Research Article

Circulating metabolic and reproductive hormone changes in laying hens kept under various heat-combating systems

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Abstract: The objective of the present study was to observe the circulating metabolic, reproductive, and stress hormones in laying hen performance under different heat-combating systems (HCSs). A total of 500 White Leghorn pullets at 24 weeks of age were subjected to four HCSs, i.e. desert cooling (DC), water sprinkling (WS), time limit feeding (TLF), and ascorbic acid (AA) supplementation, at environmental temperatures from 32.30 to 40.80 °C with 76.40% relative humidity. Blood was collected before heat stress, at peak stress, and after heat stress to collect plasma. The results of the 24-week stress period showed significantly less circulating T_3, T_4, estrogen, and progesterone in the control group in the peak stress period, whereas corticosterone was significantly higher. The plasma T_3, T_4, and estrogen and the egg production percentage remained significantly higher in the DC system as compared to other HCSs and the control. In comparison, the plasma progesterone level was significantly increased with lower T_4 in the AA supplementation group than the other HCSs. Our results validate the potential role of the DC system in protecting birds from environmental heat stress effects and subsequent improvement in egg production.

Key words: Hormones, ascorbic acid, heat stress, heat-combating system, laying hen, egg production

1. Introduction

Hormones play a vital role in the physiological growth, sexual maturity, and egg production of birds. Heat stress is a foremost concern in the egg production potential of laying hens. Heat stress can be defined as "a physiological condition when the core body temperature of a given species exceeds its range specified for normal activity, which results from a total heat load (internal and environment) exceeding the capacity for heat dissipation and this prompts physiological and behavioral responses to reduce the strain" (1). Due to higher or lower environmental temperature not only the weight of the thyroid gland but also its activity is hampered (2,3). Thyroid hormones (thyroxin, T_4; triiodothyronine, T_3) provide a major mechanism of acclimatization and hence have received extensive research consideration. The hypothalamic–pituitary–adrenal axis is activated during thermal stress (4). Corticotropin-releasing hormone from the hypothalamus induces the release of adrenocorticotropic hormone but also influences the decreased production of both T_4 and T_3

(5). Changes in adrenal gland physiology under stressful conditions have received attention because this gland plays a vital function in stress adaptation (6). This increase in corticosterone may be due to reduced feed intake in heat stress situations and ultimately less T_3 production, and this can be used as an indicator of stress in laying birds (7). Estrogen plays an important role in reproductive performance, while progesterone is related to the ovulation process of birds. The relationship of circulating levels of estrogen and progesterone was studied in adult turkey hens and in mature pullets under conditions of increasing ambient temperature (8,9). Higher temperature inhibits growth and delays the sexual maturity of fowls. Generally, a decline in egg production of heat-stressed birds is related to low feed intake, but recent research work also attributed it to the direct ovulatory hormones (10). It was proposed that ovarian function was depressed by thermal stress to the extent that progesterone secretion was virtually abolished (11). However, all of the above studies are lacking necessary information regarding hormonal

variations in the context of prolonged heat stress. This study was thus designed to determine the effect of a long-term hyperthermic environment on certain physiological changes in the plasma T_3, T_4, estrogen, progesterone, and corticosterone levels in White Leghorn layers. This study will also help to identify better heat-combating systems (HCSs) for laying hens in an effort to keep temperatures in the thermo-neutral zone.

2. Materials and methods

2.1. Poultry husbandry and experimental design
In this study 500 White Leghorn pullets at the age of 24 weeks were kept at the Poultry Experiment Station, University of Agriculture, Faisalabad, Pakistan, and were given 1 week for acclimatization. According to their initial body weight, these pullets were randomly divided into five treatment groups and four replicates to form 20 experimental units comprising 25 pullets each. All groups were maintained in different environmentally controlled houses. From the start of experiment, the birds were kept in pens bedded with wood-shavings litter (12.7 cm deep) along with automated feeders and waterers, having space of 46 cm² per bird. The experiment continued for 4 months (from May to August, the hottest months of the year). Pullets were maintained under different types of HCSs and kept in floor-type housing systems. The first HCS system was a desert cooling (DC) system operated through regular sprinkling of water on bricks using an exhaust fan of 60 cm in size at 1320 rpm. The second system was water sprinkling (WS), done by sprinkling water thrice daily on the hens' bodies with the help of a pressure spraying machine at 1100, 1400, and 1700 hours at the rate of 2 L for 20 layers. The third system was time limit feeding (TLF), in which the layers were fed twice daily during cool hours, i.e. from 0500 to 0600 and from 1900 to 2000 hours only. The fourth system was ascorbic acid (AA) supplementation, where the basal feed was mixed with AA (Sigma Chemical Co., St. Louis, MO, USA). After mixing, the total increase in AA concentration was 150 ppm as compared to regular layer hen feed. The fifth group was the control group, kept under electric ceiling fans (140 cm in size at 325 rpm) with regular layer hen feed (Table 1).

