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Original Article

Effect of Supplementation of Organic Selenium on Physiological and Production Parameters of Ghagus hens during the Early Laying Period

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ABSTRACT

Trace mineral organic selenium is known to increase egg production significantly in exotic breeds. The present study was conducted with the Ghagus native hen breed to observe the effect of organic-selenium-enriched yeast on body weight, plasma hormones, and expression of genes that influence egg production. The control (C) group was offered a corn and soybean-based basal feed without organic selenium supplementation. The other two treatment groups (T1 and T2) were offered 0.2 and 0.4 ppm of organic selenium, respectively. The results indicated that, in T1, plasma levels of ghrelin and melatonin increased (p<0.05), while progesterone levels decreased significantly (p<0.05); furthermore, the expression of melatonin, and ghrelin hormone receptors in jejunum and magnum tissues also increased (p<0.001). When compared to C and T2, the fold change expression of all amino acid transporters increased (p<0.001) in jejunum tissue, whereas in magnum tissue, the expression of only three transporters (CAT, LAT2, and LAT4) increased (p<0.001) in T1. Treatment of chickens with 0.2 ppm also increased egg production by 6±1%, and egg weight by 3± 0.5g. The study indicated that there is potential for increases in egg production in Ghagus hens with the supplementation of organic selenium during the early laying period.

INTRODUCTION

Ghagus hens are primarily reared for meat and egg purposes in backyard systems in the districts of Karnataka and Andhra Pradesh in India. The annual egg production reported is 60-80 eggs, and adult female body weight is 2.0 kg by 36 weeks of age (Vij *et al.*, 2006). Different physiological parameters influence egg parameters, and the present study aimed to observe the efficacy of organic selenium supplementation on the modulation of some physiological parameters influencing egg production. There is a relationship between gut hormones and reproduction (Comninos *et al.*, 2014), but their relation in chicken is not clear. MET has the potential to mitigate oxidative stress and inflammation in chickens and humans, whether administered by implantation or through feed. Reports also indicate that treatment with this substance increases egg parameters in chickens (Jain, 2012; Hao *et al.*, 2021; Yong *et al.*, 2021), protecting the organisms by scavenging free radicals, which helps in regulating various reproductive and growth aspects (Shao *et al.*, 2024; Khan *et al.*, 2024). When administered intraperitoneally in aged hens, melatonin increased plasma levels of LH, estradiol, and the number of immature follicles (Hao *et al.*, 2020). Ghrelin (GHL), another gut hormone, is secreted by the proventriculus of chickens (Murugesan & Nidamanuri, 2022). *In vitro* studies with chicken granulosa cells treated with human and chicken GHL fragments showed reduced secretion of progesterone (P4)

(Sirotkin & Grossman, 2015). Reports from Sirotkin & Grossman (2015) and Sirotkin *et al.* (2017) suggest that GHL has a role in integrating nutrition, metabolism and egg production. Ovarian Estradiol -17b (EST) is the most abundant estrogen and an important gonadal steroid that is involved in oviduct development and yolk precursor production (Williams *et al.*, 2004). Its concentration increases gradually until twenty weeks of age and remains high during next few weeks (Beck & Hansen, 2004). Estrogen regulates levels of gonadotropins through negative feedback effect, and is produced by the cells of less mature follicles (Porter *et al.*, 1989). Contradictory regulation of EST by MET has been reported in humans and rats (Chuffa *et al.*, 2011; Cheng *et al.*, 2020). In hens, P4 is produced by granulosa cells obtained from preovulatory follicles (Woods *et al.*, 2009) and is regulated by LH (Yang *et al.*, 1997). These studies confirm the relation between gut hormones steroids and ovarian functions.

Reports indicate that treatment with organic selenium (O Se) for naked neck chicks subjected to heat stress increased body weight, FCR, breast and thigh weight (Khan *et al.*, 2023). O Se has been demonstrated to be one of the essential trace minerals required for reproduction in Vanaraja and Aseel chickens (Nidamanuri *et al.*, 2023; 2024), as it influenced key physiological parameters. Supplementation of O Se in layers is associated with a higher efficacy in Se transfer to the egg (Tufarelli *et al.*, 2016), which is beneficial for internal egg quality. A beneficial effect of supplementation of higher doses of O Se in layers has been observed (Cobonova *et al.*, 2011; Zia *et al.*, 2018), and reports indicate that inorganic forms of Se are poorly stored and absorbed in chickens (Anizoba *et al.*, 2024). Hence the present study aimed to investigate the effect of O Se supplementation on endocrine and gene expression parameters which may affect the egg production of Ghagus hens during the early laying period (EP). Till date no work has been published with respect to supplementation of O Se in this breed.

