



Dietary ascorbic acid and betaine improve stress responses, testosterone levels and some sexual traits in male Japanese quails during the dry season

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(Received 13 May 2021; Accepted 30 May 2021)

Abstract

The experiment investigated the effects of dietary ascorbic acid and betaine stress responses, serum testosterone levels, and some sexual traits in male Japanese quails during the dry season. A total of 240 male Japanese quails (14 days old) were used and randomly assigned to four groups, each group has three replicates ($n = 20$). Birds in treatment groups were fed ascorbic acid (AA); betaine (BET); and AA + BET in their diets, whereas the control birds were fed only basal diet. Environmental conditions were predominantly outside thermoneutral zone for Japanese quails. Dietary AA ± BET increased ($p < .05$) serum catalase, reduced glutathione and testosterone, but lowered ($p < .05$) cortisol levels when compared with control group. Supplemental AA, BET, or AA + BET enhanced ($p < .05$) cloacal gland size and sexual traits. In conclusion, dietary AA and BET improved stress responses, serum testosterone levels, and some sexual traits in male Japanese quails during the dry season.

Keywords: antioxidants; heat stress; quails; sexual traits

1. Introduction

Japanese quails are good models for investigating testosterone regulation in sexual behavior, especially, in captive situation (Ball & Balthazart, 2010). However, these quails are susceptible to heat stress conditions during the dry season in the tropics (Tuleun et al., 2013). Heat stress (which could be induced in poultry birds during the dry season) activates the hypothalamus–pituitary–adrenal (HPA) axis and elevates cortisol level due to elevated adrenocorticotrophic hormone activity (Mormède et al., 2007). The increase in adrenocorticotrophic activities depresses hypothalamus–pituitary–gonad axis and lowers testosterone in Japanese quails (Tsutsui et al., 2012). Antioxidant supplementation is used to ameliorate the adverse effects of heat stress on reproductive parameters of the quail (Abd El-Hack et al., 2020). In addition, some male sexual traits, such as size of cloacal gland and testes are useful indicators of reproductive potentials of male Japanese quails (Mohan et al., 2002).pt

2. Objective

Ascorbic acid (AA) is an antioxidant which scavenges stress-induced free radicals in poultry birds (Ismail et al., 2015). Betaine (BET) exerts methyl-donor, osmolyte (Amerah & Ravindran, 2015) and antioxidant

properties in poultry birds (Alirezaei *et al.*, 2012). However, there is paucity of information on the effects of AA and BET supplementation on steroid activities and some sexual traits in male Japanese quails. The study is aimed at investigating effects of AA and BET supplementation on stress responses, testosterone levels, and some sexual traits in male Japanese quails during the dry season.

3. Methods

3.1. Experimental site

The experiment was performed during the dry season at the Animal House of Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka (6° 52' 0" N; 7° 23' E), in the Derived Savannah Ecological zone (Uguru *et al.*, 2011).

3.2. Ethical matters

The methods of handling protocol were in accordance with the Animal Use and Care Committee of University of Nigeria, Nsukka, Nigeria. All quails were managed in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1996).

3.3. Animals and experimental protocol

A total of 240 male Japanese quail chicks; 14 days old and weighing 55.7 ± 1.06 g, were sourced commercially. A total of 240 male Japanese quail (14 days old; weighing 55.7 ± 1.06 g) were used and randomly assigned to four groups, each group has three replicate ($n = 20$; Al-Salhi *et al.*, 2017). Birds in each replicate were housed in spaces (91.44 cm length \times 76.2 cm width \times 91.4 cm height) within windowed animal pen and exposed to the natural environmental conditions. Birds were fed basal diets supplemented with AA (200 mg/kg; Sahin *et al.*, 2002), BET (2 g/kg de Jong *et al.*, 2012), and a combination of AA at 200 mg/kg and BET at 2 g/kg (AA + BET) during the 49 days of study, whereas the control birds were administered basal diet only. AA used (Kempex Holland BV, Volkel, Netherlands) had a minimum purity of 99%, whereas BET (Sigma-Aldrich, St. Louis, MO) was $\geq 99.0\%$ purity. Daily dietary supplementation with AA and/or BET commenced after baseline serum testosterone values were established (when birds were 21 days old), and lasted 49 days (at 70 days old). Quails were given access to feeds and water *ad-libitum*.

