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RESPONSES OF FOLLICLE STIMULATING HORMONE, PRODUCTION PARAMETERS AND IMMUNITY TO BETAINE AND ASCORBIC ACID SUPPLEMENTATION IN JAPANESE QUAILS DURING THE DRY SEASON

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ABSTRACT

The dry season in tropical regions of the world exerts thermal stress in poultry birds. This study investigated the modulatory effects of betaine and ascorbic acid supplementation on responses in follicle stimulating hormone, production parameters and immunity of Japanese quails (Coturnix coturnix japonica) during the dry season. A total of 372 fourteen day–old female quails purchased were kept in cages. Birds were assigned by Random design to 4 groups, each having 3 replicates; after 7 days of acclimation. There were 31 birds per replicate of each group. Experimental groups include birds fed Control (basal); ascorbic acid (AA) at 200 mg/Kg; betaine (BET) at 2 g/kg and combination of ascorbic acid; 200 mg/kg and betaine; 2 g/kg (AA+BET) diets. Serum malondialdhyde (MDA), superoxide dismutase (SOD), follicle stimulating hormone (FSH) and immunity of birds were assayed at 28, 49 and 70 day–age. Egg production parameters was assessed as hen–day rate (HDEP), egg mass, feed conversion efficiency to kilogram egg mass and a dozen egg produced. During the study, ambient temperature ranged from 25.0 – 37.0 °C; relative humidity, 63.0 – 91.0 % and temperature–humidity index, 75.6 – 91.0, predominantly exceeding the thermoneutral zone for Japanese quails. In comparison with quails of control group, AA and/or betaine lowered ($P < 0.05$) MDA, but enhanced ($P < 0.05$) SOD and FSH levels. Betaine increased ($P < 0.05$) HDEP, egg mass and feed conversion efficiency compared with control quails. Compared with control, AA, either alone or combined with betaine, decreased ($P < 0.05$) heterophil, monocyte, eosinophils and heterophil/lymphocyte ratio, but betaine lowered ($P < 0.05$) leukocyte and eosinophil numbers. In conclusion, betaine and ascorbic acid supplementations modulate responses in follicle stimulating hormone, egg production parameters and immunity of Japanese quails during the dry season.

Keywords: Antioxidants, heat stress responses, quails

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INTRODUCTION

High thermal environmental condition is a major source of concern to scientists and poultry farmers in tropical and arid regions of the world. This is because heat stress results to decline in feed consumption and health, consequently reducing production in poultry production systems [1]. Heat stress causes mortality in poultry birds [2] and induces lipid peroxidation and decrease in superoxide dismutase [3]. High environmental temperature reduces egg quality and immunocompetence of laying Japanese quails [4]. However, nutrient modification is an affordable approach to ameliorate the adverse effects of heat stress [5]. Antioxidant supplementation plays a role in mitigating the deleterious effects of heat stress in Japanese quails reared in tropical regions of the world [6].

Betaine has both methyl donor and osmoregulatory properties [7] and has been used to alleviate the adverse effects of heat stress in poultry birds [8]. Bai *et al*. [9] demonstrated that some agents with antioxidant properties perform better at mitigating oxidative stress in synergistic combination than in single administration. However, there is paucity of information on the synergistic effects of betaine and ascorbic acid on responses of follicle stimulating hormone, egg laying performance and immune status of sexually mature female Japanese quails reared under thermally stressful environmental conditions. The aim of the present study is to evaluate the modulatory effects of betaine and ascorbic acid supplementation on responses of follicle stimulating hormone, production parameters and immunity of female Japanese quails during the dry season.

MATERIALS AND METHODS

Experimental Site

The experiment was performed at the Animal House of Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka (latitude 6° 52' 0" North and longitude 7° 23' East) between the months of January and March, known for the characteristic high ambient temperature and high relative humidity in the Derived Savannah Ecological zone of Nigeria [10]. The study utilized the prevailing natural environmental conditions with no simulation.

Animals, Management and Experimental Protocol

Female Japanese quail chicks totaling 372, at 14 day–old and an average weight of 56.4 ± 0.4g were sourced commercially and placed in battery cages. After acclimation for 7 days, the birds were weighed and allotted, by Complete Random design, to 4 experimental groups and 3 replicates per group [11]. Each group was made of 93 birds (with 31 quails per replicate). Quails in every replicate were housed in cages (91.44 cm × 76.2 cm × 91.4 cm) which were placed within the animal house with windows. These birds were reared and exposed to the prevailing natural environmental conditions. Birds in Control group consumed only basal diets. The treatment groups comprised of birds in AA group, which were fed diets enriched with ascorbic acid (AA; 200 mg/Kg); BET group, which consumed betaine (2 g/Kg) and AA + BET groups, fed diet enriched with a combination of AA (200 mg/Kg) and betaine (2 g/Kg). Betaine hydrochloride, sourced from Sigma-Aldrich, St. Louis, Missouri, USA, was included in diet at 2 g/Kg of feeds [12], while ascorbic acid, from Kempex Holland BV, Volkel, The Netherlands, was included at 200 mg/Kg [13].

Enrichment diets with ascorbic acid and/or betaine were fed birds in the treatment groups daily for 56 days. However, before the treatment with the agents commenced, when birds were 21 days old, baseline serum activities of follicle stimulating hormone was established via serum hormone assay. The duration of the treatment was informed by the focus of the study which investigated the responses of female quails to these supplementations at grower and early maturity phases during the thermally stressful dry season. Commercial starter diet was fed the quails between 14 and 21 days old, which was later changed to grower mash at 28 – 49 days old while layer mash was fed the quails from 49 – 70 days old. Table 1 shows the composition and proximate analysis of the basal diets consumed by the birds. Quails were given access to water and feed *ad-libitum*. The authors confirm that the animals used were kept and handled in compliance with European Union Directive 2010/63/EU for animal experiments.

