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Effect of Selenium-Enriched Azolla on Biochemical, Antioxidant, Immune, and Reproductive Responses in Japanese Quail Breeders

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ABSTRACT:

Background: Poultry diets with inorganic selenium supplementation have been controversial, as any imbalances in its normal levels may lead to health problems in quails. **Objective:** There is ample evidence showing the feasibility of enriching eggs using organic selenium. The current study was designed to assess the effect of dietary organic selenium (Se) supplementation on biochemical and reproductive performance in Japanese quail breeders. **Methodology:** A total of 525 quails aged 10 weeks were used for experimental purposes. They were divided into 7 treatments, each treatment with 75 quails, among 75 quails 50 females and 25 male birds. In each treatment, 75 quails were divided into 5 replicates. Each replicate was provided with a common feeder and water *ad libitum*. This trial continued for 4 periods, each period containing 28 days with a 16:8-hour light and dark cycle. **Results:** Feed intake, feed conversion rate, serum biochemical parameters, antioxidant enzyme activity, egg production and hatchability were recorded during the study. Dietary organic selenium significantly improved serum biochemical indices, antioxidant enzyme activities, egg production, and hatchability compared to inorganic selenium. **Conclusions:** Supplementing 0.2 ppm selenium-enriched azolla feeds is suggested to produce Japanese quail breeders with better reproductive performance and an enhanced immune system against diseases.

KEYWORDS: Immune response, Japanese quail, Organic selenium, Reproductive performance, Serological parameters.

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1. INTRODUCTION

Selenium (Se) is a micromineral that is required for many important physiological functions in farm animals, including poultry, such as reproduction, immunity, growth, and development. [1] Selenium plays many critical roles in the body, including serving as a constituent of the antioxidant defense system through glutathione peroxidase (GPx), thioredoxin reductase, and methionine sulfoxide reductase. Selenium supplementation in livestock diets is a common and well-proven practice. Poultry diets contain both organic selenium (selenomethionine) and inorganic selenium (selenite or selenate). The organic form of selenomethionine (Se-Met) provides optimal selenium reserves in the body and is effectively moved into the egg, colostrum, and milk. [1] One important application of organic selenium is to protect developing avian embryos from peroxidation during embryogenesis and hatching. [2] Previous studies reported that selenium is transferred from the hatching egg to the embryo and affects the antioxidant defense system in the body of the chick during hatching and postnatal life, egg freshness, and eggshell thickness. [1] Selenium toxicity (Selenosis) caused by high selenium concentrations has been reported in invertebrates and fish. [3] Selenium supplementation up to 0.3 ppm in feed is permitted in developed countries, and the FDA permits selenium-enriched yeast. [4] Plants cannot absorb the desired quantity of selenium from the soil in countries or areas where selenium concentration is low in the soil, and natural feed, cereal grains such as maize, rice, wheat, and soybean contain lower amounts of Se. As a result, farmers use a commercial form of organic Selenium in natural feed to meet the birds' needs. However, there is still very little information available about the optimum level of Selenium required as supplementation in Japanese quails. Considering the above facts, the present study was designed to evaluate the effects of selenium-enriched Azolla supplementation on biochemical parameters, antioxidant status, immune response, reproductive performance, and hatchability in Japanese quail breeders.

2. MATERIALS AND METHODS

Materials: Japanese quails were procured from poultry experimental station, Rajendra Nagar, Hyderabad.

Albumin estimation was done by adding the first 1ml of albumin reagent to the blank, standard, and Test. Then 0.01ml of distilled water to the blank, 0.01 ml of standard to the standard, and 0.01 ml of test sample to the test. After mixing, it was incubated for one minute at 37^0C the absorbance of the standard and test sample was read at 580nm against the blank. Then albumin concentration was calculated by the formula: OD of test /OD of standard multiplied by concentration of standard. (Erba Mannheim LIQUIXX Albumin Kit Code:120223)

Glucose assay was performed with Blank, Standard and Test. Initially 0.01ml of distilled water in blank, and same quantity of standard in standard, and test sample in Test. Later, 1 mL of Enzyme reagent was added to the blank, standard, and Test. After 10 minutes of incubation at 37^0C, standard and test were read against blank at 505nm. Then the OD values were substituted in the formula. OD of test /OD of standard multiplied by concentration of standard. (Erba Mannheim LIQUIXX Glucose Kit Code:120235), Calcium estimation was done with blank, test, and standard; then 1 mL of working reagent was added to the blank, test, and standard. Mix well, then the standard and test were read against the blank at 578 nm. Then the OD values were substituted in the formula: OD of test /OD of standard multiplied by the concentration of standard. (Erba Mannheim LIQUIXX Calcium kit Code:120225). Similarly, the phosphorus assay was performed by adding 1000 µl of reagent 1 to all tubes. To the blank, 20 µl of distilled water was added; to the standard tube, 20 µl of standard; and to the test tube, 20 µl of sample. After mixing and incubation at 37°C for 5 minutes, absorbance was measured at 340 nm. OD values were used in the formula: OD of test /OD of standard multiplied by the concentration of standard. (Erba Mannheim LIQUIXX Phosphorus kit 120229),

