation of reproductive tract and coordination of egg formation processes by mediating the upregulation of yolk proteins and recruitment of minerals for eggshell formation. The maturation of pre-ovulatory follicles and the ovulatory process are regulated mostly by the ER β [5, 6, 7].

The genistein and daidzein isoflavones present in soybean show binding affinities to both the oestrogen receptors due to their structural similarity to oestrogen (17 β -oestradiol) although it has a higher affinity to ER β (8). Through this mechanism, phytoestrogens can alter the pattern of synthesis and metabolism for endogenous hormones and also affect the physiological effects of oestrogen hormones in the body. Beyond its well-acknowledged nutritional value, due to the presence of phytoestrogens, inclusion of soybean in layer feed has raised intriguing questions about their potential impact on poultry health. Studying the effects of feeding soybean meal on the expression of oestrogen receptors in non-gonadal tissues within poultry delves into the broader implications of phytoestrogen rich diet for poultry production and welfare. Hence, the intricate relationship between soybean meal consumption and oestrogen receptor expression in non-gonadal tissues of poultry, have to be explored to unravel the multifaceted effects of this dietary component on avian health and physiology. In this context, the current investigation aims to evaluate the differential expression of mRNA of oestrogen receptor alpha (ER α) and oestrogen receptor beta (ER β) within non-gonadal tissues, such as the liver, shell gland, and oviduct, of layer chickens subjected to diets devoid of soybean and those that incorporate soybean.

Materials and Methods

Experimental design and diets

This study was approved by the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Mannuthy (IAEC/22/16 dated 06/04/2022). The study was conducted using 24 crossbred Gramasree layer birds, each 28 weeks old, procured from the University Poultry and Duck Farm, Kerala Veterinary and Animal Sciences University, Mannuthy. The birds were randomly assigned to two treatment groups, each comprising 12 birds. The experimental period lasted for 56 days. Two isocaloric and isonitrogenous experimental feeds, with different isoflavone levels, were formulated and provided to laying hens. The first group was fed a standard layer diet containing soybean meal (9), while the second group was fed a standard layer diet without soybean meal (9). The composition of experimental diet for the two experimental groups is provided in table 1.

The birds were kept at 26.5 $\pm$ 0.5°C ambient temperature and at relative humidity ranging from 70-80 per cent. A photoperiod of 16 hours per day was ensured throughout the period. The total ration was provided as a single lot in the morning for all the groups and water was provided *ad libitum* throughout the experimental period (56 days).

Sample Collection

The layer birds were slaughtered at the end of 56 days of the experiment and the non-gonadal tissues *viz.* liver, shell gland and oviduct samples (approximately 100 mg) were collected and immediately placed in cryochill vial containing RNA later (Qiagen, Life Technologies, USA). Collected samples were transported to the laboratory on ice within 30 min and stored at -80°C until total RNA extraction.

Extraction of RNA and reverse transcription

RNA was extracted from the collected tissues using RNAiso Plus according to the manufacturer's instructions and stored at -80°C. cDNA was synthesized using iscript cDNA synthesis kit (Bio Rad, USA) as per manufacturer's protocol and stored at -20°C.

Primer design and synthesis

Exon-spanning primers (Table 2) were designed using online NCBI primer design software (Primer3) and specificity of the primers was checked using BLAST primer online software.

Quantitative real-time PCR

Complementary DNA (cDNA) samples were used as templates, β -actin was used as an internal reference and the gene expression of ER α , ER β and β -Actin was detected via quantitative real-time PCR (qPCR). The reaction system consisted of 5 μ L of 2X Maxima SYBR Green qPCR Master Mix, 0.5 μ L each of forward and reverse primers, 1 μ L of cDNA, and 3 μ L of RNase-Free water (for a final volume of 10 μ L).

The program for the thermo cycler (Biorad, USA) was as follows: polymerase activity at 95°C for 2 minutes, followed by forty cycles of denaturation at 94°C for 5 seconds each, combined annealing and extension for 30 seconds at 60.8°C for ER α , 62.2°C for ER β and 64.5°C for β -Actin, tailed with a melt curve analysis at 65-95°C for 5 seconds. Three sets of each reaction were prepared.

Data Analysis

Results were expressed as mean $\pm$ S.E. The statistical significance of difference was analysed by independent T-Test using SPSS version 24.0 and the difference were considered statistically significant at 5% level ($p \leq 0.05$).

Results

Expression Pattern of ER α

In liver tissue, ER α mRNA expression was significantly upregulated ($p < 0.01$) with 6-fold increase in Group I birds compared to the control group (Group II), indicating increased estrogenic activity in the liver. This suggests that soybean meal prominently influences ER α in hepatic tissues. Similarly, in the shell gland, ER α mRNA expression was significantly ($p < 0.05$) higher in the group I than in the control group. In contrast, the upregulation of ER α mRNA in the oviduct of the phytoestrogen-fed group was not statistically significant (Figure 1).