MATERIALS AND METHODS

Approval to conduct the experiments was obtained from the Institutional Animal Ethics committee (IAEC/DPR/19/3) Regn. No.355/GO/RBi/S/01/CPCSEA).

One hundred and fifty Ghagus hens with a mean body weight of 1858 ± 11.5 g were selected and reared at the farm of the Directorate of Poultry Research, Hyderabad, India. The experiment was conducted during the months of February and March, 2021. At 22 weeks of age, hens were placed in individual California type cages, with floor space per bird of around 450-525 sq cm (0.6-0.75 sq ft). They were housed in an open side housing system. The mean shed temperature and relative humidity were 28±1 °C and 46%, respectively. All birds were allowed to acclimate towards an amount of basal feed of 90g/bird/d, based on maize and soybean, and containing 0 ppm of organic Se (O Se, selenium yeast) (Table1) for 2 weeks. Water was provided *ad libitum*. For the present experiment, experimental tenure was from 24-28 weeks of age (EP). The experiment was conducted in a Completely Randomized Design (CRD). At the beginning of 24 weeks, the hens were divided into three equal groups: Control (C), Treatment1(T1) and Treatment 2(T2), each with 50 birds and five replicates, with ten birds in each replicate. A commercially available organic product, selenium enriched yeast (O Se, Seleno Source TM AF 2000, Nurture Organics, New Delhi), was added to the basal feed (Table 1). The Control group was offered basal feed only (0ppm O Se), and groups T1 and T2 were offered basal feed mixed with 0.2ppm and 0.4ppm of O Se, respectively. All other components of the feed were supplemented according to the requirements stated in the Nutrient Requirements of Poultry -3 (ICAR, 2012), under a 16:8-h light dark cycle. During the course of the experiment, temperature ranged between 29-32 °C, and relative humidity between 60–71%.

Table 1 – Composition of the Basal Diet.

Diet	Concentration in percentage*
Maize	61.57
Soybean Meal	24.74
DORB	0.49
Stone grit	10.9
Dicalcium Phosphate	1.5
Salt	0.35
Sodium Bicarbonate	0.1
D-Methionine	0.1
Choline Chloride	0.1
Toxin binder	0.1
Tylosine	0.05

* As fed basis. Estimated values: Estimation was based on the known standard values of the feed ingredients used for feed preparation. ME-2625Kcal; CP- 17%, LYS-0.84%, MET-0.33%, Ca-3.9%, Available P-0.36%, Na-0.18%. Analyzed values: CP-16.5%, Ca-3.5%. Crude protein was estimated by the Kjeldahl procedure, which measures the total nitrogen content in the feed. Total nitrogen (%) is multiplied by 6.25 to arrive at the crude protein content (%).
Ca was measured through the titrimetric method using potassium permanganate solution.
Nutrient composition of the Premix Provided (mg/kg diet): thiamine 1; pyridoxine, 2; cyanocobalamin, 0.01; niacin, 15; pantothenic acid, 10; α-tocopherol, 10; riboflavin, 10; biotin, 0.08; menadione, 2; retinol acetate, 2.75; cholecalciferol, 0.06; choline, 650; copper, 8; iron, 45; manganese, 80; zinc, 60; Exogenous Se 0 ppm; hydrated sodium calcium aluminosilicates, 800; phytase, 375 units.

Treatment group (T1) - Basal feed + 0.2ppm Yeast enriched selenium. (T2)- Basal feed + 0.4ppm Yeast enriched selenium. Control group (C)- Basal feed containing 0.05ppm Se (As analyzed from feed ingredients).

Blood samples were collected from the wing veins of eight birds selected at random from each group, and were place in EDTA coated tubes for the estimation of hormones. The same eight birds were chosen for blood collection at weekly intervals for four weeks. After collecting blood samples, tubes were kept in ice and transported to the laboratory. The samples were further centrifuged at 3000 rpm for 15 min. The plasma obtained as supernatant was stored at -20 °C for analysis of MET, GHL, P4 and EST hormones.

