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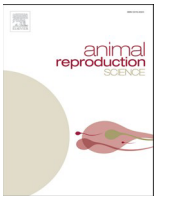

Livestock-Forest integrated system attenuates deleterious heat stress effects in bovine oocytes

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ABSTRACT

Global warming poses significant challenges to the fertility of tropical dairy cattle. One promising approach to mitigate heat stress effects on reproductive function and reduce the carbon footprint is the use of integrated livestock-forest (ILF) systems. The aim of this study was to investigate the effects of two different systems, namely Full Sun (FS) and ILF, on maternal hyperthermia and oocyte quality of Holstein and Girolando heifers during the tropical summer season. The temperature-humidity index (THI) data revealed intense heat stress during the experiment. Both the system ($P<0.01$) and the breed ($P<0.01$) factors had a significant impact on vaginal temperature, being hyperthermia more pronounced in the FS system and in the Holstein breed. Over the five time points collected at a 33-day interval, we observed distinct patterns for ILF ($P=0.65$) and FS ($P<0.001$) systems, suggesting an adaptive response in animals kept in FS systems. Furthermore, oocyte quality assessment revealed an effect of the system for oocyte diameter ($P<0.001$) and levels of IGFBP2 ($P<0.001$), and caspase 3 levels showed a decrease in ILF compared to FS for both Holstein ($P<0.001$) and Girolando ($P<0.001$) breeds. Collectively, these parameters indicate that oocyte quality during the summer months was superior in animals maintained in the ILF system. In conclusion, the ILF system demonstrated promising results in attenuating maternal hyperthermia and mitigating its effects on oocyte quality. Additionally, our observations suggest that animals in the FS system may exhibit an adaptive response to heat stress.

Abbreviations: FS, full sun; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; ILF, integrated livestock-forest; THI, temperature-humidity index.

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1. Introduction

Global warming and intense genetic selection for milk yield during the past years point out a challenging future for fertility in livestock, especially for dairy systems (Roth, 2017). Besides agroforestry ecosystem services and their innumerous environmental benefits including carbon sequestration (Jose, 2009), integrated livestock – forestry (ILF) systems are promisors to alleviate heat stress effects in animals. From an animal's physiological perspective, ILF systems are alternatives to attenuate temperature-humidity index (THI), a widely used indicator for heat stress (Khan et al., 2020), by reducing radiation charge (de Oliveira et al., 2018; Navarini et al., 2009). The shade effect decreases body temperature (de Souza et al., 2019; Lemes et al., 2021; Navarini et al., 2009), increases thermal comfort (de Oliveira et al., 2018; Giro et al., 2019; Magalhães et al., 2020; Navarini et al., 2009), improves animal welfare (Lemes et al., 2021; Reis et al., 2021) and performance (Lemes et al., 2021; Paciullo et al., 2014, 2011, 2009) in bovine. However, the role of the ILF system in fertility during the tropical summers is not well known.

Proper assessment of heat stress and efficient cooling of dairy animals irrespective of their stage of life at farm is the immediate strategy to reduce fertility declines (Sammad et al., 2020). The use of trees to provide shadow can serve this purpose while also contributing to ecosystem services and environmental benefits associated with agroforestry, and offering economic opportunities for farmers (Jose, 2009). Therefore, validating this solution to enhance fertility is essential. Regarding reproductive function, summer season thermal stress perturbs follicular environment by altered gonadotrophins secretion, follicular growth, selection, and ovulation (Boni, 2019). Competence of oocytes exposed to maternal hyperthermia during summer is impaired and embryonic development during the first cell cycles following fertilization is delayed (Khan et al., 2020). A period of 2–3 estrous cycles is demanded to the ovary restore its ability to ovulate a competent oocyte after summer heat damage, suggesting a long-term effect on ovarian oocyte pool (Roth, 2008). Also, oocytes collected during hot season have altered cleavage pattern and morpho kinetics compared to cold season-collected oocytes (Yaacobi-Artzi et al., 2022).

Impaired oocyte competence following summer heat stress is related to changes in mitochondria distribution pattern and activation of oxidative phosphorylation complexes (Roth, 2017), alterations associated with the activation of apoptosis through intrinsic pathways (Roth, 2018). Experiments have already shown increased caspase −2, −3 and −7 levels and nuclear fragmentation following heat shock in cultured oocytes (Roth and Hansen, 2004a, 2004b) and granulosa cells (Alemu et al., 2018), and its relation to oxidative stress pathway (Khan et al., 2020) and bioenergetics support mechanisms following acute heat stress (Sammad et al., 2020).