Expression Pattern of ER β

In liver tissue, although ER β mRNA expression was upregulated in Group I birds, the increase was not statistically significant ($p > 0.05$) compared to the control group. In the shell gland, ER β mRNA expression levels decreased, but this reduction was also not statistically significant ($p > 0.05$). However, in the oviduct, a significant ($p < 0.05$) downregulation of ER β mRNA expression was observed in the soyabean meal fed group compared to the control group. This downregulation may reflect a tissue-specific response to phytoestrogen exposure (Figure 2).

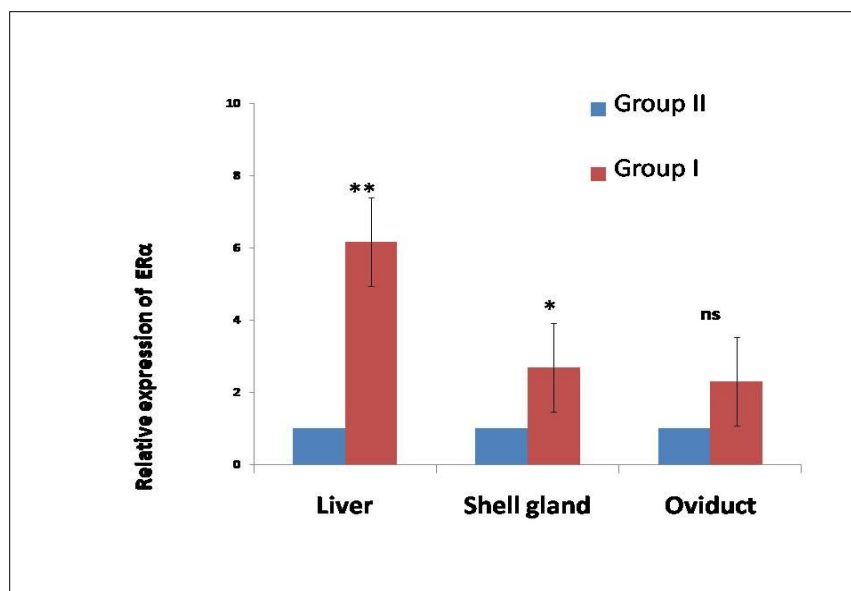


Fig 1: Expression of ERα in the liver, shell gland and oviduct of soyabean meal fed layer chickens. βactin was used as an internal reference gene to estimate the relative expression of mRNA. Data are presented as the mean ± SE. Different asterix indicate significant differences ** Highly significant difference ($P < 0.01$), * Significant difference ($P < 0.05$), ns Non significant difference ($P > 0.05$).

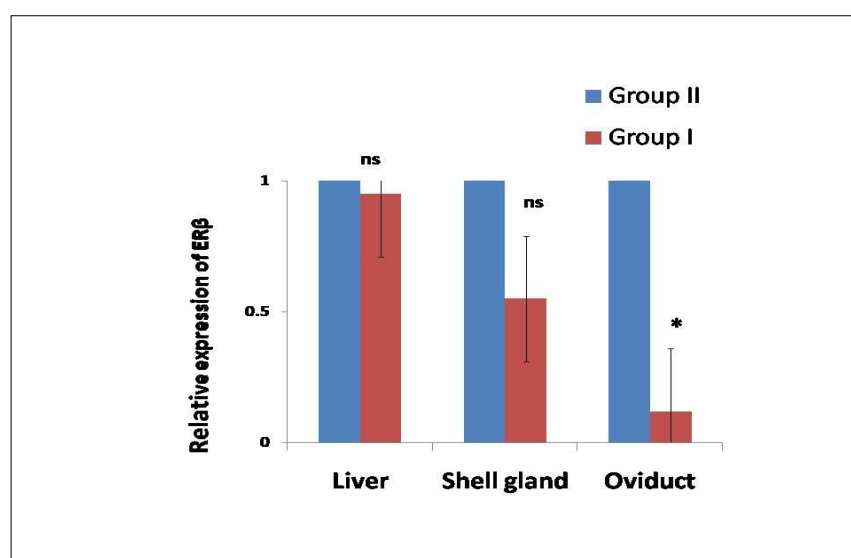


Fig 2: Expression of ERβ in the liver, shell gland and oviduct of soyabean meal fed layer chickens. βactin was used as an internal reference gene to estimate the relative expression of mRNA. Data are presented as the mean ± SE. Different asterix indicate significant differences ** Highly significant difference ($P < 0.01$), * Significant difference ($P < 0.05$), ns Non significant difference ($P > 0.05$).

