Thermal Stress Influence and Seasonal Pattern of Ovarian Follicular Growth Assessed through Ultrasonography in Sunandhini Cows under a Humid Tropical Climate

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**ABSTRACT**

The impact of thermal stress (TS) on reproductive processes starts early and persists more extended than the influence on other physiological manifestations. Since the cyclical reproductive activity begins with the growth of ovarian follicles, the present study focused on the effect of TS and its seasonal variations on the growth of ovarian follicles in ‘Sunandhini’ cows reared under the tropical climate. The year-round study was performed on 60 postpartum cows between days 28 to 91, involving eight cows at a time. The cows were replaced sequentially so that the study animals were of almost similar postpartum period throughout the year and formed a continuously changing study group. The ovarian follicular activity of each cow was monitored (nine to ten times) at weekly intervals using B mode ultrasonography. Serum samples collected during the scanning were subjected to ELISA for heat shock protein (HSP) 70 and Cortisol. A comparison of the follicle types, stress indicators, and weather parameters to assess their correlations and seasonal pattern using SPSS software. The maximum temperature of the locality was around 33°C without significant variation between seasons. Further, the extended rainy season caused moderate to high (66 to 85 %) relative humidity (RH), contributing to a temperature-humidity index (THI) exceeding 78 and putting the animals under moderate to severe TS throughout the year. HSP 70 level showed significant (P<0.001) elevation during summer (6.24 ng/mL), associated with high THI (r=0.701, P<0.001), while serum cortisol had no significant correlation with weather parameters. Only large follicles (9-14 mm) manifested the influence of TS as an increase in number (P<0.05) and size (P<0.01) together with a positive correlation (P<0.01) of HSP 70 and THI. It is inferred that TS causes stagnation of the large follicles from attaining the largest size and functional capability for ovulation. In contrast, other follicle types are unaffected even under moderate to severe TS.

**Keywords:** Follicular dynamics, season, thermal stress, tropical climate.

Long term influence of TS causes ovarian hypo-function leading to reduced growth and atresia of many follicles at the primary to tertiary stages of development [7]. Attainment of fertilization capacity of the oocytes was also adversely affected in follicles grown during TS, leading to reduced fertility of cows during summer and autumn [8], [9]. Such changes are initiated well in advance, even before detecting impairment of milk production and other physiological manifestations being the adjustment towards stress-prone situations [6], [10].

In thermo-tolerant tropical cattle breeds, TS exposure did not cause any immediate effect on reproductive performance [6]-[11]. However, long-term exposure to TS affects the growth of follicles and competence of oocytes leading to

I. INTRODUCTION

Thermal stress is one of the critical impediments to enhancing animal productivity, especially in tropical regions with high ambient temperatures (AT). While extreme cases even threaten survival, less severe cases of TS are reflected in the first instance by different types of fertility impairments [1]-[2]. Various stress alleviation measures are incorporated into the management system with variable effectiveness to restore productivity [3], [4]. However, such measurements provide little benefit for restoring impaired fertility, primarily because TS manifests early onset and prolonged consequences on the reproductive processes [5], [6].

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impairment of fertility [12], [13]. Widespread adoption of crossbreeding to increase dairy cattle productivity in the tropical climate [10] has initiated many fertility issues [1], [14]-[15]. ‘Sunandhini’ cows are the product of upgrading local non-descript cattle of the state with dairy breeds such as Brown Swiss, Jersey, and Holstein Friesian through many generations of breeding. Many fertility issues are evidenced in these animals, and one of the major causes appears to be the TS precipitated by the increase in productivity and the ongoing phenomenon of global warming [16]-[18].

On functionally normal and active ovaries of cattle, follicles grow simultaneously in two or three batches that advance one after the other during each oestrous cycle, designated by the term follicular waves [19]. TS during the follicular phase disturbs the recruitment, growth, and maturation of dominant follicles to attain the capability for ovulation [8],[20]. Such alterations of the follicular dynamics involve increased plasma FSH and decreased inhibin [8], [9] causing an inverse relationship between the follicular size and TS [21]. However, there is a shortage of studies describing the influence of TS and the seasonal pattern of follicular growth in crossbred dairy cows adapted to the tropical climate.