Protein estimation was done with blank, standard and test, initially 1ml of reagent was added to all and then 0.02ml ml

of standard in standard and then 0.02ml of test sample in test. After 10 minutes of incubation at 37^0 C, OD was read at 546nm against blank. Then the OD values were substituted in the formula: OD of test /OD of standard multiplied by the concentration of standard. ((Erba Mannheim LIQUIXX protein Kit Code :120231). Lipid parameters like cholesterol were estimated using the Allain et al and the modification of Roeschlau with further improvements to render the reagent stable in solution, as explained in the Erba commercial diagnostic kit Product (XSYS0070) instructions. In the cholesterol assay, 1 mL of working reagent was initially added to the blank, standard, and test. Then, 0.01 mL of distilled water was added to the blank,0.01 mL of standard to the standard, and 0.01 mL of test sample to the test. Mix and incubate for 5 min at 37^0C. Read the absorbance of standard and test against blank at 505 nm. OD values were substituted in the formula: OD of test /OD of standard multiplied by the concentration of standard. ((Erba Mannheim LIQUIXX Cholesterol Reagent Test Kit 14343) and Serum triglycerides were estimated using the colorimetric enzymatic method explained in (Erba Mannheim LIQUIXX Triglycerides kit 120250). Triglyceride estimation was done by using Blank, standard, and Test. Initially, 1 mL of working reagent was added to all tubes. Then 0.01ml of distilled water in the blank, 0.01 ml of standard in the standard, and 0.01 ml of test sample in the test. After mixing, incubate for 10 min at 37^0C. Read the absorbance of the standard and the test against the blank at 546nm. OD values were substituted in the formula: OD of test /OD of standard multiplied by the concentration of standard.

Experimental setup: This study was approved by the institutional ethical committee (1/2019-1/IAEC/CVSc, Hyderabad). A total of 525 quails at 10 weeks of age were used. Each experimental diet (Table 1) and water *ad libitum* were provided during the experimental period. They were further divided into 7 treatments as described in Table 2. Each treatment had 75 quails across 5 replicates, comprising 50 females and 25 males. Each replicate contains 5 cells; in each cell (cages designed for housing quails) two females and one male bird were distributed. Birds which are present in same replicate receive same diet and they were shared same feeder. The study continued for 4 periods, each period containing 28 days with a 16:8 hour light and dark cycle.

Table 1: Feed Composition of basal diet for Japanese quail breeders for 1000 Kg as per National Research Committee NRC 1994

S. No.	Ingredients	Kilograms
1	Maize	509.050
2	Rice Bran oil	11.000
3	SoyaDOC 44%	370.000
5	Stone grit	85.000
6	DCP	9.000
7	L-Lys HCl	1.100
8	DL-Methionine	3.500
9	Tryptophan	0.250
10	Salt	4.500
11	Phytase 5000 Ch, Gr	0.100
12	Galzym PB	0.350
13	Lipidin	0.500
14	Trace mineral	2.000
15	Biocholine	1.000
16	Herbal C	0.200
17	Herbal E	0.200
18	Nutriox	0.150
19	Texpro 5	0.100
20	Livoliv DS	0.500
21	Butirex C-4 (Avitech)	0.500
22	Toxin Binder	1.000
	Total	1000 Kg

Table 2: Experimental Groups

S. No.	Treatments		Dosage of Selenium
1	Treatment (T_1)	1	Basal diet
2	Treatment (T_2)	2	Basal diet +0.2 ppm org selenium
3	Treatment (T_3)	3	Basal diet +0.3 ppm org selenium

4	Treatment (T_4)	4	Basal diet +0.4 ppm org selenium
5	Treatment (T_5)	5	Basal diet + 0.2 ppm inorganic selenium
6	Treatment (T_6)	6	Basal diet + 0.3 ppm inorganic selenium
7	Treatment (T_7)	7	Basal diet + 0.4 ppm inorganic selenium

Feed intake, feed conversion rate, serum biochemical parameters, immune responses, and hatchability performance were recorded regularly throughout the experimental period (from 70 days of age to 182 days of age). At the beginning of the experiment, 10-week-old birds were chosen. The experimental duration was for four periods. Each period contains four weeks. Feed intake was measured at every week. The detailed methodology is mentioned in the following subsections.

Feed intake: Feed intake from every replicate was recorded at every week by subtracting total feed given and Feed residue. But in results we presented for one period (means 4 weeks or 28 days) by adding 4 weeks feed intake. In this we collected data for four periods,

Feed conversion rate: Formula: Feed intake /Body weight gain, so every period we got feed intake. Here we collected body weights before starting experiment and every period (every 28 days). Body weight gain is equal to the weight difference between the initial and after a period. So, in this way we got data of FCR for four periods.

Serum biochemical parameters: Quail blood was collected at the end of the experiment (26 weeks of age).

Immune responses: Humoral and cell-mediated immune responses were done at the end of the experiment (26 weeks of age)

Hatchability: At the end of every period, we collected eggs and observed Hatchability performance. In this, we collected data on hatchability for four periods.

Blood Collection: At 182 days of age, blood was collected from two birds from each replicate. For serum collection blood samples (1.5ml) were collected aseptically from birds caudal vein using sterilized needles and stored in clean sterilized glass tubes at room temperature. The serum samples were then centrifuged at 3000 rpm for 5 minutes before transferring to Eppendorf tubes and stored at -20^{0}C for the estimation of various biochemical and immunological parameters.