The IGF system is also involved in heat stress responses. Insulin growth factor could play a thermoprotective role in bovine oocytes since a decreased serum IGF level and oocyte quality impairment were described for cows after heat stress (Paula-Lopes et al., 2012). Ovarian IGF is a complex system consisting of ligands (IGF-1 and IGF-2), receptors, six IGF binding proteins (IGFBP), and IGFBP proteases. Low molecular weight IGFBP (-2, −4 and −5) have negative effects on IGFs actions by binding to them and preventing IGF binding to receptors. On the other hand, specific proteases cleave IGFBPs and increase IGF bioavailability (Fortune et al., 2004). Therefore, IGFBP levels inversely reflects IGF activity, and could represent a biomarker for negative heat stress effects on oocytes.

We employed previously validated biomarkers (Caspase-3 and IGFBP5) identified in recent research (de Silva et al., 2022), which are associated with the described pathways linked to decreased oocyte quality following heat stress, to me valuate the effects of our integrated system. This study investigated the impact of housing heifers in an integrated Livestock-Forest system during tropical summer heat stress on bovine oocytes, considering both purebred and crossbred Holstein heifers. Our hypothesis posited that the shading provided by the integrated system would mitigate exposure to harmful temperatures, thereby enhancing oocyte quality. Our findings corroborated the impact of thermal stress on the examined variables and demonstrated that the integrated Livestock-Forest system effectively mitigates maternal hyperthermia and consequent damage to bovine oocytes.

2. Materials and methods

2.1. Location of the study, ILF characterization and climatic conditions

The study was performed in an Experimental Station from Embrapa Dairy Cattle on the Innovation Center in Intensification of Sustainable Agriculture (NISA), located in southeast Brazil (Valença. Rio de Janeiro state) at coordinates 22°22'27.51"S and 43°42'29.54" W, at an altitude of 360 m above sea level.

Four hectares of pasture formed with *Urochloa decumbens v. Basilisk* on a smooth undulating terrain with an average slope of 20 % were used, two destined for the integrated livestock-forest (ILF) system, and the other two for the single pasture system in full sun. The ILF system was implemented in November 2019 by introducing a clone of hybrid *Eucalyptus urophylla* S. T. Blake x *Eucalyptus grandis* W. Hill ex Maiden (clone 1407). Trees were planted in contour lines and simple lines with 25 m spacing. Within the lines, trees were planted two meters apart, resulting in a planting density of 200 trees per hectare and a basal area of 1.33 m 2 ha^{-1} at the beginning of the experiment when trees were at 27 months of age.

Experimental procedures were carried out following ethical guidelines between December 2021 and February 2022 and were previously approved by Embrapa Dairy Cattle Ethics Committee (7374130921 protocol). Peripubertal Holstein (n=16) and Girolando (3/4 Holstein and 1/4 Gir composition, n=16) heifers aged from 14 to 18 months were selected and randomly allocated to the Integrated LF or Full Sun systems. All animals started the experiment before puberty, and at the end of experiment 43.75 % of the Girolando and 31.25 % of the Holstein heifers had reached puberty, with no effect of the system (details can be found at (Dias et al., 2023). Animals (n=32, 8 per group) were allocated into the following four experimental groups: Girolando Integrate LF, Girolando Full Sun, Holstein Integrated LF and Holstein Full Sun, at the proportion of 1.8 animal units/ha, with *ad libitum* consumption of grass, mineral salt, and water. Each system was divided into three areas used for rotational management determined by the grass height:

50 cm at entrance and 20 cm at the exit. The climatic conditions in this tropical region during the experiment was 2.54 mm mean daily rainfall; average maximum temperature of 35 °C and average minimum temperature of 14.9 °C. The relative humidity was 96 % (±13.81 %).

2.2. Vaginal temperature assessment

Vaginal temperature was assessed using iBotton data loggers (DS1921H-F5# Thermochron High Res 15–46 °C, Maxim Integrated, San Jose, CA, USA) mounted with parafilm into an internal progesterone device without the progesterone-impregnated silicone film, as described before (de Rezende Carvalheira et al., 2021). Vaginal temperature data were automatically collected at 15-minute intervals over a period of 2–4 days, with 15-day intervals between each data collection session throughout the experiment. Temperature-humidity index (THI) was calculated for each hour according to the equation: THI = (1.8 × Average Temperature + 32) − [(0.55 − 0.0055 × Relative Humidity) × (1.8 × Average Temperature − 26)] (Dikmen and Hansen, 2009). Days in which the THI remained above 78 for a minimum of 7 hours, a condition that indicates heat stress in both Girolando (Fialho et al., 2018) and Holstein