Hence, the present study attempted to assess the pattern of follicular growth during the period anticipated to have maximum follicular activity (second and third months of the postpartum period), its variation across seasons, interrelationship with weather parameters, and biological indicators of TS, in Sunandhini dairy cows maintained under the humid tropical climate of Kerala state in India.

II. MATERIALS AND METHODS

The study was at Livestock Research Station (LRS), Thiruvazhamkunnu under Kerala Veterinary and Animal Sciences University, located at 11°21’ N and 76°21’ E and an altitude of +35m MSL. The dairy farm of the station has 300 heads of “Sunandhini” (composite breed having enhanced thermo-tolerance evolved out of many generations of interbreeding local cattle with Brown Swiss, Jersey, and Holstein Friesian) dairy cattle under intensive management as per ICAR-NIANP (2013) recommendations [22], formed the study setting. Cows belonging to second or higher parity without any peri-parturient complications were selected. The study involved eight postpartum (PP) cows at a time belonging from Day 28 to Day 91, and the animals were replaced sequentially to ensure the same PP period of study animals so that 60 cows were included during the 12 months. Uniformity of the group was maintained throughout the study period by initial selection of eight cows at different PP stages and replacing two cows every month on a first-come basis with recent calved ones.

A. Ultrasonography

Monitored the ovarian follicular activity weekly using B mode ultrasound scanning (Esaote Veterinary scanner, Mylab Delta model®, Esaote S.p.A, Italy), fitted with a transrectal probe (5 MHz, SV 3513®) and recorded the types and number of follicles present on both the ovaries. Those cows bred between the two scanning periods were excluded from the subsequent weekly scanning until the declaration of non-pregnancy based on the return to oestrus or ultrasonography. The cows were scanned at their standing space within the barn, minimizing the stress of handling, facilitated by a movable trevis [23] specially designed and fabricated.

B. Ovarian Follicles

The size of the follicles identified as non-echogenic structures was assessed, taking the mean value of the largest diameters in more than one direction. Recorded the number and diameter of medium (6-8 mm), large (9-14 mm), and extra-large (15-20 mm) follicles as per the earlier reports [24]-[26]. The largest follicle was identified based on the maximum size among the follicles detected on both ovaries. Small follicles (3 to 5 mm) and the presence of cysts (more than 20 mm) were also counted.

C. Blood Sampling

Blood samples were collected (4 ml each) from each study animal at scanning. In cows bred during the study period, blood collection days were modified as Day 7, 14, and 21 post-AI and excluded those found conceived at the pregnancy diagnosis. The serum samples were separated and stored (in duplicates) frozen at -18 °C to estimate stress indicators.

D. Weather Parameters

Recorded the ambient conditions at hourly intervals using a data logger (HOBO Pro V2) installed within the barn. Daily ambient temperature (AT) recordings and RH were retrieved and stored monthly. Arrived climatic parameters such as maximum (Mx), mean (Av), and minimum (Min) of AT and RH, and also calculated the THI values using the formula of livestock and poultry heat stress index (LPHSI) [27].

\[ \text{T.H.I (LPHSI)} = T-[(0.55-[(0.55 \times RH /100) \times (T-58))] \]

where T- Average temperature (in Degree Fahrenheit), RH-Percent relative humidity.

E. Seasonal Pattern

The period of study was divided into four quarters of three months each, comprised of September to November (SON), December to February (DF), March to May (MAM), and June to August (JJA). These quarters corresponded to the region’s four seasons based on the rainfall pattern and the locality’s day length, such as Northeast monsoon, Post monsoon, Summer, and Southwest monsoon, respectively [1], [28], [29].

