HAEMATOLOGICAL RESPONSES TO DIETARY ASCORBIC ACID AND BETAINE FED TO FEMALE JAPANESE QUAILS DURING THE DRY SEASON

Ifeanyichukwu .C. Egbuniwe, Blessing Francis and Tunde Ogundeji
Department of Veterinary Physiology and Biochemistry
Faculty of Veterinary Medicine, University of Benin,
Benin City, Nigeria.
ifeanyi.egbuniwe@uniben.edu

ABSTRACT

Environmental conditions prevailing in the dry season may induce heat stress, which may be evaluated by haematological values in Japanese quails. The present study investigated the haematological responses to dietary ascorbic acid and betaine fed to female Japanese quails during the dry season. A total of 468 female Japanese quails (Coturnix japonica) at 14 days old weighing 26-28 grams were used for the study. The quails were allotted into four groups namely the control, fed basal diets only, those fed ascorbic acid, AA-group; betaine, BET-group and combination of ascorbic acid and betaine – AA+BET group. Some microclimatic variables were measured daily at 08:00 h, 13:00 h and 17:00 h for 42 days. The variables include dry-bulb temperature (DBT – in ºC), relative humidity (RH) and temperature-humidity index (THI). Blood samples were collected from each of the six birds randomly selected from each group (two per replicate) in sample bottles with and without anticoagulants (EDTA) at 28, 49 and 70 days old. Serum malondialdehyde concentrations (MDA), superoxide dismutase (SOD) and reduced glutathione peroxidase (rGSH), as well as the haematology of the birds, were evaluated. Results indicate that, whereas AA and/or betaine decreased MDA, they increased SOD and rGH activities. Betaine increased the RBC count but reduced MCV and MCH. Ascorbic acid, supplemented either alone or with betaine, reduced total leukocyte count and the number of basophils, monocytes, eosinophils and H/L ratio. It is concluded that dietary ascorbic acid and betaine modulated the haematological profile of female Japanese quails reared during the dry season.

Keywords: *Antioxidants, Haematology, Stress, Quails, Season*

INTRODUCTION

Japanese quails are an important source of affordable protein for the growing population worldwide (Jesuyon *et al*., 2021). The health and welfare of poultry birds are important for producing eggs and meat (Barbut and Leishman, 2022). Although Japanese quails are hardy to common poultry diseases, however, there is a high possibility that they may come down with disease under heat stress conditions (Singh *et al*., 2023). This is because heat stress diminishes the immunity of quails (Qin *et al*., 2023). Heat stress results from high ambient temperature and high relative humidity; the combined effect of which produces a high temperature-humidity index in quails (Gouda *et al*., 2022). Whereas some investigations have been conducted on best management practices in quail production (Lima *et al*., 2023), there is a paucity of information on the effect of ascorbic acid and betaine on the haematological profile of female Japanese quails (*Coturnix japonica*) reared during the dry season.

The haematology of quails offers an opportunity to evaluate an indicator of health of quails (Alagawany *et al*., 2020). Extreme thermal conditions elicit excess production of reactive molecules, which results in damage in cells and tissues, leading to organ failure (Ghulam Mohyuddin *et al*., 2022). During the dry season, poultry farmers utilize relatively more affordable strategies to mitigate the adverse effects of heat stress (Wasti *et al*., 2020). Dietary ascorbic acid and betaine have been utilized to improve the health of Japanese quails (Egbuniwe *et al*., 2021). Ascorbic acid and betaine exert some antioxidant capacities (Attia *et al*., 2020; Li *et al*., 2022). In In birds and mammals, AA scavenges for free radicals that are generated in abundance during stressful conditions (Bano *et al*., 2021), while betaine improves the expression of vitagenes, demonstrated to code for antioxidant enzymes (Surai *et al*., 2021). The present study aimed to evaluate the haematological responses to the supplementation of ascorbic acid and betaine in the diet of female Japanese quails during the dry season

Materials and Methods

Experimental Site

The experiment was carried out at the Animal House of the Department of Parasitology and Entomology, Faculty of Medicine, University of Nigeria, Nsukka, Nigeria. The experimental site was located in the Derived Savannah Ecological zone of Nigeria (Omaliko, 1981) with an annual rainfall ranging from 986 – 2098 mm (Momoh et al., 2010). The current study was conducted from January – March, during the peak of ambient temperature (AT) and high relative humidity (Uguru *et al*., 2011).