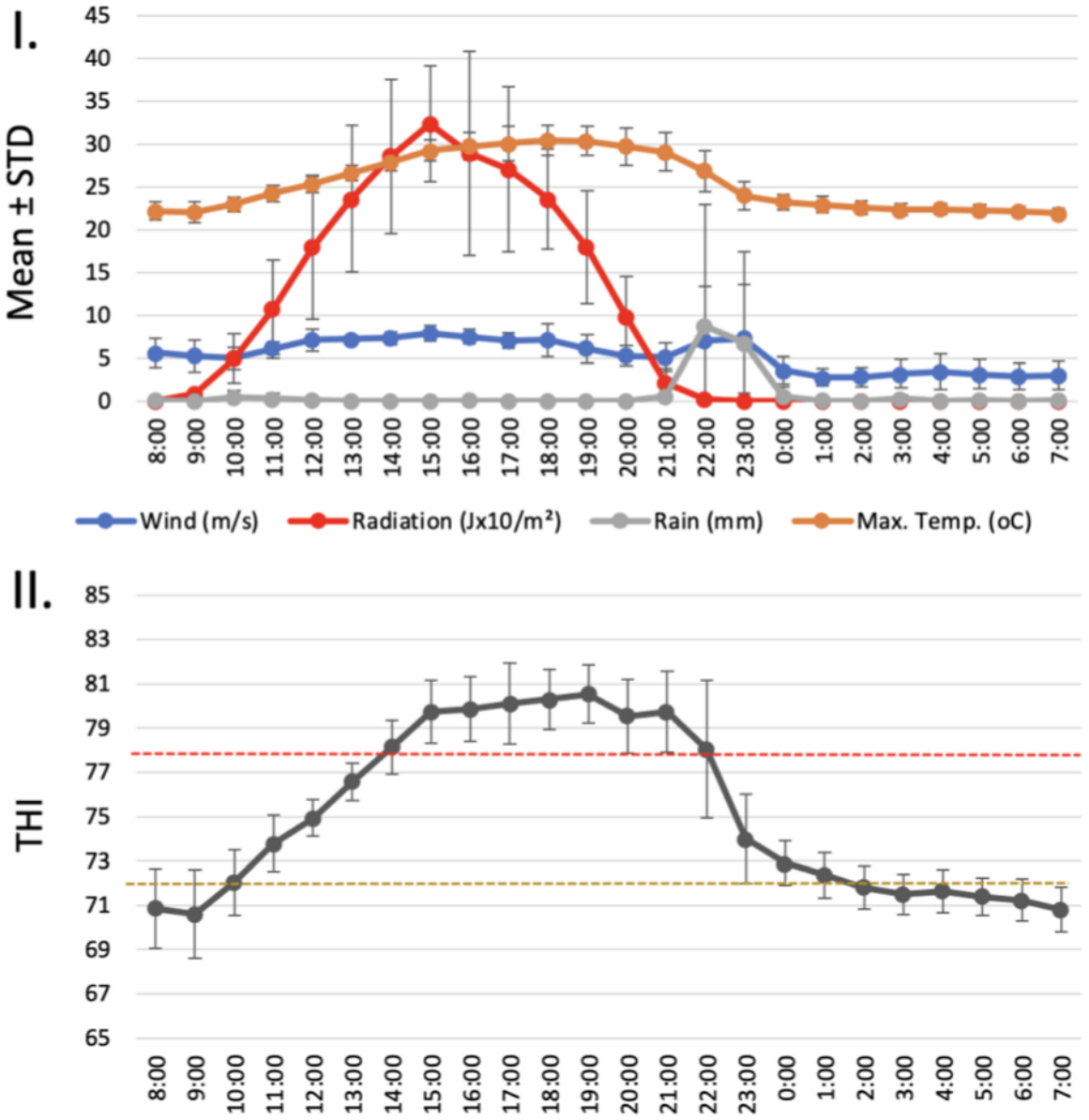

Fig. 1. Climate conditions. Graphs show average wind, radiation, rain, and maximum temperature (I) and temperature-humidity index (II) data collected by the weather station on summer days in the South hemisphere: 21st and 22nd December 2021; 5th, 6th and 23rd January 2022 (vaginal temperature assessment days, 30–60 days after animals being exposed to Integrated Livestock-Forest (Integrated IF) and Full Sun pasture systems.

