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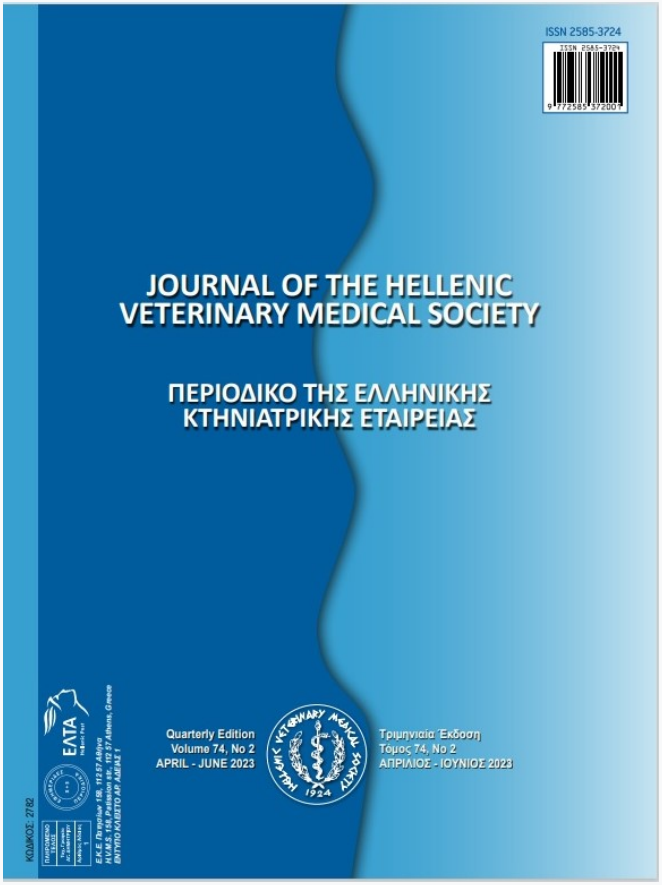

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Research article
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Effects of Calcium, Available Phosphorus and Microbial Phytase on Ovarian FSHR and LHR Expression in Laying Hens

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ABSTRACT: Folliculogenesis, steroidogenesis, ovulation, and vitellogenesis are regulated by the effect of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the hypothalamus-pituitary-ovary axis and these hormones act via follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) in the ovary. Poultry ration and food additives are essential in the regulation of reproductive activity. Phytase is a supplement frequently added to laying hen diets to increase phosphorus (P) utilization. The aim of this study was to reveal the effects of a newly isolated microbial phytase together with different concentrations of calcium (Ca^{2+}) and available phosphorus (AP) on ovarian FSHR and LHR expressions. For this purpose, 90 Lohmann LSL-White layers were first divided into three main diet groups (standard Ca^{2+} and AP, standard Ca^{2+} and low AP, low Ca^{2+} and AP) and then into three subgroups (no-phytase, commercial phytase, and microbial phytase). At the end of the experiment, all chickens were slaughtered and ovarian tissues were fixed in formalin. Routine avidin-biotin complex immunohistochemistry was performed using anti-FSHR and anti-LHR primary antibodies. Immunohistochemically, FSHR and LHR were expressed in granulosa/theca cells, oocytes, interstitial cells, and vitellus. While the expression intensity of the receptors increased in the microbial phytase-treated groups, the strongest expression was obtained in the granulosa/theca cells and oocytes in the standard Ca and low AP group. In conclusion, we suggest that the addition of newly isolated microbial phytase to diets of laying hens and feeding standard Ca and low AP may have positive effects on reproductive performance by increasing the FSHR and LHR expression in ovaries.

***Keywords*:** available phosphorus; calcium; FSHR; microbial phytase; LHR

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INTRODUCTION

Reproductive activity and egg-laying performance in poultry are regulated by the hypothalamus-pituitary-ovarian axis as in mammals (Mishra et al., 2020). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary act on the gonads and induce gametogenesis, steroidogenesis, and ovulation in females via receptors FSHR and LHR in ovarian follicle granulosa and theca cells (Grzegorzewska et al., 2009). One of the factors affecting reproductive hormone secretion and regulation in poultry is diet content (Colin et al., 2020). Calcium (Ca^{2+}) and phosphorus (P) are macro minerals and are involved in metabolic activities, eggshell formation and quality, hatchability, specific gravity, and laying performance essential for poultry production (Keshavarz and Nakajima, 1993; Jiang et al., 2013). A decrease in the absorption of Ca^{2+} and P from the intestines due to reduced amounts of Ca^{2+} and P in the diet or diseases such as Newcastle causes decreased blood serum level and affects egg production and quality (Igwe et al., 2018).