Experimental birds

A total of 468, two-week-old female Japanese quails (*Coturnix japonica*) weighing 26 to 28 grams were sourced commercially and used for the study. The quails were acclimatized for 14 days. After acclimatization, the birds were identified using number tags affixed to one leg of each bird. The birds were assigned to four experimental groups. A range of numbers were allotted to each group for identification. The experimental groups included birds fed basal diets only, as Control; birds fed diets with ascorbic acid (AA) only – AA group; betaine only – BET group and a combination of ascorbic acid and betaine – AA+BET group. Each group comprised 117 quails, with each group, subsequently, divided into 3 replicates (39 quails per replicate).

Quails in each replicate were kept in separate cages measuring 0.91 m × 0.76 m × 0.91 m. These cages were confined within a well-ventilated poultry pen. The birds were fed commercial diets which comprised starter (22 % crude protein, CP; 3100 kcal/kg of metabolizable energy/kg of feed, ME) between 14 – 28 days old; grower (15.5 % CP, 2250 ME) at 28 – 49 days old, and finisher (16.8% CP; 2680 ME), 49 – 70 days old. The birds were subjected to 17 hours of lighting, required for optimum growth, development and production of Japanese quails (Elkomy *et al*., 2019). The dietary supplementation was performed daily for 42 days.

Dietary Ascorbic acid and betaine

Betaine hydrochloride – Betaine HCl – (Sigma-Aldrich, St. Louis, Missouri, USA) and ascorbic acid – AA – (Kempex Holland BV, Volkel, The Netherlands) were used for the study. They were both commercially sourced. Betaine HCl, which is manufactured as a clear and colourless crystal with ≥ 99.0% purity, was included in the diet of experimental groups at 2 g/kg of feed (de Jong 2012). Ascorbic acid, also manufactured in crystal form, was supplemented in the feeds at 200 mg/kg of feed (Sahin et al., 2002). The supplementation of betaine HCl and AA in the diets of the treatment groups for both male and female quails was performed daily throughout the experiment.

Measurement of Parameters

Thermal environmental conditions

Environmental parameters, indicating the thermal conditions prevailing during the study period, were recorded three times daily. Ambient temperature (AT) readings were measured using Mason's Type Wet and Dry Bulb Hygrometer (Zeal, London, England). The temperature range of the hygrometer was -8 ºC to 50 ºC with an accuracy of +/-1 ºC (or +/-5%). Following AT recordings, relative humidity (RH) was obtained using Hygrometric tables for computation of relative humidity

(Zeal, London, England). Temperature – humidity index (THI) was calculated according to the formula described by Zulovich and DeShazer (1990): *THI = 0.6Tdb + 0.4Twb,* and modified by El-Tarabany (2016).

Where Tdb = dry-bulb temperature, °C, and Twb = wet-bulb temperature, °C. The THI describes the combined effects of temperature and humidity on the quails. All environmental conditions were recorded at 08:00 h, 13:00 h and 17:00 h daily throughout the duration of the study).

Blood and Serum Collection

Blood collections from quails were performed at 28, 49 and 70 days of age. A total of six birds from each group (2 birds per replicate) were randomly selected, and identified with the number of leg tags and 3 ml of blood was collected from each bird. Each quail was weighed using a digital scale to the nearest 1g (Asia Techno weigh India, Haryana, India) and slaughtered by severing the jugular vein (Erol *et al*., 2009), and its blood quickly collected into labelled sample bottles with and without anticoagulant – sodium ethylenediaminetetraacetate - (Robertson and Maxwell, 1993). Blood samples were kept at room temperature for 3 hours to allow for blood to clot properly. Thereafter, blood samples were centrifuged at 3000 x g for 10 minutes at room temperature, and sera were collected and immediately used for analysis (Lee *et al*., 2014).

Oxidative Stress Biomarkers

Serum malondialdehyde concentration

Serum malondialdehyde (MDA) concentration was determined using the method described by Stocks and Dormandy (1971). The technique is based on the principle of the quantification of thiobarbituric acid reactive substances (TBARS) formed from the reaction of thiobarbituric acid with MDA. Serum (0.5 ml) was mixed with 20 % trichloroacetic acid in a ratio of 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 a water bath (100 °C) for 15 minutes. The contents were then cooled, and centrifuged at 2500 × g for 15 minutes. The optical density of the supernatant was determined at 532 nm against blank using a spectrophotometer (Jenway 6305; Jenway, Essex, UK) and a standard curve constructed using various MDA concentrations of 0 – 20 nMoles (Sigma, St. Louis, MO, USA).