(Collier et al., 2017) cattle, were specifically chosen for comparative analysis between the groups. Data were presented as the number of hours that vaginal temperature exceeded 39.1 °C and the maximum vaginal temperature.

2.3. Oocyte recovery by ovum pick up (OPU)

Reagents were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany) unless otherwise stated**.** Oocytes were obtained from all experimental animals by OPU using a transvaginal device guiding both needle and a convex transducer coupled to ultrasound equipment. Follicles with less than 8 mm diameter were punctured and the follicular fluid was aspirated through a vacuum system (90 mmHg) and collected in a 50 ml tube containing Dulbecco PBS supplemented with 50 UI heparin and 1 % fetal calf serum (FCS) solution, at 37°C. Fluid was filtered and *cumulus*-oocyte complexes (COCs) were selected based on a compact nonatretic cumulus with at least one layer of cells and homogeneous cytoplasm or cytoplasm with minor granules (IVF-grade oocytes). Selected COCs were pooled and denuded using 200 μl TrypLE Express enzyme (Thermo Fisher Scientific, MA, USA), fixed in 4 % paraformaldehyde for 30 min at 37° C and stored at 4 °C in PBS supplemented with 3 % BSA and 0.2 % tween 20.

2.4. Caspase 3 and IGFBP2 Immunofluorescence

Oocytes were incubated in 0.5 % Triton X-100 solution for 30 min at room temperature and washed three times in PBS supplemented with 0.2 % Tween-20 (PBS-T). Then, structures were incubated with PBS-T supplemented with 3 % BSA for 4 h at 4°C for non-specific binding of antibodies blocking. Oocytes were incubated with the primary antibodies (rabbit anti-active Caspase 3, 1:1000, and mouse anti-IGFBP2, 1:50, Santa Cruz Biotechnology, TX, USA) overnight at 4°C, and then washed three times in PBS-T for 10 min and incubated with the secondary antibodies (Alexa 555-conjugated goat anti-rabbit, 1:200, and Alexa 488-conjugated goat anti-mouse, 1:200, both Invitrogen, Thermo Fisher Scientific, MA, USA,) for 1 h. Structures were washed three times for 10 mins in PBS-T and examined under a fluorescence microscope. Reactions in which the primary antibody was omitted served as negative control. Images of each structure were captured on an EVOS 5000 inverted microscope. The protocol (staining and imaging) was performed at the same time and with the same parameters for all variables (breed: Holstein, Girolando, groups: ILF, Full Sun and day of OPU: D0, D30, D60). Analysis was performed in ImageJ (version 1.53e, ©National Institutes of Health, USA) software, using ROI function to select oocyte area and mean fluorescence levels to assess and compare protein levels in oocytes among groups. The program assigns intensity values ranging from 0 and 255 to each pixel within the selected area. Subsequently, it calculates the average intensity by computing the mean of these assigned values using the ROI Measure function. Oocyte diameter was also measured during the analysis.

2.5. Statistics

The number of hours above 39.1 °C and maximum vaginal temperature were analyzed using the GLM procedure. A repeated measures analysis of variance was conducted over time, considering the factors Breed and System. The analyzed animals were included as blocks to estimate individual effects. The sphericity test was significant, indicating that the split-plot analysis was not appropriate because the covariance structure over time is not uniform. Oocyte quality parameters (diameter, caspase 3 and IGFBP2 levels) were analyzed using GLIMMIX procedure. The model included the cross-effects of Breed and System, with Day nested within Breed. For the Holstein breed, we had only two time points, and F-test itself was sufficient to check for significance. For the Girolando breed, the values were decomposed into orthogonal polynomials up to the second degree. Analyses were performed using SAS (9.4 version). Rates of IVF-grade and oocyte recovery were compared among groups in each breed using Fisher Exact test. A 5 % significance level was considered in all analysis.

Table 1
Descriptive statistics for hyperthermia variables during high THI summer days in Girolando and Holstein heifers kept at Integrated LF or Full Sun pasture.