Serum biochemical constituents: Serum biochemical parameters viz. albumin, glucose, calcium, phosphorus, total protein, cholesterol, triglycerides were determined using the instrument spectrophotometer and as per the instructions given in the Erba commercial kits.

Antioxidant activity: Antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GRx), superoxide dismutase (SOD), and catalase, as well as lipid peroxidation, were determined.

(a) Glutathione peroxidase (GPx**) enzyme activity in serum:** The method proposed by Paglia and Valentine with minor modifications was used to determine GPx activity. [5] Glutathione peroxidase activity was measured using 96-well microtiter plates. To the 12.5µl of serum, 250µl of 0.1mM PBS (pH 7.4), 12.5µl of H_2O_2, and 12.5µl of reduced glutathione were added to wells and incubated at room temperature for 5 minutes before adding 12.5µl of nicotinamide adenine dinucleotide phosphate (NADPH) solution and measured optical density at 340 nm against the blank using ELISA reader-Quant (BioTek instruments) for 5 minutes with 60 seconds interval and expressed as units/ml protein.

(b) Glutathione reductase (GRx) enzyme activity in serum: The method developed by Inger Carlberg and B Mannervik was used to estimate glutathione reductase (GRx) activity, measured in a 96-well microtiter plate. [6] 250µl of 0.1mM PBS (pH 7.4), 12.5µl of oxidized glutathione, 12.5 µl of FAD, and 12.5µl of 80 mM EDTA were added to 12.5µl of serum and incubated at room temperature for 15 minutes. Optical density was measured at 340 nm against a blank using an ELISA reader-Quant (BioTek Instruments) for 5 minutes at

60-second intervals after adding 12.5 µl of NADPH solution; values are expressed as units/ml protein.

(c) Superoxide dismutase enzyme activity in serum: The method developed by Madesh and Balsubramanian was used to estimate SOD activity. Microtiter plates (96-well) were used for assay of SOD activity [7]. To 100 µl of test sample, 6µl of 1.25 mM 3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) was added in duplicate for each sample. 15µl of 100µM pyrogallol and 29µl of 25mM PBS were added to make the volume to 150µl. Pyrogallol was freshly prepared and added after all other reagents, and the mixture was incubated for 10 minutes at room temperature. The reaction was terminated by adding 150 µl of dimethyl sulfoxide (DMSO), which arrests the reaction and dissolves the MTT formazan crystals formed. Plates were shaken well and optical density recorded at 570 nm using an ELISA reader – µ Quant (BioTek Instruments).

(d) Catalase enzyme activity in serum: The procedure proposed by Bergmeyer was used to estimate the Catalase activity, where 1 ml of 0.1 M PBS (pH 7.4) was added, 1 ml of EDTA, and 50 µl of serum were incubated at room temperature for 10 minutes. [8] After incubation, 950 µl of hydrogen peroxide was added. Optical density was measured at 240 nm against a blank using a microplate reader. The catalase activity was defined as the amount of enzyme required to decompose 1 mmol of H_2O_2 per minute, at 25^0 C at pH 7.8. Results were expressed as micromoles of H_2O_2 /min/mg of sample.

(e) Lipid peroxidation: Lipid peroxidation level was estimated by Ohkawa *etal* method. Take 100 µl of serum in the test tube and add 100 µl of distilled water and 50 µl of SDS. Incubate the mixture at room temperature for 10 minutes. [9] Add 375 µl of 20% acetic acid and 375 µl of TBA, and place in a boiling water bath for 60 minutes. Add 2.5ml of distilled water, and to that add 1.25 mL of 15:1 butanol-pyridine solution, then transfer into a 15ml centrifuge tube. Centrifuge for 10 min at 3000 rpm and collect 1 ml of supernatant in a cuvette. Read OD at 532 nm in the spectrophotometer.

Immune responses: The effect of Selenium supplementation on cell-mediated immunity (CMI) and humoral immunity (ND virus) was investigated. On the 182^{nd} day, blood samples were collected from birds of each replicate to study the immune response.

(a) Cell-mediated immune response (CMI): Two birds from each replicate were selected, and the thickness of both right and left wattles was measured using a micrometer (model no 7301, Mitutoyo, Japan). 100 µg of PHAP suspended in 0.10 ml of PBS and 0.1 ml of the PBS without PHAP injected intradermally in both (right and left wattles, respectively). The thickness of both wattles was measured 24 h post-injection of PHAP. The CMI response was calculated as the difference in thickness between right and left wattles due to PHAP injection, which is expressed in relation to the increased thickness due to PBS alone. [10]

(b) Humoral immune response: The humoral immune response was measured by antibody titer against Newcastle disease (ND titer) vaccine by collecting blood from the caudal vein, which is 12 days post-injection of the vaccine against the disease with the virus. The antibody titer against the disease was measured and expressed. The reciprocal of the highest dilution where there is complete agglutination was taken as the titer. ELISA technique was used to measure antibody titers against the ND vaccine. [11]

