



Differential Level of Hormones Amino Acids Expression of Amino Acid Transporters and Hormone Receptors during Early and Mid Laying Period and their Modulation by Organic Selenium in Vanaraja Chickens

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Abstract

The present study was conducted to compare the plasma levels of melatonin, ghrelin, steroid hormones, amino acids, expression of hormone receptors (MNTR, GHLR), and amino acid transporters (AATs, 4 nos.) during early (EP, 24-28 weeks) and mid (MP, 32-36 weeks) laying period. Hormones melatonin and ghrelin have been associated with ovarian function in chickens. Till date no reports are available with respect to mentioned physiological parameters on the production performance and their modulation by Se (selenium) treatment in Vanaraja Chickens. Vanaraja chickens at 22 weeks of age were divided equally into two groups. Control group was reared on a basal diet containing 0.3mg of inorganic Se. Treatment group was offered additional 0.3mg of organic Se enriched yeast during EP and MP. When compared between the control groups of EP and MP, the mean level of all the hormones was higher at EP. The expression of all the AATs and MNTR was higher in jejunum, compared to magnum, except for CAT and GHLR during EP. When compared to control group supplementation of selenium increased the level of plasma hormones and amino acids significantly ($P < 0.05$) during MP. When compared between EP and MP, treatment with Se increased expression of 2-3 number of aats and decreased MNTR in both the tissues significantly at EP, whereas expression of only GHLR increased in magnum. The Se treatment did not cause significant change in the expression of receptors, at MP. In conclusion, additional Se supplementation increased the utilization of hormones and modulation of the physiological parameters was higher in magnum tissue resulting in a beneficial effect on increased egg production (2%), and body weight ($P < 0.01$) during EP and MP.

Keywords: Endocrine; Gene Expression; Plasma Amino Acids; Laying Period; Vanaraja

Introduction

Vanaraja is a dual-purpose chicken variety developed by the ICAR-Directorate of Poultry Research (formerly Project Directorate on Poultry) in Hyderabad, India. It's a cross developed by mating between Red Cornish and synthetic female broilers. Vanaraja was developed for rural communities where it can be reared in backyard on natural, scavenged food with minimal supplementation. It produces brown eggs like local hens [1]. The annual egg production is 110-120 eggs.

Melatonin (MET) is not only produced by the pineal gland but also by intestinal mucosa and also in gastrointestinal tract, pancreas, intestine, retina, skin and immune cells. It reduces oxidative stress by scavenging superoxide anions [2]. Its level in the mucosa exceeds than that present in the blood [3]. MET treatment increases egg weight and egg laying rate [4,5]. One study indicated that MET may enhance levels of reproductive hormones in laying hens [2] and its binding sites have been detected in the ovaries of chickens [6]. MET significantly increases the activities of digestive enzymes

and expression of transporter genes and activity of antioxidants [4] in the jejunum, in turn helping in digestion and absorption.

It has been proposed that ghrelin is expressed in chicken ovary and majority of these effects can be mediated through ghrelin receptors [7]. It can regulate functions, such as proliferation, apoptosis, and hormone release in the chick [8]. It is proposed that the nutritional status affects mammalian reproductive processes via the metabolic hormones, leptin, ghrelin and obestatin [9,10].

The cellular signaling for the biosynthesis of albumen is regulated by estrogen, progesterone, and testosterone [11]. The function of progesterone is to modulate the function of estrogen, on growth or secretory protein production [12].

Amino acids have beneficial roles in the body in the formation of proteins, enzymes, antibodies etc [13,14]. In chickens, amino acids have important roles in minimizing oxidative stress and also influencing intestinal barrier [15,16]. The limiting essential amino acids are methionine, lysine, threonine, arginine, isoleucine and valine for broilers fed corn-soy based diets [17]. Amino acids and fatty acids are absorbed in the jejunum which is known to be either positively or negatively affected by various factors [18,19]. Different types of amino acid (AA) transporters namely, CAT 1, CAT 2, LAT 1 and LAT 4 expression [20] has been reported in chicken breeds. B⁰AT (B⁰-type AA transporter), and b^{0,+}AT (b^{0,+}-type AA transporter) are responsible for up-taking neutral and cationic amino acids such as Gly, Ser, Thr, Cys, Tyr, Asn, Gln, His, Lys and Arg [21].

Supplementation of Se (0.1–2 mg/kg diet) decreased the amount of free radicals, supported enzyme activity and improved performance in poultry [22]. Under any type of stress more amount of trace minerals is recommended than what is recommended [23]. It has been recognized that organic Se has a higher rate of tissue retention and less toxic than inorganic Se, so organic form is preferred [24]. Live yeast cells are capable of absorbing selenium and transform it into L (+) selenomethionine [25]. Skeletal muscle is the major site of selenium storage, representing about 28–46% of the total selenium pool. Selenoprotein expression is characterised by high tissue specificity, depends on Se availability, can be regulated by hormones, and contributes to various pathological conditions if compromised [26,27]. As an essential mineral element, the requirement of Se for laying hens is relatively low, about 0.3 mg/kg in diets. Role of plasma hormones, amino acids through their receptors and transporters in influencing production parameters of chicken is lacking.

The aim of the present study was to observe how the physiological levels of melatonin, ghrelin and steroid hormones varied during early (24-28 EP) and mid laying phase (32-36 MP) of the production cycle. Further their relation with receptors and transporters in influencing production parameters. Efficacy of higher dose (0.6ppm) of selenium in modulation of physiological parameters for improvement of production performance.

Material and Methods

Approval for conducting the experiments was taken from Institute Ethics committee (IAEC/DPR/19/3) Regn. No.355/GO/RBi/S/01/CPCSEA.

