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Effect of maternal heat-stress on follicular growth and oocyte competence in *Bos indicus* cattle

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Abstract

The objective was to determine whether exposure of Gir (*Bos indicus*) cows to heat-stress (HS) causes immediate and delayed deleterious effect on follicular dynamics, hormonal profile and oocyte competence. The cows were kept in tie-stalls for an adaptive thermoneutral period of 28 days (Phase I, Days −28 to −1). In Phase II (Days 0–28) cows were randomly allocated into control (CG, $n = 5$) and HS (HS, $n = 5$) treatments. The HS cows were placed in an environmental chamber at 38 °C and 80% relative humidity (RH) during the day and 30 °C, 80% RH during the night for 28 days. The CG group was maintained in shaded tie-stalls (ambient temperature) for 28 days. During Phase III (Days 28–147) animals were placed in tie-stalls (Days 28–42) followed by pasture (Days 42–147) under thermoneutrality. In each phase, weekly ovum pick up (OPU) sessions were to evaluate follicular development, morphology of cumulus–oocyte complexes (COCs), and developmental competence after *in vitro* maturation, fertilization, and culture. Serum concentrations of progesterone (P_4) and cortisol were evaluated by radioimmunoassay. Exposure of Gir cows to HS had no immediate effect on reproductive function, but exerted a delayed deleterious effect on ovarian follicular growth, hormone concentrations, and oocyte competence. Heat-stress increased the diameter of the first and second largest follicles from Days 28 to 49. Indeed, HS increased the number of >9 mm follicles (characterized as follicular codominance) during this phase. Cows exposed to HS had longer periods of non-cyclic activity ($P_4 < 1$ ng/mL), as well as shorter estrous cycles. However, HS did not affect cortisol concentration as compared to CG. Although HS had no significant effect on cleavage rate, it reduced blastocyst development during Phase III. In conclusion, long-term exposure of *B. indicus* cattle to HS had a delayed deleterious effect on ovarian follicular dynamics and oocyte competence.

Keywords: Heat-stress; *Bos indicus*; Gir; Oocyte; Ovarian follicle; IVF

1. Introduction

The reduced fertility associated with summer heat-stress (HS) is a multi-factorial problem; hyperthermia can affect cellular function in various tissues of the female reproductive tract [1,2]. Heat-stress compromised ovarian follicular dynamics [3] and the ability of

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the dominant ovarian follicle to exert dominance [4,5]. This loss of follicle dominance may be related to reduced plasma concentrations of estradiol 17-β [4] and inhibin [6], and increased plasma concentrations of FSH [6]. Heat-stress induced codominance [7], which may compromise oocyte viability; indeed, oocytes may be compromised by HS [8,9].

Oocytes harvested from follicles of Holstein cows during summer had reduced ability to develop to the blastocyst stage after *in vitro* fertilization than oocytes harvested during winter [8,9]. Moreover, exposure of Holstein heifers to HS between the onset of estrus and insemination increased the proportion of abnormal and developmentally retarded embryos as compared to heifers maintained at thermoneutrality [10]. Once the pool of ovarian oocytes was damaged by summer HS, two or three estrous cycles were required (after the end of HS) before competent oocytes were present [11]. Therefore, follicles and oocytes can be damaged by HS during early stages of folliculogenesis, with a delayed deleterious effect on ovarian function.

Direct effects of elevated temperature on oocyte competence have also been demonstrated *in vitro*. Exposure of bovine cumulus–oocyte complexes (COCs) to elevated temperature during the first 12 h of *in vitro* maturation (IVM) disrupted cytoskeleton architecture, reduced oocyte nuclear maturation [12], and induced oocyte death through apoptosis [13,14]. These deleterious effects of heat-shock decreased the proportion of oocytes that became blastocysts following *in vitro* fertilization [13,15].

Genotype is a major determinant of resistance to heat-stress. Cattle breeds of *Bos indicus* origin are more resistant to tropical conditions such as elevated temperature and humidity than breeds that evolved in a temperate climate. Most of this adaptation to elevated temperature is due to the superior ability of thermotolerant breeds to regulate body temperature [16–19]. However, breed differences in thermal resistance also extend to the cellular level. The degree by which heat-shock reduces endometrial [20], lymphocyte [21,22] and embryonic function [22,23] is greater for *Bos taurus* than *B. indicus* cattle.

Alhough *B. indicus* cattle are more resistant to HS, there is evidence that their reproductive function can also be compromised by elevated temperature. Retrospective analysis of 2395 ovum pick up (OPU) sessions demonstrated that the rate of *in vitro* embryo production was reduced when Nelore oocytes were collected during the summer [24], indicating a deleterious effect of HS on oocyte competence of *B. indicus* cattle. Therefore, the objective of the current study was to determine immediate and delayed effects of HS on follicular dynamics, oocyte competence, and hormonal profiles of Gir cows (*B. indicus*).

2. Materials and methods

2.1. Cattle

The experiment was conducted at the Santa Mônica experimental field of Embrapa's Dairy Cattle Research Center, located in Rio de Janeiro State (Brazil) using 10 multiparous non-lactating *B. indicus* cows (Gir breed) with 2.5–4.0 (2.95 ± 2.0 [25]) body condition score (BCS) and 321–476 kg (395.1 ± 16.5 kg) of body weight. Animals not previously subjected to follicular aspiration were selected (with ultrasonography) for the presence of a CL and number of follicles. The experimental protocol was reviewed and approved by the Bioethical Commission from the School of the Veterinary Medicine and Animal Science of University of São Paulo, Brazil. One cow was removed from the HS group due to an accident 21 days after the start of heat-stress.