Serum superoxide dismutase

The method described by Misra and Fridovich (1972), was used to assess superoxide dismutase (SOD) activity. The principle is based on the inhibitory effect of SOD on the autoxidation of epinephrine to form

adrenochrome at pH 10.2. 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; pH 10.2) and 0.5 mM EDTA solution (0.5 ml). Epinephrine (0.4 ml; 3 mM) – Sigma, St. Louis, Missouri, USA – was added to initiate a reaction, and the rate of change in absorbance was determined at 480 nm against a blank. A calibration curve of SOD was constructed using 0 – 195 SOD units, defined as the rate of change at 50 % SOD (Sigma, St. Louis, MO, USA) inhibition of epinephrine conversion to adrenochrome. The activity of SOD was determined as U/ml.

Serum reduced glutathione

Serum-reduced glutathione (rGSH) was evaluated by the technique described by Moron et al. (1979), and based on the principle of complex formation from the reaction of dithionitobenzene (DTNB) and acid sulfhydryl groups (non-protein thiols containing 93 % GSH-Rd). A mixture of serum (0.5 ml) and 25 % TCA (0.1 ml), kept for some minutes on ice, was centrifuged for 10 minutes at 3000 × g. In addition, 0.2 M of sodium phosphate buffer (0.7 ml, pH 8) and 0.6 mM of DTNB (2 ml; Sigma, St. Louis, MO, USA) were mixed with the supernatant (0.3 ml) for 10 minutes. Thereafter, a spectrophotometer (Jenway 6305; Jenway, Essex, UK) was used to measure the optical density of the product at 412 nm. Reduced glutathione concentration was determined from a standard graph (Sigma, St. Louis, Missouri, United States) of various GSH concentrations and expressed as nMoles/ml.

Haematological Profile

The haematological profile was determined using methods described by Cheesbrough (2006). Details of techniques used for each parameter are described below.

Packed cell volume (or hematocrit)

Blood samples (1 ml) were collected individually from 6 male and 6 female quails, into sample bottles containing anticoagulant (sodium ethylenediaminetetraacetic acid (EDTA) at a concentration of 1.5 mg/ml (Robertson and Maxwell, 1993). Thereafter, blood was drawn into capillary tubes (Fisher Scientific, Pittsburgh, Pa.) by capillary action and sealed at one end. Microhematocrit centrifuge (Hawksley, England) was used to centrifuge the blood samples at 12,000 × g for 5 minutes to obtain even packing of erythrocytes. Packed cell volume (PCV) was obtained using a microhaematocrit reader and expressed as percentage (%).

Haemoglobin concentration

Haemoglobin (Hb) concentration was determined using a cyanmethemoglobin method. The principle is based on the conversion of haemoglobin to methaemoglobin by ferricyanide action, and subsequent transformation of methaemoglobin by potassium cyanide action. The blood sample (0.02 ml) was further mixed with Drabkin'sbkins solution (1:250) for 10 minutes and absorbance was determined using a spectrophotometer (CHEM-5V3; Erba, Mannheim, Germany) at 546 nm absorbance (A). The Hb concentration was obtained using the following:

$$Hb\ concentration\ (g/dL) = \frac{(A)Sample\ x\ Concentration\ of\ standard\ x\ dilution\ factor}{(A)\ Standard}$$

Erythrocyte count

The blood sample (20µL) was thoroughly mixed with 4 ml of red blood cell dilution fluid (Dacie's solution) in a tube. The counting chamber of a Neubauer hemocytometer (Gallenkamp, United Kingdom) was filled with a small number of blood samples using a pipette and left for 2 minutes for cells to sediment. Erythrocytes (red blood cells – RBC) were counted in 5 out of 25 central squares using × 400 magnification, and the RBC count was evaluated as:

$$RBC\ count\ (\times 10^{12}\ per\ liter) = \frac{Number\ of\ cells\ counted}{100}$$