Management of birds

A sixty number of Vanaraja broiler breeder variety of chickens were selected with uniform body weight from our institute farm at about 22 weeks of age. These were housed with egg laying rate of 48%. All birds were acclimated to basal feed for 2 weeks. Water was provided ad libitum. The composition of the basal feed is as given in table 1. They were distributed into 60 individual California type cages and housed in open side housing system. National Research Council's Guide for the Care and Use of Laboratory Animals guidelines have been followed. At the beginning of 24 weeks the hens were divided in to two groups with equal number of hens. Each group had 30 birds, six replicates with five birds in each replicate. One control (CG) was offered basal feed based on maize and soybean with 0.3ppm of inorganic Se (sodium selenite) and second one treatment group (TG) which was offered basal feed with additional 0.3ppm of selenium. Selenium enriched yeast (Seleno Source™ AF 2000) a commercial organic product was purchased from Nurture Organics, Karol Bagh, New Delhi was added to basal feed to get total amount of 0.6 ppm of selenium/Kg feed. At our farm 0.3 ppm of Se is added routinely to the feed of adult birds. All other components of the feed were supplemented according to the requirements followed up for NRC 1994, under a 16:8-h light dark cycle. The temperature ranged between 27 -30°C and relative humidity at 65-70% during the course of the experiment. Under standard conditions of restricted feeding, feed was provided @ 120g/bird /day. For the present experiment, experimental tenure was divided in to two groups i e from 24-28 weeks (early laying period, EP) and then further from 32-36 weeks of age (mid laying period, MP).

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

Estimated values

ME-2625Kcal; CP- 17%, LYS-0.84%, MET-0.33%, Ca-3.9%, Available P-0.36%, Na-0.18%.

Analysed values

CP-16.5%*, Ca-3.5%

Nutrient composition

Provided (mg/kg diet): thiamin 1; pyridoxine, 2; cyanocobalamin, 0.01; niacin, 15; pantothenic acid, 10; a 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; selenium, 0.30; hydrated sodium calcium aluminosilicates, 800; phytase, 375 units* As fed basis

Collection of blood samples

Blood samples were collected from wing vein of six birds from each group at random, in to heparin coated tubes for estimation of melatonin, ghrelin, estradiol, progesterone hormones and plasma amino acids. The same six birds were chosen for blood collection at weekly intervals.

After collection of 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 amino acids and hormones.

Analysis of hormones

Hormones Melatonin (MET, E12M0005), Estradiol (EST, E12E0023), Progesterone (PROG, E12P0200) in plasma were assayed using commercial Chicken ELISA kits (BlueGene Biotech, Shanghai). Ghrelin (GHL, BC-Ech 040174) hormone was also

estimated using commercial chicken ELISA kit (Biocodon Technologies, Kansas, USA) according to manufacturer’s instructions. Briefly, The ELISA kits for the first three hormones, applied the competitive enzyme immunoassay technique utilising a respective anti –antibody and respective HRP conjugate. After the final reaction the absorbances of the colour developed in the samples was measured at 450nm in a micro plate reader (BioTek Instruments, Inc.). The intensity of the colour developed was inversely proportional to the concentration of the hormones in plasma. The concentration of each hormone in samples was interpolated from the standard curve. The concentration of standards for melatonin ranged from 50 to 1000 pg/ml. The sensitivity of the assay was 1.0 pg/ml. The concentration of standards for estradiol ranged from 250 to 5000 pg/ml. The sensitivity of the assay was 1.0 pg/ml. The concentration of standards for progesterone ranged from 2.5 to 50 ng/ml. The sensitivity of the assay was 0.1 pg/ml. The concentration of standards for ghrelin ranged from 5-1500 pg/ml. The sensitivity of the assay was 2.36 pg/ml. The intra and inter coefficients of variation were < 6 and 8% respectively. Each plasma test sample and standards were run in duplicate.

Collection of tissues

A six number of birds from each group, were sacrificed at 26 and 34 weeks of age, by cervical dislocation, for collection of jejunum (from the pancreatic loop to Meckel’s diverticulum) and magnum tissues (portion of oviduct). After collection, they were transported to lab in saline. The tissues were rinsed in PBS and placed on moistened paper towels and removed any remaining fat and connective tissue by teasing with two sets of forceps, so that fat and other tissues were not adhering to it. They were immediately stored at -80°C for further analysis. Gene expression studies were conducted for both jejunum and magnum tissues of the birds.

Analysis and estimation of plasma amino acids

The total free plasma amino acids, were analysed on HPLC equipment (WATERS Alliance Separations module 2695). Analysis of only 15 amino acids could be performed. A 100ul of each Plasma Sample was taken and 400 µl of methanol was added to precipitate the proteins. Incubated over night at -20°C. Centrifuge at 4000 rpm for 30 min and supernatant was transferred into another tube. The supernatant was evaporated under N2 at 60°C to complete dryness. Derivatizing reagent was added to the sample and incubated for 60 mins at 45°C and vacuum evaporated. The resultant pellet was dissolved in 100 µl of Buffer-A. Vortexed and centrifuged at 13000 rpm for 15 min. Supernatant was collected into the vials. 20µl was loaded on to the instrument, which was quantified using standards purchased from Sigma Co. , USA. Column used was

Luna C18 (250 x 4.6mm; 5µl). Flow rate was 1ml/min. Gradient run time was 80 min. Mobile Phase A used was : Sodium Acetate buffer and Mobile Phase B used was : Buffer A + Acetonitrile. Test samples were run in triplicates.

$$\frac{\text{Peak area of AA in Test} \times \text{Concentration of AA Standard (pm)}}{\text{Peak area of AA in Standard}}$$

X

$$\frac{\text{Molecular Weight of AA}}{\text{Concentration of Test Sample (mg)} \times 10^3}$$

RT-PCR/QPCR

Total RNA was extracted 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 suspended in diethyl pyrocarbonated (DEPC) treated water and sample purity and concentration were measured on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -80°C. The purity of the extracted RNA was assessed by UV absorbance and the OD_{260/280} ratios for all samples were >1.9. The RNA concentrations ranged from 2.0 to 2.5 µg /µl. 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 Mastercycler (Eppendorf, Hauppauge, NY) for 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and then at 4°C. Further cDNA samples were stored at -20°C. cDNA samples were diluted 1: 1 prior to RT-PCR analysis. Each reaction consisted of 1 µL diluted cDNA, 1.0 µL forward primer, 1.0 µL reverse primer, 7 µL DEPC water, and 10 µL Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher SCIENTIFIC,). Genes and the primer sequences used for the RT-PCR assays are listed in Table 2. The primers were got 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, 72°C for 30 s. Both control and treatment values were normalised against b actin and further treatment values were normalised against control. The relative quantification (RQ) was expressed as a ratio of the target gene to reference gene (b- Actin) using the delta-delta Ct (^{ΔΔ} Ct) method [28]. All samples were run in triplicate.

