



## Vitamin C supplementation ameliorates summer stress and improves antioxidative status of commercial layer reared in Mizoram

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The growth and productive performance are greatly affected by the high ambient temperature and relative humidity (Khan *et al.* 2014). The deleterious effects of high ambient temperature are more when combined with humidity (Ajakaiye *et al.* 2011). Increased mineral excretion, decreased serum vitamin, mineral, and insulin levels as well as increased serum glucose, total cholesterol, and corticosterone levels are further effects of high ambient temperatures in poultry (Gorman and Balnave 1994, Siegel 1995). The heat stress marker, HSP70, has been extensively used to analyse and estimate the impacts of thermal stress in different species of animals including chicken. Commercial layer chicken reared in Mizoram and indigenous chicken of Mizoram have been shown to suffer cold stress in winter and increase their respective concentration of heat shock HSP70 during cold stress (Khukhodziinai *et al.* 2022, Ralte 2022).

Poultry are renal synthesizers of vitamin C (Maurice *et al.* 2002) and poultry diets are not normally fortified hence, no recommended requirement is established by the NRC (1994). However, during the severe environmental conditions, the endogenous production of vitamin C is usually considered not sufficient for the biological demands in poultry and vitamin C supplementation provides benefit to poultry (Pardue and Thaxton 1986).

Mizoram located at 1020 m altitude and 23°44'12N latitude enjoys a pleasant climate throughout the year except for a brief period of winter between December and January and presence of high humidity during the months from July to August. Supplementation of vitamin C have been found to alleviate the effects of cold stress of layers reared in Mizoram (Khukhodziinai *et al.* 2022). The present study aimed to find out the impact of summer stress on commercial layer during grower stage ascertain out the effects of vitamin C supplementation on stress and

antioxidative status of layer reared in Mizoram during summer.

The study was carried out in commercial layers (BV300) reared in Poultry Unit, Instructional Livestock Farm Complex, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram. A total of 60 birds of 26 weeks old commercial layer were reared for two months during the periods from 27<sup>th</sup> June to 26<sup>th</sup> August 2022. The birds were divided into 2 groups, viz. control group and VC. VC was supplemented with vitamin C @ 250 mg/kg of basal diet. A basal diet @ 120 g/bird/day was provided to the birds during the whole period of the experiment. The basal diet consisted of 64.8% maize, 26.2% soybean meal, 3.5% fish meal, 2.21% rice bran oil, 1.26% dicalcium phosphate, 0.3% sodium chloride, 1.15% limestone powder, 0.27% methionine, 0.12% L-lysine, 0.045% L-threonine, 0.067% toxin binder, 0.067% trace mineral phosphorus, 0.017% vitamin premix and 0.067% choline chloride. Drinking water was provided *ad lib*. These birds were reared in cage rearing system. The period of study was divided into 2 phases, i.e. from 27<sup>th</sup> June to 27<sup>th</sup> July, 2022 (P1) and from 28<sup>th</sup> July to 26<sup>th</sup> August, 2022 (P2).

**Record of meteorological parameters:** The ambient temperature (AT) and relative humidity (RH) were recorded during the 2 periods and the respective values were used for calculation of temperature humidity index. THI was calculated by using the formula developed by Johnson *et al.* (1962).

$$THI = 0.08AT + RH \frac{(AT-14.4)}{100} + 46.4$$

where, AT, Temperature in °C and RH, Relative humidity in %.

**Collection of blood samples:** Blood samples were collected from each bird at three intervals, i.e. 0, 30<sup>th</sup> and 60<sup>th</sup> day of supplementation. Blood samples were collected by puncturing the wing vein of each bird by using 24 Gauge needle. Heparin coated blood collection vials were used for collection of blood.

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**Calculation of heterophil lymphocyte ratio (H:L):** Blood smears were prepared from fresh blood samples. Differential leukocyte (DLC) was conducted on blood smears of each sample by the method of Jain (1986). N:L was calculated from the result of DLC.

**Processing of blood samples:** Blood glucose was estimated by using glucometer (Accu-Check Active; Roche, Brazil) immediately after blood collection. The collected blood samples were then centrifuged at 1500 rpm for 30 min to separate the cells and plasma. Plasma was separated and harvested in fresh tubes. The packed cells left after separation of plasma was washed 3 times by centrifugation with equal volume of normal saline solution. The supernatant along with the buffy coat was discarded after every wash. To prepare 1% hemolysate, 100 µl of washed RBC was mixed with 9.9 ml of 0.05 M phosphate buffer saline, pH 7.4.