3.4. Measurements

Thermal environmental conditions, measured as ambient temperature (AT) and relative humidity (RH) were recorded daily at 08:00 hr, 13:00 hr, and 17:00 hr throughout the study period. Blood collection was performed from six quails randomly selected and identified from each group at 21, 28, 49, and 70 days old. Blood samples were centrifuged at 3,000g for 10 min (El-Tarabany, 2016) sera were harvested and immediately analyzed. Indicators of stress such as serum catalase (KU/L) and reduced glutathione (nMoles/ml) were determined spectrophotometrically, but cortisol ($\mu\text{g/dl}$); using enzyme-linked immunosorbent assay technique. Testosterone levels were determined using the microplate enzyme immunoassay kit (Monobind Incorporated, Lake Forest, CA). At 28, 49, and 70 days of age, sexual traits of quails randomly selected from each group were determined by measuring cloacal gland size ($n = 12$) and indices of testicular development ($n = 4$) such as paired testicular weight, left testicular area, and combined testicular volume. Index of gland size (or area) was determined by the product of cloacal gland width and height, and expressed in cm^2 (Siopes & Wilson, 1975). The formulas described by Chaturvedi *et al.* (1993) was used to calculate cloacal volume, expressed in cm^3 :

$$\frac{4}{3} \times 3.5414 \times a \times b^2 \text{ (where } a = 0.5 \times \text{long axis and } b = 0.5 \times \text{short axis).}$$

Measurements of length and width of left and right testes were taken to the nearest 0.01 mm using digital vernier caliper (Shanghai Techway Industrial Co., Ltd., Shanghai City, China). Bissonett's formula was used to evaluate testicular volume: $4/3\pi ab^2$ (a = half of the long axis and b = half of the short axis), and expressed in cm^3 (Banerjee & Chaturvedi, 2017).

3.5. Statistical analysis

Data obtained are expressed as mean \pm standard error of mean. The values were subjected to statistical analysis and compared using analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. Values of $p < .05$ were considered significant (Snedecor & Cochran, 1994). GraphPad Prism (GraphPad Software, Incorporated, San Diego, CA) version 6.0 was for analysis.

4. Results

4.1. Thermal environmental conditions

The range values of AT were 25.0–37.0°C, whereas those of RH were from 48.0 to 92.0% during the experimental period (Figure 1).

4.2. Indicators of stress

All serum catalase, reduced glutathione, and testosterone levels were higher ($p < .05$) in the groups treated with AA \pm BET, whereas cortisol levels were lower ($p < .05$) when compared with the control group (Table 1).

4.3. Serum testosterone levels

Serum testosterone concentrations were not detected at 21 days old in all the groups. In all groups (control and AA \pm BET), serum testosterone levels were increased as the birds grew older. This trend is observed in higher ($p < .05$) serum testosterone levels in birds at 49 and 70 days old when compared with when they were 28 days old in each of the experimental groups. At 28 and 49 days old, serum testosterone levels in quails that are fed with either enriched AA, BET, or AA + BET were higher ($p < .05$) when compared with values recorded in control group. Testosterone levels increased ($p < .05$) in birds fed either AA or AA + BET in their diets when compared with control at 70 days old (Figure 2).