The total Se content of the basal diets (Fresh) and treatment diet was analysed using inductively coupled plasma MS (ICP-MS; Agilent 7500cx, Agilent Technologies, Tokyo, Japan). The procedure followed was as given by King and Sheridan [29].

Table 2: Primer sequences of the genes.

Gene	Sequence of primers	Product bp length	NCBI Reference Sequence
CAT1	GACACTCATGATGCCT-TACTACCAG-F GAGAAGTCCATCTTCT-GCCATAGC-R	190	NM_001145490.1
b ^o AT	GCATATGATGGATG-GAATCAACTC-F GACTGCAGGAGTTCT-GTTGAAGTC-R	167	NM_001199133
y ⁺ LAT 2	CTAGCCAGGCGTTATT-GCAATCAC-F TTTACATAGGCACAATT-TACGAATG-R	145	XM_001231336.5
LAT 4	GTCTTCAGCCCATCTT-GTTGCTCAGC-F TGCTGTCAGCAGGCA-CAGCAGTTGC-R	177	XM_415803.6
MTNR1C	ATCGCAATCAACCGG-TAC-F CAAGGACCCAAC-GAAGAA-R	144	XM_013100494.3
GHSR	CTGCAAGCTCTTC-CAGTTCATCAGC-F CCAGAGGATGAGGAT-GACCAGCTTG-R	160	AB095994.1
Actin b	CGGACTACCTCATGAA-GATCCTGAC-F GCCAATGGTGATGACCT-GACCATC-R	197	NM_205518.2

F: Forward, R: Reverse

Recording of bodyweight, egg weight, egg production

Bodyweight was recorded at weekly interval during EP and MP. Mean ±SE values were estimated for twelve number of birds from each group.

Percentage of egg production for a week was calculated as Total no. 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 estimation of egg weight, 10-12 eggs were collected randomly everyday from each group, eggs were weighed on electronic balance and later mean values were calculated.

Statistical analyses

Analysis of variation (ANOVA) was computed for the data generated in completely randomized design (CRD) for all the parameters separately. Mean, Standard Error of Mean (SEM) and coefficient variations (CV%), were calculated. The least significant difference (LSD) at 5% level was used as post hoc treatment mean comparison. The statistical analysis was carried out using PROC GLM syntax in SAS software (version 9.3). t-test was also conducted for comparison of mean values of individual plasma amino acids between the control and treatment groups of EP and MP.

Results

Wherever values are mentioned as significant for hormones, BW and egg production % they are significant at least at $P < 0.05$.

During EP (24-28 weeks of age) the mean concentration of hormones when compared across the weeks, MET, EST increased by 100% and 54.5% whereas the level of plasma GHL and PROG decreased by 49.2% and 8.26% significantly within the respective CGs with increase in the age of the birds. (Table 3). Upon treatment with additional Se (TG), Mean levels of plasma MET, GHL and EST increased significantly ($P < 0.01$) by 85.7%, 55.68%, 17.90% and level of PROG decreased by 35.7% significantly across weeks. In the TG with increase in the age of the birds except for PROG mean levels of all hormones increased (Table 3). At the mid laying period (MP, 32-36 weeks), in the CG birds the mean levels of MET, GHL EST and PROG were exhibiting significant decrease, in plasma levels by 58.4%, 55.08%, 15%, 61.1% respectively across the weeks. At MP in TG birds mean MET, GHL, PROG hormones decreased except for EST from 32-36 weeks of age by 40.69%, 44.84%, 40%; whereas the conc. of estradiol increased by 11.34%. significantly, at MP, (Table 3). When compared between the CGs of EP and MP, MET, GHL, EST, PROG decreased by 7%, 27%, 41% and 38% respectively. When BW of the CGs was compared between EP and MP across weeks BW increased significantly at EP (11.0%) and MP (4.56%) with increase in age of the birds. Upon treatment with Se when compared between the weeks, the mean BW (TG) increased at EP (14.56%) and MP (6.1%) significantly. The mean egg production percentage of CG birds increased by 21% significantly ($P < 0.01$) during EP and decreased at MP by (0.3%) across the weeks (Table 3). When mean

egg production percentage of TG birds were compared across the weeks, production increased significantly by 11.76% ($P < 0.05$) at EP, whereas at MP by 1.66 % ($P < 0.05$) (Table 3).

When levels of different parameters were compared between the CG and TG groups, it was observed that supplementation of additional Se (0.3ppm) caused significant decrease in the plasma levels of MET (4.9%) at EP (Table 3). Similar effect of Se supplementation was observed on plasma GHL, estradiol and progesterone levels. The percent decrease in the concentration of GHL (33.6), estradiol (47.1) and PROG (46.0) during EP was significant, but at MP supplementation of Se (TG) caused significant increase in the levels of MET (46.02%), EST (24.56%), PROG (76.1%) whereas increase in GHL levels (4.83%) was not significant when compared to respective CGs (Table 3). The bodyweight of the birds (TG) increased significantly at both EP (3.02%) and MP (3.18%, $P < 0.01$), whereas egg production (TG) increased significantly by 4.4% at EP and 1.3% at MP when compared to respective CGs (Table 3). At EP the treatment effect was not significant on Mean \pm SEM EW (44.8vs44g) but at MP the EW increased by 2% (51.3vs.53.4,)

Footnotes for table 3

MET-melatonin, GHL-ghrelin, EST-estradiol, PROG-progesterone, BW-body weight Egg%-egg production percentage. EP- Early laying period (24-28 weeks of age), MP- Mid laying period (32-36 weeks of age). Values are expressed as Mean \pm SEM for different weeks. Mean values with different superscripts when compared between the weeks with in CG and TG for respective EP and MP with in a column are significantly different. Mean \pm SEM values with different superscripts between CG and TG of respective EP and MP with in a row are significantly different from each other. CG-Control group (basal diet including 0.3ppm of sodium selenite), TG-Treatment group (Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast). N=6 for hormone parameters. N = 12 for BW and Egg%.

