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Effects of heat stress on semen quality of Gramapriya male line roosters

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Abstract
Background: Semen quality, fertility, and hatchability were evaluated in Gramapriya male line (GML) roosters subjected to heat stress.
Methods: Twenty-four GML roosters aged 36-weeks were divided into two groups (n=12). The heat stress group was exposed to 38 ℃ temperature and 40% Relative Humidity (RH) (Temperature-Humidity Index, THI=86) for 4 hours daily for a total period of 28 days in an environment-controlled chamber, and the control group was maintained at ambient conditions throughout the trial period. The mean ambient temperature and RH during this period were 28.7 ℃ and 63.4% respectively (mean THI being 78.29). Semen samples from both the groups were evaluated for semen volume, sperm concentration, sperm viability by MTT dye-reduction assay, and magnitude of free radical damage by lipid peroxidation assay at weekly intervals during the heat exposure.
Results: Semen volume and sperm concentration did not differ significantly between the two groups except for the first week of heat exposure wherein the sperm concentration in heat-stressed roosters was significantly reduced ($P< 0.01$). Percent live spermatozoa and sperm motility were significantly reduced in heat-stressed roosters ($P< 0.01$). The values of sperm viability and magnitude of free-radical damage to the sperm, as determined by MTT dye-reduction assay and lipid peroxidation assay, respectively varied significantly between the two groups ($P< 0.01$). The heat-stressed group showed poor viability and greater lipid peroxidation than the control, suggesting greater vulnerability of GML spermatozoa to heat stress. However, there was no significant difference in fertility and hatchability between the two groups.

Keywords: Heat stress, roosters, temperature-humidity index, semen quality

Introduction
Heat stress is emerging as a serious problem in poultry production in tropical countries and gaining significance with the growing concern of global climate change. An important consequence which domestication and selective breeding have had on physiology and behaviour was reduced responsiveness to stress-evoking stimuli (*i.e.,* environmental stress) as an adaptation to living in a biologically safe, predator-free environment (Korte *et al.*, 2005) [15]. Soleimani *et al.* (2011) [29] also demonstrated reduced ability of commercial broilers to withstand high ambient temperatures when compared to village fowl and red jungle fowl. The high growth potential of domesticated genotypes is mainly attributed to lower levels of circulating cortisol. It is generally accepted that indigenous breeds of tropical countries are resistant to high ambient temperatures than faster-growing strains (Yunis and Cahaner, 1999) [35]. While semen quality and fertility are adversely affected by high ambient temperatures and humidity (Karaca *et al.*, 2002a, Shanmugam *et al.*, 2012) [12, 26], fertility and hatchability are most sensitive to environmental and genetic influences (Stromberg, 1975) [30] and have very low heritability values (0.06 to 0.13), indicating a greater influence of non-genetic factors on these traits (Sapp *et al.*, 2004) [24].
Heat stress is known to comprehensively down-grade semen production in breeder cocks (Banks *et al.*, 2005) [4]. It is known to induce multiple deleterious effects on testicular functions through inhibition of intracellular ion exchange (McDaniel *et al.*, 1995; McDaniel *et al.*, 1996; Ayo *et al.*, 2011) [19, 3, 18] and reduced seminiferous epithelial cell differentiation (Edens, 1983; McDaniel *et al.*, 1996: Obidi *et al.*, 2008) [9, 18, 22]. The motility and viability of chicken spermatozoa are affected by free radicals formed during heat stress (Surai *et al.* 2001) [31]. Elevated body temperature during heat stress and lower ion concentration in seminal plasma are attributed to lower fertility in roosters (Karaca *et al.*, 2002a, 2002b) [13].

The magnitude of heat stress effect greatly varies between breeds and varieties (Melesse *et al.*, 2013) [21] and very little work has been done in this regard on backyard varieties. Therefore, this study was taken up to assess the effects of heat stress on some of the semen quality parameters of GML roosters.

Material and Methods

Experimental birds and management

This experiment was conducted at the experimental poultry farm, ICAR- Directorate of Poultry Research, Hyderabad and was approved by the Institutional Animal Ethics Committee. Gramapriya male line (GML), a colored broiler parent line maintained at the Institute was used in the experiment. Twenty-four roosters of 36 weeks age were divided into two groups randomly (n=12). The roosters were individually caged (38 × 40 × 60 cm^3) and fed *ad libitum* with standard breeder male diet of 2600 ME (kcal/kg) and 16% crude protein. All roosters had free access to water.