A significant portion (50-80%) of P is in the form of phytic acid, which is degraded with the phytase enzyme available in very limited amounts in the digestive system of poultry (Gordon and Roland, 1998). Insufficient digestion of P generates two crucial negative consequences; (i) pollution occurs as a result of enrichment of soil and surface waters with quite a high P-rich faeces, (ii) production costs increase through the addition of expensive inorganic phosphorus sources (Abbasi et al., 2019). A solution to these negative situations is the addition of phytase to the rations exogenously (Jalal and Scheideler, 2001). Other minerals, especially Ca^{2+}, are involved in the absorption and metabolic regulation of P in laying hens. The synergistic effect of Ca^{2+} and P depends on their proper ratios in the diet (Bougouin et al., 2014; Bello et al., 2020).

In the present study, a newly isolated phytase from *Bacillus megaterium* EBD 9-1, known to have high phytase production potential, was added to the diets with different Ca^{2+} and P concentrations of laying hens. We investigated how microbial phytase (MP) affects the expression of FSHR and LHR in the ovary under diverse dietary conditions.

MATERIALS AND METHODS

Housing and management

The research was conducted at the Poultry Unit of Bursa Uludag University, Research and Application Farm after the approval of the Bursa Uludag University Animal Research Local Ethics Committee (2021-07/05). Ninety, second term Lohmann LSL-White laying hens were housed in three-tier California-type poultry cages with nipple drinkers. The light was available as 16 hours lighting:8 hours darkness and feeding was *ad libitum* group feeding.

Diet and treatments

Table 1. contains the content of the diet. The method reported in Association of Official Agricultural Chemists (AOAC) International was used for the nutrient analysis of the feed ingredients and mixed rations used in the preparation of the diets (AOAC, 2000) and the starch content was determined according to a previously reported method (Bal et al., 2000). The metabolizable energy levels were calculated using the formula developed by the Turkish Standardization Institute.

The chickens were randomly distributed into three

Table 1. Ration ingredients and chemical composition (g/kg) of the basal ration of standard calcium and available phosphorus (Ca+AP) group.

Ingredients	g/kg
Corn (7.5% CP)	565.70
Soybean meal (44% CP)	50.00
Full fat soybean	177.00
Corn gluten	50.00
Sunflower meal	30.00
Limestone	97.00
Dicalcium phosphate	19.50
Salt	2.50
Vitamin-mineral premix*	3.50
DL-methionine	1.00
L-lysine HCl	1.00
Sodium bicarbonate	1.00
Choline chloride	0.80
Antioxidant	1.00
Analysed concentration	
Metabolizable energy, kcal/kg	2793
Crude protein	173.00
Calcium	42.00
Available phosphorus	4.30
Sodium	1.70
Lysine	8.30
Methionine	4.10
Threonine	5.70

*: 3.5 kg premix contains 10,000,000 IU vitamin A; 2,500,000 IU vitamin D3; 20,000 mg vitamin E; 3,000 mg vitamin K3; 1,000 mg vitamin B1; 4,000 mg vitamin B2; 3,000 mg vitamin B6; 25 mg vitamin B12; 22,500 mg niacine; 10,000 mg calcium D-pantothenate; 500 mg folic acid; 50 mg biotin; 400,000 mg choline; 100,000 mg Mn; 25,000 mg Fe; 60,000 mg Zn; 5,000 mg Cu; 200 mg Co; 500 mg I; 200 mg Se.

diet groups as (i) standard levels of Ca^{2+} and standard levels of available phosphorus (Ca^{2+}+AP), (ii) standard levels of Ca^{2+} and low levels of AP (Ca^{2+}+low AP), (iii) low levels of Ca^{2+} and low levels of AP (low Ca^{2+}+low AP). Each diet group had three phytase subgroups as without phytase (Phy-), commercial phytase (CP), and microbial phytase (MP).

A total of 300 FTU/kg dose of the commercial phytase (6-phytase derived from *E. coli*) or microbial phytase (3-phytase derived from *Bacillus megaterium* EBD9-1, isolated from the soil of Trabzon province in Turkey) were added to the relevant diets. Phosphorus was available as 0.10% in a diet containing 300 FTU/kg phytase. The same amount of phytase equalled to 1 g/kg matrix value for Ca^{2+}. The calcium and AP levels in the Ca+AP-containing groups were arranged to be 4.20% and 0.43%, respectively, whereas in the low Ca+low AP diet groups these values were 4.10% and 0.33%, respectively.

Histomorphology and immunohistochemistry

Birds were slaughtered at the end of the experiment and ovarian samples were fixed in 10% neutral buffered formalin. After routine histological procedures, ovarian tissues were embedded in paraffin. Five µm thick sections were obtained from the paraffin blocks and cuts were taken to lysine slides. After deparaffinization and rehydration, sections were stained with immunohistochemistry for detection of FSHR and LHR expressions in ovaries.