Mean values with different superscripts are significant at least at $P < 0.05$.

Plasma amino acids

When compared between CGs of EP and MP, out of 15 plasma amino acids analysed, the concentration of alanine, threonine, tryptophan, histidine, phenyl alanine was more at EP and was significant whereas at MP concentration of glutamic acid, threonine, valine, serine, asparagine, methionine, tyrosine and lysine increased significantly (Figure 1a and b). The difference in concentration of other as was not significant.

Table 3: Level of plasma hormones melatonin, ghrelin, estradiol, progesterone, body weight, egg production during early and mid laying period of Vanaraja breeder hens.

Age(wks)	Early Laying Period (EP)											
	MET (pg/ml)		GHL (ug/ml)		EST (pg/ml)		PROG (ng/ml)		BW (g)		Egg %	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
24	302.3 ^a	276.7 ^a	40.2 ^c	14.1 ^a	110.5 ^a	71.5 ^b	2.42 ^c	1.4 ^c	2336.6 ^a	2352.8 ^a	50.9 ^a	59.3 ^a
26	407.4 ^b	477 ^b	25.6 ^b	19.3 ^b	139.7 ^b	67.4 ^a	1.90 ^a	1.22 ^b	2488.1 ^b	2588.4 ^b	70.7 ^b	74.3 ^b
28	623.2 ^c	513.9 ^c	19.9 ^a	21.9 ^c	169.1 ^c	84.4 ^c	2.22 ^b	0.92 ^a	2593.8 ^c	2695.6 ^c	71.5 ^c	70.5 ^b
SEM	2.60	2.57	1.07	0.48	2.6	1.67	0.06	0.03	8.58	8.47	1.12	1.03
LSD	7.86	7.76	3.41	1.58	7.92	5.05	0.20	0.11	24.91	24.6	3.25	3.02
Mean	444.3 ^a	422.5 ^b	28.48 ^a	18.89 ^b	141.03 ^a	74.59 ^b	2.17 ^a	1.17 ^b	2470.7 ^a	2545.6 ^b	64.1 ^a	68.5 ^b
SEM	1.49		0.65		1.26		0.03		6.36		0.62	
LSD _{0.05}	4.33		1.91		3.67		0.09		13.97		1.38	
Age (wks)	Mid Laying Period (MP)											
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
32	599.3 ^c	764.1 ^c	35.4 ^b	26.7 ^b	88.4 ^c	98.7 ^a	1.88 ^c	2.5 ^b	2768.2 ^a	2806.1 ^a	61.9 ^a	62.4 ^a
34	387.8 ^b	588.0 ^b	13.3 ^a	24.9 ^b	87.1 ^b	99.7 ^b	1.51 ^b	3.0 ^b	2812.3 ^b	2961.9 ^b	63.9 ^a	65.3 ^b
36	249.05 ^a	453.1 ^a	15.9 ^a	14.1 ^a	74.8 ^a	109.9 ^c	0.73 ^a	1.5 ^a	2896.5 ^c	2979.4 ^b	61.7 ^a	64.2 ^b
SEM	2.58	4.90	0.92	1.14	1.63	1.76	0.05	0.17	8.46	17.9	0.76	0.78
LSD	7.71	14.8	2.95	3.63	4.9	5.30	0.18	0.55	24.58	52.06	2.21	2.28
Mean	412.1 ^a	601.7 ^b	21.5 ^a	21.9 ^a	82.6 ^a	102.9 ^b	1.34 ^a	2.4 ^b	2825 ^a	2915 ^b	62.5 ^a	63.8 ^b
SEM	2.26		0.51		0.97		0.07		10.45		0.22	
LSD _{0.05}	6.54		1.50		2.83		0.22		22.96		0.94	

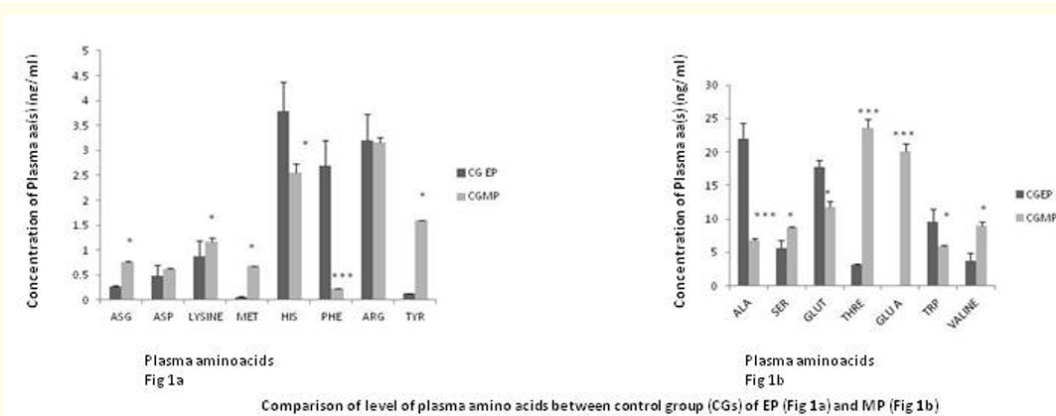


Figure 1a and b: Comparison of level of plasma amino acids between control groups (CGs) of early (EP, 26 weeks) and mid laying (MP, 34 weeks) period of Vanaraja breeder hens. ASG-Asparagine, ASP- Aspartic acid, LYS-Lysine, MET-Methionine, HIS- Histidine, PHE-Phe-nylalanine, ARG-Arginine, TYR-Tyrosine, ALA-Alanine, SER-Serine, GLUT- Glutamine, THRE- Threonine, GLU.A- Glutamic acid, TRP- Tryptophan, VAL-Valine, *P<0.05, **P<0.01, ***P<0.001 CG-Control group(basal diet including 0.3ppm of sodium selenite),

Upon treatment with Se, concentration of eight plasma amino acids, methionine, tyrosine, tryptophan, glutamic acid, histidine, arginine, valine, glutamine increased significantly at EP; six amino acids were not significantly different from CG and one decreased

(Figure 2a and b), whereas at MP, eleven number of amino acids increased, two decreased and rest without any significant change. Treatment with selenium had similar effect in increasing the number of essential amino acids (Figure 3a and b).