Table 1: Composition and proximate analysis of quail diets

Feed Composition	Starter	Grower	Layer
Ingredients, %			
Maize	30	0	34
Sweet potato meal	30	60	26
Blood meal	5	5	5
Groundnut cake	29.7	29.7	29.7
Wheat offal	1	1	1
Bone meal	3.25	3.25	3.25
dl-Methionine	0.25	0.25	0.25
Lysine	0.25	0.25	0.25
Vitamin Premix	0.3	0.3	0.25
Salt	0.25	0.25	0.25
Proximate analysis			
Metabolizable energy, Kcal/kg	2940	2877	3000
Crude protein, %	22.58	21.49	22
Crude fiber, %	4.88	6.84	4.3
Calcium, %	1.35	1.41	1.2
Phosphorus, %	0.45	0.45	0.45
Lysine, %	1.22	1.22	1.3
Methionine, %	0.53	0.5	0.56
Cystine, %	0.36	0.36	0.3
Dry matter, %	93.84	93.73	94.14
Ether extract, %	3	2.75	3
Ash, %	11.96	6.95	7.4
Nitrogen-free extract, %	54.07	67.97	69.21

Vitamin premix supplied per kg diet: vitamin A: 10,000IU; vitamin D3: 2,000 IU, vitamin E: 51, vitamin K: 2.34 mg; riboflavin: 5.5 mg, calcium pantothenate: 10 mg, niacin: 25 mg, chlorine chloride: 250 mg, folic acid: 1 mg, manganese: 56 mg, zinc 50 mg, copper: 10 mg, iron: 20 mg and cobalt: 1.25 mg.

Thermal Environmental Conditions

Thermal environmental conditions prevailing during the study period were measured as dry– bulb temperature (DBT) using Mason's Type Wet and Dry Bulb Hygrometer [Zeal, London, England; with accuracy of +/-1 ºC (or +/-5%)]; relative humidity (RH), obtained with Hygrometric Tables for computation of relative humidity (Zeal, London, England) and temperature–humidity index (THI), calculated with formula as initially described [14] and modified [15]:

Temperature-humidity index (THI) = 0.6Tdb+0.4Twb,

Where, *Tdb* = dry-bulb temperature (°C) and *Twb* = wet-bulb temperature (°C). All environmental recordings were done three times daily at 08:00 h, 13:00 h and 17:00 h on throughout the study (Table 2).

Blood and Serum Collection
During the morning hours (between 09:00 h – 11:00 h; [16], blood samples were individually collected from six (6) quails in each group (2 birds per replicate) selected by random sampling method after identification with leg number tags. Blood collection was performed by sacrificing [17] at 21, 28, 49 and 70 days old. Blood samples were quickly collected into properly labelled sample bottles with and without anticoagulant (sodium ethylenediamintetraacetate). Thereafter, blood in plane bottles were centrifuged at 3000 × *g* for 10 minutes under room temperature [15] were harvested and immediately analyzed.

Biomarkers of Oxidative Stress
Serum malondialdhyde concentration (MDA) was determined [18] for all the groups. Briefly, 0.5 mL serum was mixed with 20 % trichloroacetic acid in ratio 1:1, incubated at room temperature (25°C) and centrifuged at 2500 × *g* for 10 minutes. Following the addition of 1 % thiobarbituric acid to the supernatant, samples were warmed in water bath (100 °C) for 15 minutes. The contents were then cooled, centrifuged at 2500 × *g* for 15 minutes. The optical density of the supernatant was determined at 532 nm against the blank, using spectrophotometer (Jenway 6305; Jenway, Essex, UK) and a standard curve constructed using various MDA concentrations of 0 – 20 nMoles.

The method described by Misra and Fridovich [19], was used to assess serum superoxide dismutase (SOD) activity. Briefly, ice-cold ethanol (0.25 mL) and chloroform (0.15 mL) were added to serum (0.5 mL) following its dilution with 0.5 mL water. The solution was mixed thoroughly and centrifuged at 2500 × g for 10 minutes. The supernatant was further mixed with 0.05 M carbonate buffer (1.5 mL; pH10.2) and 0.5 mM ethylenediethyltetraacetate (EDTA) solution (0.5 mL). Epinephrine (0.4 mL; 3 mM) from Sigma (St. Louis, Missouri, USA) was added to initiate a reaction, and the rate of change in absorbance determined at 480 nm against a blank. A calibration curve of SOD was constructed using 0 – 195 SOD units, defined as rate of change at 50 % SOD (Sigma, St. Louis, MO, USA) inhibition of epinephrine conversion to adrenochrome. Activity of SOD was determined as U/mL.

Follicle Stimulating Hormone Assay
Serum follicle stimulating hormone (FSH) were determined by solid phase enzyme-linked immunosorbent assay (ELISA) technique based on principle as described by Tietz [20]. Microplate immune–enzymatic assay kits (Monobind Incorporated, Lake Forest, California, USA) were used for the assays. Briefly, following addition of 50 µL of standards, controls and serum samples into assigned wells, 100 µL of enzyme conjugate reagent were dispensed into each well. The contents of the wells were mixed gently for 30 seconds and incubated at room temperature (25 °C) for 60 minutes. The wells were then decanted, rinsed with 350 µL of wash buffer and blot dried with absorbent paper. A working substrate (100 µL) was then added to each well, incubated for 15 minutes at room temperature and the reaction stopped by adding into each well 50 µL of stop solution. Within 30 minutes, absorbance was measured spectrophotometrically using microplate reader (StatFax 4200; Awareness Tech, Palm City, Florida, USA) at absorption wavelength of 450 nm and reference wavelength of 620 – 630 nm. A graph was plotted between mean absorbance from each reference standard against its concentration, forming a standard curve. Concentration of FSH were determined from the standard curve and expressed as mIU/mL of serum. The lower limit of detection for quantitative FSH assay was 0.134 mIU/mL.

Production parameters
Production parameters are defined as hen–day egg production (HDEP), egg mass and feed conversion efficiency of birds in each group to 1 kg egg mass and per dozen eggs. Eggs laid were collected from each experimental group once daily at 15:00 h. The collection of egg commenced when the birds began laying at 49 days old until 70 days of age. All eggs laid were weighed using digital milligram scale (WAOAW, Lexington, Kentucky), to the nearest 0.001g. Average egg weight per replicate was determined on daily basis, HDEP was calculated as the number of eggs produced daily divided by number of hens alive daily [21]. Egg mass was obtained as average egg weight multiplied by the number of eggs laid daily in each replicate. Feed–to–egg mass ratio was determined as the ratio between the daily feed consumption and the daily mass of eggs laid in each group during the study period [22]. Feed efficiency per dozen eggs produced was calculated using the following formular [23]:

$$\text{Feed–to–per dozen eggs} = \frac{\text{Kilogram of feed consumed} \times 12}{\text{Total number of eggs produced}}$$

Immune Responses

Immune responses of the birds were evaluated using total and differential leukocyte counts as described by Cheesbrough [24]. Leucocyte

Statistical Analysis

Data obtained were expressed as mean ± standard error of mean (mean ± SEM). The values were subjected to statistical analysis and compared using analysis of variance (ANOVA), followed by Tukey's post-hoc test. Values of $p < 0.05$ were considered significant [25]. GraphPad Prism (GraphPad Software, Incorporated, San Diego, California, USA) version 6.0 was used for analysis.