F. Stress Indicators

Frozen stored serum samples were subjected to enzyme-linked immune-sorbent assay (ELISA) as per standard procedure using specific ELISA Kits for HSP 70 (Chongqing Biospes Co Ltd, China) and Cortisol (Neogen – USA). Estimated the HSP 70 concentration from the serum samples collected weekly from all the study cows (except during the weeks after breeding) and the post-AI samples (3 each) from the cows inseminated during the study period. The ELISA for serum cortisol was performed only from the weekly samples (excluding post-AI samples) after ether extraction of Cortisol from individual samples as per the kit instructions.
G. Statistical Analysis

Analyzed the number and types of ovarian follicles for descriptive details, monthly, quarterly and half-yearly variations, and correlation with other study variables such as weather parameters and stress indicators using SPSS software (SPSS V. 24.0.) to identify the seasonal pattern and influence of TS on ovarian follicular activity.

III. RESULTS AND DISCUSSION

The study was initiated from d28 pp to avoid the stress of the post-calving transition period and to ensure the completion of uterine involution. Further, the study was limited to 91 days pp anticipating insemination and conception of more cows within three months, leading to their exclusion, since the primary focus of the study was stress influence on follicular growth during the postpartum period.

A. Ultrasonography Findings on Ovaries

The number of different types of follicles detected on both the ovaries at each scanning, categorized by subjective assessment of the appearance and ultrasonographic measurement of the diameter, are compared between seasons in Table I. The total number of follicles per scanning in a season was highest during MAM than in other seasons, even though the difference was statistically non-significant.

The numbers of different follicle types detected during a particular season are compared in Table II. The numbers of extra-large follicles were very few (Av. 1.67±0.36) irrespective of the seasons, while there were many small follicles (Av. 145.66±12.85) during all the seasons. Small follicles were comparatively high (163.00±35.13) during MAM, and the lowest was during SON (114.33±27.39). The number of medium (Av. 43.00±2.99) and large-sized (45.00±3.45) follicles was almost equal during the three seasons except for MAM.

The total count of different types of follicles varied significantly within each of the seasons (P<0.001), concurring with the report of [24]. However, the difference was not significant between seasons for the same types of follicle types except for large follicles. During MAM, together with a significantly more number of large follicles (P<0.001), all types of follicles were more. This increase indicates that climate plays a regulatory role in the growth pattern of large follicles, as reported by [7]. While the lowest numbers of extra-large follicles were in DJF (Table II), all other follicle types were lowest during SON, and the difference was significant only for large follicles.

A. Weather Parameters

The combined influence of AbT, RH, and THI of the study locality was compared between seasons to understand the effect of various climatic factors on the animals' TS. Across seasons THI was more than 78, indicating exposure of the animals to TS throughout the year, and a comparison with thermo-neutral conditions could not be made in the study. Moderately high AbT associated with annual mean MxT of 33.64 °C without significant seasonal variation formed the major contributor to the prevalence of such high THI in the locality. MnT also showed substantial variation between seasons, with the lowest seasonal mean value of 22 °C. However, the daily mean of AT did not fall below 27 °C in any of the seasons. In addition, there was moderate to high RH (66 to 85 %) contributed by the extended rainy season in the area. Hence, even in seasons with slightly lower AbT, THI was high enough, so the animals suffered moderate to severe TS throughout the year.

### TABLE I: MEAN ± SE OF DIFFERENT TYPES OF FOLLICLES DETECTED AT EACH USG EXAMINATION DURING THE FOUR SEASONS

| Season | Number of Cows | Large (9-14 mm) | Medium (6-8 mm) | Small (3-5 mm) | Total number of follicles |
|--------|----------------|----------------|----------------|----------------|--------------------------|
| SON    | 93             | 1.17 ± 0.84    | 1.11 ± 0.12    | 3.69 ± 0.31    | 6.03 ± 0.31              |
| DJF    | 106            | 1.06 ± 0.74    | 1.16 ± 0.12    | 3.91 ± 0.23    | 6.17 ± 0.27              |
| MAM    | 129            | 1.42 ± 0.81    | 1.19 ± 0.11    | 3.79 ± 0.24    | 6.45 ± 0.26              |
| JJA    | 117            | 1.09 ± 0.06    | 1.05 ± 0.11    | 3.72 ± 0.256   | 5.91 ± 0.30              |
| Overall| 445            | 1.12 ± 0.39**  | 1.13 ± 0.06**  | 3.78 ± 0.13**  | 6.16 ± 0.14**            |