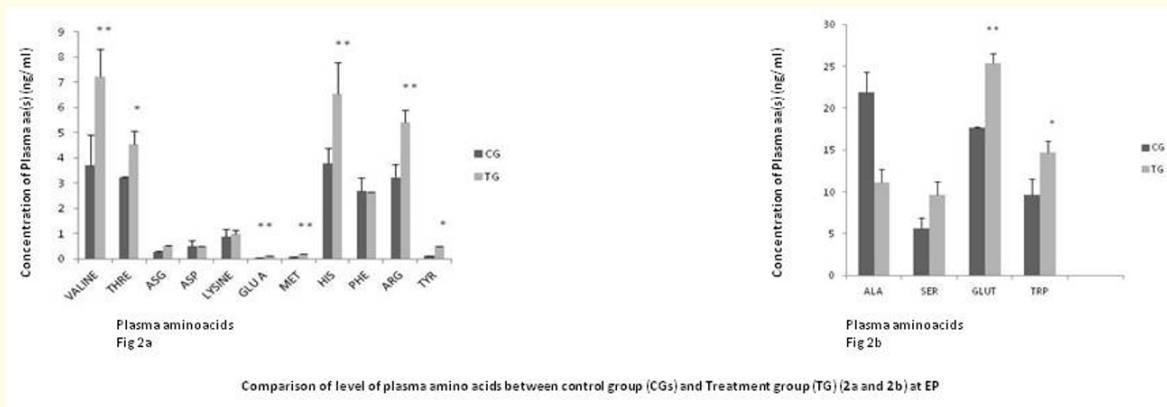


Figure 2a and b: Comparison of level of plasma amino acids between control (CG) and treatment group (TG) during early (EP, 26 weeks) laying period * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 6$. CG-Control group(basal diet including 0.3ppm of sodium selenite), TG-Treatment group(Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast).

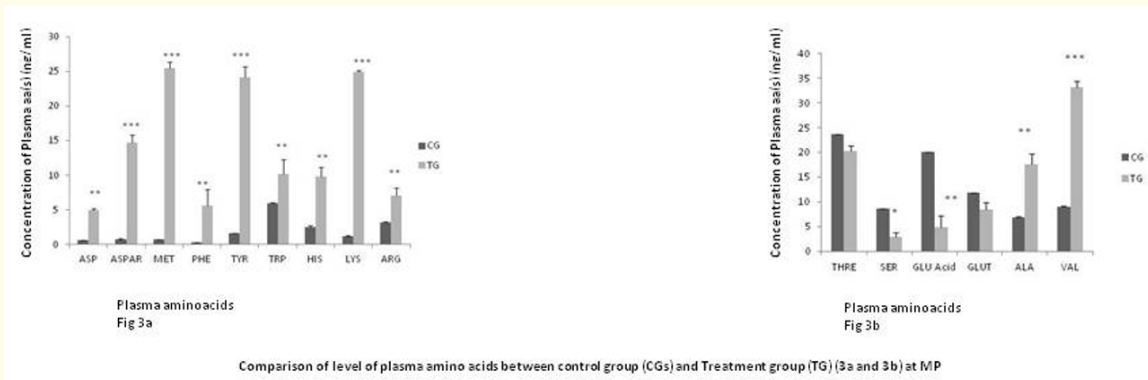


Figure 3: Comparison of plasma amino acids between control (CG) and treatment group (TG) during mid (MP, 34 weeks) laying period * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 6$. CG-Control group(basal diet including 0.3ppm of sodium selenite), TG-Treatment group(Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast).

Expression of amino acid transporters and hormone receptors

When fold change expression of amino acid transporters namely LAT 4, LAT2, CAT and b^oAT and hormone receptors for MET and GHL between CGs of EP and MP were analysed for jejunum and magnum tissue, it was observed that in the jejunum, relative expression of all aats except CAT and MNTR ($P < 0.05$) was significantly more where as expression of GHRL ($P < 0.01$) was less at EP compared to gene expression of respective CGs at MP (Figure 4a). In the magnum tissue the relative fold change expression of LAT4 ($P < 0.01$) and b^oAT ($P < 0.001$) and also expression of MNTR ($P < 0.01$) GHLR ($P < 0.001$) receptors in the CG birds decreased at EP whereas expression of only CAT increased, when compared to respective CGs at MP (Figure 4b and 4c).

When compared between CG and TG (Se treated group), fold change expression of CAT ($P < 0.001$) and LAT2 ($P < 0.05$) increased significantly, whereas expression of LAT4 ($P < 0.05$) and

boAT decreased in the jejunum tissue in the TG. Relative expression of MNTR and GHRL also decreased in jejunum tissue at EP in the TG (Figure 5a). In the magnum tissue, expression of LAT2, LAT4 ($P < 0.01$) and CAT ($P < 0.001$) increased and that of boAT decreased in the TG. The fold change expression of MNTR decreased and that of GHLR increased ($P < 0.001$) when compared to CG at EP (Figure 5b).

At MP, with Se treatment, in jejunum, significant increase ($P < 0.001$) in the expression of LAT4 and decrease in the relative expression of other aats observed was not significant. Upon treatment expression of hormone receptors also did not significantly change with respect to respective expression of receptors in CG (Figure 6a). In the magnum tissue of TG except for LAT2 ($P < 0.001$), expression of other aats decreased ($P < 0.01$) with no significant effect on the expression of hormone receptors when compared to CG at MP (Figure 6b).