Leucocyte count

A blood sample (100 µL) was added to 1.9 ml of 1 % ammonium oxalate solution (white blood cell – WBC diluting fluid) in a tube. A small quantity was filled into a Neubauer haemocytometer (Gallenkamp, United Kingdom) using a pipette, and allowed to sediment for 2 minutes. The number of leukocytes (WBC was counted in the four large corner squares of the chamber (64 small squares) using a microscope under × 100 magnification. The determination of WBC count was by formula:

$$WBC\ count\ (\times 109\ per\ litre) = \frac{Number\ of\ cell\ counted}{20}$$

Differential leukocyte Count

The blood sample was smeared thinly on a microscope slide, air-dried and stained using Leishman's stain. The stained thin blood smear was examined using a Motic B3 Microscope (Motic, Carlsbad, CA, USA) under oil immersion, at × 1000 magnification. The differential count of WBCs was performed in each field using a blood cell counter (Durga, Delhi, India) until 100 cells were counted. Each WBC type counted

was expressed as a per cent of the total WBC.

Erythrocytic indices

The indices of erythrocyte (RBC) were calculated with the corresponding formula stated below:

$$\textit{Mean Cell Volume (MCV; fl)} = \frac{Hematocrit\ x\ 10}{Red\ blood\ cell}$$

$$\textit{Mean Cell hemoglobin (MCB; pg/red cell)} = \frac{Hemoglobin\ x\ 10}{Red\ blood\ cell}$$

$$\textit{Mean Cell hemoglobin concentration (MCHC; g/dl)} = \frac{Hemoglobin\ x\ 100}{Hematocrit}$$

$$\textit{RBC distribution width (RDW-CV; \%)} = \frac{Standard\ deviation\ of\ MCV\ x\ 100}{MCV}$$

Statistical Analysis

Data obtained were expressed as mean ± standard error of the mean (± SEM). The values obtained 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 (Snedecor and Cochran, 1994). GraphPad Prism (GraphPad Software, Incorporated, San Diego, California, USA) version 6.0 will be used for data analysis.

Results

Environmental parameters

Tables 2 and 3 show the weekly and diurnal fluctuation of the microclimatic conditions measured during the study period. The minimum and maximum values of mean RH of 59.5 ± 1.88 % and 79.6 ± 1.25 % were obtained after the first week, when the birds were 35 days old and 4th week, when the birds were 56 days old, respectively. The mean minimum DBT of 30.2 ± 0.73 ºC and THI of 78.5 ± 1.11 were recorded during the first week (when the birds were 35 days old), while the mean maximum values of 32.1 ± 0.80 ºC for DBT and 85.5 ± 1.07 for THI were obtained at the 7 weeks of the study (when the birds were 70 days old). The DBT, RH and THI ranged from 25.0 – 37.0 ºC, 48.0 – 91.0 and 69.8 – 91.0, respectively (Table 3). The mean maximum DBT value of 32.6 ± 0.2 ºC and the mean minimum of 27.3 ± 0.2 ºC were recorded at 13:00 h and 08:00 h, respectively. Whereas the mean minimum and maximum RH, of 69.9 ± 1.7% and 80.6 ± 1.9% were obtained at 13:00 h and 08:00 h, respectively, the values of 77.8 ± 0.6 and 84. 7 ± 0.4 recorded at 08:00 h and 17:00 h were the mean minimum and maximum THI, respectively.

Stress biomarkers of female birds

The biomarkers of stress are shown in Table 4. The MDA concentration in the serum of birds fed diets with either AA, BET or AA+BET was significantly ($P < 0.05$) lower when

compared with the values of the control birds. However, serum SOD was significantly ($P < 0.05$) higher in the AA, BET and AA+BET-fed birds when compared with those of the control. Similarly, there were significantly ($P < 0.05$) higher values of rGSH in the birds fed dietary BET, at 28 day-old and AA+BET, at 70 day-olds in comparison with those of the control.

Female erythrocytic characteristics

Table 5 describes the erythrocytic characteristics of the female quails. The erythrocyte profile obtained from the birds was predominantly within physiological reference values. There was no significant ($P > 0.05$) difference in PCV and Hb concentration between female quails in treatment groups when compared with that in birds of the control group during the study period.

The RBC count was significantly ($P < 0.05$) higher in the female birds fed dietary betaine, either alone or in combination with AA when compared with those of the control especially in 49-day-old birds. The values of erythrocytic indices, namely MCV, MCH and MCHC, varied in response to the supplementation with AA and betaine, either alone or in combination. The values of MCV and MCH were significantly ($P < 0.05$) lower in the female birds fed dietary ascorbic acid and/or betaine when compared with the corresponding values recorded in the control groups. However, there was no significant ($P > 0.05$) difference in MCHC recorded in treated birds when compared with those of control groups in birds.