Induction of heat stress

One group of roosters was exposed to 38℃ temperature and 40% Relative Humidity (RH) (Temperature-Humidity Index, THI=86) for 4 hours duration every day for a total period of 28 days in an environment-controlled chamber (Newtronic® Walk-in humidity chamber). The control group was maintained at ambient conditions throughout the trial period. The mean ambient temperature (T_a) and RH during this period were 28.7˚C and 63.4%, respectively. THI was calculated as per the formula: THI = (0.8 × T_a) + [(RH/100) × T_a -14.3)] + 46.3 (Mader *et al.*, 2010) [17]. The mean THI was 78.29 during the experiment period.

Semen collection

Semen was collected for analytical purposes at weekly intervals from roosters by abdominal massage method (Burrows and Quinn, 1937) [5], once before heat exposure and for three consequent weeks during the experiment period. Semen was individually collected in a sterile glass funnel and diluted 4 times using a diluent described by Sasaki *et al.* (2010) [25] and immediately subjected to analysis.

Semen evaluation

The volume of semen ejaculate was measured by drawing the sample into a 1mL syringe with an accuracy of 0.02 mL. Motility of the sperm was subjectively assessed as percentage progressively motile sperm by placing a drop of diluted semen on a clean, grease-free glass slide, overlaid with a coverslip, and examined under high-power objective (40×). Concentration of spermatozoa was estimated as per Taneja and Gowe (1961) [32] using a spectrophotometer at a wavelength of 540 nm. Sperm viability was estimated using tetrazolium dye reduction test in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used in duplicates and absorbance was taken using a colorimeter (CL 157, Elico Ltd, Hyderabad, India) at 570 nm (Hazary *et al.*, 2001) [10]. The percentage live sperm was estimated by differential staining technique using eosin-nigrosin stain (Campbell *et al.*, 1953) [6]. One smear was prepared for each sample and 200 sperm were counted in each.

Lipid peroxidation assay

Seminal plasma was separated by centrifuging the neat semen at 1000 × g for 15 min. The clear supernatant was analyzed for lipid peroxidation by thiobarbituric acid method (Hsieh *et al.*, 2006) [11]. 0.1 mL of seminal plasma was added to 0.9 mL of distilled water, and then 0.5 mL of thiobarbituric acid reagent was added and heated for 1 hour in a water bath. This mixture was then cooled and centrifuged at 1500 × g for 10 min and the absorbance of the supernatant was read at 534 nm.

Fertility and hatchability

Semen was collected from the roosters just before insemination and was pooled group-wise and sperm concentration was adjusted to approximately 200 million spermatozoa/ 0.1 mL with an insemination dose of 0.1 mL per hen. A total of twenty females per group were inseminated with pooled-cum-standardized semen thrice at 4-day intervals. Eggs were collected for 14 consecutive days from the second day after the first insemination and stored at 15 ℃ for a maximum period of 7 days until incubated in two separate batches. The eggs were incubated at standard conditions in an automatic setter. The incubated eggs were candled on the 18th day of incubation for embryonic development and fertile eggs were transferred into setter compartment. Eggs that hatched on the 21st day of incubation were counted to calculate the hatchability. The infertile eggs were broken and observed to confirm the absence of embryonic development. Fertility and hatchability percentages were calculated as follows:

$$\text{Fertility \%} = \frac{Number\ of\ fertile\ eggs}{Total\ number\ of\ eggs\ obtained} \times 100$$

$$\text{Hatchability \%} = \frac{Number\ of\ eggs\ hatched}{Total\ number\ of\ fertile\ eggs\ set} \times 100$$

Testicular histology

Two roosters each from both groups were humanely sacrificed at the end of the experiment. Testes were collected and fixed in 10% neutral buffered formalin. Sections were made from fixed and paraffin-embedded tissue and stained with hematoxylin and eosin (H&E) stain (Luna, 1968) [16]. The slides were examined by AX70 microscope and DP27 Digital Camera System (Olympus, Tokyo, Japan).

Statistical analysis

The data on semen characteristics *viz.*, ejaculate volume, sperm concentration, sperm motility, percent live sperm, MTT dye reduction, lipid peroxidation scores, and fertility and hatchability were subjected to two-sample t-test (Snedecor and Cochran, 1989) [28] assuming equal variances. 'p' values ≤ 0.05 were considered significant. Data are presented as mean ± standard error.

Results and Discussion

The gross morphology and architecture of testes after heat stress exposure did not change significantly. However, heat-stressed roosters revealed a striking increase in the cellular density and size along with a marked reduction in the amount of adipose tissue (Fig. 1 a & b).