Standard avidin-biotin complex technique was used for immunohistochemical staining. Sections were boiled in sodium citrate buffer in microwave (1 M, pH 6.1, for 3x5 minutes, 750 W) for the antigen retrieval. After treating with H_2O_2 for the elimination of endogenous peroxidase activity, sections were covered with blocking solution for 20 min to reduce nonspecific antibody binding. Later, sections were incubated overnight at 4 °C with anti-rabbit FSHR (1:250, 22665-1-AP, Proteintech, rabbit polyclonal) and LHR (1:250, NLS1436, Novus Biologicals, rabbit polyclonal) primary antibodies. Samples were then rinsed with PBS and incubated with ImmPRESS-HRP anti-rabbit IgG (peroxidase) reagent (NC9027342, Vector Laboratories) for 30 min and incubated 5 minutes with DAB for colour development. Harris hematoxylin was used for counterstaining. The intensity and localization of FSHR and LHR expressions were assessed by two blinded observers, and five different areas per slide were scored with an H-scoring system: 0: no immunoreaction; 1+: weak immunoreaction; 2+: moderate immunoreaction; 3+: strong immunoreaction. The percentage of cells at different staining intensities was determined by visual assessment, with the score calculated using the formula 1x (% of 1+ cells) + 2x (% of 2+ cells) + 3x (% of 3+ cells) (Mazieres et al., 2013). All micrographs were taken with a microscope (Nikon 80i, JP).

Statistical analysis

FSHR and LHR expressions were investigated by using the non-parametric Kruskal Wallis test. The Mann-Whitney U test was used to analyze statistical significance between the two groups ($p<0.05$). Statistical analysis of the study was performed using SPSS 23.0 (IBM).

RESULTS

The effects of the different doses of Ca^{2+} and AP, and the use of phytase on FSHR and LHR expressions in the ovaries of laying hens are presented in Tables 2 and 3, and Figures 1 and 2.

Table 2. H-scores of follicle stimulating hormone receptor (FSHR) immunoreactivity after Ca^{2+} and available phosphorus (AP) variable diet and phytase application.

Groups (n=10 each)		Oocyte (mean±SE)	Vitellus (mean±SE)	Granulosa/ Theca Cells (mean±SE)	Interstitial Cells (mean±SE)
Ca+AP	Phy-	$2.20\pm0.13^{b,A}$	$1.40\pm0.16^{b,A}$	$1.90\pm0.18^{c,A}$	$2.60\pm0.16^{a,AB}$
	CP	$1.90\pm0.28^{c,B}$	$1.70\pm0.15^{a,B}$	$2.20\pm0.20^{b,A}$	$2.30\pm0.15^{b,BC}$
	MP	$2.50\pm0.17^{a,B}$	$1.60\pm0.22^{a,B}$	$2.70\pm0.10^{a,A}$	$2.80\pm0.13^{a,A}$
Ca+low AP	Phy-	$1.40\pm0.27^{b,B}$	$1.50\pm0.22^{b,A}$	$1.90\pm0.18^{c,A}$	$2.40\pm0.27^{b,AB}$
	CP	$2.90\pm0.10^{a,A}$	$2.60\pm0.16^{a,A}$	$2.10\pm0.10^{b,A}$	$2.90\pm0.10^{a,A}$
	MP	$3.00\pm0.01^{a,A}$	$3.00\pm0.01^{a,A}$	$2.90\pm0.15^{a,A}$	$2.90\pm0.10^{a,AB}$
Low Ca+low AP	Phy-	$1.10\pm0.23^{c,B}$	$1.40\pm0.22^{bc,A}$	$1.90\pm0.18^{bc,A}$	$2.10\pm0.17^{b,BC}$
	CP	$2.50\pm0.22^{b,B}$	$1.70\pm0.21^{ab,B}$	$2.10\pm0.18^{ab,A}$	$2.60\pm0.16^{a,AB}$
	MP	$2.90\pm0.10^{a,A}$	$1.80\pm0.13^{ab,B}$	$2.20\pm0.20^{ab,B}$	$2.50\pm0.18^{a,B}$

[a, b, c]; express the significant difference between subgroups in each main group; [A, B, C]; express the significant difference within the subgroups themselves, $p<0.05$.

Ca: Calcium, AP: Available phosphorus, Phy-: Phytase not added, CP: Commercial phytase, MP: Microbial phytase

Table 3. H-scores of luteinizing hormone receptor (LHR) immunoreactivity after Ca^{2+} and available phosphorus (AP) variable diet and phytase application.