Female leukocytic characteristics

Table 6 describes the leukocytic features of female quails during the study period. The total and differential leukocytic counts obtained during the study period were predominantly within the physiological range for quails. There was significantly lower TLC in the birds fed betaine when compared with the value obtained in the control, AA and AA+BET. Heterophil count was significantly ($P < 0.05$) reduced in the birds that were fed dietary AA, either alone or in combination with betaine in comparison with those of the control. There was a significantly ($P < 0.05$) higher number of lymphocytes in the AA-treated birds when compared with those observed in the control groups. The monocyte count in quails fed dietary AA, either alone or in combination with betaine was significantly ($P < 0.05$) lower when compared with the values obtained in birds in the control group. The birds fed supplemental AA or betaine recorded significantly ($P < 0.05$) lower eosinophils in comparison with the values obtained in the control quails. There was a significantly ($P < 0.05$) lower H/L ratio in the AA-fed quails when compared with those of the control birds. Basophil count did not differ significantly ($P > 0.05$) when the

treated birds were compared with the control and with one another.

Discussion

Environmental parameters

The results of the present study showed that the prevailing microclimatic conditions during the study period exceeded the thermoneutral zone of DBT, RH and THI recommended for Japanese quails. The thermoneutral zone for quails is 23.8 ± 0.7 °C for AT and 58.5 ± 5.7% for RH (El-Tarabany, 2016). The findings indicate wide fluctuations in these environmental conditions during the day and the period of the study, with the hottest period being the afternoon hours in the dry season. Thus, the dry season could be thermally stressful for female Japanese quails. Güngören *et al*. (2023) reported that wide fluctuations in environmental conditions could be thermally stressful to Japanese quails in tropical climate. Abuoghaba *et al*. (2021) showed that high AT and high RH resulted in heat stress in poultry birds, produce high THI exceeding the zone of thermal comfort for quails (de Oliveira Castro *et al*., 2023).

Heat stress conditions, such as those observed in the study, elicit the production of reactive molecules known to be detrimental to the health and welfare of quails (Chauhan *et al*., 2021). Hence, it is necessary to initiate measures to mitigate the adverse effects on the health and well-being of female quails that are inevitably reared under thermally stressful conditions of the environment. Some affordable measures may include dietary supplementation with agents that have antioxidant properties (Hassan *et al*., 2021) such as AA and BET.

Stress biomarkers of female birds

The study shows that AA and BET, supplemented either alone or in combination, decreased serum levels of MDA in female Japanese quails during the dry season. This may be due to the decrease in lipid peroxidation in the studied birds in either AA or BET groups. The study showed that AA and BET improved the activities of antioxidant enzymes, such as SOD and rGSH. The results agree with those of Karageçili *et al*. (2022) who showed that AA decreased lipid peroxidation in quails. The findings of Özbilgin *et al*. (2021) corroborate the study. Sahebi-Ala *et al*. (2021) showed that betaine supplementation in diets of heat-stressed broiler chickens reduced MDA concentration.

The role of AA and BET in reducing lipid peroxidation, evidenced by deceased MDA and, increasing SOD and rGSH activities could be that they enhanced the antioxidant defences in the female quails exposed to the thermally stressful dry season. Ascorbic acid can scavenge free radicals, and consequently, exerts an antioxidant capacity in heat-stressed birds (Sun *et al*., 2023). The dietary supplementation with antioxidants

offers a relatively more affordable approach to mitigating heat stress in poultry birds, including Japanese quails (Vandana *et al*., 2021).

Female erythrocytic characteristics

Dietary supplementation with betaine; alone and in combination with AA enhanced red cell count in female quails during the thermally stressful dry season. The findings of the study indicate that supplemental betaine decreased the erythrocytic indices in the birds. This is in agreement with Alsulami and El-Saadony (2023) who reported that bacteriogenic selenium nanoparticles enhanced RBC count in poultry. Uyanga *et al*. (2022) showed that betaine improved the erythrocytic indices in poultry birds.