Variable	Breed	System	D1	D2	D3	D4	D5
Hours of hyperthermia	Girolando	Full Sun	17.1±1.1	13.8±1.6	12.8±1.8	12.2±1.9	7.7±1.2
		Integrated LF	12.0±2.4	10.7±2.0	11.2±2.0	8.3±1.6	8.2±4.0
	Holstein	Full Sun	19.8±1.3	18.8±1.2	15.0±2.0	14.5±2.6	13.2±1.46
		Integrated LF	13.1±2.1	15.3±2.5	13.0±1.2	12.4±1.7	16.2±1.6
Max. Temperature	Girolando	Full Sun	40.2±4.4	40.4±6.5	39.9±7.3	40.0±7.8	39.8±4.8
		Integrated LF	39.8±9.6	40.2±8.1	39.8±5.6	39.8±6.5	39.8±6.1
	Holstein	Full Sun	40.9±5.1	41.2±4.7	40.5±7.9	40.5±10.6	40.5±5.8
		Integrated LF	40.5±8.4	41.0±10.2	40.2±4.7	40.4±6.6	40.9±6.6

Results are presented as means and standard errors.
The days of data collection, namely D1, D2, D3, D4, and D5, corresponded to 22, 23, 37, 38, and 55 days after the start of the experiment.

3. Results

3.1. Vaginal temperature in response to climate conditions

In Fig. 1, we present data collected from the INMET (National Institute of Meteorology, Brazil) weather station, situated approximately 1.5 km away from the experiment site. This data is depicted in terms of the average and standard deviation for the five days during which vaginal temperature was analyzed in this study. Wind, radiation, rain, and maximum temperature are presented hourly (Fig. 1.I). Temperature-humidity index (THI) is shown as a separate graph (Fig. 1.II), with yellow (72) indicating a mild heat stress threshold, and red (78) marking a moderate heat stress threshold (Armstrong, 1994). During the days that vaginal temperature was collected, the average of hours for mild and moderate stress was 7.40 ± 1.51 and 11.6 ± 6.46 per day, and average THI was 75.24 ± 4.00 (STD).

Table 1 displays the mean values of hyperthermia variables for each group assessed in the experiment. No effects related to individual animals were observed for either variable ($P>0.05$). The effect of each main factor on vaginal temperature is illustrated in Fig. 2. For both the number of hours above 39.1° C and the maximum temperature, no significant interactions were found between Breed and System ($P=0.66$ and 0.84) or Breed and Day ($P=0.20$ and 0.12). However, the Day did significantly influence System for both variables ($P=0.03$ and 0.03), and therefore, the results are presented separately for the Integrated LF and Full Sun systems. The effect of the System was significant, with heifers kept in Integrated LF exhibiting fewer hours above 39.1 °C (11.9 vs 14.47, $P=0.001$) (Fig. 2.I) and lower maximum temperatures (40.24 vs 40.41, $P= 0.003$) (Fig. 2.V) compared to Full Sun heifers.

Breed also played a significant role in vaginal temperature, as Girolando heifers exhibited fewer hours above 39.1 °C (11.59 vs 14.79, $P<0.001$) (Fig. 2.II) and lower maximum temperatures (40.00 vs 40.65, $P<0.001$) (Fig. 2.VI) than Holstein heifers.

Furthermore, the assessment day had a notable impact on vaginal temperature, affecting both the number of hours above 39.1 °C ($P<0.001$) and maximum temperatures ($P<0.001$). In the Full Sun system, a decrease ($P<0.001$) was evident from D1 to D5 in terms of the number of hours above 39.1 C (Fig. 2.III). However, such trend was not detected ($P=0.65$) in the Integrated LF system (Fig. 2.IV). Maximum temperatures also differed ($P<0.001$) across days for both the Full Sun (Fig. 2.VII) and the Integrated LF (Fig. 2.VIII) systems.

3.2. OPU recovery rate and IVF-grade oocytes percentage

All animals were submitted to OPU for oocyte recovery at the beginning of experiment (D0), after 30 (D30) and 60 (D60) days. Table 2 shows the number of aspirated follicles, oocytes and recovery rates, and IVF-grade percentages for groups of Girolando and Holstein heifers located at Integrated LF or Full Sun pasture at D0, D30 and D60. For D0 OPU, no data for the number of aspirated follicles were collected.

No effect ($P>0.05$) was detected from beginning (D0) to the end (D60) of experiment for IVF-grade oocytes rate in Girolando in the Full Sun group. In the ILF group, the IVF-grade oocytes rates were lower ($P<0.05$) in D30 than in D0 and D60. A decrease ($P<0.01$) was noticed in IVF-grade oocytes rate in Holstein Integrated LF group. No effect ($P>0.05$) was detected for Holstein Full Sun group, but a small IVF-grade oocyte number was recovered (n=15), which might have interfered with these results.