**Analysis of plasma and hemolysate:** Concentration of HSP70, thiobarbituric acid reactive substances (TBARS), ferric reducing antioxidant power (FRAP) were estimated in plasma while superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase were estimated in hemolysate. HSP70 in plasma was estimated by using Chicken HSP70 (Heat Shock protein 70 high sensitivity) ELISA Kit (Wuhan Fine Biotech Co., Ltd.). Activity of SOD was estimated by nitro blue tetrazolium reduction method described by Nishikimi (1972). GPx activity was estimated by the measure of rate of oxidation of glutathione by  $H_2O_2$  as described by Rotruck *et al.* (1973). Catalase activity was determined by method of Aebi (1984). TBARS was estimated by following the method of Nwanjo and Ojiaka (2005). FRAP was estimated by the method of Benzie and Strain (1999). Protein concentration of plasma and hemolysate was estimated by Biuret method (Lubran 1978). Total cholesterol content of blood was estimated by the method of King as described by Wooten (1964) with certain modifications.

**Statistical analysis:** Statistical analyses were done using the protocol reported by Snedecor and Cochran (1994). Univariate analysis was applied to find out the differences between the different days of blood collection and independent Student's t-test was applied to find out the differences between the groups by using SPSS version 16.

Meteorological parameters recorded during the period

Table 1. AT, RH and THI during different periods of experiment

| Parameter |                    | P1         | P2         | Overall    |
|-----------|--------------------|------------|------------|------------|
| AT        | T <sub>av</sub>    | 23.37±0.21 | 23.38±0.20 | 23.37±0.14 |
|           | T <sub>min</sub>   | 20         | 20         | 20         |
|           | T <sub>max</sub>   | 30         | 28         | 30         |
| RH        | RH <sub>av</sub>   | 78.75±1.00 | 83.50±0.89 | 81.13±0.68 |
|           | RH <sub>min</sub>  | 45         | 49         | 45         |
|           | RH <sub>max</sub>  | 94         | 95         | 95         |
| THI       | THI <sub>av</sub>  | 55.58±0.14 | 55.70±0.13 | 55.64±0.09 |
|           | THI <sub>min</sub> | 53.04      | 53.09      | 53.04      |
|           | THI <sub>max</sub> | 59.87      | 60.27      | 60.27      |

of study are presented in Table 1. Effect of vitamin C supplementation on stress markers, viz. HSP70, H:L and antioxidative status markers, viz. SOD, GPx, catalase, TBARS and FRAP of commercial layer during P1 and P2 are presented in Table 2. Effect of vitamin C supplementation blood biochemical parameter, viz. plasma total protein, total protein of hemolysate and total cholesterol of commercial layer during P1 and P2 are presented in Table 3.

The highest T<sub>max</sub> was recorded during P1 and the lowest T<sub>min</sub> was recorded in both P1 and P2. The highest RH<sub>max</sub> was recorded during P2 and the lowest RH<sub>min</sub> was recorded during P1. The highest THI<sub>max</sub> recorded during the whole period was lower than the stressful range as THI value from 76 to 81 is stressful for poultry (Tao and Xin 2003). However, AT and RH were above the optimal AT (15 to 20°C) for poultry (Kocaman *et al.* 2005) and optimal RH (60-70%) for poultry (Mashaly *et al.* 2004). It was therefore more likely that these birds suffered from mild degree of stress.

**Effect of vitamin C supplementation on stress markers:** HSP70 level of control group was found to increase from 30<sup>th</sup> to 60<sup>th</sup> day of experiment. The present finding could be the effect of continuous exposure of the birds during summer months. It could be due to mild heat stress experienced by layer during the experimental period as the highest THI (THI<sub>max</sub>) of 60.27 during the whole period of study was recorded during P2. HSP70 expression also increased in heat stressed broilers (Mahmoud and Edens 2003). On the contrary, there was no change in HSP70 levels of VC at different intervals of vitamin C supplementation.

The H:L ratio has been a good indicator of stress in poultry (Gross and Siegel 1983). In the present experiment, there was no significant change in H:L of control and VC during different phases of experiment.

**Effect of vitamin C supplementation on antioxidative markers:** Acute heat stress has been found to increase SOD activities of broilers (Altan *et al.* 2003, Lin *et al.* 2008). In the current experiment, there was significant increase in SOD activities in control group from 0 to 60<sup>th</sup> day (Table 2). Increase in SOD activity could be due to summer stress. In VC, there was decrease in SOD activity. The decrease in SOD activity of VC could be the response of amelioration of stress by vitamin C.