2.2. Experimental design

A schematic representation of the experimental design is shown (Fig. 1). A group of 10 Gir cows were initially kept in tie-stalls under thermoneutral temperature for management adaptation from Days −28 to −1 (Phase I). During this period, cows were subjected to two weekly OPU sessions (Days −14 and −7). On Day 0, cows were randomly allocated into two treatment groups: control (CG/$n = 5$) and heat-stress (HS/$n = 5$) for 28 days (Phase II, Days 0–28). The HS cows were placed in an environmental chamber at 38 °C and 80% relative humidity (RH) under a cold fluorescent light from 6:00 to 18:00 h, followed by 30 °C and 80% RH from 18:00 to 6:00 h for 28 days. The CG group was maintained in shaded tie-stalls under ambient thermoneutral temperature for 28 days. During this phase, cows were subjected to weekly OPU sessions (five OPU sessions/cow/group). On Day 28, HS cows were removed from the environmental chamber (Phase III) and maintained in tie-stalls for 14 days (Days 28–42) under thermoneutrality. At Day 42, HS and CG cows were placed on pasture (*Brachiaria brizantha*) supplemented with minerals and water *ad libitum* (Days 42–147). During Phase III (Days 28–147), cows were subjected to weekly OPU sessions (17 OPU sessions/cow/group). In Phases I, II, and III, the number follicles/ovary and the diameter of the two biggest follicles were

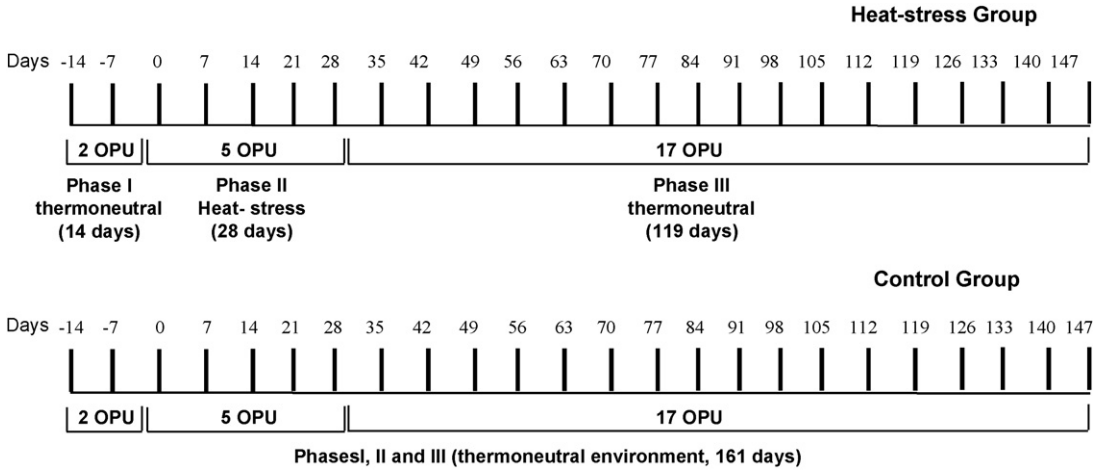

Fig. 1. Schematic representation of the experimental design.

evaluated once a week in each OPU session. All follicles ≥3 mm were aspirated and the oocytes were morphologically evaluated, selected and subjected to *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC).

2.3. Nutritional management and measurements

Cattle were fed corn silage and supplemented with concentrate (2 kg/cow) and water *ad libitum* from Days −14 to 42. During this period, dry matter intake was individually monitored for three consecutive days every week.

2.4. Environmental and physiological measurements

Daily dry and humid bulb temperatures were recorded at 08:00 and 15:00 h using a psychrometer. Relative humidity was measured in CG and HS groups. The temperature and humidity index (THI) was calculated to measure thermal loading [26,27]. Respiration rate (RR), rectal (RT), and cutaneous temperature (CT) were recorded twice a week at 08:00 and 15:00 h.

2.5. Follicular measurements and OPU procedures

Ovum pick up procedures alternated between CG and HS animals. Follicle population was identified, measured, and recorded according to follicle diameter (<6 mm, 6–9 mm, and >9 mm). Follicular codominance was characterized by the presence of two or more follicles >9 mm in one or both ovaries from the same donor cow [28].

Each OPU session was conducted with a portable ultrasonographic device equipped with a sectorial intravaginal 7.5 MHz probe (Scanner 200S, Pie Medical, Maastricht, Netherlands) using disposable 1.0 mm × 40 mm 19-gauge needles (BD Precision Guide®, São Paulo, SP, Brazil) and a vacuum pressure of 70 mmHg, equivalent to a flow rate of 10 mL/min. Follicular aspirate was recovered via a 2 mm internal diameter and 80 cm length Teflon circuit (Handlle Cook®, Ribeirão Preto, SP, Brazil) directly connected to a needle and 50 mL conical tube containing 15 mL Dulbecco's phosphate buffered saline (DPBS; Nutricell® Nutrientes Celulares, Campinas, SP, Brazil) supplemented with 1% (v/v) fetal calf serum (FCS) and 125 IU/mL heparin (Liquemine®, Roche Laboratory, São Paulo, SP, Brazil) at 35–37 °C. The conical tube containing follicular aspirate was transported to the laboratory and COCs were washed using an 80 μm filter (Millipore®, Bedford, USA). Cumulus–oocyte complexes were harvested, washed once in DPBS supplemented with 1% (v/v) FCS at 37 °C and morphologically evaluated under stereomicroscope.