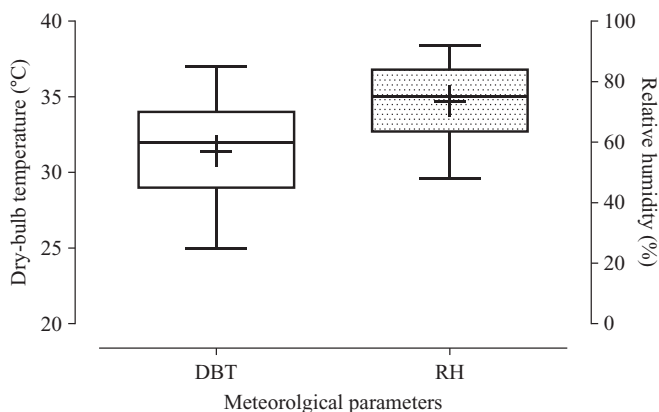


Figure 1. Boxplot describing micro-environmental conditions within the poultry house during the study period. DBT, dry bulb temperature (°C); RH, relative humidity (%).

Table 1. Indicators of stress in Japanese quails reared during the dry season ($n = 6$)

Stress indicators	Experimental groups			
	Control	AA	BET	AA + BET
Catalase (KU/L)	32.4 ± 2.0 ^a	51.6 ± 3.3 ^b	45.4 ± 1.3 ^b	47.1 ± 2.1 ^b
Reduced glutathione (nMol/ml)	6.2 ± 0.4 ^a	8.2 ± 0.4 ^b	8.2 ± 0.5 ^b	8.6 ± 0.4 ^b
Cortisol (µg/dl)	1.0 ± 0.2 ^a	0.7 ± 0.2 ^a	0.5 ± 0.1 ^b	0.5 ± 0.1 ^b

Abbreviations: AA, ascorbic acid; AA + BET, combination of ascorbic acid and betaine included in diets; BET, betaine; Control, basal diet. ^{a,b}Mean values with different superscript letters within the same row are significantly different ($p < .05$).

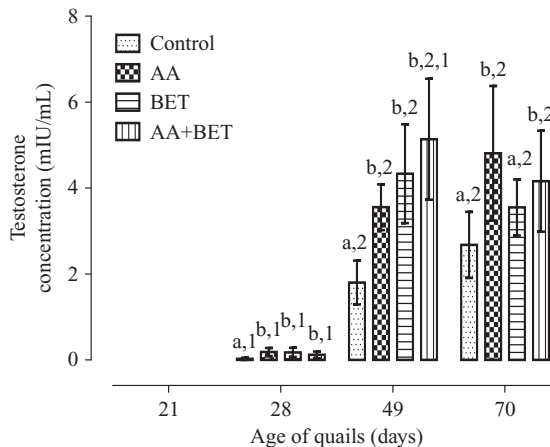


Figure 2. Changes in serum concentration of testosterone with age of Japanese quails during the study period. Control, basal diet; AA, ascorbic acid; BET, betaine; and AA + BET, combination of ascorbic acid and betaine included in diets. ^{a,b,1,2}Mean values with different superscript letters and numbers between and within groups, respectively, are significantly different ($p < .05$).

4.4. Sexual traits of male quails

Cloacal gland area increased ($p < .05$) in the treated quails when compared with those of the control, especially, in 28 days old birds. In addition, cloacal volume was higher ($p < .05$) in the treated quails when compared with values obtained in the control, especially at 28 and 49 days of age. Furthermore, quails of the treated group recorded increased ($p < .05$) paired testicular weight when compared with birds of control group at 28 days old. Testicular area and combined volume were higher ($p < .05$) at 28 and 49 days old, when compared with the corresponding values obtained in the control group (Table 2).