Groups (n=10 each)		Oocyte (mean±SE)	Vitellus (mean±SE)	Granulosa/ Theca Cells (mean±SE)	Interstitial Cells (mean±SE)
Ca+AP	Phy-	$1.20\pm0.32^{bc,AB}$	$1.80\pm0.13^{a,B}$	$1.80\pm0.29^{bc,A}$	$1.20\pm0.13^{b,AB}$
	CP	$1.60\pm0.16^{ab,C}$	$1.50\pm0.34^{b,B}$	$2.00\pm0.3^{ab,B}$	$1.70\pm0.15^{a,A}$
	MP	$1.90\pm0.28^{ab,B}$	$1.90\pm0.31^{a,B}$	$2.20\pm0.20^{ab,B}$	$1.70\pm0.15^{a,A}$
Ca+low AP	Phy-	$1.00\pm0.26^{b,AB}$	$2.20\pm0.13^{bc,A}$	$1.80\pm0.29^{b,A}$	$1.50\pm0.17^{a,AB}$
	CP	$2.60\pm0.16^{a,A}$	$2.30\pm0.21^{ab,A}$	$2.60\pm0.16^{a,A}$	$1.70\pm0.15^{a,A}$
	MP	$2.60\pm0.16^{a,A}$	$2.70\pm0.15^{ab,A}$	$2.70\pm0.15^{a,A}$	$1.90\pm0.10^{a,A}$
Low Ca+low AP	Phy-	$0.90\pm0.23^{c,BC}$	$1.90\pm0.30^{a,B}$	$2.00\pm0.15^{ab,A}$	$0.60\pm0.16^{bc,BC}$
	CP	$2.00\pm0.21^{b,B}$	$1.10\pm0.23^{a,B}$	$1.90\pm0.23^{bc,B}$	$1.00\pm0.26^{ab,B}$
	MP	$2.60\pm0.16^{a,A}$	$2.40\pm0.22^{a,A}$	$2.40\pm0.22^{ab,B}$	$0.80\pm0.20^{ab,B}$

[a, b, c]; express the significant difference between subgroups in each main group; [A, B, C]; express the significant difference within the subgroups themselves, $p<0.05$.
Ca: Calcium, AP: Available phosphorus, Phy-: Phytase not added, CP: Commercial phytase, MP: Microbial phytase