Girolando heifers exhibited an increase ($P<0.05$) in the recovery rate between the D30 to D60 evaluations in both groups. An opposite effect was seen for Holstein heifers, in which a decrease ($P<0.05$) was noticed in recovery rate from D30 to D60 evaluation, in both groups. This decrease affected the number of recovered and IVF-grade oocytes obtained for Holstein groups at D60 OPU, which

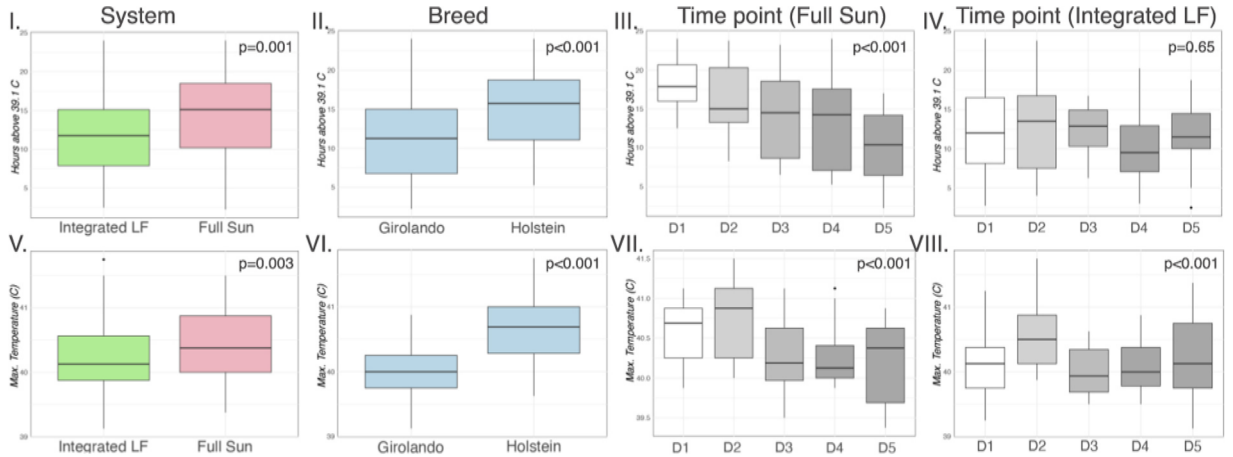

Fig. 2. Vaginal temperature assessment 30–60 days after animals being exposed to Integrated Livestock-Forest (Integrated IF) and Full Sun systems during summer. Boxplot graphs show number of hours above 39.1 Celsius degrees (I, II, III and IV) and maximum vaginal temperature (V, VI, VII and VIII) grouped by factors: System (Integrated LF vs Full Sun, I and V), Breed (Girolando vs Holstein, II and VI) and Day (D1, D2, D3, D4, D5, IV and VIII).

Table 2
Oocyte recovery by ovum pick up during summer days (0, 30 and 60) in Girolando and Holstein heifers kept at Integrated Livestock-Forest or Full Sun pasture.

Groups		Aspirated follicles (n)	Recovered oocytes (n)	IVF-grade oocytes (n)	IVF-grade oocytes (%)	Recovery rate (%)
Girolando						
Integrated LF	D0	N.A.	122	91	74.59[a]	N.A.
Integrated LF	D30	156	64	33	51.56[b]	41.02[a]
Integrated LF	D60	164	107	78	72.89[a]	65.24[b]
Full Sun	D0	N.A.	110	84	76.36[a]	N.A.
Full Sun	D30	198	123	82	66.66[ab]	62.12[c]
Full Sun	D60	160	114	77	67.54[a]	71.25[d]
Holstein						
Integrated LF	D0	N.A.	75	38	50.66 [A]	N.A.
Integrated LF	D30	153	79	39	49.36 [A]	51.63 [A]
Integrated LF	D60	109	40	9	22.50[B]	36.69[B]
Full Sun	D0	N.A.	81	42	51.85 [A]	N.A.
Full Sun	D30	113	73	42	57.53 [A]	64.60 [C]
Full Sun	D60	133	27	15	55.55 [A]	20.30[D]

N.A.: Missing data (D0 sampling).
[a,b,c,d,A,B,C,D] Distinct superscript letters in same column indicate percentages are not equal (comparison inside breeds. a-d, Girolando; A-D, Holstein)

was below the minimum for immunofluorescence evaluation (n=9 and 15).