Heat-stress affects all phases of semen production and decreases semen quality and quantity with time (Banks *et al.*, 2005; Ayo *et al.*, 2011) [4, 3]. A THI threshold value of 70 was earlier reported in chickens, both layers and broilers (Tao and Xin, 2003) [33]. In broiler breeders, the sperm quality index declined, and dead sperm count increased upon exposure to 32℃ (McDaniel *et al.*, 2004) [20]. In this study, the major

semen parameters like ejaculate volume and sperm concentration remained unaffected between the groups. However, the sperm concentration was significantly lower in heat-stressed roosters at the end of the first week. An earlier study in broiler breeders also showed that heat exposure to 32℃ did not affect the gross semen parameters. However, it affected the sperm nuclear status to a significant extent (McDaniel *et al.*, 1995) [19]. Edens (1983) [9] also showed non-significant changes in gross semen parameters and stated that roosters may adapt to short-term thermal stress. In the present study also, the exposure was for only four hours duration in order to mimic the natural thermal fluctuations. A significant reduction in the mitochondrial enzyme activity as assessed by the MTT dye reduction test was observed in heat-stressed roosters from the first week of exposure and was consistently lower during the entire experiment. There was also a significant reduction in the percentages of live and progressively motile sperm in heat-stressed roosters. Several workers have also reported a decrease in number of live sperm and motility when males were subjected to heat stress (McDaniel *et al.*, 1995; McDaniel *et al.* 2004) [19, 20].

Spermatozoa from several species are highly susceptible to oxygen-induced damage mediated by lipid peroxidation because of their high content of polyunsaturated fatty acids and relatively low levels of antioxidants (Aitken *et al.*, 1989) [2]. Exposure to fatty acid peroxides results in rapid loss of motility and viability (Aitken *et al.*, 1989) [2]. Free radicals generated during heat-stress induce lipid peroxidation in the membranes of avian spermatozoa which adversely affects sperm viability and motility (Surai *et al.*, 2001) [31]. In addition, lipid peroxidation has also been shown to decrease sperm-oocyte interaction and fertilizing potential of spermatozoa (Aitken *et al.*, 1989) [2]. Determination of malondialdehyde (MDA), a toxic by-product of lipid peroxidation offers a facile means of assessing lipid peroxidation in biological materials. Rui *et al.* (2017) [23] observed MDA induced decline in chicken sperm function under *in vivo* conditions. MDA may be generated during hydrolysis by the oxidation of polyunsaturated fatty acids (Draper and Hadley, 1990) [8] owing to a higher proportion of polyunsaturated fatty acids. In this study, sperm motility and viability were affected by heat-stress and its lipid peroxidative effect on the spermatozoal membrane as reported earlier by McDaniel *et al.* (1995 and 1996) [19, 18].

Despite the significant influence of heat stress on percent live sperm, sperm motility and lipid peroxidation, no significant effect on fertility (p = 0.150) and hatchability (p = 0.360) were observed. On the contrary, few works on broiler breeder roosters have demonstrated a decline in the fertility of heat-stressed birds which was attributed to a drop in sperm storage and sperm penetration ability (McDaniel *et al.* 1995, 1996) [19, 18]. However, in these experiments, the heat exposure period was relatively longer. Aengwanich (2008) [1] reported an important relation between the body size and heat tolerance in three different varieties of chicken (Broiler, Thai Indigenous and Thai Indigenous Crossbred), where the broilers failed to perform on par with the indigenous varieties under heat stress conditions owing to their relatively higher body size and growth/ metabolic rates. The same analogy may be applied in the current study to explain the non-significant effects of heat stress on fertility and hatchability of GML roosters *i.e.* due to their medium-sized bodies and low growth/ metabolic rates.

The percent fertility was 80.58 ± 4.09 and 69.22 ± 6.50 and hatchability was 95.44 ± 3.97 and 91.21 ± 3.97 in heat-stressed and control groups, respectively with no significant differences. Fertility was highest in 39-weeks old roosters and progressively declined until 72 weeks (Cerolini *et al.*, 1997; Kelso *et al.*, 1997) [7, 14]. The semen quality indicated by volume and sperm concentration were improved in White Leghorn and Dahlem Red roosters reared in hot tropical climate from early to mid-age (24 to 48 weeks) and then declined as age advanced (Shanmugam *et al.* 2012, 2014) [26, 27]. Age of roosters may be a reason for non-significant effect on fertility observed in the present study. Global gene and protein expression analysis showed some genes and proteins are associated with thermotolerance and differentially expressed in the testes of heat-stressed Taiwanese native roosters (Wang *et al.*, 2018) [34]. Hence, the effect of heat stress on semen quality varies to a great extent depending on the duration of stress, age, body weight and breed which greatly influence the process of physiological adaptation to heat-stress.