Birds from each group were sacrificed at 28 weeks of age by cervical dislocation for the collection of jejunum (from the pancreatic loop to Meckel's diverticulum) and magnum tissues (portion of oviduct) (Reicher *et al.*, 2020). After collection, they were transported to the lab in saline. The tissues were rinsed in PBS, placed on moistened paper towels, and any adhering fat and connective tissue were removed. They were immediately stored at -80 °C for further analysis. Gene expression studies for MET, GHL hormone receptors (MNTR, GHLR), and four amino acid transporters were conducted for both jejunum and magnum tissues of the birds.

The blood samples collected at weekly intervals were analyzed for different hormones separately. Mean±SE values for each hormone per bird were calculated for each week, and then Mean±SEM was calculated for the EP phase. Hormones MET (E12M0005), Estradiol (EST, E12E0023), Progesterone (P4, E12P0200) were assayed in the plasma using commercial Chicken ELISA kits (BlueGene Biotech, Shanghai). The Ghrelin (BC-ECh 040174) hormone was also estimated using commercial chicken ELISA kits (Biocodon Technologies, Kansas, USA) according to manufacturer's instructions. The ELISA kits used for the estimation of the first three hormones were based on the competitive enzyme immunoassay technique, while those used for GHL measurements were based on a non-competitive assay. At the final step, the absorbance's of the color developed in the samples was measured at 450nm in a ELISA plate reader (BioTek Instruments, Inc.). From the standard curve, the concentration of hormones was estimated. The intra and inter coefficients of variation were <7 and 8% respectively. Each plasma test samples and standards were run in duplicate.

Total RNA was extracted from approximately 100mg of tissues, using Trizol reagent (Invitrogen, Carlsbad, CA) purified with RNeasy Mini Kit (Qiagen, Valencia, CA), and treated with RNAse-Free DNAse kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was dissolved in diethyl pyro carbonate (DEPC) treated water. The sample purity and concentration were measured on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The purity of the extracted RNA was assessed by taking the ratio (OD 260/280) for all samples. Values ranged between 1.9 -2.0, while the RNA concentrations ranged from 2.0 to 2.5 µg /µl. RNA samples were stored at –80°C until further assay. A total of 2 µg of total RNA was reverse transcribed with a cDNA reverse transcription kit according to manufacturer's protocol (Thermo Scientific, Verso cDNA synthesis kit), using a Gradient Master cycler (Eppendorf, Hauppauge, NY). Further cDNA samples were stored at –20°C. cDNA samples were diluted 1: 1 prior to RT-PCR analysis with the help of an Applied Biosystems RT-PCR machine. Each reaction consisted of 1 µL diluted cDNA, 1.0 µL forward primer, 1.0 µL reverse primer, 7uL DEPC water, and 10 µL Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher SCIENTIFIC). The genes and primer sequences used for the RT-PCR assays are listed in Table 2. The primers were synthesized by Chromous Co. Bangalore, India. The qPCR conditions were 95°C for 5 min, followed by 40 cycles of 95 °C for 30 s and 55 °C for 30 s, followed by 72 °C for 30 s. All samples were run in triplicate. Both control and treatment (T1, T2) values were normalized against the b actin reference gene. The relative quantification (RQ) was expressed as a ratio of the target gene to the control gene using the delta-delta Ct method (Rao *et al.*, 2013). The difference in values was considered significant at the level of $p<0.05$.

The total Se content of the basal diets (Fresh) and treatment diet was analyzed using inductively coupled plasma MS (ICP-MS; Agilent 7500cx, Agilent Technologies, Tokyo, Japan) (Ohki *et al.*, 2016).

Body weight (BW) was recorded at a fortnight interval during the study. Mean ±SEM values were estimated for twenty birds from each group.

Percentage of weekly egg production (EP%) was calculated as Total number of eggs for a week/Actual no. of hen days X 100.

Actual no. of hen days = Total number of hens in a week – loss of hens in that week.

For the estimation of egg weight (EW), 20 -30 eggs were randomly collected every day from each group. Eggs were weighed on an electronic balance to the nearest g, and mean and Mean±SEM values were subsequently calculated.

Table 2 – Sequence of primers for different genes.