GPx is a key enzyme in the antioxidant defense system of glutathione and plays a fundamental role in the elimination of ROS by the organism. It has been demonstrated that high environmental temperature affects the activity of GPx (Tan *et al.* 2010) with increase in GPx activity (He *et al.* 2020). In the present experiment, GPx activity of control group increased significantly from 0 day to 30<sup>th</sup> day of experiment and remained stable till 60<sup>th</sup> day of experiment. In the birds supplemented with vitamin C, the GPx activity remained stable throughout the period of study.

Catalase is an enzyme present in the cell, which protects the cells against hydrogen peroxide toxicity and lipid peroxidation (Yamaguchi 1991). There was significant increase in CAT activity of control group from 0 day to

Table 2. Effect of vitamin C supplementation on stress and antioxidative biomarkers of commercial layer

| Parameter | Group   | Day of supplementation       |                              |                              | P value            |
|-----------|---------|------------------------------|------------------------------|------------------------------|--------------------|
|           |         | 0                            | 30 <sup>th</sup>             | 60 <sup>th</sup>             |                    |
| HSP70     | Control | 19.84 <sup>a</sup> ±0.55     | 20.34 <sup>a</sup> ±1.60     | 26.26 <sup>b</sup> ±0.88     | 0.00**             |
|           | VC      | 21.78±1.78                   | 24.90±0.67                   | 21.71±1.34                   | 0.14 <sup>NS</sup> |
|           | P value | 0.31 <sup>NS</sup>           | 0.01*                        | 0.02*                        |                    |
| H:L       | Control | 0.35±0.02                    | 0.36±0.02                    | 0.38 <sub>B</sub> ±0.01      | 0.36 <sup>NS</sup> |
|           | VC      | 0.34±0.01                    | 0.33±0.01                    | 0.30 <sub>A</sub> ±0.01      | 0.06 <sup>NS</sup> |
|           | P value | 0.62 <sup>NS</sup>           | 0.19 <sup>NS</sup>           | 0.00***                      |                    |
| SOD       | Control | 334.11 <sup>a</sup> ±15.73   | 368.47 <sup>b</sup> ±9.71    | 426.60 <sup>c</sup> ±7.52    | 0.00***            |
|           | VC      | 364.19 <sup>b</sup> ±21.4    | 302.02 <sup>a</sup> ±11.75   | 277.58 <sup>a</sup> ±9.20    | 0.00***            |
|           | P value | 0.26 <sup>NS</sup>           | 0.00***                      | 0.00***                      |                    |
| GPx       | Control | 101.16 <sup>a</sup> ±7.94    | 155.00 <sup>b</sup> ±9.91    | 165.16 <sup>b</sup> ±5.01    | 0.00***            |
|           | VC      | 124.87±6.01                  | 127.54±6.86                  | 118.46±7.30                  | 0.62 <sup>NS</sup> |
|           | P value | 0.02*                        | 0.03*                        | 0.00***                      |                    |
| CAT       | Control | 2810.10 <sup>a</sup> ±381.67 | 5227.30 <sup>b</sup> ±781.76 | 5925.30 <sup>b</sup> ±648.25 | 0.00**             |
|           | VC      | 4123.10 <sup>b</sup> ±361.42 | 3067.10 <sup>a</sup> ±363.42 | 2805.80 <sup>a</sup> ±323.57 | 0.02*              |
|           | P value | 0.01*                        | 0.02*                        | 0.00***                      |                    |
| TBARS     | Control | 2.54 <sup>a</sup> ±0.18      | 2.63 <sup>a</sup> ±0.15      | 3.13 <sup>b</sup> ±0.17      | 0.04*              |
|           | VC      | 2.33±0.20                    | 1.98±0.13                    | 1.87±0.10                    | 0.08 <sup>NS</sup> |
|           | P value | 0.47                         | 0.00**                       | 0.00***                      |                    |
| FRAP      | Control | 4.45 <sup>b</sup> ±0.13      | 3.61 <sup>a</sup> ±0.13      | 3.40 <sup>a</sup> ±0.12      | 0.00***            |
|           | VC      | 4.58 <sup>a</sup> ±0.16      | 4.40 <sup>a</sup> ±0.14      | 6.49 <sup>b</sup> ±0.23      | 0.00**             |
|           | P value | 0.55 <sup>NS</sup>           | 0.00***                      | 0.00***                      |                    |

Means in the same row with different superscripts differ significantly. <sup>NS</sup>Indicates non-significant difference.