Hatchability performance: Hatchability is the percentage of fertile eggs that hatch. After collecting the eggs from the pen, the number of eggs laid each day was recorded. The hatchability of Japanese quails was determined in two steps by exposing the fertile eggs; i.e., a total of 100 eggs from each treatment were collected and incubated at a constant temperature of 99.5°F to 100°F with 85-86% humidity during the setter days (15 days) and 98.1°F-99°F with 85-90 % relative humidity during hatcher days (3 days), respectively. An electric balance was used to weigh the hatched-out quail chicks (Setra, M-EL-410s, and USA). The percentage of hatchability was calculated based on the total number of eggs placed in the incubator.

$$\text{Hatchability (\%)} = \frac{\text{No. of chicks hatched out}}{\text{Total number of eggs set}} \times 100$$

Egg quality: Ten eggs were collected from each treatment at regular intervals throughout the experiment to assess egg quality. Egg length, egg width, egg weight, egg density, egg strength, albumen length, albumen width, yolk height, yolk width, yolk weight, and Haugh unit (HU) were measured. Egg length, egg width, albumin length, albumin width, yolk width was determined by using vernier calipers. Egg weight, egg density, and yolk weight were determined by an electronic balance. Egg strength was determined by using a material testing machine with a 100-kg loading cell and speed. Yolk height, albumin height, and Haugh unit (HU) were determined by using a micrometer. The albumin height is correlated with the egg weight, which determines the Haugh unit (HU).

Statistical analysis: One-way analysis of variance (ANOVA) on SPSS was used for the statistical analysis by performing Duncan's Multiple Range Test (DMRT) to analyze the significance ($p<0.05$) between control and treatment groups (SPSS 16.0). For each parameter, after SPSS analysis, results were presented with mean value, N represents Replicate number, P represents significance, and SEM represents Standard error of the mean. Data represented using graphs and tables wherever required.

3. RESULTS

Serum Biochemical Parameters: Serological parameters like, glucose, calcium and cholesterol were shown significant ($P<0.05$) changes in T2 group when compared to control group. Cholesterol levels are elevated in the control group and decreased in selenium treatments. Whereas calcium levels are high in organic selenium-treated groups, followed by inorganic and control groups. Interestingly, glucose levels increased in organic selenium-treated groups, followed by inorganic selenium and control groups (Table 3).

Table: 3 Serum Biochemical Parameters in Japanese Quail Breeders

Groups	Albumin g/dl	Glucose mg/dl	Calcium (mg/dl)	Phosphorous (mg/dl)	Protein (gm/dl)	Cholesterol (mg/dl)	Triacyl glcerides(mg/dl)
T1	2.375± 0.15	$233.2^{b}\pm0.06$	$16.67^{c}\pm0.81$	8.30±0.20	3.723±0.50	$199.84^{a}\pm3.06$	155.3±4.36
T2	2.308±0.07	$244.7^{ab}\pm0.04$	$18.83^{a}\pm0.75$	8.40±0.21	3.806±1.27	$180.00^{c}\pm9.8$	145.8±3.54
T3	2.350±0.02	$251.7^{a}\pm0.03$	$18.17^{ab}\pm0..75$	8.44±0.17	3.765±0.77	$181.68^{c}\pm14.72$	146.8±3.60
T4	2.357±0.04	$253.5^{a}\pm0.03$	$18.00^{ab}\pm1.41$	8.37±0.12	3.716±0.99	$181.68^{c}\pm14.72$	147.2±2.13
T5	2.337±0.08	$228.2^{b}\pm0.08$	$17.00^{bc}\pm0.89$	8.42±0.10	3.736±0.98	$184.85^{bc}\pm1.91$	150.5±1.45
T6	2.305±0.06	$239.0^{ab}\pm0.07$	$17.67^{abc}\pm1.03$	8.32±0.19	3.435±0.57	$188.76^{abc}\pm2.99$	147.5±1.62
T7	2.390±0.04	$237.2^{ab}\pm0.05$	$17.50^{bc}\pm1.04$	8.45±0.17	3.395±0.61	$196.46^{ab}\pm13.87$	120.8±20.6
N	6	6	6	6	6	6	6
P	0.491	0.017	0.011	0.665	0.966	0.004	0.099
SEM	0.012	2.333	0.175	0.0263	0.125	1.772	3.195

N= Number of replicates= 6; P= Significant level; SEM= Standard error of the mean. Values in the same column not sharing a common superscript (a–c) differ significantly $P<0.05$) from each other. a, b, c, d superscripts refer to comparisons of performance among different treatments. Where from superscript "a" to "d" performance will be decreased. Parameters that have significance at $P<0.05$ have been marked with superscripts.

Effect of selenium enriched feed on antioxidant parameters: Antioxidant enzymes showed significant differences among treatments ($P<0.05$). Moreover, the antioxidant status of the whole body system can modulate the optimum functioning of the body and improve the performance of birds. Glutathione peroxidase (GPx) and glutathione reductase (GRx) activities were significantly ($P<0.05$) higher in quails treated with organic selenium than

in the control group. The SOD and catalase levels were significantly higher in quails treated with organic selenium than in the control group, with decrease in MDA levels (Table 4).