RESULTS

Thermal Environmental Conditions

Figure 1shows a boxplot describing the thermal environmental conditions recorded during the study period. Mean values of dry bulb temperature (DBT), relative humidity (RH) and temperature-humidity index (THI) obtained were 31.5 ± 0.40 °C, 79.6 ± 0.80 % and 84.8 ± 0.50, respectively. The minimum and maximum DBT values were 25.0 – 37.0 °C; RH, 48.0 – 91.0% and THI, 75.6 – 91.0. The DBT and RH recorded fluctuated widely and were negatively skewed with the median values for DBT (33.0 °C) and THI (86.4) higher than the mean DBT and mean RH. Table 2 shows the diurnal fluctuation of meteorological parameters. The extreme minimum DBT (25.0 °C) and THI (69.8) were recorded at 08:00 h. Extreme maximum DBT (37.0 °C) and THI (90.3) were obtained at 13:00 h and 17:00 h respectively. Conversely, the extreme minimum and maximum RH of 48.0 % and 92.0 % were obtained respectively at 13:00 h and 08:00 h. The highest mean DBT (33.7 ± 0.32 °C) and THI (86.0 ± 0.55), and the lowest mean RH of 67.9 ± 1.6 were recorded at 13:00h. The lowest mean DBT (27.8 ± 0.26 °C) and THI (78.5 ± 0.62), and the highest mean RH (79.6 ± 1.80 %) were obtained at 08:00 h.

Biomarkers of Stress

The responses of biomarkers of stress are shown in Table 3. The female quails at 28 and 49 days old recorded significantly lower MDA in those fed either AA or AA+BET compared with values observed in either control ($p < 0.01$) or BET ($p < 0.05$) groups. At 70 days old, MDA was significantly ($p < 0.05$) lower in female quails fed AA and/or BET diets when compared with the control groups. Serum concentrations of MDA rose with increase in age, especially, for control birds fed basal diets with no supplementation. Female birds at 70 days old, fed AA and/or BET – enriched diets recorded significantly ($p < 0.05$) higher serum SOD when compared with values observed in the birds fed basal diets only. Serum SOD was not significantly ($p > 0.05$) different when the treated groups were compared with one another.

Serum Follicle Stimulating Hormone

Figure 2 shows the changes in serum FSH levels in female Japanese quails during the study period. Serum levels of FSH did not differ significantly ($p > 0.05$) at 21 day – old, when values obtained in the treatment groups were compared with those of either the control group or with one another. Serum FSH levels in 28 days old female quails were significantly higher when fed either AA ($p < 0.01$) or BET ($p < 0.05$) in diets than that recorded in control quails, but no significant ($p > 0.05$) differences were observed between AA and BET groups. In addition, 28 day–old birds in AA groups recorded significantly ($p < 0.05$) higher serum FSH compared with that in the AA+BET group. Serum FSH levels in 49 days old quails fed BET diets increased significantly ($p < 0.05$) when compared with that of control quails. Female quails fed either AA, betaine or AA + betaine, at 70 days old, recorded significantly ($p < 0.05$) higher serum FSH when compared with that in control birds. Serum level of FSH in AA supplemented birds were significantly ($p < 0.05$) higher than those observed in either BET or AA+BET.

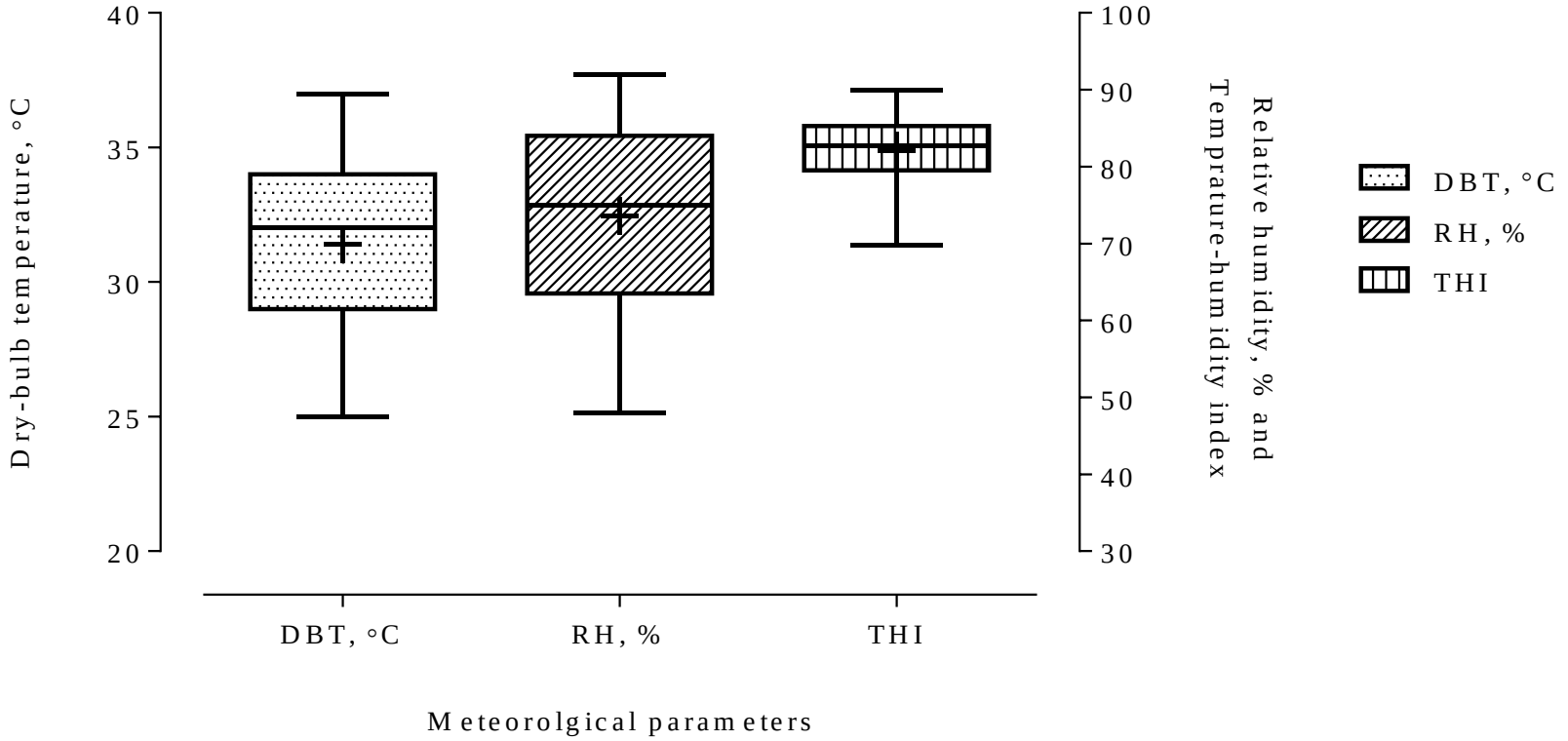

Figure 1: Meteorological parameters obtained during the study period.
Note: Plus sign (+) indicates the mean values of DBT, Dry–bulb temperature; RH, Relative humidity and THI, Temperature–humidity index.