Parame	Sequence of primers	Product bp length	NCBI Reference Sequence
CAT1	GACACTCATGATGCCTTACTACCAG-F	190	NM_001145490.1
	GAGAAGTCCATCTTCTGCCATAGC-R		
BAT	GCATATGATGGATGGAATCAACTC-F	167	NM_001199133
	GACTGCAGGAGTTCTGTTGAAGTC-R		
y^+LAT 2	CTAGCCAGGCGGTTATTGCAATCAC-F	145	XM_001231336.5
	TTTACATAGGCACAATTTACGAATG-R		
LAT 4	GTCTTCAGCCCCATCTTGTTGCTCAGC-F	177	XM_415803.6
	TGCTGTCAGCAGGCACAGCAGTTGC-R		
MTNR1C (MNTR)	ATCGCAATCAACCGCTAC-F	144	XM_013100494.3
	CAAGGACCCAACGAAGAA-R		
GHSR (GHRL)	CTGCAAGCTCTTCCAGTTCATCAGC-F	160	AB095994.1
	CCAGAGGATGAGGATGACCAGCTTG-R		
Actin b	CGGACTACCTCATGAAGATCCTGAC-F	197	NM_205518.2
	GCCAATGGTGATGACCTGACCATC-R		

F-Forward Primer; R- Reverse Primer

Genes - CAT1 – Cationic amino acid transporter, BAT- Branched chain amino acid transporter, LAT – Large amino acid transporter, MTNR1C (MNTR) – Melatonin hormone receptor, GHSR (GHRL) – Ghrelin hormone receptor

Statistical Analysis

Data are presented as Mean ± SEM. All data were analyzed using the SPSS statistical software (SPSS for Windows, version 16.0; SPSS Inc. Chicago, IL). One-way analysis of variance followed by Duncan's multiple comparison test was used to identify differences in means among treatments. The hormonal data were analyzed using repeated measures ANOVA. Data were assumed to be statistically significant at $p<0.05$ or $p<0.01$ for gene expression parameters.

RESULTS AND DISCUSSION

The present study mainly aimed to investigate the effect of treatment with O Se on the modulation of egg production for the Ghagus breed. Furthermore, its effect in modulating the ovarian and gut hormones and gene expression of hormone receptors and amino acid transporters was also investigated.

Effect on BW, EW, EP%: While supplementation with 0.2 ppm (T1) and 0.4 ppm (T2) of organic selenium (O-Se) from 24 to 28 weeks of age did not significantly ($p>0.05$) affect body weight compared to the control group, the T1 group exhibited a significant increase in egg production percentage (6 ± 1%, $p<0.01$) and an increase in egg weight (3 ± 0.5 g, $p<0.01$) compared to both the control and T2 groups (Table 3). Previous studies have demonstrated that organic selenium supplementation can enhance growth and reproductive performance in poultry, with increased growth rates in birds supplemented with O-Se (Khan *et al.*, 2018; Bora *et al.*, 2024). In the present study neither of the treatments caused an increase in body weight or feed intake of the laying birds. Similar results have been reported for broiler chicks (Bakhshalinejad *et al.*, 2019). When birds reach adult age, they maintain relatively stable body weight, and when they enter the egg production stage, the energy and nutrients are also diverted for egg production, which may explain these results. The two doses of O Se supplemented in the present study could not increase body weight, as the difference in the values for egg production and weight between control and treatment groups was significant. Most of previous studies have been restricted to amelioration of heat stress upon supplementation of SY (Mohamed *et al.*, 2024). It has been reported that selenium doses have to be increased during the laying period for higher egg production (Gul *et al.*, 2021). Further research is required to formulate the optimal dose of O Se in feed for different applications in chicken according to the state of physiological functions.

Table 3 – Effect of selenium supplementation on egg production, weight, and level of plasma hormones.