Table 4: Antioxidant activity in Japanese Quail Breeders

Groups	GPx (Units/ml)	GRx (Units/ml)	SOD (U/mg of protein)	Catalase (units/g Hb)	Lipid peroxidation (nmol MDA/mg protein)
T1	$396^{d}\pm15.07$	562±36.02	$3.043^{bc}\pm0.20$	$5.96^{bc}\pm0.83$	1.863±0.11
T2	$454^{a}\pm28.78$	608±48.30	$3.665^{a}\pm0.44$	$6.99^{a}\pm0.77$	1.686±0.04
T3	$445^{ab}\pm14.97$	584±13.21	$3.240^{abc}\pm0.48$	$6.80^{ab}\pm0.88$	1.738±0.14
T4	$422^{bcd}\pm24.62$	579±20.65	$3.381^{ab}\pm0.43$	$6.43^{abc}\pm0.69$	1.773±0.25
T5	$444^{abc}\pm32.96$	567±8.72	$3.296^{abc}\pm0.31$	$6.88^{ab}\pm0.79$	1.766±0.09
T6	$414^{cd}\pm20.84$	574±5.84	$3.006^{bc}\pm0.27$	$6.23^{abc}\pm0.56$	1.808±0.11
T7	$415^{cd}\pm21.78$	580±13.03	$2.871^{c}\pm0.32$	$5.75^{c}\pm0.39$	1.791±0.16
N	6	6	6	6	6
P	0.01	0.081	0.013	0.032	0.536
SEM	4.53643	4.21521	0.06498	0.124	0.02238

GPx: Glutathione peroxidase; GRx: Glutathione reductase; SOD: Superoxide dismutase; MDA: Malonaldehyde. N= Number of replicates= 6; P= Significant level; SEM= Standard error mean. Values in the same column not sharing a common superscript (a–d) differ significantly P<0.05 with each other. a, b, c, d superscription refers comparison of performance among different treatments. Where from superscript “a” to “d” performance will be decreased. Parameters that have significance at P<0.05 have been marked with superscripts.

Effect of selenium-enriched feed on immune responses: Selenium is essential for optimal immune response and has an impact on both the innate and acquired immune systems. Table 5 shows the effect of dietary selenium concentration on humoral immune response with antibody titers against Newcastle disease (ND) vaccine and cell-mediated immunity with phytohemagglutinin-P (PHAP) test in breeders. Antibody titers against the ND vaccine and PHAP test were unaffected by feeding the diet with varying concentrations of selenium. In the present study, ND titer test results showed that 0.2 and 0.3 ppm organic selenium showed slight improvement in performance when compared with other treatments. PHAP test results showed that 0.2 ppm organic selenium performed well when compared with other treatments.

Table 5: Antibody titer to Newcastle disease vaccine and lymphocyte proliferation ratio in Japanese quail breeders fed with different concentrations of Selenium in the diet.

Groups	ND titre value	Difference in thickness
T1	$4.167^{b}\pm0.98$	0.048±0.02
T2	$6.333^{a}\pm0.51$	0.102±0.09
T3	$6.333^{a}\pm1.75$	0.087±0.07
T4	$6.500^{a}\pm2.50$	0.063±0.05
T5	$5.667^{ab}\pm1.75$	0.093±0.07
T6	$5.667^{ab}\pm1.36$	0.073±0.05
T7	$5.166^{ab}\pm0.40$	0.042±0.03
N	6	6
P	0.124	0.619
SEM	0.245	0.010

N= Number of replicates= 6; P= Significant level; SEM= Standard error mean. Values in the same column not sharing a common superscript (a–b) differ significantly P<0.05 with each other. a, b, c, d superscripts refer to comparisons of performance among different treatments. Where from superscript “a” to “d” performance will be decreased. Parameters that have significance at P<0.05 have been marked with superscripts.

Effect of selenium-enriched feed on Feed intake, feed conversion rate and egg production

Table 6 shows the effect of selenium supplementation on quail-day feed intake, feed conversion rate and egg production. Selenium treatment had a significant ($p<0.05$) effect on quail-day egg production. Feed intake data from four periods clearly show that inorganic selenium treatments have higher feed intake than control and selenium-enriched azolla treatments. Feed conversion rate observations show that the 0.4 ppm organic selenium treatment showed significantly higher egg production compared with the control ($P<0.05$). Egg production was highest in T2, i.e., 0.2ppm selenized azolla, with a significant ($P<0.05$) difference when compared to other treatments. T2 and T3 groups, approximately 2.3 % and 0.8% of the eggs were found to be abnormal in terms of size and eggshell thickness, respectively.