3.3. Oocyte diameter from D0 to D60

Oocytes collected during summer at D0, D30 and D60 from Girolando (n= 387, 30–80 per group/day) and at D0 and D30 from Holstein (n= 153, 32–43 per group/day) heifers in Integrated LF and Full Sun groups were measured and immunostained for caspase 3 and IGFBP2 proteins. No assessment was performed for Holstein D60 oocytes due to the low number of recovered IVF-grade oocytes (Table 1). An effect on oocyte diameter was detected for the breed ($P<0.01$), the system ($P<0.01$) and the day ($P<0.01$) variables, and a significant ($P<0.01$) interaction between factors was present. Details of the orthogonal decomposition analysis can be found in Table 3.

Holstein oocytes exhibited smaller mean diameters compared to Girolando oocytes (100.72 vs 116.89, $P<0.001$), irrespective of the system. The system factor was significant: Girolando oocytes exhibited lower ($P<0.05$) diameters in the Full Sun system compared to the integrated LF system. However, this reduction in diameter was not observed for Holstein oocytes (Girolando: 111.14 vs 122.63, $P<0.01$; Holstein: 97.92 vs 103.52).

Over time, Girolando oocytes in the Full Sun system displayed a reduction in diameter ($P<0.001$), with a decrease of approximately 0.41 units per day) (Fig. 3.I and 3.III). In the Integrated LF system, oocyte diameter followed a quadratic equation pattern (Fig. 3.III). It decreased from D0 to D30 and then increased from D30 to D60 ($y=129{,}13893–0{,}66923x+0{,}00905x^2$). Notably, oocyte diameter was higher ($P<0.001$) in the integrated LF system compared to the Full Sun system at D60.

Holstein oocytes exhibited a lower ($P<0.01$) diameter from D0 to D30 in both the Full Sun (108.48 vs 87.36) (Fig. 3.II) and the Integrated LF (112.86 vs 94.18) (Fig. 3.III) systems. At D30, Holstein oocytes were also smaller ($P=0.02$) in the Full Sun system compared to the Integrated LF system.

Table 3
Statistics for orthogonal polynomial's decomposition of oocyte quality variables during high THI summer days in Girolando and Holstein heifers kept at Integrated LF or Full Sun pasture.

Group	Variable	Type of regression	Regression equation	R2
Girolando				
Full Sun	Diameter	Linear	Y=123.38–0.408x	38.65
	Caspase3	Quadratic	y=123.74+2.618x−0.04x2	39.66
	IGFBP5	Quadratic	y=5.05+0.0066x−0 00010 x2	26.37
Integrated	Diameter	Quadratic	$Y=129.14–0.67x+0.009x^2$	12.65
	Caspase3	Quadratic	y= 132.41+0.747x−0. 016x2	22.74
	IGFBP5	Quadratic	y=4.99+0.003x−0.00006x2	11.78
Holstein				
Full Sun	Diameter	Linear	y=108.48–0.7038x	48.98 %
	Caspase3	Linear	y=103.12+0.937x	68.07
	IGFBP5	Linear	y=123.324 + 0.607x	59.90
Integrated	Diameter	Linear	y=112.861 – 0.623x	38.40 %
	Caspase3	Linear	y=141.53 – 0.733x	39.72
	IGFBP5	Linear	y=139.78 – 0.427x	35.08