Cumulus–oocyte complexes were morphologically classified based upon oocyte cytoplasmasmic characteristics and the number of cumulus cell layers as follows: Grade I, compact COCs with more than three layers of cumulus cells and oocyte with homogeneous cytoplasm; Grade II, compact COCs with three or less layers of cumulus cells and oocyte with slightly heterogeneous cytoplasm; partially denuded, oocytes showing complete removal of cumulus cells from less than 1/3 of zona pellucida (ZP) surface; denuded and/or degenerated, oocytes with no cumulus cells over most of ZP surface and/or vacuolization and shrinkage of

cytoplasm, and expanded cumulus: COCs with expansion of cumulus cells. Selected Grades I and II COCs were transported to *in vitro* production (IVP) laboratory in 1.5 mL cryotubes containing tissue culture medium (TCM) 199 supplemented with 25 mM HEPES (Gibco®, Rockville, USA), 10% (v/v) FCS, 49.4 μg/mL pyruvate, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37–39 °C.

Unless otherwise stated, all chemicals and media used for oocyte/embryo holding, manipulation, and culture were purchased from Sigma® Chemical Co. (St. Louis, MO, USA).

2.6. In vitro oocyte maturation, fertilization and culture

For each replicate COCs were pooled per treatment. Groups of 4–18 COCs (the number of COC was balanced within replicates) were matured in 100 μL drop TCM 199 supplemented with 20 μg/mL FSH and 10% (v/v) estrous cow serum overlaid with mineral oil for 22 h at 39 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. After maturation, COCs were placed in 100 μL drop of IVF-medium [29] supplemented with 20 μg/mL heparin and 6 mg/mL of essentially fatty acid-free BSA overlaid with mineral oil. Frozen–thawed Gir or Holstein semen was purified by swim-up procedure as described before [30]. The sperm pellet was reconstituted in IVF-medium and oocytes were fertilized with a sperm suspension solution ($\sim 2 \times 10^6$ sperm/mL) at 39 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. *In vitro* fertilization was performed with same batch of frozen–thawed Gir or Holstein semen for both treatments in each OPU session. Approximately 18 h after fertilization, presumptive zygotes were partially stripped of cumulus cells by mechanical pipetting in TALP medium [29]. Groups of 12–18 presumptive zygotes surrounded by one or two cumulus cells layers were co-cultured in 50 μL drop CR2aa medium [31], supplemented with 10% (v/v) FCS and 1 mg/mL BSA covered by a granulosa cell monolayer under mineral oil at 39 °C in an atmosphere of 5% (v/v) CO_2 in humidified air. The proportion of cleaved oocytes, 4–8 cell and >8-cell stage embryos was recorded at 3 days and blastocyst development at 7 days post-insemination.

2.7. Blood samples and hormonal assays

Blood samples were weekly collected 1 day prior to each OPU procedure by venipuncture of the coccygenal vessels using vacutainer tubes (Vaccutainer®, Becton-Dickinson, Franklin Lakes, NJ, USA). Blood samples were collected and centrifuged for 15 min at 750 × *g* at 4 °C. Serum samples were frozen and stored at −20 °C until analysis. Serum progesterone (P_4) and cortisol concentrations were determined by radioimmunoassay using specific I^{125} RIA Kits (Coat-a-Count®, MedLab, São Paulo, SP, Brazil) as previously validated at the LDH laboratory (Laboratório de Dosagens Hormonais) from the University of São Paulo. The intra-assay coefficient of variation was 10.96 and 4.53% and assay sensitivity was 0.05 ng/mL and 0.2 μg/dL for P_4 and cortisol, respectively. Luteal activity during the experimental period was evaluated. Females with serum $P_4 < 1.0$ ng/mL were considered in a follicular phase, whereas those with >1.0 ng/mL were considered in a luteal phase. The estrous cycle was defined as the period between two OPU sessions when serum P_4 concentrations were <1.0 ng/mL.

2.8. Statistical analysis

The effect of the treatment on THI, feed intake, physiological responses, ovarian follicular population, endocrine pattern and COC number for each week and experimental phase was analyzed by ANOVA for repeated measures, using the MIXED model procedure of SAS (SAS Institute Inc., Version 8.02) [32]. For comparisons between treatments, the two-sample Student's *t*-test was used for continuous variables. This analysis included fixed effects of treatment, phase, OPU session week, and their interactions (treatment × phase and treatment × week), as well as the random effect of animal. A Chi-square test was used to compare binomial variables, including COC morphology and developmental potential; this analysis included fixed effects of treatment, phase and OPU session week. Oocyte competence was determined as the percentage of oocytes that cleaved and reached the blastocyst stage following *in vitro* fertilization. Regression analyses were performed to determine the best-fit line for COCs developmental potential over experimental weeks. Results were expressed as mean ± S.E.M. Treatment differences with $P < 0.05$ were considered significant, whereas $0.05 < P < 0.10$ were considered a tendency.