Table 6: Japanese Quail Breeders Feed intake, FCR and Egg production

Period	Groups	Feed intake/bird	FCR	Egg production
First	T1	$31.00^{c}\pm0.15$	4.19 ± 0.11	250.4 ± 5.89
	T2	$31.50^{bc}\pm0.76$	4.09 ± 0.16	260.6 ± 8.53
	T3	$31.20^{c}\pm0.38$	4.09 ± 0.14	258.0 ± 7.34
	T4	$32.10^{ab}\pm0.60$	4.25 ± 0.07	255.0 ± 6.40
	T5	$32.20^{ab}\pm0.69$	4.24 ± 0.15	256.6 ± 4.21
	T6	$32.26^{a}\pm0.32$	4.23 ± 0.06	258.0 ± 3.39
	T7	$32.00^{ab}\pm0.22$	4.28 ± 0.03	252.4 ± 2.60
	N	5	5	5
	P	0.001	0.058	0.13
	SEM	0.112	0.022	1.057
	Groups	**Feed intake/bird**	**FCR**	**Egg production**
Second	T1	$30.83^{b}\pm0.47$	$4.11^{bc}\pm0.08$	$253^{bc}\pm4.39$
	T2	$31.89^{a}\pm0.43$	$4.04^{c}\pm0.08$	$266^{a}\pm8.46$
	T3	$31.20^{b}\pm0.59$	$4.10^{bc}\pm0.10$	$257^{ab}\pm10.01$
	T4	$32.02^{a}\pm0.32$	$4.43^{a}\pm0.22$	$244^{c}\pm12.54$
	T5	$31.27^{b}\pm0.60$	$4.26^{b}\pm0.10$	$247^{bc}\pm5.85$
	T6	$32.00^{a}\pm0.27$	$4.23^{b}\pm0.12$	$255^{bc}\pm7.52$
	T7	$30.95^{b}\pm0.48$	$4.12^{bc}\pm0.05$	$253^{bc}\pm2.19$
	N	5	5	5
	P	0.001	0.01	0.05
	SEM	0.108	.02867	1.66868
	Groups	**Feed intake/bird**	**FCR**	**Egg production**
Third	T1	26.92 ± 1.38	$3.586^{c}\pm0.16$	$253.6^{ab}\pm1.67$
	T2	27.28 ± 0.29	$3.612^{c}\pm0.17$	$255.6^{a}\pm11.19$
	T3	26.74 ± 0.27	$3.932^{ab}\pm0.14$	$230^{d}\pm7.03$
	T4	27.74 ± 1.20	$4.031^{a}\pm0.17$	$232.8^{cd}\pm11.60$
	T5	27.04 ± 0.32	$3.934^{ab}\pm0.25$	$233^{cd}\pm15.06$
	T6	27.42 ± 0.30	$3.822^{abc}\pm0.21$	$243^{bcd}\pm13.91$
	T7	$26.88\pm0,12$	$3.666^{bc}\pm0.20$	$248.4^{abc}\pm14.24$
	N	5	5	5
	P	0.355	0.004	0.001
	SEM	0.125	0.041	2.442
	Groups	**Feed intake/bird**	**FCR**	**Egg production**
Fourth	T1	$26.08^{b}\pm0.40$	3.604 ± 0.06	$244^{a}\pm4.56$
	T2	$28.27^{a}\pm1.69$	3.992 ± 0.25	$248^{a}\pm2.73$
	T3	$26.58^{b}\pm1.98$	3.784 ± 0.24	$239^{a}\pm3.28$
	T4	$25.94^{b}\pm1.25$	3.884 ± 0.17	$237^{a}\pm4.38$
	T5	$26.38^{b}\pm0.92$	3.784 ± 0.29	$225^{b}\pm4.39$
	T6	$28.34^{a}\pm0.59$	3.898 ± 0.07	$236^{a}\pm20.26$
	T7	$27.61^{ab}\pm0.95$	3.764 ± 0.15	$245^{a}\pm2.30$
	N	5	5	5
	P	0.011	0.109	0.005
	SEM	0.250	0.0362	1.759

FCR= Feed conversion rate; N= Number of replicates= 6; P= Significant level; SEM= Standard error of the mean. Values in the same column not sharing a common superscript (a–

d) differ significantly P<0.05) from each other. a, b, c, d superscripts refer to the comparison of performance among different treatments. Where from superscript “a” to “d” performance will be decreased. Parameters that have a significance level less than P<0.05 have been marked with superscripts.

Effect of selenium-enriched feed on egg quality: The analysis of variance (ANOVA) showed a significant influence of the treatment groups on egg weight (P < 0.05) across all periods of the experiment, confirming a consistent and reliable effect of dietary treatment on egg weight throughout the study. This affirms that egg weight is the most sensitive and consistent characteristic indicating the influence of the treatments.

In the first period, the egg length (P = 0.006), egg weight (P = 0.01), length of albumin (P = 0.01), width of albumin (P = 0.05), and Haugh unit (P = 0.013) showed significant variation. This shows good early reactions in external egg quality (size and shape) and internal quality characteristics. The fact that there are several important parameters implies that treatments affected the physiological state broadly at the early production stage. During the second period, the traits of significance became fewer; egg weight (P = 0.049) and yolk width (P = 0.002) were significant. The change indicates that the effects of treatment were more concentrated on internal egg elements, especially yolk development, and less on external egg characteristics.

In the third period, variability went up once more, and the significant effects were on the egg width (P = 0.001), egg weight (P = 0.01), albumin length (P = 0.003), and yolk height (P = 0.05). This is a stage of maximum differentiation of treatments, primarily in the structural characteristics of eggs and their albumin properties, which reflects active physiological adaptation in laying performance.

Egg weight (P = 0.001), egg strength (P = 0.013), and Haugh unit (P = 0.041) were found to have significant effects in the fourth period. This indicates a change towards stability in egg quality especially the shell strength and internal freshness (albumin quality). These results indicate enhanced structural integrity and inner egg quality at later phases of the experiment.

The density of eggs and most of the yolk-related characteristics were non-significant across all periods, and so they were fairly constant and not highly sensitive to changes in treatment. (Figure 1A to 1D).