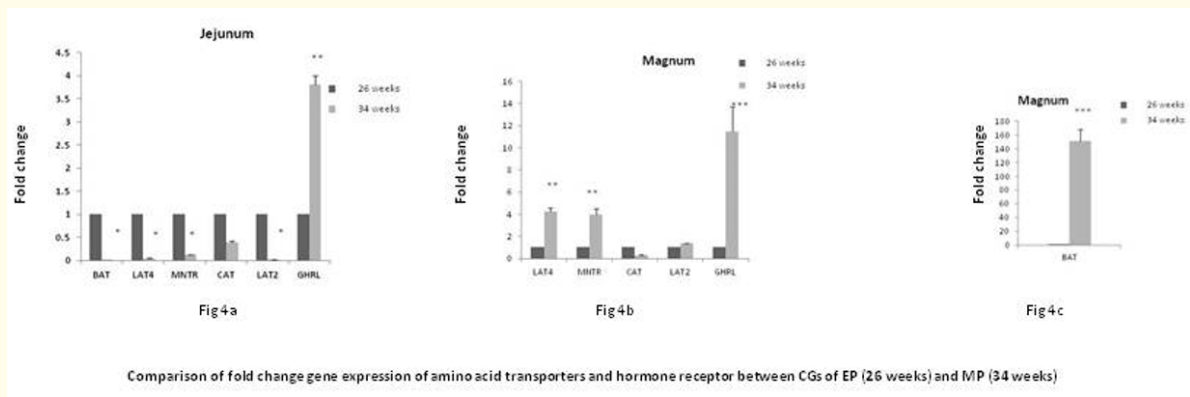


Figure 4a,b and c: Comparison of fold change gene expression of amino acid transporters (boat, CAT, LAT2, LAT4) and melatonin receptor (MNTR) and ghrelin receptor (GHLR) in the(a) jejunum and (b&c) magnum tissues between control groups (CGs) of early (EP, 26 weeks) and mid laying (MP, 34 weeks) period of Vanaraja breeder hens. N=6. Reference gene – b Actin, *P<0.05, **P<0.01, ***P<0.001. CG-Control group(basal diet including 0.3ppm of sodium selenite), TG-Treatment group(Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast)

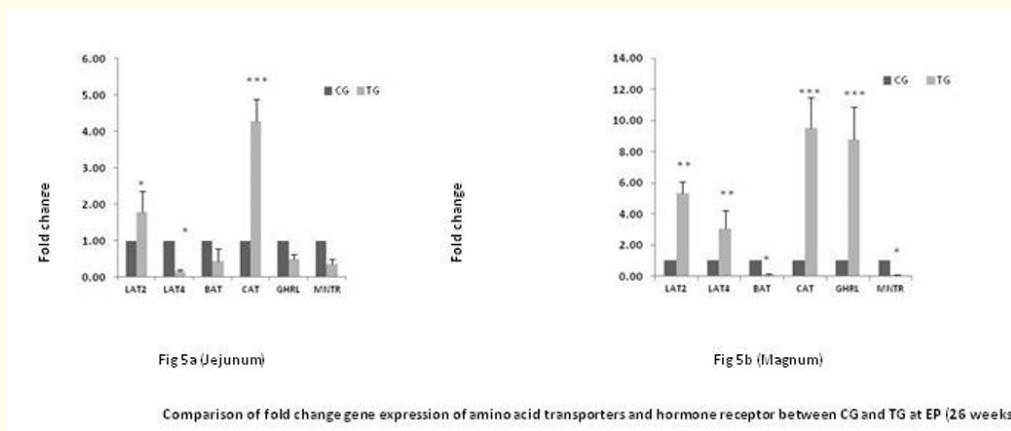


Figure 5a and b: Comparison of fold change gene expression of amino acid transporters (boat, CAT, LAT2, LAT4) and melatonin receptor (MNTR) and ghrelin receptor (GHLR) in the(a) jejunum and (b) magnum tissues between control (CG) and treatment group (TG) during early (EP, 26 weeks) laying period *P<0.05, **P<0.01, ***P<0.001. N=6. Reference gene – b Actin. CG-Control group(basal diet including 0.3ppm of sodium selenite), TG-Treatment group(Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast).

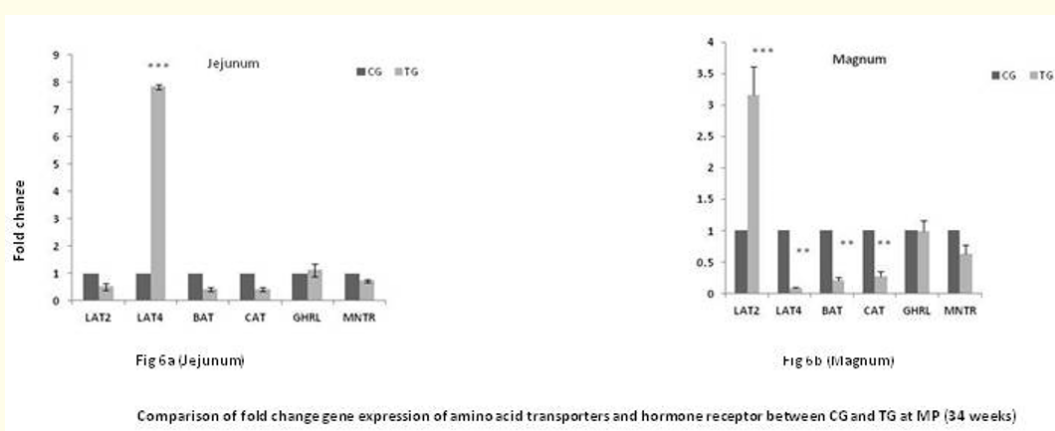


Figure 6a and b: Comparison of fold change gene expression of amino acid transporters (boat, CAT, LAT2, LAT4) and melatonin receptor (MNTR) and ghrelin receptor (GHLR) in the(a) jejunum and(b) magnum tissues between control (CG) and treatment group (TG) during mid (MP, 34 weeks) laying period *P<0.05, **P<0.01, ***P<0.001. N=6. Reference gene – b Actin. CG-Control group(basal diet including 0.3ppm of sodium selenite), TG-Treatment group(Same as feed offered to CG with additional supplementation of 0.3ppm of selenium enriched yeast).