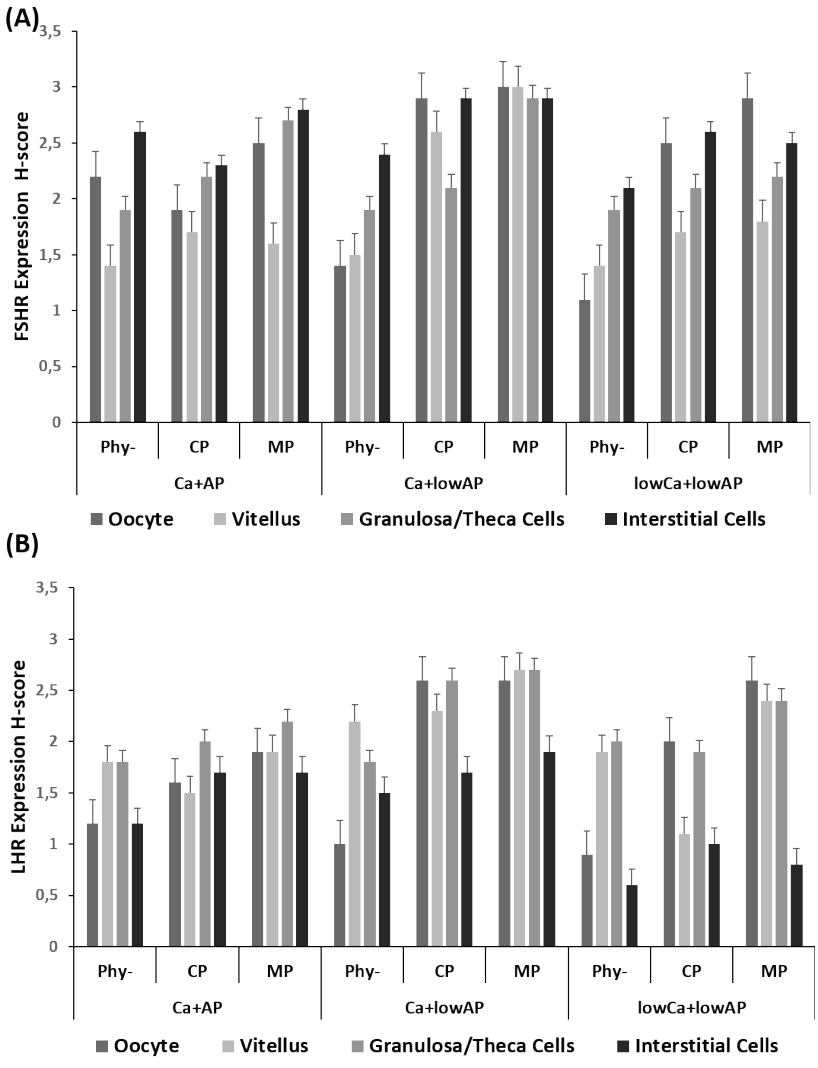

Figure 1. H-scores of follicle stimulating hormone receptor (FSHR)(A) and luteinizing hormone receptor (LHR)(B) expressions after Ca^{2+} and available phosphorus (AP) variable diet and phytase application. Statistical differences among the groups are given in Table 4 and 5. Ca: Calcium; AP: Available phosphorus; Phy-: Phytase not added; CP: Commercial phytase; MP: Microbial phytase.