Table 2: Diurnal fluctuations of environmental conditions recorded during the study period.

	Environmental Parameters		
Hour of day (h)	**DBT, °C**	**RH, %**	**THI**
08:00	27.8 ± 0.26 (25.0 – 33.0)	79.6 ± 1.80 (54.0 – 92.0)	78.5 ± 0.62 (69.8 – 84.2)
13:00	33.7 ± 0.32 (28.0 – 37.0)	67.9 ± 1.56 (48.0 – 87.0)	86.0 ± 0.55 (77.7 – 86.0)
17:00	32.9 ± 0.26 (29.0 – 36.0)	72.7 ± 1.24 (55.0 – 88.0)	85.7 ± 0.46 (78.8 – 90.3)

DBT = Dry–bulb temperature; RH = Relative humidity and THI = Temperature – humidity index

Table 3: Level of serum oxidative markers in female Japanese quails fed diets enriched with betaine and/or ascorbic acid (n = 6).

Oxidative markers	Age of birds (days)	Experimental groups			
		Control	AA	BET	AA+BET
Malondialdhyde concentration, nMol/mL	28	0.57 ± 0.02^{a}	0.47 ± 0.01^{b}	0.52 ± 0.05^{a}	0.47 ± 0.02^{b,a}
	49	0.72 ± 0.04^{a}	0.54 ± 0.02^{b,a}	0.61 ± 0.02^{a}	0.48 ± 0.03^{b}
	70	0.77 ± 0.04^{a}	0.52 ± 0.03^{b}	0.60 ± 0.04^{b}	0.41 ± 0.03^{b}
Superoxide dismutase, U/mL	28	0.81 ± 0.03^{a}	0.91 ± 0.08^{a}	0.89 ± 0.03^{a}	0.98 ± 0.08^{a}
	49	0.60 ± 0.02^{a}	0.81 ± 0.02^{a}	0.76 ± 0.03^{a}	0.78 ± 0.02^{a}
	70	0.53 ± 0.02^{a}	0.87 ± 0.02^{b}	0.78 ± 0.02^{b}	0.91 ± 0.01^{b}

Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets; ^{a,b}Mean values with different superscripts between groups are significantly different ($p < 0.05$).

Egg Laying Performance of Japanese Quail Hens

Egg laying by quails in all groups commenced at 46.25 ± 0.62 days of age with a range of 45 – 48 days old. Thereafter, the HDEP was consistently ($p < 0.05$) higher in hens fed BET supplements during the study period at the 56th and 63rd days of age compared with either the control or other treated groups (Fig. 3). The general laying pattern, that is HDEP, increased significantly ($p < 0.05$) as the birds grew older (Fig. 3). A similar trend was observed in the egg mass during the same period (Fig. 4).

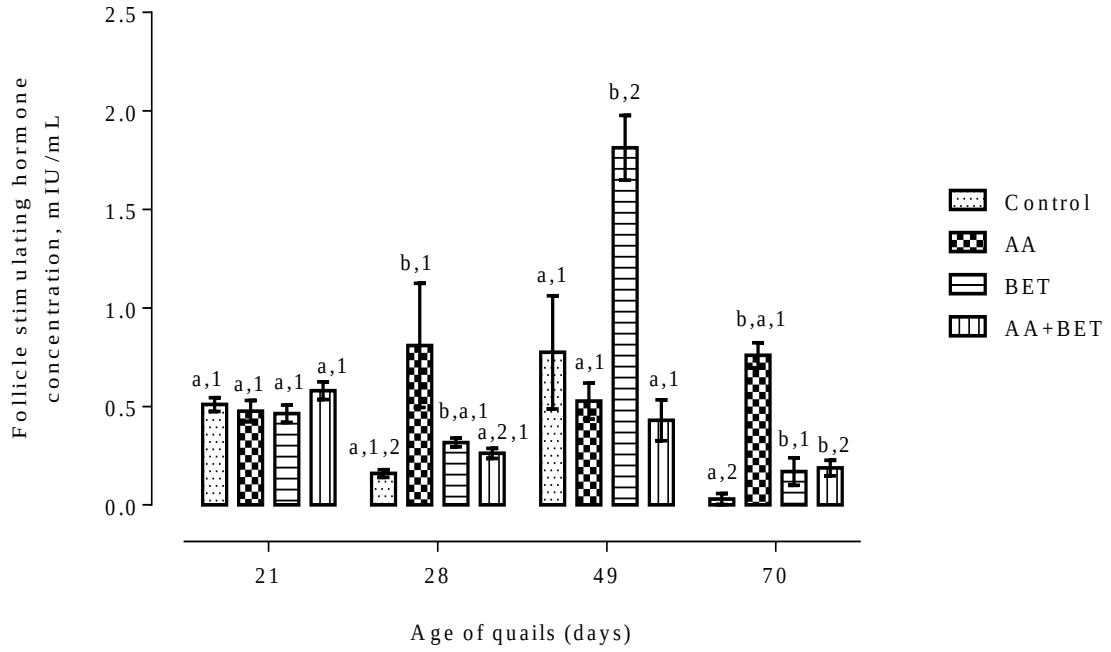

Figure 2: Changes in serum concentration of follicle stimulating hormone in female Japanese quails fed dietary betaine and/or ascorbic acid.

Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. [a,b,1,2] Mean values with different superscripts and numbers between and within groups, respectively, are significantly different
($P < 0.05$).