Table 1: Percent ingredient composition of experimental diet

No	Feed ingredients	Group I diet percentage	Group II diet percentage
1	Yellow maize	53.16	53.65
2	De-oiled rice bran	8.85	--
3	Soybean meal	26.86	--
4	Groundnut Cake	--	20.58
5	Dried fish	--	3.78
6	Wheat bran	--	7.07
7	Gingelly oil cake	--	5.00
8	Calcite powder	3.98	4.00
9	Grit	5.00	4.23
10	Dicalcium phosphate	1.29	1.04
11	DL - Methionine	0.21	0.19
12	L- Lysine	0.20	0.20
13	Soda bicarb	0.05	0.05
14	Salt	0.40	0.21
	Total	100.00	100.00

Table 2: Primers used for quantitative real-time PCR analysis

Gene	Gene Accession no (NCBI):	Primer name	Primer Sequence (5'-----3')	Primer Length (bp)	product Size (bp)
ERα	NCBI Reference Sequence: NM_205183.2	ERα F	CTGGAAGCTGAGCCACCTAT	20	101
		ERα R	TCTCTGTCTGCAAGGTTGGT	20	
ER β	GenBank: AB036415.1	ERβ F	GCTGGTTCACATGATTGGCT	20	101
		ERβ R	AGACTTCCATCCAGCAGCTT	20	
β ACTIN	NCBI Reference Sequence: NM_205518.2	C ACTB F	TGACAATGGCTCCGGTATGT	20	115
		C ACTB R	GCCCATACCAACCATCACAC	20	

Discussion

The present study explored the effects of soybean meal, rich in phytoestrogens, on ERα and ERβ expression in non-gonadal tissues like liver, shell gland and oviduct of layer chicken.

The observed upregulation of ERα in the liver and shell gland suggests that soybean meal can enhance estrogenic activity in these tissues. This is of particular interest, as the liver plays a crucial role in the metabolism and homeostasis of oestrogen, while the shell gland is involved in the production of eggshell.

In case of avian species, oestrogen plays a crucial role in the communication between liver and ovary for the development of eggs. In addition to the impacts on the glucose and lipid metabolism oestrogen also control the production of yolk and eggshell proteins [10]. The significant upregulation of ERα mRNA in the liver is consistent with estrogen's and oestrogen agonist's role in promoting synthesis of vitellogenin and vitelline envelop protein, where the liver synthesizes yolk precursors under oestrogen control [11]. Oestrogens also regulate hepatic lipid metabolism, which is crucial for oogenesis [12]. This suggests that phytoestrogens in the soybean meal may mimic oestradiol and can enhance reproductive functions by increasing ERα-mediated transcription in liver tissues.

In the current study, the observed upregulation of ERα mRNA in the shell gland, aligns with a previous report suggesting that daidzein causes upregulation of ERα mRNA along with improvement in eggshell thickness [13]. Additionally, research has shown that ERα expression predominates over ERβ in the shell glands of laying hens, reinforcing the receptor's central role in reproductive tissue response to oestrogen [4]. However, contrasting findings in a study involving daidzein supplementation demonstrated a downregulation of ERα in the shell gland, yet with improved eggshell quality during the late laying stage [14]. These contrasting results shows the complexity of soyabean meal effects on shell gland function and highlight the need to consider factors such as dosage, species specific mechanisms phytoestrogen type and reproductive stage in evaluating oestrogen receptor activity in laying hens.

The downregulation of ERβ mRNA in the oviduct might reflect a tissue-specific response to phytoestrogens. It is reported that phytoestrogens exhibit higher affinity for ERβ than ERα [15]. Unlike ERα, which is upregulated to support egg production, ERβ may be downregulated as part of a feedback mechanism to maintain hormonal balance in tissues predominantly expressing this receptor subtype [16].

The modulation of ERα and ERβ by phytoestrogens has practical implications for poultry nutrition, as dietary soybean meal could be leveraged to enhance reproductive performance by supporting liver and uterine function. However, the tissue-specific effects, particularly the downregulation of ERβ in the oviduct, suggest that careful

consideration is required to balance dietary phytoestrogens for optimal health outcomes in layer chickens. Further research is needed to explore the long-term effects of phytoestrogen exposure on reproductive and metabolic health in poultry.

In conclusion, this study demonstrates that soybean meal, a rich source of phytoestrogens, can modulate the expression of oestrogen receptors α and β in non-gonadal tissues of layer chickens. The upregulation of ERα in the liver and shell gland, and the downregulation of ERβ in the oviduct, suggest tissue-specific responses to phytoestrogen exposure. These findings highlight the importance of considering the impact of phytoestrogens on the endocrine system of layer chickens, which may have implications for reproductive performance and egg quality.

Acknowledgments: The authors acknowledge the Dean of the College of Veterinary and Animal Sciences, Mannuthy, for providing the opportunity and necessary infrastructure to conduct this research

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