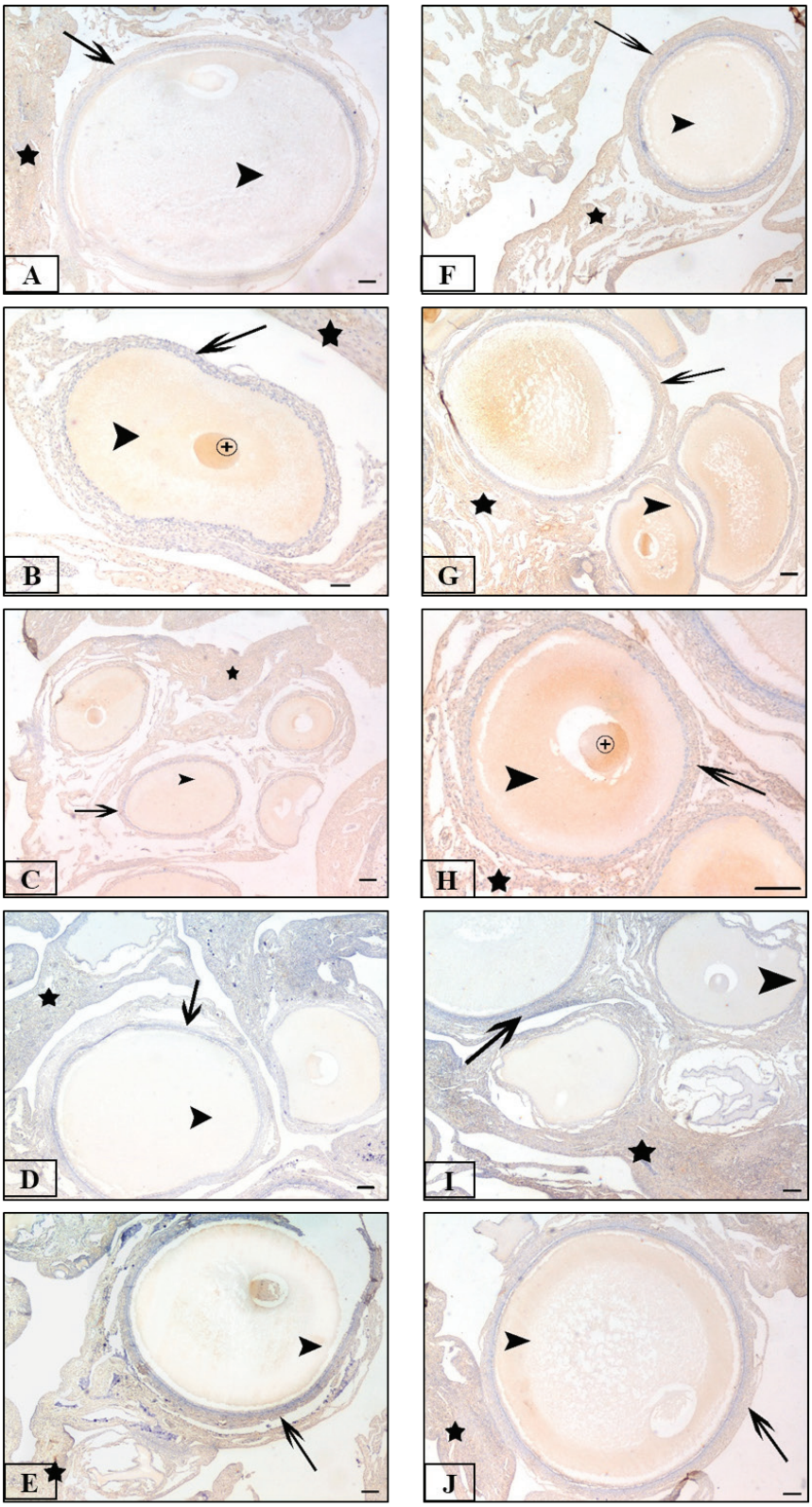

Figure 2. Follicle stimulating hormone receptor (FSHR)(A-E) and luteinizing hormone receptor (LHR)(F-J) immunohistochemistry results. Granulosa/theca cells, oocyte, vitellus, and interstitial cells are labeled with arrow, plus, arrowhead, and star, respectively. (A) Ca+AP+MP; (B) Ca+low AP+CP; (C) Ca+low AP+MP; (D) low Ca+low AP+Phy-; (E) low Ca+low AP+MP; (F) Ca+AP+MP; (G) Ca+low AP+CP; (H) Ca+ low AP+MP; (I) low Ca+low AP+CP; (J) low Ca+low AP+MP, where Ca: Calcium; AP: Available phosphorus; Phy-: Phytase not added; CP: Commercial phytase; MP: Microbial phytase. Bars= 100 μm.

Expression of FSHR was observed in the cell membrane of the granulosa/theca cells, oocyte, vitellus, and interstitial cells in different growing stages of the ovarian follicles (Fig. 2A-E). When the ratio between Ca and AP increased (Ca+low AP group), the severity of the FSHR expression in oocyte, vitellus, and granulosa/theca cells increased; no increase was observed in the interstitial cells (Fig. 2B, 2C). The FSHR expression decreased when chickens were fed with low doses of either Ca or AP (Fig. 2D). The FSHR expression increased after phytase administration, especially the MP (Fig. 2E). The strongest FSHR expression was observed in the ovarian components after feeding chickens with standard Ca and low AP diet group and with MP (Fig. 1, Fig. 2C, Table 2).

Expression of LHR was observed in the cytosol of the oocytes, vitellus, and granulosa/theca cells of the follicles, and the interstitial cells (Fig 2F-J). The LHR immunoreactivity in theca cells increased after feeding with standard Ca+low AP and MP (Fig 2H). Similar to FSHR expression, when the Ca and AP were decreased, the LHR immunoreactivity was also less prominent (Fig 2I). After MP administration in the low Ca and low AP diet group, the reaction of LHR increased (Fig 2J). Interstitial cells were slightly stained for both FSHR and LHR. The statistical differences of FSHR and LHR expressions among the groups are shown in Tables 4 and 5.

DISCUSSION

The hormones FSH and LH, which play an effective role in folliculogenesis, steroidogenesis, and ovulation in the ovary, act through their receptors FSHR and LHR (Liu et al., 2019). In the literature, there are studies about the mRNA expressions of FSHR and LHR in poultry ovaries (Hu et al., 2021; Liu et al., 2007), but the localization of these receptors has not been investigated so far. In this study, we investigated the FSHR and LHR expressions in ovaries under the effects of different concentrations of Ca and P, and newly isolated MP.

In many species, it is known that FSH acts on the granulosa cells of growing follicles, while LH acts on theca cells of large antral follicles (Chen et al., 2018). However, the results of recent studies have demonstrated that FSHR and LHR can be localized also in other areas (oocyte, blood vessels, interstitial cells) of ovaries (Burns et al. 2001; Meduri et al., 2002). Meduri et al. (2002) demonstrated the direct effect of FSH on oocyte development by observing the expression of FSHR in human and porcine oocytes. In the same study, LH expression was not observed in the oocyte of the follicle at any developmental stage (Meduri et al., 2002). Unlike Meduri et al. (2002), we observed expression of both FSHR and LHR in oocytes in our study. In a study performed in weaned gilts, authors have demonstrated LHR expression in oocytes, and they suggested that the expression of FSHR and LHR in oocytes is important for activating the intracellular steroidogenic signalling pathway (Wan et al., 2021). Some researchers have proposed that the FSHR and LHR genes have a key regulative role during vitellogenesis in chub mackerel fishes (Nyuji et al., 2013). Similarly, we observed FSHR and LHR expressions in the vitellus of the follicles in our study.

Phytase is frequently added to poultry diets to increase P utilization (Um and Paik, 1999). This enzyme enhances the reproductive performance and egg quality characteristics (Sahara et al., 2018; Shet et al., 2018). In our study, FSHR and LHR were more intensely expressed in granulosa/theca cells and oocytes in the groups supplemented with MP and CP compared to the group without phytase. There was inconsistency between the phytase-untreated and -treated groups regarding the FSHR and LHR expressions in the interstitial cells and vitellus. Similar inconsistencies about the effect of additional phytase have been reported also in other studies. While the addition of 250 and 300 FTU/kg phytase to diets with low levels of AP was suggested to improve feed intake and egg mass layers by some researchers (Jalal and Scheideler, 2001), no effect was observed by others (Zyła et al., 2011; Tischler et al., 2015; Shet et al., 2018). In a study performed using very high levels of phytase (5000 FTU/kg) compared to other studies, Saleh et al. (2021) demonstrated improved eggshell quality and yolk cholesterol level in a 30% less AP diet.