3. Results

3.1. Environmental and physiological responses

During Phases I and III, HS and CG cows were maintained under the same environmental thermoneutral

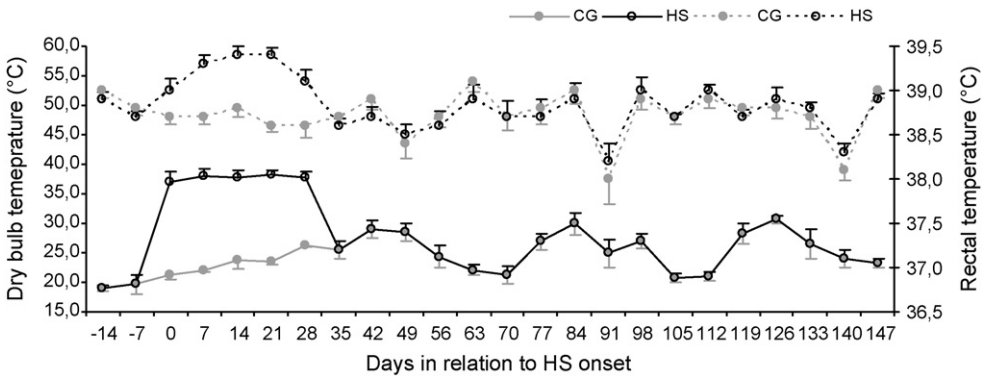

Fig. 2. Environmental dry bulb temperature in thermoneutral control (CG: solid gray line with solid circles) or heat-stress (HS: solid black line with open circles) treatments and rectal temperature in thermoneutral control (CG: dotted gray line with solid circles) or heat-stress (HS: dotted black line with open circles) treatments. Results are means ± S.E.M. Dry bulb temperature means were higher for HS cows on Days 0, 7, 14, 21, and 28 ($P < 0.05$) and rectal temperature means were higher for HS cows on Days 0 ($P = 0.07$), 7, 14, 21, and 28 ($P < 0.05$).

condition. Therefore, there was no difference between HS and CG treatments for dry bulb temperature (Fig. 2) and THI (69.8 ± 0.9 and 74.3 ± 0.5) for Phases I and III, respectively. However, during the stress period (Phase II), the dry bulb temperature (Fig. 2; $P < 0.05$) and THI (92.8 ± 0.2 vs. 70.7 ± 0.6, $P < 0.0001$) were greater in the HS than CG group. Similarly, dry matter intake (DMI) was similar between HS and CG groups during Phases I (6.7 ± 0.4 vs. 6.9 ± 0.4 kg/day, respectively) and III (8.0 ± 0.4 vs. 8.4 ± 0.3 kg/day, respectively). However, during the stress period (Phase II), DMI was lower ($P < 0.05$) in HS versus CG cows (6.9 ± 0.2 vs. 8.0 ± 0.3 kg/day, respectively). Heat-stress also increased (Fig. 2) RT (39.3 ± 0.05 vs. 38.7 ± 0.0 °C, $P < 0.0001$), CT (36.1 ± 0.1 vs. 31.0 ± 0.2 °C, $P < 0.0001$) and RR (79.2 ± 1.9 vs. 20.4 ± 0.5 breaths/min, $P < 0.0001$) as compared to CG cows.

3.2. Follicular measurements and endocrine profiles

There was a negative effect of OPU session in the number of visualized follicles and recovered COCs along the trial period ($P < 0.05$). Heat-stress did not affect follicular recruitment, since the population of >3 mm follicles and the number of recovered COCs were not affected by treatment (Table 1). There was no effect of HS on the pattern of follicular growth in Phases I and II. However, HS increased ($P < 0.05$) the number of large follicles (Table 1) and diameter of the 1st and 2nd largest follicles in Phase III (Fig. 3). Indeed, HS increased ($P < 0.05$) the number of follicles >9 mm (characterized as follicular codominance; Table 1). Follicular codominance and increased follicular diameter were associated with a sustained reduction in plasma P_4 for 21–28 days after cessation of HS

Table 1
Mean (±S.E.M.) follicular end points of Gir (*Bos indicus*) cows exposed to thermoneutral (CG) and heat-stress (HS) treatments

Variable	Phase I		Phase II		Phase III	
	CG	HS	CG	HS	CG	HS
Total follicles (>3 mm)	25.5 ± 2.5	28.5 ± 2.8	24.2 ± 1.1	24.0 ± 1.9	15.3 ± 0.6	15.8 ± 0.8
Small follicles (<6 mm)	23.9 ± 2.4	26.9 ± 3.1	22.6 ± 1.1	22.1 ± 2.0	13.8 ± 0.7	13.6 ± 0.8
Medium follicles (6–9 mm)	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Large follicles (>9 mm)	1.1 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	1.5 ± 0.1	1.0 ± 0.1[b]	1.6 ± 0.1[b]
Diameter of the 1st largest follicle (mm)	12.1 ± 1.5	11.1 ± 1.7	13.3 ± 0.8	13.0 ± 0.6	11.4 ± 0.4[b]	14.0 ± 0.4[b]
Diameter of the 2nd largest follicle (mm)	6.2 ± 1.3	6.0 ± 1.2	5.9 ± 0.6	7.1 ± 0.8	6.3 ± 0.3[b]	8.7 ± 0.5[b]
Recovered COCs	11.2 ± 2.8	14.3 ± 2.5	9.6 ± 1.0	11.0 ± 1.3	8.6 ± 0.7	7.9 ± 0.6
Recovery rate[a]	112/220 (50.9%)	143/273 (52.4%)	241/491 (49.1%)	265/480 (55.2%)	712/1076 (66.2%)[b]	535/897 (59.6%)[b]

[a] Number of recovered COCs per number of aspirated follicles.
[b] Treatments differed ($P < 0.05$).