Groups	Body weight (g)	Egg production (%)	Egg weight (g)	Melatonin (pg/ml)	Ghrelin (pg/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
Control (C) 0ppm	2113.7^a	43.5^a	39.9^a	317^a	19.6^a	16.7^a	7.35^a
Treatment 1 (T1, 0.2ppm)	2093.5^a	50.9^b	43.2^b	345^b	29.0^b	16.6^a	5.23^b
Treatment 2 (T2, 0.4ppm)	2088.6^a	41.3^a	39.6^a	311^a	17^a	15.8^a	6.84^a
SEM	9.75	0.9	1.28	2.05	1.75	0.4	0.4
p-value	$p>0.05$	$p<0.01$	$p<0.01$	$p<0.05$	$p<0.05$	----------	$p<0.05$

Values are presented as means. Values with different superscripts in a column are significantly different from each other at least at $p<0.05$, For eggs N=20-25, For Hormones N=8

Effect on Hormones: Supplementation with O-Se (T1) significantly increased ($p<0.05$) plasma concentrations of MET and GHL while concurrently decreasing ($p<0.05$) plasma progesterone (P4) hormone levels compared to the control and T2 groups. No significant differences in plasma estradiol (EST) levels were observed among the treatment groups (Table 3). Hormones like LH and FSH have been associated with reproduction or egg production. It has been reported that *in vivo* treatment with exogenous MET increased egg production (Jia *et al.*, 2016; Kahraman *et al.*, 2022), and *in vitro* treatment with ghrelin on ovarian cells also modulated ovarian hormones (Schalla & Stengel, 2021; Sirotkin *et al.*, 2023). Reports on the physiological levels of MET and GHL and their modulation by organic selenium (O-Se) supplementation are scarce in backyard native chickens, with limited data available specifically for Aseel and Vanaraja breeds, as reported in our previous

studies (Nidamanuri *et al.*, 2023; 2024). Compared to treatment with inorganic Se, the organic form proved to be safer, presenting less toxicity and a higher utilization (Liu *et al.*, 2020; Kim *et al.*, 2020). Hence, in the present study, O Se was used as a supplement. A study utilizing ghrelin analogs on ovarian granulosa cells *in vitro* indicated that ghrelin directly affected ovarian functions, especially steroid and peptide hormone secretions (Sirotkin & Grossman, 2007; Nidamanuri *et al.*, 2023). In the present study, the increase in the levels of ghrelin upon treatment did not modulate the levels of steroid hormones significantly compared to the control and T2 groups (Table 3). Nevertheless, significant increases in egg production and weight were observed (Table 3). This indicates that treatment with 0.2ppm of O Se and higher ghrelin levels might have had a direct influence on egg production and jejunum and magnum tissues. Although ghrelin acts as an anorexigenic hormone in chickens, this study found that elevated ghrelin (GHL) levels did not reduce feed intake (data not presented) or body weight. Additionally, our results do not indicate a beneficial effect of organic selenium (O Se) on steroid hormones. Therefore, the improvements in production observed in the present study are likely to be a direct effect of ghrelin and other metabolic hormones.

Gene Expression: Compared to the control and T2 groups, supplementation with O-Se (T1) significantly increased (p<0.001) the fold change in expression of the hormone receptors MNTR and GHRL in both jejunum and magnum tissues (Fig. 1 & 2). Similarly, in jejunum tissue, T1 significantly increased (p<0.001) the fold change in expression of all four amino acid transporters studied (BAT, LAT4, LAT2, and CAT) compared to the other groups (Fig. 1). However, in magnum tissue, only the expression of LAT4, LAT2, and CAT was significantly increased (p<0.001) in T1 compared to the control and T2 groups (Fig. 2). Treatment with 0.4ppm of O Se (T2) decreased the concentration of all the hormones, fold change in the expression of the receptors, and amino acid transporters when compared to control, but the difference was not significant (Fig 1 & 2). Moreover, it led to decreased egg production compared to the control (2±0.4%, p<0.01) and T1 group (8±0.6%, p<0.001) (Table 3). Similarly, upon comparison between groups, the weight of eggs only increased for the T1 group (Table 3) (p<0.05). The significant increase in the fold change expression of GHRL and MNTR in both jejunum and magnum tissues in the T1 group suggests that lower doses of O-Se may enhance egg production and weight by increasing plasma ghrelin and melatonin levels. However, higher doses of O-Se (0.4 ppm) significantly decreased egg production compared to both the T1 and control groups (p<0.001). The elevated levels of melatonin and the increased expression of its receptors in the ovary and jejunum, as reported by Holt *et al.* (2021) and Mandal (2023), may have contributed to improved nutrient absorption, jejunum integrity, and magnum function in the T1 group. Hence, supplementation of 0.2 ppm of O Se affected egg production and weight positively. Studies in aquaculture indicate that selenium supplement plays an important role in the composition of enzymes, leading to the synthesis and release of more nutrients from the intestinal epithelium (Zhu *et al.*, 2014). Selenium (Se) is primarily absorbed in the small intestine and plays a crucial role in maintaining the integrity of vital tissues (Ozgul & Naziroglu, 2012). The observed increase in the expression of certain amino acid transporters in the T1 group suggests enhanced nutrient absorption, which may have contributed to the improved egg production. Furthermore, organic selenium (O-Se) is well-known for its antioxidant properties, effectively mitigating oxidative stress in poultry, a common challenge during the production period (Elgendy *et al.*, 2022; Bora *et al.*, 2024).