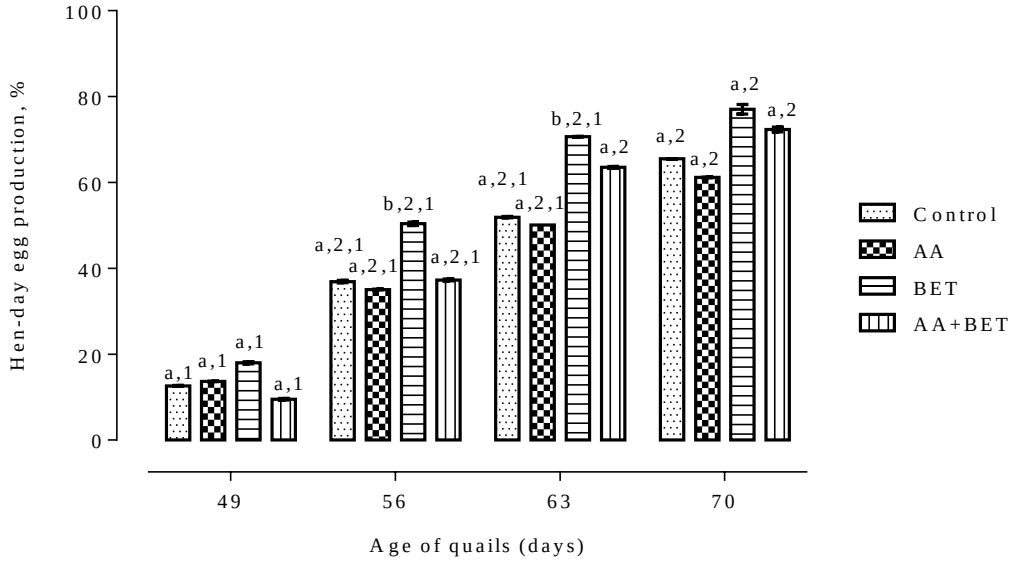

Figure3: Hen–day egg production of female Japanese quails fed dietary betaine and/or ascorbic acid.

Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. [a,b,1,2] Mean values with different superscript letters and numbers between and within groups, respectively, are significantly different
($P < 0.05$).

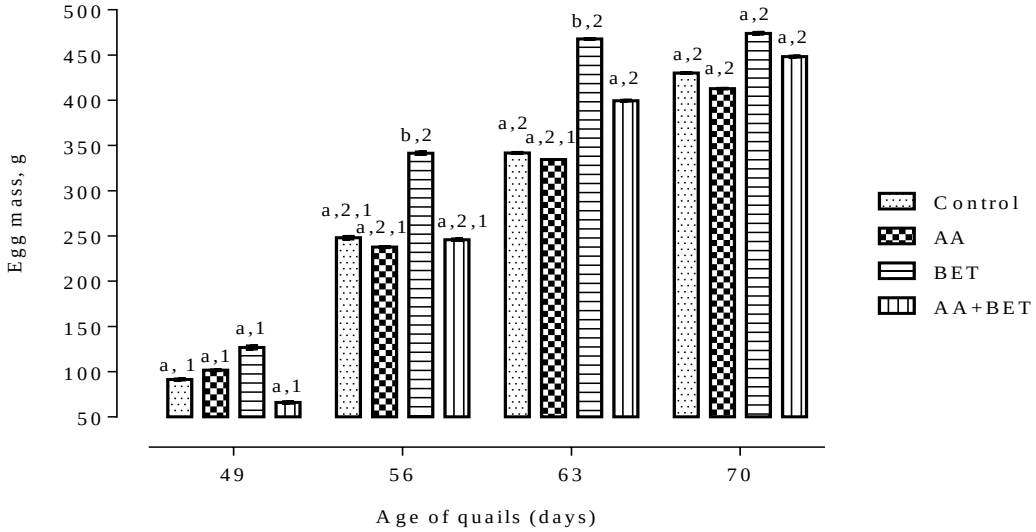

Figure 4: Mass of eggs laid by Japanese quail hens fed dietary betaine and/or ascorbic acid.
Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. [a,b,1,2] Mean values with different superscript letters and numbers between and within groups, respectively, are significantly different ($p < 0.05$).

The lowest feed-to-egg ratio of 4.85 % was obtained in quails in the BET group, closely followed by 5.69 % recorded in AA+BET group (Fig. 5). The feed-to-egg mass ratio in the groups fed BET and AA+BET was lower (by 1.04 % and 0.2 % respectively) than that of the control value of 5.89 % during the period (Fig. 5). Similarly, the quails which consumed BET and AA+BET in diets recorded lower values (by 0.1 % and 0.01 % respectively) of feed conversion efficiency to dozen eggs produced (0.57 % and 0.66 %, respectively) compared with 0.67 % obtained in the control group (Fig. 6).

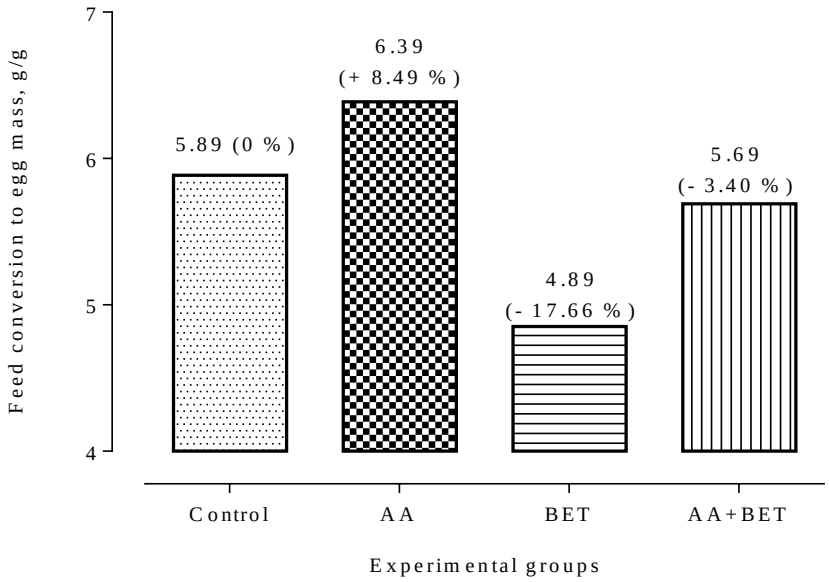

Figure 5: Feed conversion to egg mass of eggs laid by 372 Japanese quail hens fed diets enriched with betaine and/or ascorbic acid during the study period.
Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. Positive (+) or negative (-) sign indicate the degree to which feed –to–egg

mass ratio in the treatment groups are greater or less than those obtained in the control group, which serve as baseline value.