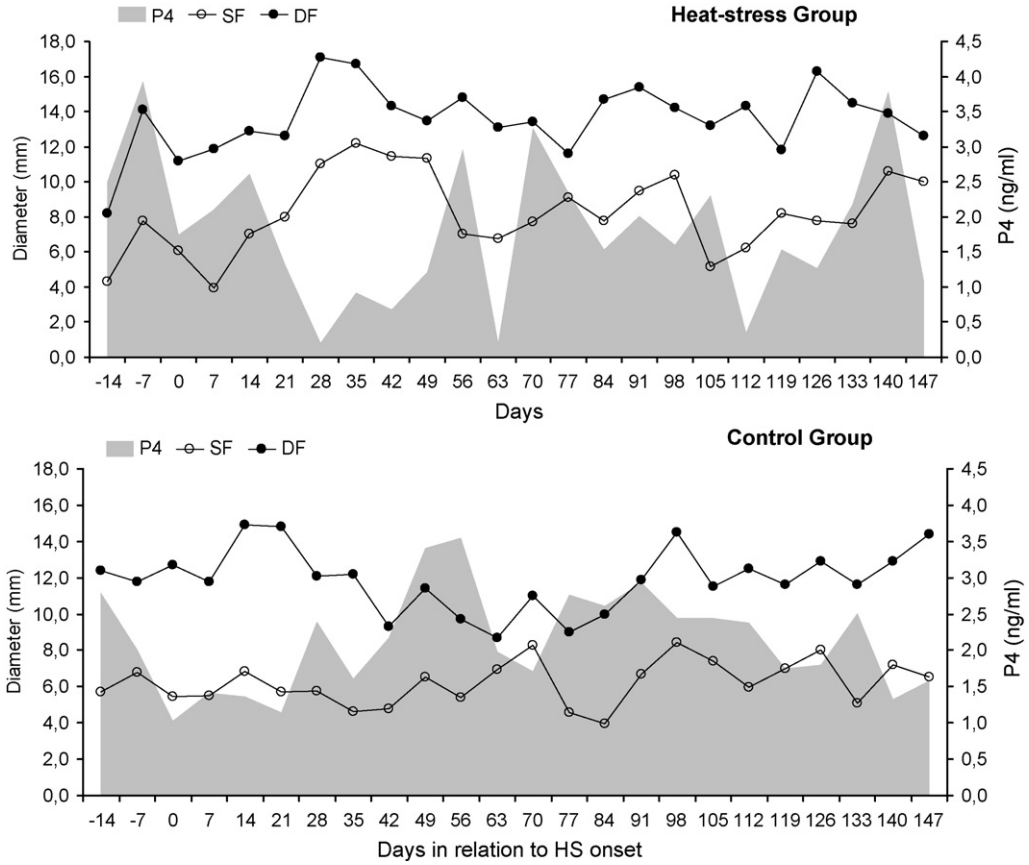

Fig. 3. Diameter of the dominant follicle (DF: black line with solid circle) and the largest subordinate follicle (SF: black line with open circle) and serum progesterone (P_4) concentration (gray area) of Gir (*Bos indicus*) cows exposed to thermoneutral (CG) (bottom panel) or heat-stress (HS) (top panel) treatments. Heat-stress increased ($P < 0.05$) the diameter of the dominant (Days 28, 35, 42, and 56) and largest subordinate (Days 28, 35, 42, and 49) follicles and reduced P_4 concentration up to Day 28, as compared to thermoneutral control group.

treatment (Fig. 3). There was no effect of HS on P_4 concentrations in Phases I and II (Table 2). However, P_4 concentrations were reduced ($P < 0.05$) by HS in Phase III (Table 2). Heat-stress reduced ($P < 0.05$) the percentage of cows with 21-day estrous cycles and increased ($P < 0.01$) the percentage of cows with short (<21 days) cycles (Table 3). However, there was no effect of HS on the proportion of cows with >21-day cycles (Table 3). Cows exposed to HS had longer periods of non-cyclic activity ($P_4 < 1$ ng/mL). The reduction in the length of the estrous cycle in the HS group occurred during Phase III, when P_4 concentrations were the lowest ($P < 0.05$; Table 2). There was no effect of HS on cortisol concentrations as compared to CG (Table 2). However, overall cortisol concentration was higher ($P < 0.0001$) in Phases I and II than Phase

Table 2
Mean (±S.E.M.) of the estrous cycle length, serum P_4 and cortisol concentrations in Gir (*Bos indicus*) cows exposed to thermoneutral (CG) or heat-stress (HS) treatments in each experimental phase

Variable	Phase I		Phase II		Phase III	
	CG	HS	CG	HS	CG	HS
Estrous cycle (days)	–	–	19.8 ± 1.2	21.0 ± 0.0	20.7 ± 0.5*	17.5 ± 0.8*
Progesterone (ng/mL)	2.4 ± 0.7	3.1 ± 1.2	1.5 ± 0.3	1.7 ± 0.5	2.3 ± 0.2*	1.7 ± 0.3*
Cortisol (μg/dL)	2.3 ± 0.5 A	3.2 ± 0.8 A	2.4 ± 0.2 A	2.4 ± 0.2 A	1.9 ± 0.2 B	1.3 ± 0.1 B

A, B: Within a row, experimental phases without a common letter differed ($P < 0.0001$).
* Treatments differed ($P < 0.05$).