5. Discussion

5.1. Thermal environmental conditions

The results of the present study indicate that the recorded AT and RH fluctuated widely and predominantly exceeded the thermoneutral zone for Japanese quails. The thermoneutral zone for quails are $23.8 \pm 0.7^\circ\text{C}$ for AT and $58.5 \pm 5.7\%$ for RH (El-Tarabany, 2016). Thus, the results of the current study suggest that the natural environmental conditions during the dry season are potentially stressful for Japanese quails. The findings of the present study agree with those of Minka and Ayo (2013), who showed that the dry season in the Northern Guinea Savannah, with mean AT and RH of $32.5 \pm 1.4^\circ\text{C}$ and $66.9 \pm 5.7\%$, respectively, were stressful to Japanese quails. In addition, Mehaisen et al. (2017) demonstrated that AT values exceeding 30°C may be stressful for Japanese quails (Mehaisen et al.,

Table 2. Variations in sizes of cloacal gland and testes in male Japanese quails during the dry season

Cloacal gland parameters	Age of birds (days)	Experimental groups			
		Control	AA	BET	AA + BET
Cloacal gland area (n = 12; cm ²)	28	0.43 ± 0.07 ^{a,1}	0.85 ± 0.12 ^{b,1}	0.73 ± 0.09 ^{b,1}	0.85 ± 0.11 ^{b,1}
	49	1.33 ± 0.11 ^{a,2,1}	2.32 ± 0.28 ^{b,2,1}	2.00 ± 0.15 ^{a,2,1}	1.96 ± 0.35 ^{a,b,2,1}
	70	2.67 ± 0.30 ^{a,2}	3.30 ± 0.15 ^{a,2}	3.36 ± 0.11 ^{a,2}	3.75 ± 0.23 ^{a,2}
Cloacal gland volume (n = 12; cm ³)	28	1.18 ± 0.28 ^{a,1}	3.54 ± 0.74 ^{b,1}	2.66 ± 0.47 ^{b,1}	3.46 ± 0.65 ^{b,1}
	49	6.34 ± 0.77 ^{a,1}	14.85 ± 2.57 ^{b,2,1}	11.05 ± 1.57 ^{b,2,1}	11.86 ± 3.29 ^{b,1}
	70	19.09 ± 2.54 ^{a,2}	25.63 ± 1.57 ^{a,2}	26.32 ± 1.62 ^{a,2}	32.43 ± 3.10 ^{b,2}
Paired Testicular weight (n = 4; g)	28	0.02 ± 0.01 ^{a,1}	0.12 ± 0.7 ^{b,1}	0.06 ± 0.01 ^{b,a,1}	0.13 ± 0.06 ^{b,1}
	49	2.77 ± 0.94 ^{a,2}	3.28 ± 0.25 ^{a,2}	3.57 ± 0.59 ^{a,2}	3.61 ± 0.47 ^{a,2,1}
	70	4.40 ± 0.26 ^{a,2}	3.82 ± 0.22 ^{a,2}	4.63 ± 0.37 ^{a,2}	4.93 ± 0.25 ^{a,2}
Left testes area (n = 4; cm ²)	28	0.19 ± 0.07 ^{a,1}	0.40 ± 0.09 ^{b,1}	0.27 ± 0.05 ^{a,1}	0.38 ± 0.08 ^{b,1}
	49	1.91 ± 0.34 ^{a,2,1}	2.61 ± 0.12 ^{b,2}	2.87 ± 0.42 ^{b,2}	2.86 ± 0.07 ^{b,2}
	70	3.19 ± 0.21 ^{a,2}	2.89 ± 0.13 ^{a,2}	3.83 ± 0.30 ^{a,2}	3.42 ± 0.19 ^{a,2}
Combined testicular volume (n = 4; cm ³)	28	0.05 ± 0.03 ^{a,1}	0.21 ± 0.06 ^{b,1}	0.11 ± 0.02 ^{b,1}	0.17 ± 0.05 ^{b,1}
	49	2.43 ± 0.79 ^{a,2}	3.70 ± 0.34 ^{b,2,1}	3.57 ± 0.64 ^{b,2}	3.65 ± 0.41 ^{a,2}
	70	4.40 ± 0.22 ^{a,2}	3.84 ± 0.21 ^{a,2}	5.24 ± 0.31 ^{a,1,2}	5.22 ± 0.42 ^{a,2}

Abbreviations: AA, ascorbic acid; AA + BET, combination of ascorbic acid and betaine included in diets; BET, betaine; Control, basal diet. ^{a,b,1,2}Mean values with different superscript letters and numbers between and within groups, respectively, are significantly different (*p* < .05).