The Seleno yeast content in the feed samples (Fresh) ranged from 0.25-0.28mg/kg for CG where as for TG the concentration ranged from 0.54-0.58 mg/kg.

Discussion

In the present study significant differential levels of hormones, expression of receptors, amini acid transporters was observed between two different laying periods. Further selenium could efficiently modulate the levels of mentioned physiological parameters affecting production performance positively. It is known that Se and MET when administered together it reduces oxidative injury and increases antioxidant status in tissues [2,30]. During the early laying period, the concentration of melatonin was more when compared with the mid laying period when compared between respective control groups but was not significant. The concentration of plasma MET increased with increase in age of birds during EP but decreased during MP with increase in age of birds. The concentration of MET in the blood was inversely proportional to the age of chickens, In the present study, with increase in age from early to mid-laying period the level of plasma hormone might have decreased. The influence or turnover of MET hormone may be more and synthesis less at MP than at EP when compared between respective CGs resulting in less concentration of hormone at MP. The level of plasma MET decreased during MP in CG, so also the expression MET receptors, in jejunum but increased in magnum during MP when compared between respective CGs of EP and MP, indicating up regulation of receptors and making magnum more sensitive to the hormone. Hence Se treatment (TG) did not have significant effect on MET receptors in jejunum and magnum during MP. Literature with respect to regulation of MLTR1c (melatonin receptor in birds) by MET is not available. Melatonin treatment increases egg weight and egg laying rate [31]. Melatonin has been shown to have direct effects on ovarian function and microbiota [5]. In the present study also upon treatment with Se, decrease in the plasma level of hormones at EP as envisaged as more activity of hormones for its functions, and increase at MP might have affected the production parameters positively. Treatment with Se, caused significant increase in bodyweight with increase in plasma MET concentration, it can be suggested that in CG at MP, MET might have greater role in regulation of BW and EW than for egg production in Vanaraja chickens leading to decrease in plasma levels of MET.

At EP in CG, level of GHL hormone decreased as the age of the birds increased and vice versa in TG, when compared between the groups at respective weeks, the concentration was less in TG compared to CG at respective weeks. The expression of GHLR also

decreased in jejunum in TG during EP, while in the magnum the expression of GHLR increased in TG indicating more sensitivity for hormone by the magnum tissue. The concentration of hormone decreased in both the CG and TG at MP with increase in age of birds with no significant change between mean plasma levels when compared between the groups. The Fold change in the expression of GHL receptor was also not significant when compared between CG and TG. When compared between the CGs of EP and MP, expression of GHLR was significantly upregulated at MP in both tissues where the mean level of plasma GHL was also significantly less. Hence treatment with Se did not have significant effect on GHLR during MP. It is reported that ghrelin is required for reproductive functions [32], and further may be independent of its receptor expression [4].

It is well known that when hormone levels increase expression of receptor decreases. When compared between the CGs of EP and MP, the level of respective hormones decreased during MP when compared to EP phase, hence the expression of GHLR and METR increased during MP. The turnover or utilisation of hormones for production activity may be more at MP than EP for growth, as revealed in the present study.

Laying hens absorb lot of nutrients to form eggs during the peak laying period, which commonly results in intestinal villus damage and intestinal mucosa shedding [33]. Ghrelin and melatonin are known to have protective effects on the gastrointestinal tract [34,35]. Hence the utilisation of these hormones may be more during MP compared to EP, hence level of hormones when compared between respective CGs, may be less at MP compared to EP. Hence increased levels of MET and GHL upon supplementation during MP, may have beneficial effects on the functions of digestive tract also helping in more digestion and absorption of nutrients.

EST a steroid hormone has a role in regulating liver, bone metabolism and inflammation besides maintaining quality of eggs [11,36]. The majority of plasma estrogen is from the ovarian follicle secretion. During EP, treatment with Se lead to less concentration of plasma hormone, indicating sufficient availability or more utilisation of Estrogen hormone. Body weight and egg weight increase during MP compared to EP, hence less metabolic energy is required during EP compared to MP. When compared between respective CGs, the circulatory levels of estrogen are decreasing at MP when compared to EP and egg production was marginally less (0.5%). This may also indicate more utilisation of estrogen hormone during MP for other physiological functions and increase due to Se supplementation during MP, might prove beneficial for

maintaining shell thickness and other factors as regulated by estrogen. Estrogen is required for activity of carbonic anhydrase enzyme and shell thickness also. The rise in circulating estrogen is helpful in switching from bone formation to medullary bone [37]. in laying hens, and increase in estradiol levels upon Se supplementation during MP may be beneficial. When compared across weeks both EST and MET is increasing in CG and TG at EP being favourable for increase in percent egg production and BW at EP. At MP treatment is increasing EST hormone levels when compared to CG across weeks and also when compared between respective weeks, which might have increased egg production, egg weight and body weight significantly. Estradiol, progesterone and testosterone have been detected in the eggs of different breeds [38]. Although in the present study effect on egg parameters have not been conducted, and egg production was similar at EP and MP in CGs, requirement of these hormones for maintaining egg quality might have increased as the weight of the eggs increased significantly (44vs.51g) as the birds reached MP stage and additional Se supplementation proved beneficial in increasing level of the steroid hormone and egg weight during MP.

When analysed for PROG hormone the concentration decreased during EP and MP for both CG and TG across the weeks. Supplementation decreased the level during EP but increased during MP when compared between respective weeks, this is because at MP the levels of CG is less when compared to EP, hence supplementation might have increased the levels at MP. Similar were the observations when mean levels of hormone were compared between CG and TG at EP and MP respectively. Supplementation is increasing utilisation of PROG at EP and increasing the lower levels of hormone at MP. This in turn increased egg production % and BW at EP and at MP in addition to the two production parameters mentioned even the EW increased. A positive feedback mechanism exists between PROG and LH for ovulation, which results in positive correlations between PROG and egg production in layers [39]. However decrease in plasma PROG at EP upon supplementation with Se can be considered as resulting in greater utilisation of hormone for physiological activities, which might have led to higher mean egg production percentage and mean total body weight.