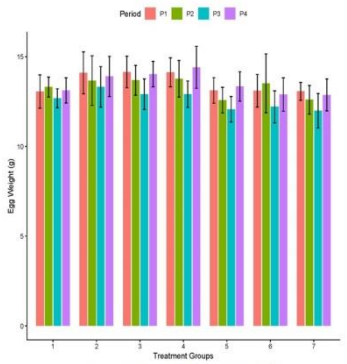

Figure 1A: Egg weight (period wise)

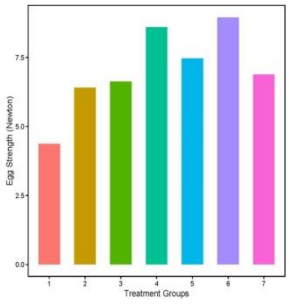

Figure 1B: Egg Strength (Period 4)

The bar chart indicates that eggs weight was different between treatment groups in each period. Groups 2-4 (0.2 to 0.4 ppm organic selenium) tended to have higher weights of eggs in the early periods, whereas minor decreases took place in the late periods. The error bars show that there are variations within treatments (organic and inorganic selenium), although there are general trends that egg weight was consistently affected by treatments during all periods which support the results of ANOVA. The final period indicates a significant difference in the strength of eggs between groups that is a sign of better quality of shell in some organic selenium treatments. An increase in strength values indicates an increase in calcium use and hardening of the shell which is essential to the protection of the egg and quality (Figure A&B).

The Haugh unit comparison between Period 1 and Period 4 shows that there is a variation in internal egg quality. Increases in early stages indicate fresher and better albumin, whereas slight decreases or levels in later ones indicate physiological adjustments.

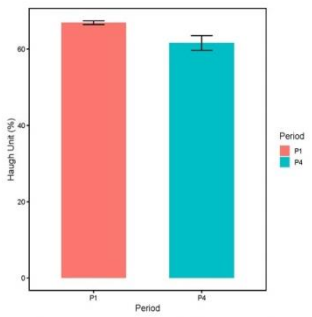

Figure 1C: Haugh Unit (period 1 vs Period 4)

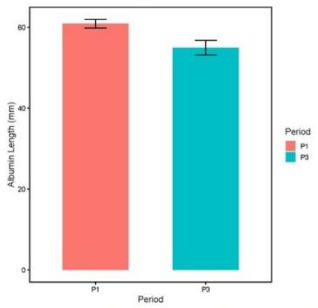

Figure 1D: Albumin length(period 1 vs Period 4)

Variations in treatment can still be seen, which affirm strong impacts during early and late production stages. There is a definite response to treatment in albumin length during the early and mid-stages. Period 1 values are higher in the chosen groups, which means that there is more protein deposition in albumen, whereas Period 3 is more variable, which means that the body is adjusting itself physiologically and reallocating the internal egg (Figure 1C and D).

Effect of selenium-enriched feed on hatching performance

The data in Table 7 show that 0.2ppm selenized supplementation had a beneficial effect on hatchability in the T2 bird group, i.e., selenized azolla showing improvement in hatchability percentage (average 91%).

Table 7: Hatchability performance in Japanese quail breeders

Groups	First period % of hatch	Second period % of hatch	Third period %of hatch	Fourth period %of hatch	Average %
T1	70	72	69	67	69.50
T2	90	92	94	88	91.00
T3	83	70	80	80	78.25
T4	72	68	68	75	70.75
T5	62	57	70	70	64.75
T6	69	70	73	68	70.00
T7	65	71	62	66	66.00

4. DISCUSSION:

To attain more profitability in the poultry industry, efficient growth of the livestock has to be maintained. Hence, the present work focuses on the evaluation of selenium-enriched Azolla supplementation on biochemical changes, antioxidant activity, immune response, reproductive performance, and hatchability in Japanese quail breeders.

Serum Biochemical Parameters: Reduced levels of calcium, cholesterol, and glucose were observed in the serum of the organic selenium-treated group of Japanese quails. This may be due to increased mineral bioavailability and enhanced immune protein synthesis, as previously reported in selenium-supplemented poultry. [12] Enhanced calcium levels in organic selenium groups are responsible for mineral absorption, intestinal integrity, and reduced oxidative stress levels in gut epithelium. [12] Elevated glucose levels of organic selenium groups associate with selenium dependent enzymes role in carbohydrates metabolism and its association with insulin sensitivity. [13] Selenium has a role in antibody production and lymphocyte proliferation by maintaining redox balance in immune cells. [14] Selenium has a role in improving lipid utilization and reducing cholesterol synthesis by regulating lipid metabolism and lipid peroxidation. [15]

Antioxidant activity: Reactive oxygen species produced in the animal body due to metabolic reactions are harmful to the body, and their effects were prevented by antioxidant activity as one of the primary defense systems. The constant production of hydrogen peroxide in all cell types, including intestinal epithelial cells, causes gastrointestinal injury. [16] Intestinal nutrient uptake and its requirements were affected by gastrointestinal health issues. [17] Oxidative stress was elevated with metabolic reactions in the liver, muscles, and immune cells, leading to free radical production. SOD converts free radicals into hydrogen peroxide, which is further removed by glutathione

peroxidase and catalase. Improvement in malondialdehyde production and lipid peroxidation was regulated by SOD. [18]

In the current study, malondialdehyde levels were decreased and elevated levels of GPx, GRx, SOD, and catalase activities were observed in quails treated with organic selenium; this indicates the improvement in whole bird antioxidant status. Current study findings are in agreement with the results of Nasiroleslami et al., [19] who has reported an improvement in liver GPx activity and decrease in serum malondialdehyde content. Reduction in Lipid peroxidation and improvement in Glutathione peroxidase and Glutathione reductase activities of serum were in agreement with results of Rao et al. [20] The cellular antioxidant enzyme system regulates the overall redox balance. [14] SOD, GPx, and catalase were upregulated in a coordinated manner and create an efficient antioxidant network. They reduce oxidative stress and support metabolic and immune functions. The current findings revealed a positive correlation between fertility and the hatchability rate of fertile eggs, indicating that hatchability improves as fertility increases.