2.2. Stress phases
For the experiment, birds at 24 weeks of age were maintained in their comfort zone for 1 week during the acclimatization period, named as the prestress phase (PSS), where the environmental temperature was 27 to 30 °C. The peak-stress phase (PSP) followed, in which laying hens were exposed to high environmental temperatures and humidity with HCSs (Table. 2). After the PSP these birds underwent the poststress (PS) phase, in which the environmental temperature returned back to that of the comfort zone.

Table 1. The formulation of diet used in the experimental layer ration.

Ingredients	Inclusion level (%)
Corn	22
Rice tips	26
Rape seed meal	04
Sunflower meal	02
Soybean meal	05
Fish meal	05
Meat meal and poultry byproducts	08
Rice polishing	13.38
Molasses	05
Marble chips	07
Bone meal	02
Methionine	0.12
Vitamin and mineral premix supplement	0.50
Chemical composition of the diet	
Metabolizable energy (ME), kcal/kg	2740
Crude protein (CP), %	17.41
Crude fiber (CF), %	3.54
Calcium	3.40
Phosphorus	0.45

2.3. Blood collection
In all phases of stress, the blood was collected at 0430 hours, before feeding, from five randomly selected birds from each replicate. A quantity of 1 mL of blood was collected from the brachial vein in heparinized vacutainers to collect plasma. Blood samples were centrifuged at 5000 rpm for 5 min in a temperature-controlled centrifuge (Thermo Scientific Benchtop Centrifuge) and the plasma was stored at –20 °C in polyethylene tubes for further analysis of hormones.

2.4. Plasma hormone analysis
All the hormones, including T_3, T_4, estrogen, progesterone, and corticosterone, were detected by the use of commercially available ELISA kits according to the manufacturer's instructions. Each of the samples was prepared in duplicate to enhance precision. Furthermore, an appropriate software package was used to facilitate analysis, data generation, and quality control. The determination of plasma T_4 was carried out by ELISA kit (Autobio Diagnostics, Co., China, E-1002), with assay sensitivity of 0.4 μg/dL, intraassay precision of >3.58%, and interassay precision of >9.64%. The kits used for plasma T_3 determination were of commercial ELISA origin (Autobio Diagnostics, Co., China, E-1001). The sensitivity of the

Table 2. Mean weekly minimum and maximum room temperature (°C) and relative humidity (%) under various heat combating systems throughout the experimental period of 24 weeks. HCS: Heat-combating system; RH: relative humidity.

HCS	Temperature (°C)		Range	RH (%)
	Maximum	Minimum		
Control	40.80 ± 0.90	32.30 ± 1.29	8.50	76.40 ± 1.25
Desert cooling	35.60 ± 2.24	30.30 ± 4.11	5.30	88.70 ± 2.14
Water sprinkling	37.70 ± 2.80	31.80 ± 1.95	5.90	78.90 ± 0.69
Time limit feeding	39.00 ± 2.05	31.40 ± 3.35	7.60	80.20 ± 0.56
Ascorbic acid	39.00 ± 1.58	31.90 ± 2.03	7.10	78.90 ± 0.69

assay was 0.2 ng/mL, intraassay precision was >3.94%, and interassay precision was >7.83%. Plasma corticosterone was measured by the Enzo Life Sciences ELISA kit (Enzo, Inc. USA, Catalog No. ADI-900-097). The sensitivity of the kit was 26.99 pg/mL, interassay precision was >7.8%, and intraassay precision was >6.6%. Plasma progesterone concentration was determined by solid-phase ELISA (Bio Check, Inc. USA, Lot. RN-28387). The sensitivity of the assay was 0.3 ng/mL, intraassay precision was >7.1%, and interassay precision was >12.6%. Circulating estrogen concentrations were determined by ELISA (Bio Check, Inc. USA, Lot. RN-27637). The sensitivity of the estradiol ELISA was 10 pg/mL, intraassay precision was less than 24.1%, and interassay precision was less than 26.7%.

2.5. Production performance
For the whole experiment, the production performance of the laying hens was recorded for a period of 24 weeks. The egg production rate was recorded during the PSP and PS.