O-Se supplementation can also positively influence reproductive capacity (Jlali *et al.*, 2013). While the current study demonstrated beneficial effects of O-Se supplementation at lower doses, higher doses may have detrimental effects on egg production, suggesting the need for optimizing the supplementation level for optimal performance.

Estimation of Selenium in feed: The estimated selenium concentration in the basal diet was 0.05 ppm (from the feed constituents only), and it was observed to be respectively 0.19 ppm and 0.36 ppm in T1 and T2.

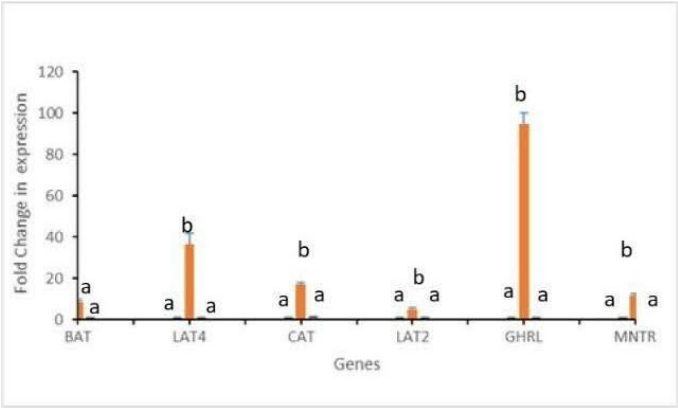

Figure 1 – Fold change in the expression levels of mRNA of hormone receptors (MNTR and GHRL) and amino acid transporters (BAT, LAT4, CAT, LAT2) of jejunum tissue during the early laying period. Bars representing mean values with different superscripts are significantly different from each other at least at p<0.01, N=6.

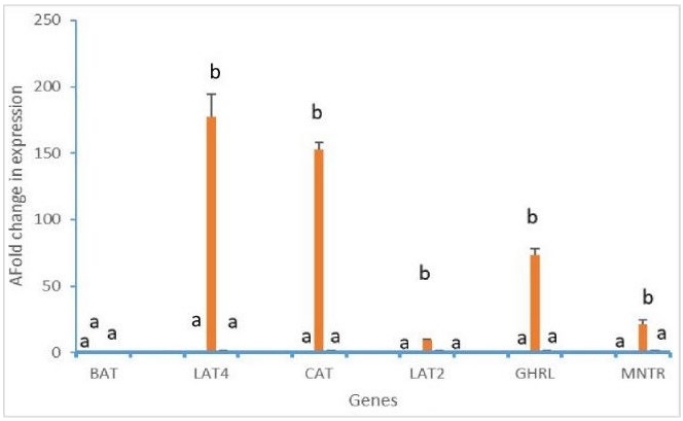

Figure 2 – Fold change in the expression levels of mRNA of hormone receptors (MNTR and GHRL) and amino acid transporters (BAT, LAT4, CAT, LAT2) of magnum tissue during the early laying period. Bars representing mean values with different superscripts are significantly different from each other at least at $p<0.01$, N=6.

CONCLUSION

From the present study, it can be concluded that 0.2ppm O Se supplementation is beneficial for egg production in the native Ghagus poultry breed during the early laying period through modulation of physiological parameters, without adversely affecting bodyweight.

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None.

AUTHOR CONTRIBUTIONS

All authors have made equal contributions for this study.

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DATA AVAILABILITY STATEMENT

Data will be available upon request.

CONFLICT OF INTEREST

All authors report no conflicts of interest.

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