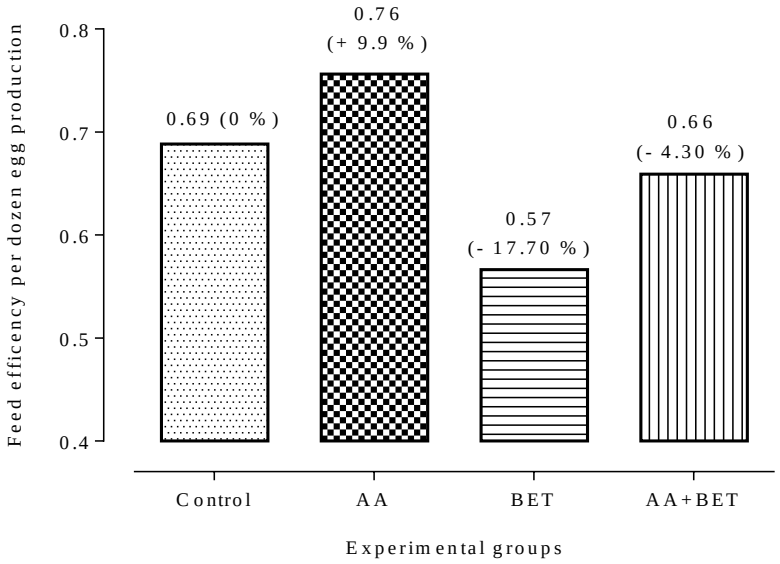

Figure 6: Feed conversion efficiency per dozen eggs produced by 372 Japanese quail hens fed diets enriched with betaine and/or ascorbic acid.
Control = basal diet; AA = Ascorbic acid, BET = Betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. Positive (+) or negative (-) sign indicate the degree to which feed –to–egg mass ratio in the treatment groups were greater or less than those obtained in the control group, which serve as baseline value.

Immune Status

Immune status of female quails as described by total and differential leukocyte counts is shown in Table 4. The TLC in BET fed quails was significantly ($p < 0.05$) lower than that of the control group at 70 days old. Total leukocyte counts did not differ significantly ($p > 0.05$) in quails fed treated diets when compared with those of control group at 28 and 49 days old. The TLC in the quails were predominantly within the normal values of 1.3 – 2.5 for Japanese quails, with the exception of those obtained in control, AA and BET groups at 49 day–old. There was significantly ($p < 0.05$) lower percentage heterophil in quails fed AA at 28 and 70 day – old and AA+BET, at 70 day – old, compared with values obtained in control birds. In 49 day–old female quails, percentage heterophil were above the normal range of 25.0 – 50.0, except in birds fed diets enriched with AA. Generally, percentage lymphocytes were within normal values of 50.0 – 70.0 for Japanese quails, except values recorded in control at 28 and 49 day–old and either BET or AA+BET at 49 day–old. Percentage lymphocytes in 28 and 70 day–old female quails fed AA enriched diets were significantly ($p < 0.05$) higher than those of control birds of these ages. There were no significant differences ($p > 0.05$) in percentage lymphocytes when birds in either AA, BET or AA+BET groups were individually compared with values obtained in the control birds at 49 day–old. The quails in either the AA or AA+BET groups recorded significantly ($p < 0.05$) lower percentage of monocytes than the control birds. The percentage monocytes obtained at 28, 49 and 70 day–old birds in all groups were within the normal range of 0.0 – 4.0 for Japanese quails. At age 28, 49 and 70 day–old, there were significantly ($P < 0.05$) lower percentage eosinophil in female quails fed diets enriched with AA or betaine compared with those of the control groups.

Table 4. Leukocytic parameters at different ages of female Japanese quails fed dietary ascorbic acid and/or betaine (n = 6)

Leukocytic parameters	Age of birds (days)	Experimental groups				
		Control	AA	BET	AA+BET	Standard*
Leukocyte count, $\times 10^9$/L	28	1.65 ± 0.42	1.57 ± 0.49	1.32 ± 0.25	1.34 ± 0.42	1.3 – 2.5
	49	0.89 ± 0.06	1.06 ± 0.15	1.05 ± 0.17	1.54 ± 0.17	
	70	2.11 ± 0.54[a]	2.00 ± 0.39[a]	1.66 ± 0.37[b]	1.83 ± 0.16[a]	
Heterophils, %	28	51.25 ± 3.20[a]	37.00 ± 2.50[b]	45.50 ± 4.91[a]	44.25 ± 3.33[a]	25.0 – 50.0
	49	56.25 ± 1.75	45.75 ± 0.34	55.25 ± 2.70	58.30 ± 0.88	
	70	45.75 ± 2.53[a]	30.50 ± 1.85[b]	40.00 ± 1.47[a]	36.75 ± 3.43[b,a]	
Lymphocyte, %	28	46.00 ± 3.56[a]	60.00 ± 2.08[b]	52.00 ± 5.40[a]	53.75 ± 3.30[a]	50.0 – 70.0
	49	42.25 ± 1.49	53.01 ± 6.40	43.25 ± 2.59	40.00 ± 0.58	
	70	52.01 ± 2.35[a]	66.25 ± 2.10[b]	57.50 ± 1.50[a]	61.25 ± 3.97[a]	
Monocytes, %	28	1.50 ± 0.50	1.67 ± 0.33	1.00 ± 0.00	1.00 ± 0.00	0.0 – 4.0
	49	1.75 ± 0.48[a]	1.00 ± 0.00[b]	1.75 ± 0.48[a]	1.00 ± 0.41[b]	
	70	1.75 ± 0.48	2.00 ± 0.71	1.50 ± 0.29	1.25 ± 0.48	
Eosinophils, %	28	1.25 ± 0.25[a]	0.33 ± 0.33[b]	0.67 ± 0.33[b]	0.25 ± 0.25[b]	0.0 – 15.0
	49	0.25 ± 0.25[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.25 ± 0.25[a]	
	70	0.75 ± 0.25[a]	0.50 ± 0.29[b]	0.50 ± 0.29[b]	0.75 ± 0.48[a]	
Basophils, %	28	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00	0.0 – 2.0
	49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
	70	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.25	0.00 ± 0.00	
Heterophil/Lymphocyte ratio	28	1.15 ± 0.14[a]	0.62 ± 0.06[b]	0.95 ± 0.24[a]	0.84 ± 0.11[a]	N/A
	49	1.32 ± 0.08	0.93 ± 0.19	1.31 ± 0.16	1.46 ± 0.04	
	70	0.89 ± 0.09[a]	0.46 ± 0.04[b]	0.70 ± 0.04[a]	0.62 ± 0.09[a]	