Table 3
Length of the estrous cycle in Gir (*Bos indicus*) cows exposed to thermoneutral (CG) or heat-stress (HS) treatments

Estrous cycle (days)	CG, no./total (%)	HS, no./total (%)	*P*-value
21	31/35 (88.6)	15/24 (62.5)	0.0200
<21	3/35 (8.6)	9/24 (37.5)	0.0086
>21	1/35 (2.9)	0/24 (0.0)	0.2018
Mean ± S.E.M.	20.6 ± 0.4	18.4 ± 0.7	0.0048

Table 4
Distribution of COCs (various morphological categories) recovered from Gir (*Bos indicus*) cows exposed to thermoneutral (CG) or heat-stress (HS) conditions

COCs	Phase I		Phase II		Phase III	
	CG, no./ total (%)	HS, no./ total (%)	CG, no./ total (%)	HS, no./ total (%)	CG, no./ total (%)	HS, no./ total (%)
Grade I	31 (27.7)	45 (31.5)	64 (26.6)	65 (24.5)	118 (16.6)	76 (14.2)
Grade II	38 (33.9)	63 (44.1)	100 (41.5)	107 (40.4)	308 (43.3)	229 (42.8)
Partially denuded	13 (11.6)	8 (5.6)	22 (9.1)	23 (8.7)	83 (11.7)	71 (13.3)
Denuded/degenerated	25 (22.3) a	18 (12.6) b	38 (15.8) b	61 (23.0) a	168 (23.6)	143 (26.7)
Expanded cumulus	5 (4.5)	9 (6.3)	17 (7.1) x	9 (3.4) y	35 (4.9)	16 (3.0)
Selected for IVF	69 (61.6) b	108 (75.5) a	164 (68.0)	172 (64.9)	426 (59.8)	305 (57.0)
Total (no.)	112	143	241	265	712	535

Columns without a common letter differ (a, b: $P < 0.05$; x, y: $P < 0.1$).

III, indicating that cows kept in tie-stall or environmental chamber had higher cortisol than those kept on pasture (Table 2).

3.3. Follicular aspirations and oocyte recovery

The total number of follicles (18.3 ± 0.6 vs. 19.6 ± 0.9 for CG vs. HS, respectively), the percentage of aspirated follicles [82.7% (1895/2292) vs. 83.9% (1758/2094) for CG vs. HS, respectively] and oocyte recovery rate [60.1% (2195/3653) vs. 60.0% (1137/1895) for CG vs. HS, respectively] were not affected by HS. However, HS reduced ($P < 0.05$) COCs recovery rate in Phase III (Table 1). Heat-stress increased ($P < 0.05$) the proportion of denuded and/or degenerated COCs (Table 4). There was no effect of HS on the percentage of COCs selected for IVF.

3.4. Oocyte developmental capacity

The data shown in this section refers to 17 OPU–IVP procedures. Out of 17 OPU–IVP sessions, seven were disregarded due to laboratory problems. Cleavage rate was not affected by OPU session (Fig. 4). There was no effect of HS on cleavage rate or the proportion of embryos reaching the 2-cell, 4–8 cell or >8-cell stages at 3 days post-insemination in Phases I, II, and III (Table 5). During Phase II, HS had no immediate effect on the total blastocyst rate nor on the percentage of embryos reaching the early, normal and expanded blastocyst stages at 7 days post-insemination (Table 5). However, HS reduced ($P < 0.05$) the total blastocyst rate and the proportion of embryos reaching the normal ($P < 0.05$) and expanded blastocyst ($P < 0.1$) stages in Phase III (Table 5). There was a negative effect of HS ($P < 0.001$) on blastocyst production and a tendency for a greater difference between treatments over time (Fig. 5).

4. Discussion

The current study demonstrated that HS exerted a delayed deleterious effect on follicular development, progesterone profile and oocyte competence in Gir cows (*B. indicus*). Moreover, the lack of immediate effect of HS on reproductive function suggested that susceptibility of this thermotolerant breed to HS required long-term exposure to elevated temperature. It is well known that *B. indicus* cattle are more resistant to elevated temperature and humidity than *B. taurus* cattle. Most of this adaptation to elevated temperature is due to superior ability of thermotolerant breeds to regulate body temperature [16–19] as well as intrinsic cellular resistance to elevated temperature [20–23].