** Significant (P<0.01)  NS - Non-significant.

### TABLE II: MEAN ± SE OF THE TOTAL COUNT OF DIFFERENT FOLLICLE TYPES DURING THE FOUR SEASONS

| Seasons | Extra Large (15-20 mm) | Large (9-14 mm) | Medium (6-8 mm) | Small (3-5 mm) |
|---------|------------------------|----------------|----------------|----------------|
| SON     | 1.67 ± 1.67            | 36.33 ± 8.67*  | 34.67 ± 7.80   | 114.33 ± 27.39|
| PDF     | 1.33 ± 0.67            | 40.00 ± 1.00*  | 45.00 ± 1.00   | 160.00 ± 6.00  |
| MAM     | 2.00 ± 1.15            | 61.00 ± 1.15*  | 51.33 ± 7.69   | 163.00 ± 35.13|
| JJA     | 1.67 ± 0.67            | 42.67 ± 5.90*  | 41.00 ± 1.53   | 145.33 ± 29.54|
| Total   | 1.67 ± 0.36*           | 45.00 ± 3.45*  | 43.00 ± 2.99*  | 145.66 ± 12.83**|

* Significant (P<0.05), F-value 5.911, ns - Non-significant.

Means having different superscripts vary significantly across seasons.

### TABLE III: CORRELATION COEFFICIENT (AND P-VALUE) OF DIFFERENT FOLLICLE TYPES WITH BIOLOGICAL PARAMETERS AND THI

| Parameters | Extra Large (15-20 mm) | Large (9-14 mm) | Medium (6-8 mm) | Small (3-5 mm) |
|------------|------------------------|----------------|----------------|----------------|
| HSP70      | 0.250 (-0.434)         | 0.861          | 0.630 (-0.28)* | 0.371 (-0.235)|
| Cortisol   | 0.512 (-0.089)         | 0.177 (-0.582) | 0.013 (-0.969) | -0.206 (-0.520)|
| THI        | 0.323 (-0.307)         | 0.351 (-0.063) | 0.209 (-0.515)*| -0.0210 (-.394)|

* Significant (P<0.05).  ** Significant (P<0.01).
A. Stress Indicators in Serum

The biological response of animals exposed to TS-prone climate was assessed by comparing HSP 70 and Cortisol levels in serum with weather parameters. Estimated the HSP 70 concentration of 544 serum samples, including 445 weekly intervals and 99 post-AI samples (from 33 cows inseminated during the study period). The serum cortisol assay involved 445 weekly samples, which were subjected to ether extraction of Cortisol preceding the assay as per the kit instructions.

Values of HSP 70 in 544 samples ranged from 0.17 to 31.99 ng/mL, with a mean value of 3.54 ± 0.16 ng/mL. HSP 70 showed a significant positive association (P<0.001) with major determinants of TS such as THI and AbT. HSP 70 levels varied significantly between months (Fig. 1), quarters, and half years, with a major hike (6.24 ng/Ml) during the period of maximum TS (MAM), indicating the involvement of HSP in the process of getting adapted to extreme TS. During the other three seasons of lesser THI, the elevation of HSP 70 was less, attributable to the lower levels of TS, to which the animals have already been adapted.