Control = basal diet; AA = Ascorbic acid, BET = betaine and AA+BET = Combination of ascorbic acid and betaine included in diets. [a,b] Mean values with different superscript letters within the same row are significantly different ($P < 0.05$).
* Reference: Pollack *et al*., 2005

The percentage eosinophil recorded in all groups were within the normal range of 0.0 – 15.0 for Japanese quails at all ages. However, at 28 days old, the percentage eosinophil of group AA+BET was significantly ($p < 0.05$) lower than that of the control group. Percentage basophil of birds in all groups were within the normal range of 0.0 – 2.0 for Japanese quails. There was no significant ($p > 0.05$) difference in percentage basophil in treated groups were compared with those of the control during the study period. In female quails which consumed either AA or BET – enriched diets, H/L ratio were significantly ($p < 0.05$) lower than the value obtained in the control group at 28 day–old.

DISCUSSION

Thermal Environmental Conditions

The results of this study implies that the Japanese quails were exposed to chronic high DBT and high RH during the dry season, and that these environmental conditions (DBT, RH and THI) were predominantly outside the thermoneutral zone [DBT (23.8 ± 0.7 °C), RH (58.5 ± 5.7%) and THI (76.0 – 80.0)] for Japanese quails [15]. The results also demonstrate that there were wide fluctuations in environmental parameters, evidenced by the wide range between minimum and maximum temperatures recorded during the study period. The results of the present study suggest that the hottest parts of the day were observed in the afternoon and evening periods during the dry season. These findings agree with those of Minka and Ayo [26,27] who demonstrated that the hot–dry season with mean DBT and RH recordings of 32.5 ± 1.4 °C, 66.9 ± 5.7 % respectively, constituted thermal stress to the quails. However, although their studies [26, 27] were on transportation of quails in the Northern Guinea Savannah zone, the present study emphasized the effects of thermal stress during growing and mature phases (sexual development) of Japanese quails in the Derived Guinea Savannah zone. The results also agree with those of Sinkalu *et al*. [28] who demonstrated that wide fluctuations in environmental conditions, such as DBT, may result in heat stress in poultry birds. In addition, heat stress may occur in Japanese quails, specifically, when AT is above 30 °C [29] and generally in poultry birds, when they are exposed to high AT and high RH [30]. Furthermore, chronic stress also results when high AT and high RH persists for a prolonged period of days or weeks [1].

Consequently, the results of this study suggest that the thermally stressful environmental conditions prevailing during the dry season in the study area may be detrimental to the health and welfare of Japanese quails. These stressful environmental conditions which prevailed during the dry season, as shown by high AT, RH and THI, may increase thermal load, impair the capacity to dissipate excess heat to the environment by thermoregulatory mechanisms and consequently, result in heat stress in the Japanese quails. It has been reported that heat stress impairs ability for heat loss in poultry [31], and in Japanese quails [15].

Thermal Condition and Biomarker of Stress

The results of the present study suggests that female quails were exposed to some levels of oxidative stress induced by chronic high AT and high RH, but AA and/or betaine supplementation ameliorated the deleterious effects of the thermal environmental conditions prevailing at the time. This finding was demonstrated by the significantly ($p < 0.05$) lower serum levels of MDA and the higher antioxidant enzyme (SOD), obtained at both grower and adult phases of growth of the birds. This result agrees with those of Dalkilic et al. [32] who demonstrated that the antioxidant property of orange peel oil enhances thermotolerance by decreasing MDA concentrations in Japanese quails. The findings of this study agree Mehaisen et al. [33] which showed that propolis attenuated lipid peroxidation by reducing serum MDA levels in heat stressed Japanese quails as well as with those of Omid et al. [34] showed that the amino acid, N–acetyl cysteine, enhanced the activities of antioxidant enzymes, thus improving the antioxidant status of heat stressed breeder quails. Heat stress results in excessive generation of reactive oxygen species (ROS) free radicals, lipid peroxidation and oxidative stress in quails [35]. Therefore, it is recommended that adequate preventive measures (like dietary enrichments with agents with antioxidant properties) be utilized to ameliorate the adverse effects of heat stress when rearing Japanese quails during the thermally stressful dry season period as obtained in the study area during this study.

It has been reported that excessive generation of ROS, due to high ambient temperature (AT) and high RH [1] result in oxidative stress when the levels of pro–oxidant molecules produced exceed the antioxidant capacity of the body system [36]. In addition, chronic stress depletes the antioxidant defenses in poultry birds making them susceptible to the deleterious effects of thermally stressful environmental conditions [37]. However, from the results of the present study, dietary supplementation with AA and/or betaine reduced the adverse effects of heat stress in female quails, as shown by the enhanced serum levels of SOD, especially, in 70 day–old female Japanese quails. The antioxidant, AA, is known to scavenge free radicals in birds under stressful conditions [38] while betaine acts at both cellular and gene levels via its osmoregulation and methyl donor capabilities, may be responsible for the results obtained in the present study. Surai *et al*. [39] demonstrated that betaine is involved in DNA methylation at genes, including vitagenes, which are families of genes responsible for encoding for components of antioxidant defense system. The vitagenes, consequently, enable animals adapt to stress. Furthermore, the methyl donor property of betaine enhances protein synthesis, especially through endogenous synthesis of methionine from homocysteine, which is essential in achieving optimal physiological performance in Japanese quails. The osmotic balance induced by betaine in the enterocytes may enhance nutrient utilization during thermally stressful dry season, to ensure the uninterrupted supply of resources needed for protein synthesis including, antioxidant enzymes in Japanese quails [40]. The results of the present study suggest that AA and/or betaine supplementation exerted antioxidant potentials and improve thermotolerance in the female Japanese quails, especially at maturity, during the dry season. This implies that supplementation of quail diets with AA and/or betaine enhances the dismutase pathway to degrade reactive superoxide molecules to non-reactive species. The SOD enzyme retards the progression of lipid peroxidation in tissues and ameliorates the adverse effects in the birds.