The effects of betaine on some erythrocytic parameters in female quail may be attributed to its capacity for DNA methylation. The methylation of DNA may enhance the synthesis of proteins involved in haemopoiesis (Saunderson *et al*., 2023). This requires further investigation since the role of DNA methylation in blood formation was not evaluated in the current study. Additionally, betaine has been shown to enhance the utilization of nutrients (Park and Kim, 2019) including iron, which is involved in haemopoiesis. The probable improvement in nutrient utilization of betaine is due to its osmoregulatory property, which stabilizes various cells in the body including erythrocytes (Alagawany *et al*., 2022). The improved erythrocytic characteristics are beneficial for the female quails in meeting the high oxygen demand required for growth and production. The higher the RBC count the more capacity for the quails to increase the circulation of oxygen in the birds (Truong *et al*., 2023).

Female leukocytic characteristics

The findings of the present study showed that AA modulated immune responses to the thermally dry season. These results are evidenced by lower values of total leukocyte count, and the number of heterophils, monocytes and eosinophils, especially in birds supplemented with AA in diets, either alone or in combination with BET, when compared with those of the control birds. In addition, AA decreased the H/L ratio and increased lymphocyte count when compared with the values obtained in the control birds. It could be suggested that the supplementation with AA in the diets of female Japanese quails would improve the immune responses to infectious agents, tissue damage and parasitism, especially when reared during the thermally stressful dry season in tropical regions.

Gouda *et al*. (2020) showed that AA improved the immunity of broiler chickens during the thermally stressful dry season. Yousefi *et al*. (2023) demonstrated the antioxidant property of betaine to enhance the immune responses to thermal stress. The mechanism underlying the role of AA and BET improve immune

responses may be attributed to the ability to enhance nutrient utilization.

Thus, from the results of the study, it has been shown that AA and BET betaine supplemented, either alone or in combination, may decrease lipid peroxidation, by decreasing serum MDA concentrations, and affect erythrocytic and leukocytic characteristics by improving RBC counts and the immunity of female quails when reared during the stressful conditions prevailing during the dry season.

It could be concluded that dietary ascorbic acid and betaine influence haematological responses by decreasing MDA concentration and immune cells, but increasing RBC count in female Japanese quails reared during the dry season.

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Table 1: Composition and proximate analysis of quail diets

Feed Composition	Starter	Grower	Finisher
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)	3100	2250	2680
Crude protein (%)	22.0	15.5	16.8
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.

Table 2: Weekly variations in environmental conditions recorded during the study period

Experimental period (Week)	Age of quails (days)	Environmental conditions		
		DBT (° C)	RH (%)	THI
0	28	31.7 ± 0.54 (28.0 – 36.0)	78.0 ± 2.06 (57.0 – 92.0)	84.8 ± 0.57 (80.2 – 88.2)
1	35	30.2 ± 0.73 (25.0 – 35.0)	59.5 ± 1.88 (48.0 – 80.0)	78.5 ± 1.11 (69.8 – 84.2)
2	42	31.2 ± 0.74 (25.0 – 36.0)	61.0 ± 2.12 (50.0 – 85.0)	80.4 ± 1.24 (71.2 – 90.3)
3	49	31.4 ± 0.82 (25.0 – 37.0)	78.9 ± 2.14 (62.0 – 91.0)	84.4 ± 1.01 (75.6 – 90.7)
4	56	31.6 ± 0.70 (25.0 – 36.0)	79.6 ± 1.25 (71.0 – 91.0)	84.9 ± 0.96 (75.6 – 91.0)
5	63	32.0 ± 0.63 (28.0 – 35.0)	78.1 ± 1.52 (79.5 – 90.0)	85.4 ± 0.82 (79.0 – 90.3)
6	70	32.1 ± 0.80 (27.0 – 36.0)	78.6 ± 1.81 (68.0 – 91.0)	85.5 ± 1.07 (77.0 – 91.0)

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

Table 3: Hourly fluctuations in mean values of microenvironmental conditions during the study period

Microclimatic Conditions	Hour of the Day		
	08:00 h	13:00 h	17:00 h
Dry-bulb temperature (°C)	27.3 ± 0.2	32.6 ± 0.2	32.1 ± 0.2
Relative humidity (%)	80.6 ± 1.9	69.9 ± 1.7	73.5 ± 1.3
Temperature-humidity	77.8 ± 0.6	84.6 ± 0.4	84. 7 ± 0.4

Table 4: Serum levels of stress indicators in female Japanese quails fed dietary ascorbic acid and/or betaine during the study period (n = 6).