2017). The high AT and RH which prevailed during the dry season, when the present study was conducted, have detrimental to the health, welfare, and reproductive performance of Japanese quails.

5.2. Heat stress and indicators of stress

The result of the present study demonstrates that supplementations with AA and/or BET in diets of male Japanese quails improved serum antioxidant enzyme activities when reared during the thermally stressful dry season. The findings of the present study agrees with those of Alirezaei et al. (2012) who demonstrated that BET improved the antioxidant status of meat of broiler chicken subjected to heat stress conditions. Khan et al. (2012) also showed that AA plays a role in enhancing antioxidant defenses in poultry birds. The findings of the current study suggest that AA and BET supplementation would enhance antioxidant defenses and improve thermotolerance in Japanese quails during the dry season.

5.3. Heat stress and testosterone levels

The results of the current study show testosterone synthesis in testes may have been too minimal to be detected at 21 days old male Japanese quails. The inability to detect testosterone at 21 days of age could be due to heat stress–induced delay in sexual maturity. The developmental phases of quails are classified as

chick (0–21 days [or 0–3 weeks]); grower (28–35 days [or 4–5 weeks]); and adult (above 35 days [or 5 weeks]; Puspamitra *et al.*, 2018). The results of the present study are in agreement with Min *et al.*, 2016, who showed stress results in decline in testosterone levels in serum, but that AA and vitamin E ameliorated this decline in rooster breeders. Parisi and Guerriero (2019) showed that sex steroids, such as testosterone, are susceptible to oxidative stress due to thermal stress. The susceptibility of testosterone to the deleterious effects of free radicals requires antioxidants to ameliorate these effects (Sahin *et al.*, 2017). The increased serum testosterone observed in the present study in birds fed dietary AA, both alone and in combination with BET, may be attributed to the antioxidant potentials of AA, acting in synergy with the methyl donor properties of betaine, when incorporated in the diets of male Japanese quails. These mechanisms of action of AA and BET could be beneficial to improve gonadal activities, libido, and fertility in male Japanese quails when exposed to the thermally stressful dry season.

5.4. Stress and sexual traits

The findings of the current study indicate that the size of cloacal gland increased in birds of the treated groups. The results of the present study agree with those of Biswas *et al.* (2017) who demonstrated that selenium, an antioxidant, incorporated in diets of Japanese quails increased the size of cloacal gland and production of cloacal foam. Biswas *et al.* (2007) also showed vitamin E improved cloacal gland size. Cloacal gland is a peculiar anatomical feature of *Coturnix* quails which is an indication of testicular size and function. Therefore, the assessment of cloacal gland size is referred to as a noninvasive tool to evaluate testicular activity and maturity in Japanese quails (Dominchin *et al.*, 2016, 2017). Momoh *et al.*, (2014) showed that the age of sexual maturity in Japanese quails reared under hot–humid condition is 5–6 weeks old. The present study did not evaluate the age of sexual maturity. This is one limitation of the present study. However, the result of the present study suggests that the bigger cloacal gland observed in AA and/or BET fed quails when compared with control group could be due to antioxidant effects of AA and BET on testosterone activity.

Similarly, the results of the present study show that testicular development increased with increase in age, especially, in those fed diets enriched with AA, alone and its combination with BET. The findings of the present study agree with those of Tsutsui *et al.* (2015), who demonstrated that rosemary oil exerted antioxidant protection against oxidative damage in the testes of heat-stressed Japanese quails. Heat stress–induced lipid peroxidation may result in developmental and functional damages in testes (Türk *et al.*, 2016). It is suggested that improved testicular development in male quails fed diets enriched with AA and/or BET in the present study could improve fertility during the dry season. The results of the current study and its potential benefits to reproductive responses in the birds corroborate with those of Egbuniwe *et al.*, 2020, who demonstrated that diets enriched with BET and AA promote reproductive potentials as well as, sex and stress hormones (Egbuniwe *et al.*, 2021) in Japanese quails during the dry season.