2.6. Data analyses
The experimental design was a completely randomized design with temperature, HCS, and their interactions with time of stress (PSS, PSP, and PS). Data were analyzed with a mixed model using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). All data are presented as mean ± SEM. The differences between treatments were subjected to Duncan's multiple range test and P ≤ 0.05 was considered statistically significant.

3. Results

3.1. Plasma triiodothyronine (ng/mL)
The effect of the HCSs and the phases of heat stress were significant for the plasma T_3 concentration of the experimental hens. The plasma T_3 concentration was found to be significantly increased at PS as compared to the respective values at PSS and PSP. During PSS and PSP the DC and TLF systems remained statistically different; however, the plasma concentration of T_3 remained significantly higher in the PS phase (Figure 1A). A

significant effect of HCS and stress phase interaction was observed on mean plasma T_3 concentration.

3.2. Plasma thyroxine (µg/dL)
The data on statistical analysis revealed a significant effect of HCSs and various phases of heat stress on the mean plasma T_4 concentration of the experimental hens (Figure 1B). There was a significant difference among various HCSs, except between the DC and TLF systems. The maximum concentration of T_4 (45.05 ± 0.40 µg/dL) was found in TLF hens and the minimum (35.96 ± 1.195 µg/dL) was found for control hens. The concentration of T_4 was lower at PSP and remained higher at PS. The interaction between the HSCs and heat stress phases was also found to be significant in this respect.

3.3. Plasma estrogen (pg/mL)
Statistically there was a significant effect of HCSs, phases of heat stress, and the interaction of both on the plasma estrogen concentration (Figure 1C). Heat stress induced a significant decrease of circulating estrogen concentration, but it remained higher in the DC system. Comparison of the means indicated that the plasma estrogen value observed in the control group was significantly higher than that of WS and TLF, but it was not different in comparison to those in the DC and AA supplementation groups. The results further showed significant differences among the plasma estrogen concentrations at various phases of heat stress. The level of estrogen declined to a minimum (170.9 ± 0.06 pg/mL) at PSP from a maximum (189.6 ± 0.06 pg/mL) at PSS, again giving rise to a higher level (175.0 ± 0.03 pg/mL) at PS irrespective of the treatment.

3.4. Plasma progesterone (ng/mL)
The effects of HCSs and the phases of heat stress as well as their interaction were found to be significant on the plasma progesterone concentration of the experimental hens (Figure 1D). At PSP, the maximum plasma concentration of progesterone was seen with AA supplementation and the minimum with TLF and DC, while the difference remained nonsignificant between the control and WS
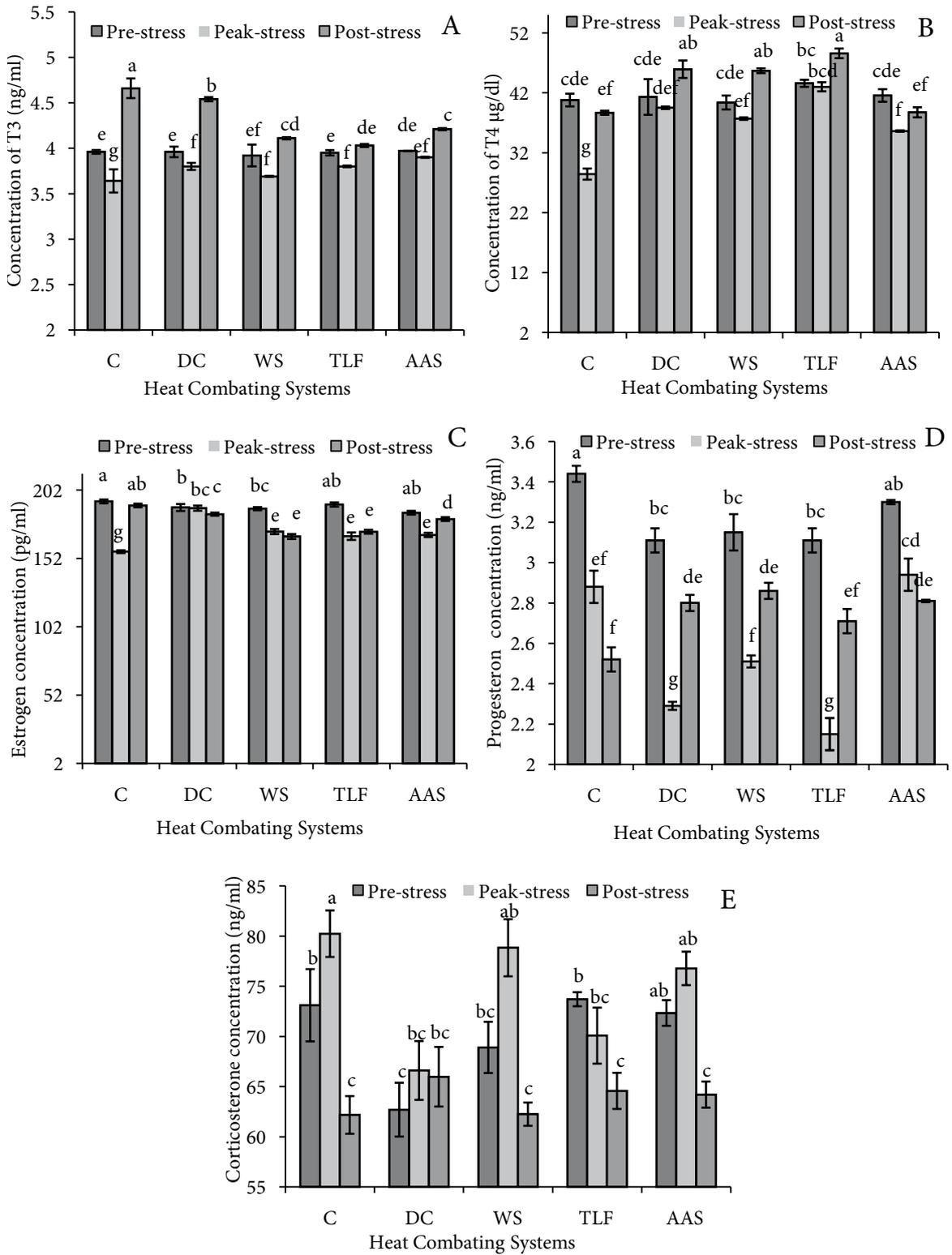
Figure 1. Plasma hormone concentrations of laying hens exposed to prestress, peak stress, and poststress conditions. The hens underwent different heat-combating system treatments, i.e. C = control, DC = desert cooling system, WS = water sprinkling system, TLF = time limit feeding system, and AAS = ascorbic acid supplementation. A) Concentration of T_3, B) concentration of T_4, C) concentration of estrogen, D) concentration of progesterone, E) concentration of corticosterone. Bars with similar letters above them do not differ significantly (P ≤ 0.05). All data are presented as mean ± SEM.