The increased THI and animal physiological responses, e.g. rectal and cutaneous temperatures as

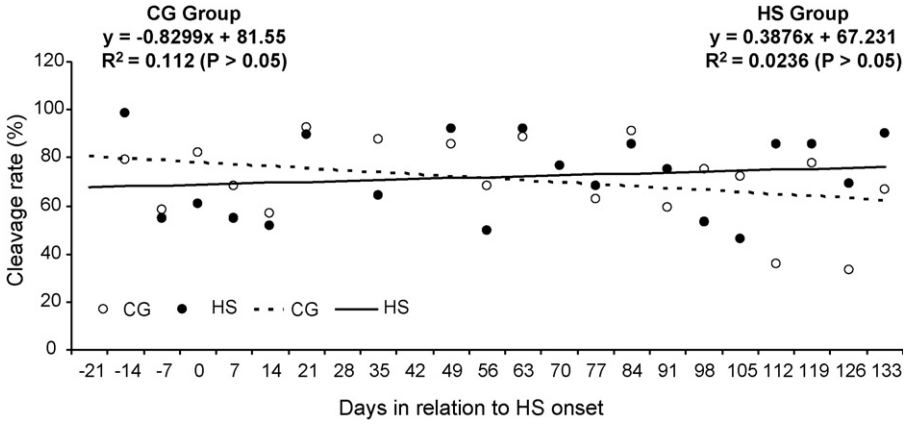

Fig. 4. Percentage (CG, open circles and HS, solid circles) and regression equation's adjusted lines (CG, dotted lines and HS; solid lines) of cleaved Gir (*Bos indicus*) COCs.

well as respiration rate, indicated that HS treatment (38 °C and 80% RH from 6:00 to 18:00 h and 30 °C and 80% RH from 18:00 to 6:00 h) for 28 days effectively induced hyperthermia in Gir cows. For example, rectal temperature was 0.6 °C higher in the HS group than control. As an immediate result of hyperthermia, there was a reduction in feed intake. It has been widely accepted that HS reduces immediate voluntary feed intake under both experimental and field conditions [33–35] as a primary mechanism to balance heat production with heat loss.

There was no immediate effect of HS on the population of small, medium, and large follicles in Gir cows. However, exposure to HS increased the number and diameter of large follicles during Phase III of the trial. This pattern of follicular growth suggested a delayed and ineffective mechanism of follicular selection and dominance resulting in follicular codominance [7], delayed [7] or double ovulations [36], and reduced fertility. Furthermore, HS caused an immediate [3,37] as well as a delayed [6,11] effect on ovarian follicular growth in Holstein cows. However, this is the first study demonstrating a carry-over effect of HS on follicular dynamics of *B. indicus* cattle.

Heat-stress also reduced the length of the estrous cycle and P_4 concentration in Phase III. Earlier studies were inconsistent regarding the effect of HS on steroidogenesis [38–40]. However, in most recent studies, plasma steroid concentrations were reduced during HS in lactating cows and dairy heifers [41]. *In vivo* and *in vitro* studies demonstrated that elevated temperature reduced theca and granulosa cell viability, resulting in low androstenedione and estradiol production due to low androgen substrate and aromatase activity [1].

Table 5
Developmental potential of oocytes collected from Gir (*Bos indicus*) cows exposed to thermoneutral (CG) and heat-stress (HS) treatments

	Phase I		Phase II		Phase III	
	CG, no./ total (%)	HS, no./ total (%)	CG, no./ total (%)	HS, no./ total (%)	CG, no./ total (%)	HS, no./ total (%)
Early cleavage stages (Day 3)						
2-Cell stage	9/59 (15.3)	9/105 (8.6)	21/101 (20.8)	18/121 (14.9)	27/317 (8.5) y	30/230 (13.0) x
4–8 Cell stage	19/59 (32.2)	36/105 (34.3)	37/101 (36.6)	41/121 (33.9)	143/317 (45.1)	88/230 (38.3)
>8-Cell stage	16/59 (27.1)	39/105 (37.1)	14/101 (13.9)	15/121 (12.4)	56/317 (17.7)	41/230 (17.8)
Cleavage rate	44/59 (74.6)	84/105 (80.0)	72/101 (71.3)	74/121 (61.2)	226/317 (71.3)	159/230 (69.1)
Blastocyst stage (Day 7)						
Early blastocyst	11/59 (18.6)	16/105 (15.2)	2/31 (6.5)	5/52 (9.6)	27/279 (9.7)	11/188 (5.9)
Normal blastocyst	8/59 (13.6)	17/105 (16.2)	6/31 (19.4)	6/52 (11.5)	41/279 (14.7) a	13/188 (6.9) b
Expanded blastocyst	4/59 (6.8)	8/105 (7.6)	3/31 (9.7)	2/52 (3.8)	8/279 (2.9) x	1/188 (0.5) y
Blastocyst rate	23/59 (39.0)	41/105 (39.0)	11/31 (35.5)	13/52 (25.0)	76/279 (27.2) a	25/188 (13.3) b
No. embryos/donor (mean ± S.E.M.)	3.8 ± 1.3	4.6 ± 0.9	3.7 ± 0.9	2.6 ± 0.9	3.0 ± 0.5 a	1.7 ± 0.5 b

Columns without a common letter differ (a, b: $P < 0.05$; x, y: $P < 0.1$).