Heat Stress and Serum Follicle Stimulating Hormone

The findings of the present study show that the supplementation with AA and/or betaine improved serum FSH of female Japanese quails of different ages during the dry season. The findings imply that during the thermally stressful dry season, dietary enrichment with either AA and/or betaine improves the activity of gonadotropin, FSH in female Japanese quails. These results agree with those of Al-Salhie *et al*. [41] who showed that vitamin E, alone and in combination with propolis, improved FSH in Japanese quails exposed to heat stress conditions. Their study [41] assessed the antioxidant properties of vitamin E and proplis in male adult quails, but the present study evaluated the antioxidant properties of AA and/or betaine on FSH in female grower and adult quails. Karanth *et al*. [42] demonstrated that AA induces gonadotropin release by autocrine activity of nitric oxide. The findings of the present study suggest that supplementation with AA and/or betaine in diets fed quails reversed the adverse effects of thermal stress on FSH activity and, their subsequent physiologic roles in avian reproduction.

Pu *et al*. [43] showed that stressors like heat stress activate the hypothalamus–pituitary–adrenal axis via the central effect of corticotrophin–releasing hormone (CRH) which inhibits the secretion of gonadotropin–releasing hormone (GnRH), and consequently, FSH. This implies that there would be a decline in oogenesis in sexually mature female Japanese quails. The CRH and GnRH were not evaluated in the current study. Other peptides such as RF-amide peptides, kisspeptin and RF amide-related peptide-3, also known as gonadotropin inhibiting hormone (GnIH) may inhibit GnRH activity under heat stress conditions [44]. Therefore, optimum reproduction may decline when quails are subjected to thermally stressful dry season as observed in the current study. In the poultry industry, AA is used to ameliorate the deleterious effects of heat stress owing to its free radical–scavenging property [45]. Betaine, may have enhanced the activity of hypothalamic secretion of gonadotropins via DNA methylation. Methylation of DNA by betaine has been shown to influence hypothalamic secretion of hormones [46]. The results of the present study further suggest that feeding female Japanese quails diets enriched with AA and/or betaine may mitigate the disruption of critical events required for gametogenesis during the thermally stressful dry season.

Heat Stress and Egg Laying Performance

The results of this study demonstrated that dietary supplementation with betaine, either alone or in combination with AA, enhanced the HDEP, egg mass, and feed conversion efficiency to 1 kg egg mass and one dozen eggs produced. This is demonstrated by significantly ($p < 0.05$) higher values of HDEP, egg mass, but lower values of FCR and FEPD in betaine fed quails compared with those fed only basal diet without supplementation during the dry season. It implies that in quails fed dietary supplementation with betaine and its combination with AA, will require less quantity of feed to produce heavier eggs and a dozen eggs. These findings also agree with those of Ratriyanto and Prastowo [47] that betaine and floor space improve egg laying performance of Japanese quails reared under tropical conditions. The osmolyte function of betaine ensures osmotic balance in intestinal epithelium [40] thus, enhancing nutrient utilization in poultry birds, particularly Japanese quails [47]. Furthermore, betaine functions as a methyl donor and plays a role in protein synthesis through DNA epigenesis [48]. This implies that the combined functions of betaine as an osmolyte and methyl donor may be responsible for the improved laying pattern in Japanese quails during the dry season.

The findings are of particular importance because poultry birds (including Japanese quails) lay fewer and smaller sized eggs at the commencement of egg production [49], especially under thermally stressful environmental conditions. The results suggest that diets enriched with betaine, fed to sexually mature Japanese quails, may increase the production of more acceptable eggs in terms of egg size and number. The improvement in HDEP, egg mass, FCR and FEPD are beneficial to quail farmers because of the expected increase in profit margin arising from increase in egg production and reduction in cost of feed due to high feed conversion efficiency in female Japanese quails reared during the dry season.

Heat Stress and Immune Response

The findings of the present study show that AA and/or betaine supplementation modulated immune responses to thermally dry season. The findings of the present study showed that betaine significantly ($p < 0.05$) lowered leukocyte count when compared to control birds, especially, in 70 day–old female Japanese quails. This implies that betaine may have improved the protection of the birds against infectious agents. The results of the present study indicate that AA supplementation, either alone or in combination with betaine, reduced the risk of heat stress induced inflammation during the dry season. Though, rarely seen in peripheral circulation, few circulating monocytes are normal in Japanese quails. However, 49 day–old birds in either AA or AA+BET groups recorded significantly ($p < 0.05$) lower monocytes compared to those not fed supplemental diets. Monocyte numbers increased in response to inflammatory reactions [50,51]. The findings of the present study also indicates that AA enriched diet has the potential to ameliorate the adverse effects of heat stress in female quails. Evidence of tissue damage may have been lowered in quails fed AA enriched diets as shown by the lower, but normal, eosinophil counts recorded in the present study. Eosinophils are involved and increase in number in tissue damage that may result from lipid peroxidation due to excessive generation of free radicals. This is shown by the significantly ($p < 0.05$) lower heterophil/lymphocyte ratio. The results of the present study also show that AA decreases heterophil, but increases lymphocyte percentages, hence, the decrease in H/L ratio. The findings of the present study agree with those of Scanes *et al*. [52] who demonstrated that AA decreased corticosteroid–induced elevation of H/L ratio in male turkey.

The results of the present study suggest that the female quails were generally in good health conditions although the potential risks for inflammation, infections and tissue damage in female quails were reduced during the dry season with the supplementation of AA and/or betaine in diets of the birds. These findings showed that leukocytic parameters recorded in female quails were mostly within normal ranges for Japanese quails. However, total leukocyte, heterophil and eosinophils counts was significantly ($p < 0.05$) lower while lymphocyte numbers were higher when compared with control birds, particularly, in AA fed quails. The antioxidant, AA, improves the antioxidant defenses by scavenging free radicals [53], consequently, ameliorating the adverse effects of stress on leukocytes due to the thermally stressful dry season. From the

results of the present study, it is apparent the AA can act in synergy with betaine to improve immune responses of female quails to heat stress. Betaine, as a methyl donor, methylates DNA and modifies the expression of vitagenes, which are genes which code for proteins which are involved in antioxidant defense [54]. These vitagenes facilitate reduction of the adverse impact of heat stress on cellular components of the immune system. Thus, it is suggested that supplementation with AA, either alone or in combination with betaine may improve the health status of the female Japanese quails, especially at maturity, when reared in thermally unfavourable environments. The use of betaine and/or AA supplementations in diets of quails may be a recommended management practice to enhance thermotolerance, health and productivity of laying Japanese quail hens during the dry season.

It is therefore, concluded that betaine and ascorbic acid exert modulatory effects on responses to follicle stimulating hormone, production parameters and immunity of female Japanese quails during the dry season.

Declarations of Competing Interest

The authors do not have any conflict of interest to declare.

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