Stress indicators	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}
	49	0.72 ± 0.04^{a}	0.54 ± 0.02 ^{b}	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	0.91 ± 0.08	0.89 ± 0.03	0.98 ± 0.08
	49	0.60 ± 0.02	0.81 ± 0.02	0.76 ± 0.03	0.78 ± 0.02
	70	0.53 ± 0.02^{a}	0.87 ± 0.02^{b}	0.78 ± 0.02^{b}	0.91 ± 0.01^{b}
Reduced glutathione (nMol/ml)	28	6.94 ± 0.76^{a}	8.85 ± 0.61^{a}	9.85 ± 0.57^{b}	8.93 ± 0.51^{a,b}
	49	6.27 ± 0.77	7.70 ± 0.64	7.98 ± 0.80	7.98 ± 0.80
	70	5.58 ± 0.42^{a}	8.09 ± 0.88^{a}	7.32 ± 0.81^{a}	8.76 ± 0.79^{b,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).*

Table 5: Responses in red blood cells of female Japanese quails fed dietary ascorbic acid and/or betaine during the study period (n = 6)

Erythrocytic parameters	Age of birds (days)	Experimental groups				Reference values *
		Control	AA	BET	AA+BET	
PCV (%)	28	29.50 ± 1.50	32.00 ± 2.42	30.60 ± 1.29	31.75 ± 0.85	30.0 – 45.1
	49	43-00 ± 0.85	44.33 ± 0.25	45.25 ± 0.48	44.50 ± 0.48	
	70	32.00 ± 0.58	35.33 ± 2.73	35.25 ± 1.32	34.25 ± 1.25	
RBC count ($\times 10^6/\mu l$)	28	3.76 ± 0.32	4.47 ± 0.34	4.33 ± 0.15	4.24 ± 0.13	4.0 – 5.2
	49	3.28 ± 0.16[a]	3.89 ± 0.12[a]	4.50 ± 0.40[b]	4.24 ± 0.10[b]	
	70	3.76 ± 0.05	4.12 ± 0.13	3.65 ± 0.16	3.92 ± 0.28	
Hb concentration (g/dl)	28	8.70 ± 0.39	9.12 ± 0.73	8.92 ± 0.47	9.57 ± 0.27	10.7 – 14.3
	49	12.24 ± 0.36	12.20 ± 0.19	12.92 ± 1.02	12.47 ± 0.15	
	70	9.86 ± 0.27	10.95 ± 0.25	10.59 ± 0.45	10.75 ± 0.59	
MCV (fl)	28	79.70 ± 5.92	71.74 ± 1.50	73.15 ± 4.72	74.99 ± 2.44	60.0 – 100.0
	49	131.50 ± 3.12[a]	114.10 ± 0.75[a]	102.10 ± 5.78[b]	105.00 ± 1.15[b]	
	70	85.08 ± 1.24	85.54 ± 3.95	96.79 ± 1.35	88.62 ± 6.62	
MCH (ρg)	28	52.43 ± 3.76	45.55 ± 1.48	47.73 ± 3.71	50.37 ± 1.60	N/A
	49	83.43 ± 1.47[a]	70.06 ± 1.03[b]	64.47 ± 3.25[b]	65.60 ± 0.76[b]	
	70	58.47 ±	59.35 ±	64.77 ±	62.10 ±	

		1.09	0.64	0.91	5.63	
		29.52 ±	28.46 ±	29.12 ±	30.13 ±	28.0 –
MCHC (g/dl)	28	0.31	0.48	0.50	0.26	38.5
		28.47 ±	27.54 ±	28.45 ±	28.03 ±	
	49	0.20	0.34	1.42	0.10	
		30.81 ±	31.29 ±	30.01 ±	31.35 ±	
	70	0.32	1.86	0.17	0.88	

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). PCV = packed cell volume; RBC count = erythrocyte count; Hb = haemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin and MCHC = mean corpuscular haemoglobin concentration

**Reference: Pollack et al., 2005*

Table 6: Responses in white blood cells of female Japanese quails fed dietary ascorbic acid and/or betaine during the study period (n = 6)

		Experimental groups				
Leukocytic parameters	Age of birds (days)	Control	AA	BET	AA+BET	Reference values*
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]	
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)*