groups. At PS the circulating progesterone concentration was significantly higher in all of the HCSs as compared to the control group. The results further indicated significant differences in the plasma progesterone concentrations of the hens at different heat stress phases. Plasma progesterone concentration was highest at PSS, then declined at PSP and again increased at PS.

### 3.5. Plasma corticosterone (ng/mL)

The HCSs and phases of heat stress as well as their interactions exhibited significant effects on the mean plasma cortisol concentration of the experimental hens (Figure 1E). The maximum concentration of corticosterone was noted at PSP and minimum during the PS phase. The best results were obtained with the DC system, where at PSS, PSP, and PS the plasma concentration of corticosterone remained nonsignificant. A maximum level of this stress hormone was observed during PSP of the control and the WS and AA supplementation groups, which decreased and became nonsignificant at PS irrespective of the treatment group.

3.6. Production percentage

During PSP and PS the mean egg production percentage was generally superior under the DC system as compared to AA and control birds (Figure 2). However, during PS the egg production enhanced nonsignificantly in birds under TLF and control treatments, while it decreased in WS. AA supplementation did not influence egg production during PSP or PS.

4. Discussion

To our knowledge, there are still no data available on the comparison of different HCSs to mitigate the effects of heat stress in laying hens. Heat stress induces a decrease in the production of laying hens. This decrease may be due to decreased production of T_3, estrogen, and progesterone or maybe the decreased release of GnRH-induced FSH and LH. The common complaint during the heat stress months is the decrease in egg production. However, there was no effect of circulating LH and FSH, while the high ambient temperature in laying hens affects the ovaries. Lower decrease in T_3 concentration was observed in the DC and TLF systems and this may be due to less stressful conditions induced by heat stress. To combat the drastic effects of heat stress and decreased egg production, different types of HCSs are used in the world. Among them, the DC system is one that provides cool air with water and is commonly used in the Indian subcontinent during periods of high temperature. Meanwhile, in the TLC system, there is an increase in feeding gap and most of the feed is provided during cool hours of the day. The WS system acts like perspiration in mammals and induces a cooling effect and relief from heat stress, and AA is as an antioxidant that is supplemented to decrease the
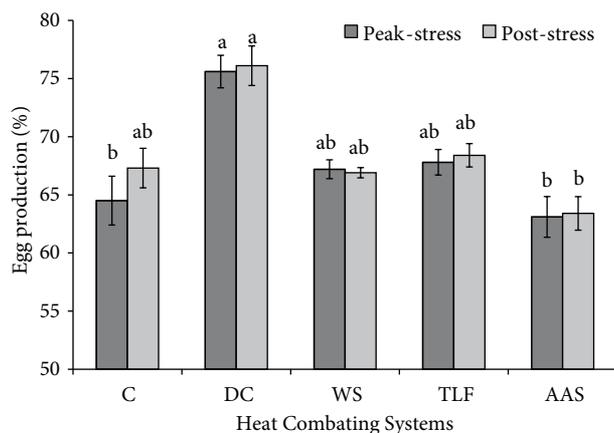
Figure 2. Egg production percentage of laying hens kept under heat-combating systems and in the poststress phase. Bars with similar letters above them do not differ significantly (P ≤ 0.05). All data are presented as mean ± SEM.