The concentration of most of the mentioned plasma amino acids increased in Se treated groups during EP (8/15) and MP (11/15), greater during MP, indicating provision of more amino acids for metabolic processes. This indicates that upon Se supplementation, concentration of most of plasma amino acids increased during MP including essential aas out of which essential amino acids like MET,

VAL, LYS, TYR, TRP increased, hence expression of most of the aats in the jejunum and magnum might have decreased when compared to the fold change expression of respective aats at EP, during which more number of aats were expressed. Expression of amino acid exchanger CAT and LAT2 in jejunum, where as in magnum, LAT2, LAT4 and CAT increased significantly in TG indicating the requirement for increased egg production and body weight during EP when compared to CG. Even though the percentage of egg production was not significantly different between the CGs of both EP and MP, the increase in the expression of amino acid transporters and hormone receptors was already significantly more in the magnum of CG during MP compared to respective CG of EP hence stimulatory effect of Se on aats might not be evident during MP. It was observed that without supplementation (CGs) fold change in the gene expression of all the parameters increased in the magnum tissue, as the birds moved from EP to MP period, indicating the greater requirement for maintenance of magnum structural integrity and production performance at MP. At MP upon supplementation availability of greater concentration and more number of aas might have been used for growth as evident by significant increase in BWs and also egg weight when compared to respective weights of CG. When compared between TGs of EP and MP expression of more number of aats in jejunum and magnum during EP was observed, might have been beneficial for increase in egg production and body weight. However production of eggs is not solely dependent on amino acid transport but they are required for protein synthesis in egg formation. It is reported that methionine, lysine, threonine, arginine, isoleucine and valine with the last three being limited essentially equally [17] based on maize and soy fed chickens. However, because the L system usually has a high transport capacity, presumably due to a high level of expression, even a poor substrate may achieve a significant flux via system [40]. The same at 34 weeks of age expression of only one L system aat increased upon Se supplementation, hence might have not contributed to deficiency of amino acids. Mucins are glycoproteins that contain high amounts of threonine, and serine in their peptide backbones and are the major components of the intestinal mucosal barrier responsible for protecting the gastrointestinal tract [41]. It was observed that upon supplementation the concentration of the mentioned aas increased in plasma at EP whereas decreased at MP this may be because their concentration in plasma is already significantly more in CG of MP when compared with the respective CG of EP, indicating the homeostasis of aas maintained for the integrity of tissues. It can also be that, their expression is at a sufficient level at the later stage.

Since the BW of CG increased significantly at MP when compared between the CGs of EP and MP, aas might have been used more for growth during MP besides egg production, it is also known that as the age advances BW increases. Hence might have led to marginal decrease in percent of egg production in CG of MP. Lu et al [42]. reported that diets containing with excess Se addition in the diet upto 3mg/kg, to layers from 30-42wks of age, did not adversely affect the various physiological parameters. In the present study also, doubling the amount of Se @ 0.6ppm in the diet, might not have caused adverse effects. Since Vanaraja is a cross between Red Cornish and PD2 (Lineage of synthetic broilers), it has a broiler gene, hence additional supplementation of Se might have greater impact on body weight. Literature available with respect to role of selenium in influencing the growth of layers or breeder hens is less. Se is known to deposit in muscles, promote growth performance and improve feed efficiency, nutrient digestion etc [22,43]. Hence in the present experiment also in the TG Se supplementation might have increased body weight.

Selenium may be affecting body weight of hens indirectly by modulating the levels of plasma hormones and antioxidant enzymes [44-46]. which are known to have important role in regulating the production parameters. Similar may be the effect of Selenium in the present study in chickens.

In the present study supplementing Se for five weeks during EP increased mean percentage of egg production and was also maintained during MP without causing any adverse effect and also as reported by Thiry, *et al.* [47]. However in the present experiment, additional dose of selenium supplemented was less. The present study is the first report that Se supplementation affects genes associated with amino acid transporters and hormone receptors in Vanaraja variety of chickens. Very little information is available defining the metabolic and role of Se with endocrine hormones in female reproductive tissues. Data from the present study support the hypothesis that Se, especially in the organic form, is necessary for optimizing energy production, growth reproduction and gene expression in Vanaraja breeder reproductive tissues. As an essential mineral element, the requirement of Se for laying hens is relatively low, about 0.3 mg/kg in diets, whereas once as a nutritional additive, its supplementation can be elevated to increase bio efficiency as suggested by Gladyshev, [48].

Conclusion

It can be concluded that the concentration of plasma hormones varied between EP and MP and also the gene expression of receptors and transporters. An additional amount of Selenium modulated all the physiological parameters differentially at early and

mid laying period, resulting in increase in production performance significantly at EP and MP respectively.

Limitation of the study was literature available with respect to mentioned endocrine and gene expression studies with respect to production performance is scanty. Number of parameters studied was more, so, single dose of selenium was used. Hence in future, studies should be replicated with larger number of birds with greater number of doses.

Author Contributions

NAL, KSRR, SJK, MRK, MS: Study concept, design, interpretation of data; NAL,MRK, SJK: drafting of the manuscript and preparation of figures; MRK, MS: Analysis of hormones and participated in writing of manuscript; KSRR: Provided facilities for conducting experiments. SJK: Statistical analysis of gene expression data, preparation of graphs for gene expression studies. NAL: Overall supervision and conducting experiments.

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Institutional Review Board Statement

Approval for conducting the experiments was taken from Institute Ethics committee IAEC-DPR, 2019 (IAEC/DPR/19/3). Regn. No.355/GO/RBi/S/01/CPCSEA.

Data Availability Statement

The data presented in the study are included in the article/ Supplementary material, further enquiries can be directed to the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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