30<sup>th</sup> day that remained stable till 60<sup>th</sup> day of experiment. As per the reports of Altan *et al.* (2003), exposure of birds to heat stress resulted in a significant increase in CAT activity. In the present study, vitamin C supplemented birds were found to decrease CAT activity from 0 day to 30<sup>th</sup> day. The study revealed lower antioxidative status in control birds and better antioxidative status in VC. This finding could be due the beneficial effect of vitamin C.

Free radicals cause lipid peroxidation in an organism and malondialdehyde (MDA) is one of the byproducts of polyunsaturated fatty acid peroxidation in cells. Heat stress increases the MDA concentration as a lipid peroxidation indicator (Halliwell and Gutteridge 1989, Sahin *et al.* 2002). In the present experiment, TBARS concentration of control group increased significantly from 0 day to 30<sup>th</sup> day and remained stable till 60<sup>th</sup> day of experiment while in VC, the MDA concentration remained stable throughout the period of experiment. Albokhadaim *et al.* (2019) reported higher MDA concentration in heat stressed birds as compared to the birds reared in thermoneutral condition. Further, it has also been reported that supplementation of vitamin C caused reduction in MDA concentration of heat stressed birds (El-Din *et al.* 2008, Jang *et al.* 2014, Albokhadaim *et al.* 2019).

FRAP assay measures directly the reducing capacity of the substance, which is an important parameter for a compound to be good antioxidant (Benzie and Strain 1999). The FRAP concentration of control group decreased significantly on 60<sup>th</sup> day of experiment while the reverse was true for VC. Perai *et al.* (2015) reported reduction in

FRAP of broilers after transportation during summer stress. Jena *et al.* (2013) reported beneficial effects of vitamin C supplementation in broiler breeder hens during summer by increasing FRAP concentration.

*Effect of vitamin C supplementation on blood biochemical parameter:* The blood glucose of control group showed no significant change from 0 to 60<sup>th</sup> day of experiment (Table 3). In VC, there was significant decrease in blood glucose from 0 day to 30<sup>th</sup> day and remained stable till 60<sup>th</sup> day of experiment. Elevation of blood glucose has been reported during heat stress in broilers (Kutlu and Forbes 1993) and cold stress in layers (Khukhodziaini *et al.* 2022). In the present study, the initiation of the experiment was on 27<sup>th</sup> June, 2022. In Aizawl, Mizoram during the period from 8 AM to 2 PM in months from April to May, THI<sub>av</sub> recorded was 72.27±0.50 while THI<sub>max</sub> and THI<sub>min</sub> were 74.22±0.50 and 70.40±0.50 respectively (Ralte 2016). Ajakaiye *et al.* (2010) and Attia *et al.* (2015) also reported reduction of blood glucose by vitamin C supplementation in heat stressed layers.

The total plasma protein of control group remained stable from 0 day to 30<sup>th</sup> day and decreased significantly from 30<sup>th</sup> to 60<sup>th</sup> day of experiment. This could be the effect of summer stress. These results agree with those of Seliem (2011) and Ezzat *et al.* (2017) who observed a significant decrease in total protein in heat stressed chicken. Total plasma protein of VC group increased significantly from 0 day to 60<sup>th</sup> day of experiment while there was no significant difference between the values on 30<sup>th</sup> and 60<sup>th</sup> day. Such findings could be due to the antioxidative property of

Table 3. Effect of vitamin C supplementation on blood biochemical parameters of commercial layer

| Parameter                   | Group   | Day of supplementation                |                          |                         | Significance       |
|-----------------------------|---------|---------------------------------------|--------------------------|-------------------------|--------------------|
|                             |         | 0                                     | 30 <sup>th</sup>         | 60 <sup>th</sup>        |                    |
| Glucose                     | Control | 178.27±2.46                           | 183.77±3.40              | 186.77±3.18             | 0.14 <sup>NS</sup> |
|                             | VC      | 183.73±1.64                           | 177.13±2.29              | 170.70±2.91             | 0.00**             |
|                             | P value | 0.07 <sup>NS</sup>                    | 0.11 <sup>NS</sup>       | 0.00***                 |                    |
| Total protein               | Control | 5.56 <sup>b</sup> ±0.20               | 5.40 <sup>b</sup> ±0.20  | 4.89 <sup>a</sup> ±0.11 | 0.03*              |
|                             | VC      | 5.16 <sup>a</sup> ±0.20               | 5.55 <sup>ab</sup> ±0.19 | 5.90 <sup>b</sup> ±0.12 | 0.02*              |
|                             | P value | 0.18 <sup>NS</sup>                    | 0.61 <sup>NS</sup>       | 0.00***                 |                    |
| Total protein of hemolysate | Control | 1.54 <sup>b</sup> <sub>AB</sub> ±0.03 | 1.49 <sup>b</sup> ±0.04  | 1.36 <sup>a</sup> ±0.02 | 0.00**             |
|                             | VC      | 1.58 <sup>a</sup> <sub>B</sub> ±0.03  | 1.72 <sup>b</sup> ±0.05  | 1.79 <sup>b</sup> ±0.03 | 0.00**             |
|                             | P value | 0.46 <sup>NS</sup>                    | 0.00**                   | 0.00***                 |                    |
| Total cholesterol           | Control | 164.71±4.53                           | 185.40±3.61              | 184.14±4.22             | 0.00**             |
|                             | VC      | 160.68±2.93                           | 161.54±2.56              | 154.46±2.84             | 0.15 <sup>NS</sup> |
|                             | P value | 0.46 <sup>NS</sup>                    | 0.00***                  | 0.00***                 |                    |