detrimental effects of heat stress (12). In our experiment, T_4 concentration was highest in DC and TLC in the PS phase. Thyroid hormones (T_3 and T_4) are important in regulating metabolic heat production activities and maintenance of body temperature in mammals and birds. Heat stress induces a continuous decrease in T_3 production (13), while the production of T_4 is also affected by high ambient temperatures either towards increase (14) or decrease (15), or no change (16). Less production of T_3 is either due to less fabrication or less conversion of T_4 into T_3. Our results indicating that T_3 concentration declined under heat stress conditions in all the groups are due to high temperature, and then it again regained its blood concentration in the PS phase. This gain in T_3/T_4 has already been reported (2). The highest T_3 concentration in the blood during PS may correspond to maximum production among the phases, as the level of T_3 in the blood is considered to be related to the rate of egg production (14,17), being the reservoir of its potential. At PSS the blood concentration of T_3 remained lower due to the initial phase of production and in PSP due to high environmental temperature causing low egg production (18), but as birds were relieved of summer stress it led to a maximum concentration of T_3 in the blood. The lowest T_3 concentration in the TLF system may be under the influence of two types of stresses, heat stress and fasting stress. Buyse et al. (19) reported a decrease in circulating T_3 and insulin-like growth factor in birds subjected to TLF. The lowest plasma thyroxin was produced by the nontreated hens under the influence of heat stress conditions.

Heat stress causes a decrease in circulating estrogen levels, resulting in decreased egg production percentage (20). However, maximum estrogen level in PSS and minimum during PSP was observed, and this level returned

to a peak at PS. A decrease in plasma levels of estrogen was reported to be affected by high ambient temperature (21). As estrogen plays an important role in the reproduction performance of the fowl, its decreased serum concentration adversely affects the laying performance. Another reason for this decrease in egg production may be the hampered uptake of gut calcium (21) or the decreased production of cholecalciferol-1-hydroxylase, which has a momentous role in calcium homeostasis (22). The modification in the reproductive hormone's role is attributed to stress-linked changes in the neuro-hormone pathway. The stress-induced hindrance in pathways of PRL, FSH, and LH was already reported in laying hens (23) and turkeys (8) and this can be easily correlated with the decreased egg production in the stress phase. However, a previous study (9) clamed no effect of heat stress on the serum levels of LH and FSH, suggesting no change in ovarian function due to heat stress. This change in ovarian steroid production is due to the debilitating effect of heat stress on decreased ovarian blood flow in laying hens (24). The induction of the DC system clearly acted on and decreased the devastating effect of heat stress on ovarian function. The literature also indicates that due to high levels of heat stress there is low feed intake, triggering low body weight

(11) and less availability of calcium. Lower feed intake has an important correlation with circulating progesterone contents (25) and disappearance of preovulatory follicles after a few days of feed withdrawal (26). Birds have another peculiarity in that they can control their body temperatures by controlling their feed intakes. Along with other factors, corticosterone is another hormone that helps to control metabolism and body heat. In birds the best indicator of stressful conditions is circulating cortisol or corticosterone (27). The hypothalamic–pituitary–adrenal axis is important in body integration but has deleterious effects on health (28). Due to these devastating effects of high circulating corticosterone there is a great need to keep the circulating levels of corticosterone in the physiological range. All the HCSs exhibited an effect on the corticosterone level of the hens but the maximum decrease of corticosterone was observed in the DC system.

In conclusion, the results of this study show that the DC system works better as compared to all other used systems to decrease the effects of heat stress. This system also proved to be the best in keeping the blood plasma concentrations of circulating corticosterone, estrogen, and thyroxine within physiological ranges and ultimately hens provided better egg production percentages.

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