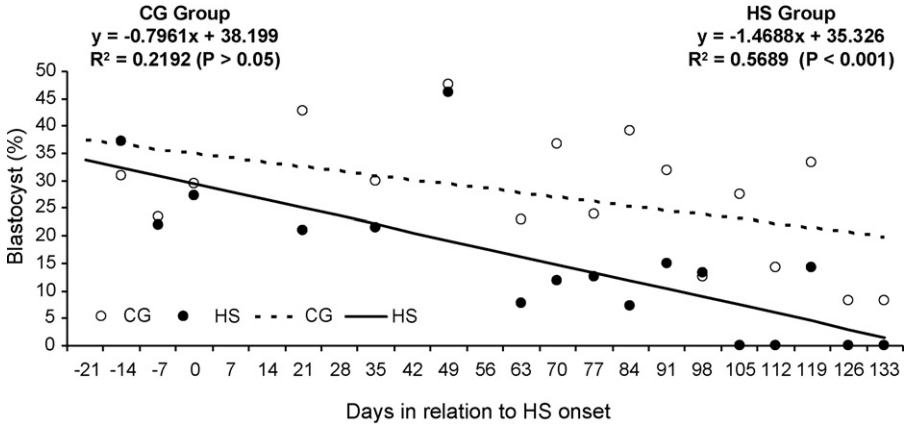

Fig. 5. Percentage (CG, open circles and HS, solid circles) and regression equation's adjusted lines (CG, dotted lines and HS, solid lines) of Gir (*Bos indicus*) COCs that reached the blastocyst stage following *in vitro* fertilization.

Steroidogenesis can also be affected by oxidative status of follicular and/or luteal cells [42,43]. It has been suggested that HS increased reactive oxygen species (ROS) production due to the increased metabolic rates associated with cellular hyperthermia. Elevated temperature increased liver peroxidation [44] and activity of enzymes involved in ROS production such as cyclooxygenase [45] and xanthine oxidase [46]. Moreover, exposure of dairy cows to HS decreased total antioxidant activity in blood [47]. This increase in ROS affected P_4 production by impairment of LH receptors [48,49] or inhibition of cholesterol translocation to the mitochondria [50] or cytochrome P450scc enzyme activity [51].

The endocrine profile of lactating Holstein cows was also affected by steroid metabolism. High DMI increased steroid metabolic rate in high-producing cows [6,7,52,53]. Moreover, the deleterious effect of HS on fertility was greater for high- versus low-producing cows [54]. Perhaps the synergistic deleterious effect of HS and lactation on fertility acted through different mechanisms, with similar effects on follicular dynamics, oocyte competence, and embryonic development. Furthermore, HS may have reduced steroidogenesis and inhibin synthesis due to damage in follicular and luteal cells, compromising follicular and oocyte development starting in the preantral period. Reduced steroidogenesis and increased steroid metabolism during follicular selection and dominance reduced estradiol and P_4 availability in HS lactating cows, and compromised FSH and LH secretion, follicular dominance and the preovulatory LH surge. In the present experiment, the reduced plasma P_4 concentration in the HS group during Phase III may also be explained by the reduction in ovulation rate and subsequent CL formation [36].

The regular cyclic activity observed in thermoneutral cows subjected to repeated OPU sessions was in agreement with other studies [11,55,56]. In those studies, the follicular phase interval was 20–22 days following OPU sessions conducted between Days 3 and 15 of consecutive estrous cycles. Our hypothesis is that the increase in P_4 concentration and cyclicity maintenance in the CG was due to the occurrence of ovulations between aspiration sessions or luteinization of aspirated follicles [11,55,56].

Blood cortisol profile was not affected by immediate or delayed HS. However, the management conditions had a major effect on this response. Cortisol concentration was higher in Gir cows kept in tie-stalls either inside or outside the environmental chamber as compared to cows grazing in pasture. Therefore CG and HS cows were exposed to a similar management stress load.

Exposure of Gir cows to HS did not affect oocyte quality (Grades I and II) and cleavage rate. Indeed, a series of *in vivo* [8,10,57] and *in vitro* [58–61] studies demonstrated that elevated temperature did not affect oocyte cleavage rate. In the present study, HS compromised oocyte developmental capacity in Phase III, as reflected by reduced blastocyst production. This carry-over effect of HS on blastocyst production was maintained during the entire experimental period and up to 105 days after the end of HS. In contrast, seasonal experiments demonstrated that once the ovarian follicular pool was damaged by summer HS, it took two or three estrous cycle after HS ended to restore the follicular pool and oocyte quality [11]; this difference

was probably associated with the severity of HS treatment between studies. The current study suggested that HS exerted a negative effect on follicular growth and oocyte function even before the antral phase (42 days [62]) or primary follicle (85 days [63]) reducing oocyte developmental potential for a period longer than two or three estrous cycles.

Roth et al. [11] demonstrated that the carry-over effect of HS on the quality of Holstein oocytes was reduced after removal of impaired follicles by repeated OPU. However, in the current study, weekly OPU did not restore oocyte quality. This lack of OPU beneficial effect was probably due to the increased intensity and duration of HS. Moreover, the number of ovarian follicles was greater in *B. indicus* than *B. taurus* cattle [64]. Therefore, the large number of follicles aspirated over a prolonged interval might have caused morphological and functional alterations in the ovary, further compromising oocyte competence [65,66].

The mechanisms by which HS compromised function of Gir oocytes are not known. However, *in vitro* studies demonstrated that elevated temperature reduced oocyte protein synthesis [59], disrupted microfilament, and microtubule architecture, disorganized oocyte meiotic spindle, reduced oocyte nuclear maturation [12], and induced oocyte death through apoptosis [13,14]. Moreover, exposure of germinal vesicle oocytes to heat-shock induced pre-mature translocation of the cortical granules to the oolemma and reduced the ability of the oocyte to reach the metaphase II stage [60].

In conclusion, the present study demonstrated that exposure of Gir cows to a 28-day period of HS exerted a delayed effect on reproductive function, manifested by an increased incidence of large follicles, more follicular codominance, and reductions in estrous cycle length, progesterone concentrations, and oocyte developmental capacity.

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