Immune responses: In the current study, elevated antioxidant activity reduces stress in Japanese quails due to the immunomodulator activity of selenium. Deficiency of selenium in the diet leads to increased levels of oxidative stress and is also associated with diseases. [20] The current study shows that 0.2 ppm organic selenium performed well among all treatments in the ND titer and PHAP test. Selenium plays a crucial role in elevated GPx activity and inhibits arachidonic acid peroxidation. Hence, it protects cells and tissues of the immune system from free radicals. Therefore, it can be stated that Selenium enhances birds' immunity, antioxidant activities and reduces inflammation. [21] Therefore, deficiency of selenium is associated with changes and damage in the bursa of Fabricius, which is the locus for lymphocyte production. Selenium may be involved in lowering of glucocorticoids, decrease in duration and rate of intramammary infections were associated with selenium, and also regulates the function of lymphocytes, neutrophils and natural killer cells. [22] Hence, the above results suggest that feeding a diet enriched with organic selenium might have an immunostimulatory effect on quails.

Feed intake, feed conversion rate and egg production: Higher feed intake was observed in four periods of duration in inorganic selenium treatments, whereas significant feed conversion rate and egg production were observed in organic selenium treatment. Chantiratikul et al. [23] reported that selenium supplementation had no impact on egg production in laying hens. However, Mobaraki and Shahryar [24] stated that selenium and vitamin E supplementation significantly improve egg production in Japanese quails. Adebiyi et al. [25] found a significant increase in egg production in turkeys supplemented with Selenium (0.3mg/kg feed), Mobaraki and Shahryar [24] found that selenium supplementation had no effect on feed intake. In the case of feed conversion ratio, Mohiti-Asli et al. [26] observed a similar effect, which aligns with our findings. Cruz and Fernandez [27] found that selenium supplementation improved feed intake and daily egg production. We noticed that, improvements in egg production and hatchability were associated with selenium-enriched treatments, and these results coincided with Surai PF et al. [1]

Egg quality: Egg weight reflects overall nutrient deposition. In the present study, organic selenium treatments showed higher egg weight. Due to the higher bioavailability of organic selenium in the form of selenomethionine, this is effectively absorbed and incorporated into body proteins and eggs. [28] Hence, this improves protein synthesis, nutrient utilization and nutrient deposition, finally improving egg weight in the organic selenium treatments. [13]

Haugh unit indicates the albumin quality and freshness. In the study, Haugh unit was better in organic selenium treatments. In organic selenium treatments, selenium modulates antioxidant activity, thereby protecting albumin

proteins from oxidative damage during egg production and storage. [14] Organic selenium improves the antioxidant enzyme activity, structural integrity, and viscosity of albumin proteins, thereby resulting in thicker albumin and higher Haugh unit. [28]

Hatchability performance: In the current study, 0.2 ppm organic selenium treatment performs better improvement in hatchability percentage. Mobaraki and Shahryar [24] reported a significant improvement in hatchability with various levels of selenium supplementation in breeding Japanese quails after 7 and 10 days of storage. Hoffman and Heinz [29] observed beneficial effects of organic selenium supplementation on reproduction. Moreover, in our previous study we observed that, supplementation of azolla enriched selenium influences the growth, performance and biochemical parameters in a positive way. In performance trials, we observed that 0.2ppm organic selenium significantly impacts body weight gain ($P<0.013$) and feed conversion rate ($P<0.02$). Biochemical parameters like albumin ($P<0.017$), phosphorous ($P<0.014$) and protein ($P<0.013$) levels were increased at significantly. [30]

5. CONCLUSIONS:

The experimental observations of Japanese quail breeders from 10 to 26 weeks of age concluded that dietary supplementation of 0.2 ppm selenium-enriched Azolla significantly enhanced antioxidant status, reproductive performance, and hatchability in Japanese quail breeders, suggesting its potential as an effective organic selenium source. Therefore, supplementing 0.2 ppm selenium-enriched Azolla in feeds is proposed to produce Japanese quail breeders with higher performance and an improved immune system. Future insights of the work include transcriptome analysis to understand the molecular insights of the chelated organic selenium mechanism of action and its distribution in the Japanese quails.

Limitations, future scope and recommendations: A limitation of the present study is the absence of molecular data to explain the observed biochemical changes. To validate the translational potential of our findings, we are looking to investigate the efficacy of selenium supplementation in other animal models.

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Declaration of Generative AI

The authors declare this manuscript was written without the use of generative artificial intelligence tools. All the content, including text generation, data analysis and references was developed and reviewed by the author without assistance from AI technologies.

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