The overall mean value of serum cortisol in 445 samples was 8.27±0.20 ng/mL. The highest level was during MAM (9.44±0.25 ng/mL), which did not vary significantly from the value during SON, unlike the other two seasons. Similarly, the lowest level (6.84±0.61 ng/mL) during DJF did not vary significantly from the level of JJA. Even though cortisol levels also differed significantly between seasons, there was no significant correlation with any of the determinants of TS studied, including THI. Despite the highest levels of both HSP 70 and Cortisol attained in MAM, the pattern of variation was different during other seasons, as shown in Fig. 1, and there was no significant correlation between HSP 70 and Cortisol across the seasons. Thus, it is evident that even though Cortisol is an indicator of physiological stress, it is not specific to TS. At the same time, HSP 70 appears more specific as an indicator of TS, even though its usefulness for early detection necessitates further studies.

Table III shows the correlation of the numbers of different types of follicles with THI, HSP 70, and Cortisol. There was no significant correlation between small and extra-large follicles with climatic variables or biological indicators. Whereas large-sized follicles showed a significant positive correlation with HSP 70 (P<0.001), and medium-sized follicles maintained a moderate negative association (P<0.05) with THI and HSP 70. Further, the average numbers of different follicles at each scanning showed no significant correlation with weather parameters and stress indicators estimated in the serum.

A significant correlation of HSP 70 with counts of medium and large follicles indicates that TS enhances the growth of such follicles, which agrees with the earlier finding of more follicular development during TS [6]. A positive correlation of THI with the count of medium follicles also indicates enhancement of follicular growth during summer, concuring with the report of [8]. However, serum cortisol did not show a significant correlation with follicular growth parameters in the present study, contrary to the earlier reports of Cortisol and other glucocorticoids influencing the growth and luteinization of ovarian follicles [30], [31]. This finding agrees with the report of reduced cortisol concentration in cows under chronic TS [32].

Many studies report an increase, decrease, and no change in the number of small follicles (3 to 5 mm) in response to TS [18], [33], [34]. In the present study, there was no evidence of TS influence on the growth of small follicles, which concurs with the report [6] that TS did not affect follicular recruitment so that the population and growth pattern of small follicles are unaffected. Thus, it can be inferred that follicular recruitment and growth occur irrespective of the season and predominant TS factors in cattle, and various factors acting at the intra-ovarian level regulates them [24], [35].

Unlike the early phases of follicular growth, little affected by TS, there was a climatic influence on advanced phases, evidenced by the significant variation of large follicles across seasons. TS increased the number of large follicles leading to follicular co-dominance and enhanced growth of a few larger follicles instead of increasing the diameter of the most prominent follicle alone, as reported earlier [6], [24]. Thus, TS-mediated impairment of fertility is reflected by the stagnation of medium and large follicles without completion of final growth and maturational changes leading to ovulation.

The presence of more numbers of all types of follicles during the period of maximum TS (MAM) indicates enhancement of follicular growth by TS and agrees with the report by [6]. TS was reported to cause an increase in the level of FSH and a decrease of inhibin, leading to enhancement of follicular growth in cows during summer and autumn [8]. Reduced expression of signs and resultant late detection of oestrus during summer, leading to delayed breeding and inadequate stimuli for ovulation, can be another mechanism [36] so that follicles continue to grow and attain a larger size than during other seasons.

Reference [37] reported an increase in the incidence of ovulatory failure and persistence of preovulatory follicles during the warm season. Poor dominance of early recruited follicles causes more preovulatory follicles to grow [38]. Thus, more numbers of smaller follicles grow simultaneously and lead to manifestations such as failure of ovulation, co-dominance, multiple ovulations, and cyst formation [37], [39]. Even though follicular growth is more during MAM, oestrus detected were less during the season attributable to poor manifestation and failure of detection caused by functional incompetency of the dominant follicles.
IV. CONCLUSION

The adverse influence of TS on the fertility of cows occurs partly due to the impact of stress factors on follicle growth and maturation. The development of ovarian follicles was affected only at the phase of large follicles (9-14 mm). There was a significant increase in the number of large follicles during the period of maximum TS, with a positive correlation of HSP 70. It appeared that TS causes stagnation of follicle growth at the stage of large follicle since further maturation to attain the largest size and functional capability for ovulation gets affected. In contrast, other phases of follicle recruitment and growth continue unaffected, even under the situations of moderate TS.

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