Formulating proper ratios of Ca^{2+} and AP in the diet is crucial on the reproductive performance parameters (Lim et al., 2003; Liu et al., 2007; Ziaei et al., 2009). Egg production was completely stopped in diets with Ca^{2+} levels below 0.5 g/kg because Ca^{2+} is needed for gonadotropin (especially LH) synthesis from the pituitary (Gilbert et al., 1981). The Ca^{2+} and AP content affected the FSHR and LHR immunoreactivities in our study. Strong FSHR and LHR staining were seen in the standard Ca^{2+} and low AP groups, whereas in the low Ca^{2+} and AP diet group, the immunostainings for both receptors were slight. Addition of MP to the diets enhanced the signal in all groups sug-

gesting that additional phytase increases the FSHR and LHR expressions in ovaries, even in diets with insufficient Ca^{2+} and P levels.

CONCLUSION

In conclusion, in this study, FSHR and LHR immunoreactivities were shown for the first time in granulosa/theca, oocyte, vitellus, and interstitial cells of laying hens. The strongest FSHR and LHR expressions were observed in standard Ca^{2+} and low AP diet supplemented with a newly isolated MP source.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abbasi F, Fakhur-un-Nisa T, Liu J, Luo X, Hussain I, Abbasi R (2019) Low Digestibility of Phytate Phosphorus, Their Impacts on The Environment, and Phytase Opportunity in The Poultry Industry. Environ Sci Pollut Res Int 26(10):9469-9479.

AOAC (Association of Official Agricultural Chemists) International. Official Methods of Analysis. Arlington, VA, USA, Association of Official Analytical Chemists; 2000.

Bal MA, Shaver RD, Jirovec AG, Shinners KJ, Coors JG (2000) Crop Processing and Chop Length of Corn Silage: Effects on Intake, Digestion, and Milk Production by Dairy Cows. J Dairy Sci 83(6):1264-1273.

Bello A, Dersjant-Li Y, Korver DR (2020) Effects of Dietary Calcium and Available Phosphorus Levels and Phytase Supplementation on Performance, Bone Mineral Density, and Serum Biochemical Bone Markers in Aged White Egg-Laying Hens. Poult Sci 99(11):5792-5801.

Bougouin A, Appuhamy JADRN, Kebreab E, Dijkstra J, Kwakkel RP, France J (2014) Effects of Phytase Supplementation on Phosphorus Retention in Broilers and Layers: A Meta-analysis. Poult Sci 93(8):1981-1992.

Burns KH, Yan C, Kumar TR, Matzuk MM (2001) Analysis of Ovarian Gene Expression in Follicle-Stimulating Hormone β Knockout Mice. Endocrinology 142(7): 2742-2751.

Chen H, Cui Y, Yu S (2018) Expression and Localisation of FSHR, GHR and LHR in Different Tissues and Reproductive Organs of Female Yaks. Folia Morphol (Warsz) 77(2):301-309.

Colin G. Scanes, Leasea D. Butler, Michael T. Kidd (2020) Chapter 20: Reproductive Management of Poultry, (Eds. Bazer FW, Lamb GC, Wu G), pp. 349-366. (Academic Press)

Gilbert AB, Peddie J, Mitchell GG, Teague PW (1981) The Egg-Laying Response of The Domestic Hen to Variation in Dietary Calcium. Br Poult Sci 22(6):537-548.

Gordon RW, Roland DA (1998) Influence of Supplemental Phytase on Calcium and Phosphorus Utilization in Laying Hens. Poult Sci 77(2): 290-294.

Grzegorzewska AK, Sechman A, Paczoska-Eliasiewicz HE, Rzasa J (2009) The Expression of Pituitary FSHβ and LHβ mRNA and Gonadal FSH and LH Receptor mRNA in the Chicken Embryo. Reproductive Biology 9(3):253-269.

Hu S, Zhu M, Wang J, Li L, He H, Hu B, Hu H, Xia L (2021) Histomorphology and Gene Expression Profiles During Early Ovarian Folliculogenesis in Duck and Goose. Poult Sci 100(2):1098-1108.

Igwe AO, Ihedioha JI, Okoye JOA (2018) Changes in Serum Calcium and Phosphorus Levels and Their Relationship to Egg Production in Laying Hens Infected with Velogenic Newcastle disease virus. J Appl Anim Res 46(1):523-528.

Jalal MA, Scheideler SE (2001) Effect of Supplementation of Two Different Sources of Phytase on Egg Production Parameters in Laying Hens and Nutrient Digestiblity. Poult Sci 80(10):1463-1471.