The results of this study may be useful to quail breeders who could use AA and BET to ameliorate heat stress effects on male sexual traits and optimize reproductive performance of male quails reared during the dry season.

6. Conclusion

From the results of the current study, it is demonstrated that heat stress conditions prevailing during the dry season in the zone adversely impact on some sexual traits of male Japanese quails. However, dietary enrichments with AA and/or BET could be utilized to ameliorate the negative impacts of dry season–induced heat stress in male Japanese quails. In conclusion, dietary AA and BET improve steroidal responses and some sexual traits in male Japanese quails during the dry season.

Acknowledgment. The authors thank Drs. Oguejiofor, Akogwu, and Obetta for their assistance during the period of the current study.

Funding statement. None to declare.

Conflict of interest. The authors declare no conflict of interest.

Data availability statement. The data that support the findings of this study will be openly made available in University of Nigeria, Nsukka Repository at <https://oer.unn.edu.ng/reports?rdr=1>.

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Cite this article: Egbuniwe IC, Uchendu CN, Obidike IR (2021). Dietary ascorbic acid and betaine improve stress responses, testosterone levels and some sexual traits in male Japanese quails during the dry season *Experimental Results*, *2*, e23, 1–11. <https://doi.org/10.1017/exp.2021.10>

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This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

doi:10.1017/exp.2021.10.pr1

Review 1: Dietary ascorbic acid and betaine improve stress responses, testosterone levels and some sexual traits in male Japanese quails during the dry season

Reviewer: Dr. Tuncay Tufan 

Siirt University

Date of review: 28 May 2021

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Conflict of interest statement. I did a peer review for this article. I do not have any conflict of interest.

Comments to the Author: It is understood that a correction was made by the author in this research article. It is recommended to rewrite the title, associating it with "heat stress". In the "Materials and Methods" section, it is recommended to write the method a little bit abbreviated. Also, due to the low number of results in this article, it is recommended to be published as "short communication".

Score Card

Presentation



Is the article written in clear and proper English? (30%)

3/5

Is the data presented in the most useful manner? (40%)

3/5

Does the paper cite relevant and related articles appropriately? (30%)

4/5

Context



Does the title suitably represent the article? (25%)

3/5

Does the abstract correctly embody the content of the article? (25%)

3/5

Does the introduction give appropriate context? (25%)

3/5

Is the objective of the experiment clearly defined? (25%)

4/5

Analysis



Does the discussion adequately interpret the results presented? (40%)

4/5


Is the conclusion consistent with the results and discussion? (40%)

4/5

Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)

3/5

Review 2: Dietary ascorbic acid and betaine improve stress responses, testosterone levels and some sexual traits in male Japanese quails during the dry season

Reviewer: Dr. Mehran Mehri 

University of Zabol, Animal Science, Zabol, Iran (the Islamic Republic of)

Date of review: 26 May 2021

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Conflict of interest statement. Reviewer declares none.

Comments to the Author: This manuscript has been written very poor and needs to be thoroughly revised by native English speaker. A lot of grammatical syntax issues are in the text that should be amended before scientific revision.

Score Card

Presentation



Is the article written in clear and proper English? (30%)

1/5

Is the data presented in the most useful manner? (40%)

1/5

Does the paper cite relevant and related articles appropriately? (30%)

4/5

Context



Does the title suitably represent the article? (25%)

4/5

Does the abstract correctly embody the content of the article? (25%)

1/5

Does the introduction give appropriate context? (25%)

1/5

Is the objective of the experiment clearly defined? (25%)

1/5

Analysis



Does the discussion adequately interpret the results presented? (40%)

1/5

Is the conclusion consistent with the results and discussion? (40%)

1/5

Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)

3/5