Table 1: Semen characteristics: Ejaculate volume, sperm concentration, sperm motility and percent live sperm

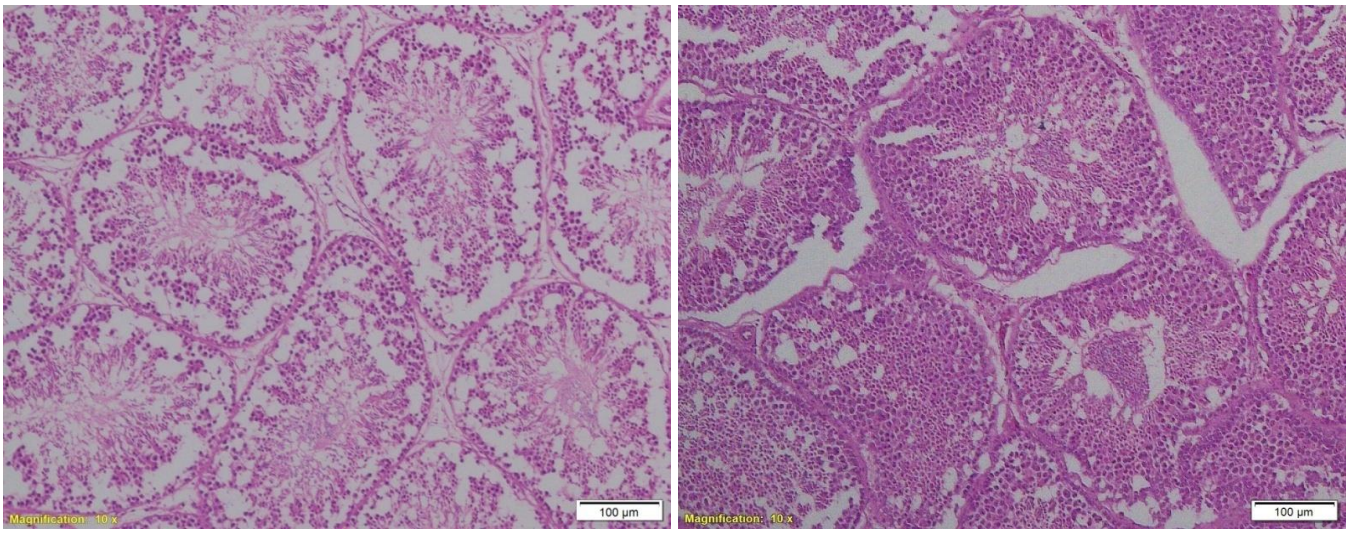
Week	Ejaculate volume (mL)			Sperm concentration (10^9/mL)			Sperm motility (%)			Live sperm (%)		
	HS	C	p- value	HS	C	p- value	HS	C	p- value	HS	C	p- value
0	0.43±0.04	0.52±0.06	0.226	3.45±0.28	4.14±0.29	0.114	70	70	-	79.95±0.94	82.41±1.00	0.089
1	0.38±0.021	0.40±0.02	0.483	3.21±0.44	4.18±0.17	0.054	45±4.35	70	0.000	44.22±5.85	84.48±1.64	0.000
2	0.49 ± 0.04	0.57± 0.06	0.271	5.04±0.36	4.76±0.38	0.608	48.33±1.66	70	0.000	64.04±3.96	84.04±0.09	0.000
3	0.45±0.04	0.56±0.06	0.126	4.20±0.23	4.36±0.33	0.709	50	70	0.000	66.01±1.53	88.48±1.0	0.000

Week: 0- before heat stress; 1, 2 and 3- first, second- and third-week post heat exposure
HS- Heat stress group; C-Control group

Table 2: Semen characteristics: MTT dye reduction and lipid peroxidation scores

Week	MTT dye reduction (nM of MTT formazan/min/ million sperm)			Lipid peroxidation (nM of MDA/ mL of seminal plasma)		
	HS	C	p value	HS	C	p value
0	24.93±2.06	20.86±1.14	0.098	0.34 ± 0.04	0.43 ± 0.04	0.144
1	6.83±0.85	21.25±1.89	0.000	1.98 ± 0.20	0.51 ± 0.02	0.000
2	7.09 ± 1.05	17.71 ± 1.16	0.000	2.79 ± 0.22	0.48 ± 0.01	0.000
3	6.52 ± 0.59	25.26 ± 3.01	0.000	3.16 ± 0.13	0.45 ± 0.01	0.000

Week: 0- before heat stress; 1, 2 and 3- first, second and third weeks post heat exposure
HS- Acute heat stress group; C-Control group



a) Control roosters b) Heat-stressed roosters

Fig 1: Histological sections of testicular tissue (H&E; 10×) showing seminiferous tubules and their constituent cells

Conclusion

This study mimics the natural thermal fluctuations observed during tropical summers and therefore it may seem appropriate to draw conclusions about the reproductive performance of GML roosters during summer months in India. The results of this study suggest an apparent deterioration in the semen quality of GML roosters during hot summer months, for which the remediation could be managemental and/ or nutritional. However, there were no significant effects on the overall fertility and hatchability rates along with the absence of lesions within the testicular tissues of GML roosters, indicating these changes are transient.

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