Jiang S, Cui L, Shi C, Ke X, Luo J, Hou J (2013) Effects of Dietary Energy and Calcium Levels on Performance, Egg Shell Quality and Bone Metabolism in Hens. Vet J 198(1):252-258.

Keshavarz K and Nakajima S (1993) Re-evaluation of Calcium and Phosphorus Requirements of Laying Hens for Optimum Performance and Eggshell Quality. Poult Sci 72 (1):144-153.

Lim HS, Namkung H, Paik IK (2003) Effects of Phytase Supplementation on the Performance, Egg Quality, and Phosphorous Excretion of Laying Hens Fed Different Levels of Dietary Calcium and Nonphytate Phosphorous. Poult Sci 82(1):92-99.

Liu N, Liu GH, Li FD, Sands JS, Zhang S, Zheng AJ, Ru YJ (2007) Efficacy of Phytases on Egg Production and Nutrient Digestibility in Layers Fed Reduced Phosphorus Diets. Poult Sci 86(11):2337-2342.

Liu Z, Xue J, Luo Y, Wang Q, Zhong H, Liang M, Wang C (2019) Effects of Dietary Energy Concentration on Reproductive Hormone Secretion and Gene Expression in the Hypothalamus-Pituitary-Gonad Axis in Laying Geese. Braz J Poult Sci 21(03).

Méduri G, Charnaux N, Driancourt MA, Combettes L, Granet P, Vannier B, Loosfelt H, Milgrom E (2002) Follicle-Stimulating Hormone Receptors in Oocytes? J Clin Endocrinol Metab 87(5):2266-2276.

Mishra SK, Chen B, Zhu Q, Xu Z, Ning C, Yin H, Wang Y, Zhao X, Fan X, Yang M, Yang M, Yang D, Qingyong N, Li Y, Zhang M, Li D (2020) Transcriptome Analysis Reveals Differentially Expressed Genes Associated with High Rates of Egg Production in Chicken Hypothalamic-Pituitary-Ovarian Axis. Sci Rep 10(1):1-8.

Nyuji M, Kitano H, Shimizu A, Lee JM, Kusakabe T, Yamaguchi A, Matsuyama M (2013) Characterization, Localization, and Stage-Dependent Gene Expression of Gonadotropin Receptors in Chub Mackerel (Scomber Japonicus) Ovarian Follicles. Biol Reprod 88(6):148.

Sahara E, Despedia F, Raden A (2018) The Influence of Phytase Enzyme to Laying Performance and Quality of Egg Shell of Golden Arabian Chicken. In 'Proceedings of the 1st Sriwijaya International Conference on Environmental Issues'. (Ed. Amin M) 68. 01022.

Saleh AA, Elsawee M, Soliman MM, Elkon RYN, Alzawqari MH, Shukry M, Abdel-Moneim AME, Eltahan H (2021) Effect of Bacterial or Fungal Phytase Supplementation on the Performance, Egg Quality, Plasma Biochemical Parameters, and Reproductive Morphology of

Laying Hens. Animals 11(2):540.
Shet D, Ghosh J, Ajith S, Awachat VB, Elangovan A V (2018) Efficacy of Dietary Phytase Supplementation on Laying Performance and Expression of Osteopontin and Calbindin Genes in Eggshell Gland. Anim Nutr 4(1):52-58.
Tischler A, Halas V, Tossenberger J (2015) Effect of Dietary NPP Level and Phytase Supplementation on the Laying Performance Over One Year Period. Poljoprivreda 21(1):68-72.
Um JS, Paik (1999) Effects of Microbial Phytase Supplementation on Egg Production, Eggshell Quality, and Mineral Retention of Laying Hens Fed Different Levels of Phosphorus. Poult Sci 78(1):75-79.
Wan B, Yuan X, Yang W, Jiao N, Li Y, Liu F, Liu M, Yang Z, Huang L, Jiang S (2021) The Effects of Zearalenone on the Localization and Expression of Reproductive Hormones in the Ovaries of Weaned Gilts. Toxins (Basel) 13(9):1-14.
Ziaei N, Shivazad M, Mirhadi SA, Gerami A. (2009) Effects of Reduced Calcium and Phosphorous Diets Supplemented with Phytase on Laying Performance of Hens. Pak Jof Biol Sci 12:792-797.
Zyła K, Mika M, Duliński R, Świątkiewicz S, Koreleski J, Pustkowiak H, Piironen J (2012) Effects of Inositol, Inositol-Generating Phytase B Applied Alone, and in Combination with 6-Phytase A to Phosphorus-Deficient Diets on Laying Performance, Eggshell Quality, Yolk Cholesterol, and Fatty Acid Deposition in Laying Hens. Poult Sci 91(8):1915-1927.