Means with different superscripts in the same row differ significantly. <sup>NS</sup>Indicates non-significant difference.

vitamin C. Vitamin C could ameliorate heat stress effects and maintained stable total plasma protein and vitamin C further increased total plasma protein of VC. These results are in agreement with previous reports of El-Din *et al.* (2008) who reported that vitamin C supplemented local laying hen was having higher total protein than that of heat stressed control group. Sahin *et al.* (2003) observed that vitamin C supplemented broiler chickens were having higher total protein as compared to birds without supplement during heat stress. Torki *et al.* (2014) also reported that dietary vitamin C supplementation significantly increased the total protein concentration in laying hens as compared to birds without supplement during heat stress condition.

In control group, the total cholesterol concentration was found to increase significantly from 0 day to 30<sup>th</sup> day and remained stable till 60<sup>th</sup> day of experiment. Increase in total cholesterol could be due to summer stress as Kutlu and Forbes (1993) had reported earlier that the total cholesterol of broiler chicks increased upon exposure to heat stress. In VC there was no significant change in total cholesterol from 0 to 60<sup>th</sup> day of experiment. It could be due to the beneficial effect of vitamin C supplementation in these groups. This findings are supported by Sahin *et al.* (2003) who reported that total cholesterol of vitamin C supplemented broiler birds were significantly lower as compared to heat stressed control birds. El-Din *et al.* (2008) also reported that total cholesterol of vitamin C supplemented local laying hen was significantly higher as compared to heat stressed control birds.

Commercial layers experienced mild degree of summer stress during the months of July and August 2022. Summer stress caused significant increase in HSP70, SOD, GPx, CAT, TBARS, total cholesterol and decrease in FRAP, total plasma protein, total protein of hemolysate of layer. Supplementation of vitamin C (@250 mg/kg feed) could ameliorate summer stress in commercial layer. Amelioration of summer stress by vitamin C supplementation could be measured by estimation of stress biomarkers, viz. HSP70,

antioxidative enzyme activity, viz. SOD, GPx and CAT, biochemical parameters viz. blood glucose, total plasma protein and total cholesterol.

#### SUMMARY

The present study aimed to investigate the effects of vitamin C supplementation on stress and antioxidative status of sixty 26 weeks old commercial layer birds during summer in Mizoram. Two groups of birds were reared for a period of 2 months, viz. control group and VC. Control group was on basal diet @120 g/bird/day while VC was supplemented with vitamin C @ 250 mg/kg basal diet. THI was calculated from the records of AT and RH during the period. Blood samples were collected from wing vein of each bird on 0, 30<sup>th</sup> and 60<sup>th</sup> day of treatment for estimation of HSP70, H:L, SOD, GPx, CAT, TBARS, FRAP, blood glucose, total plasma protein, total protein of hemolysate and total cholesterol. The THI recorded during the period of study was not indicative of heat stress while T<sub>max</sub> of 30°C and RH<sub>max</sub> of 95% were above the optimal ranges for poultry. Presence of heat stress and decrease in antioxidative status of control group was detected by increase in HSP70, SOD, GPx, CAT, TBARS and total cholesterol and decrease in FRAP, total plasma protein and total protein of hemolysate. Upon vitamin C supplementation, HSP70, GPx and TBARS became stable, SOD and CAT activity and total cholesterol decreased and FRAP, total plasma protein, and total protein of hemolysate increased. The estimates of heat stress and antioxidative markers indicated presence of stress in commercial layers in summer and vitamin C supplementation was found to ameliorate the stress and improve the antioxidative status.

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