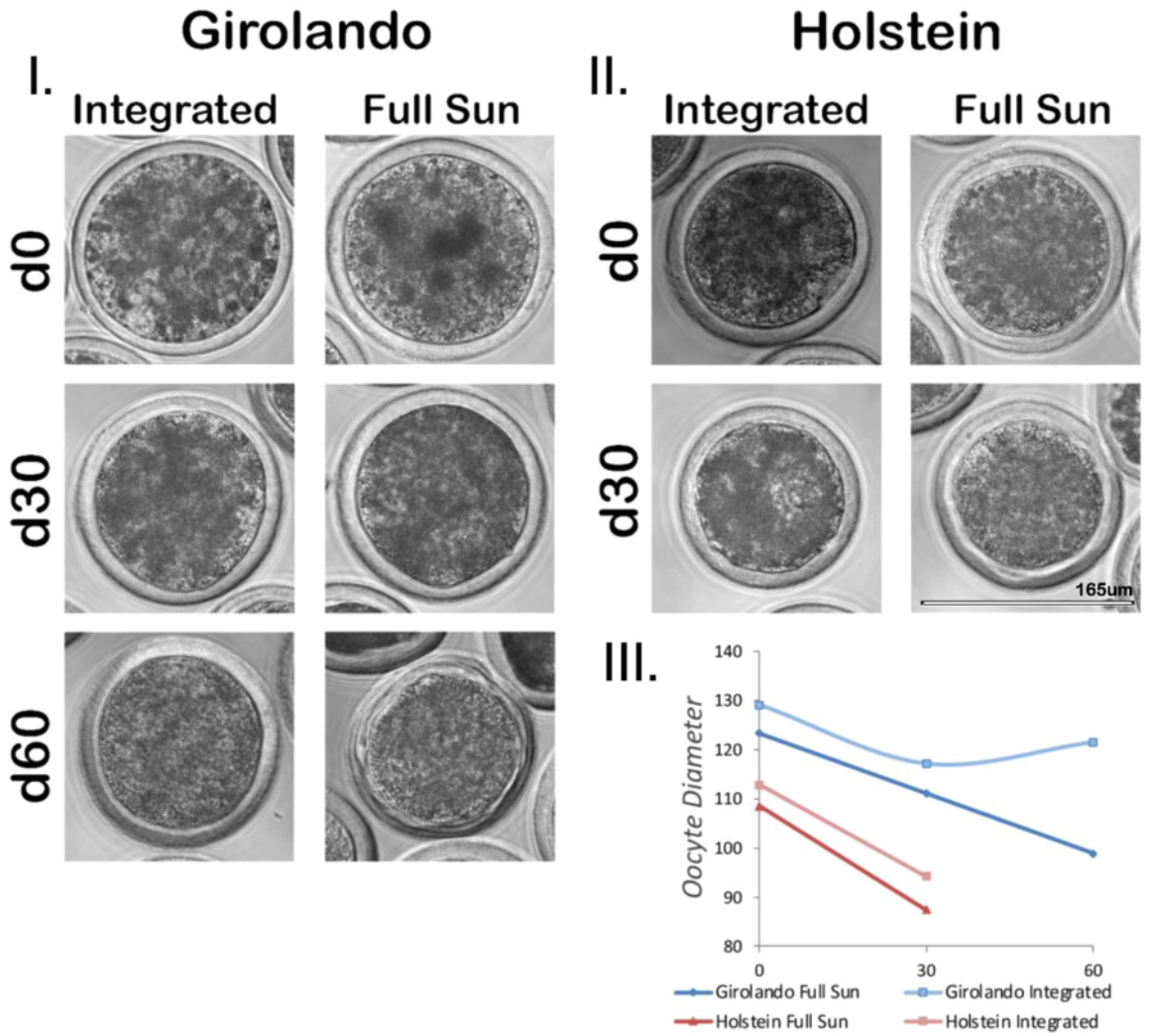

Fig. 3. Oocyte diameter variation during summer days (0, 30 and 60) in heifers kept at Integrated Livestock-Forest (Integrated LF) or Full Sun pasture. Phase contrast images of Girolando (I) and Holstein (II) oocytes are shown. Graph shows Girolando and Holstein mean oocyte diameter (excluding zona pellucida) inside system and day factors (III).

3.4. Caspase 3 levels from D0 to D60

The assessment of caspase 3 protein expression is presented in Fig. 4. There was an effect observed for the breed ($P<0.01$) and the day ($P<0.01$) variables. No overall effect was detected for the group ($P=0.89$), possibly due to the lower caspase 3 levels detected at D0 (before system allocation) in Full Sun oocytes. Furthermore, a notable($P<0.01$) interaction between factors was identified. Details of the orthogonal decomposition analysis can be found in Table 3.

Holstein oocytes exhibited lower caspase 3 levels compared to Girolando oocytes (123.86 vs 136.58, $P<0.001$), regardless of the system. Within Girolando breed, Integrated LF oocytes displayed a decrease in caspase 3 levels (130.89 vs 142.28, $P<0.01$) (Fig. 4.I), a trend not observed for Holstein oocytes (130.53 vs 117.18) (Fig. 4.II).

For the Girolando breed, a quadratic equation aptly described the pattern of oocyte caspase 3 levels. These levels increased from D0 to D30 and subsequently decreased from D30 to D60 (ILF: $y = 132.41+0.75x-0.016x^2$; FS: $y = 123.74+2.62x-0.04x^2$). Furthermore, caspase 3 levels were lower at D60 in the integrated LF system (136.80 vs 119.53, $P<0.001$) compared to the Full Sun system (Fig. 4. III).

Regarding Holstein oocytes, their caspase 3 levels increased from D0 to D30 in the Full Sun system (103.12 vs 131.25, $P<0.001$), but decreased from D0 to D30 in the integrated LF (141.53 vs 119.53, $P<0.001$) system. At D30, Holstein oocytes in the Integrated LF system exhibited lower ($P<0.01$) levels of caspase 3 compared to those in the Full Sun system (Fig. 4.III).

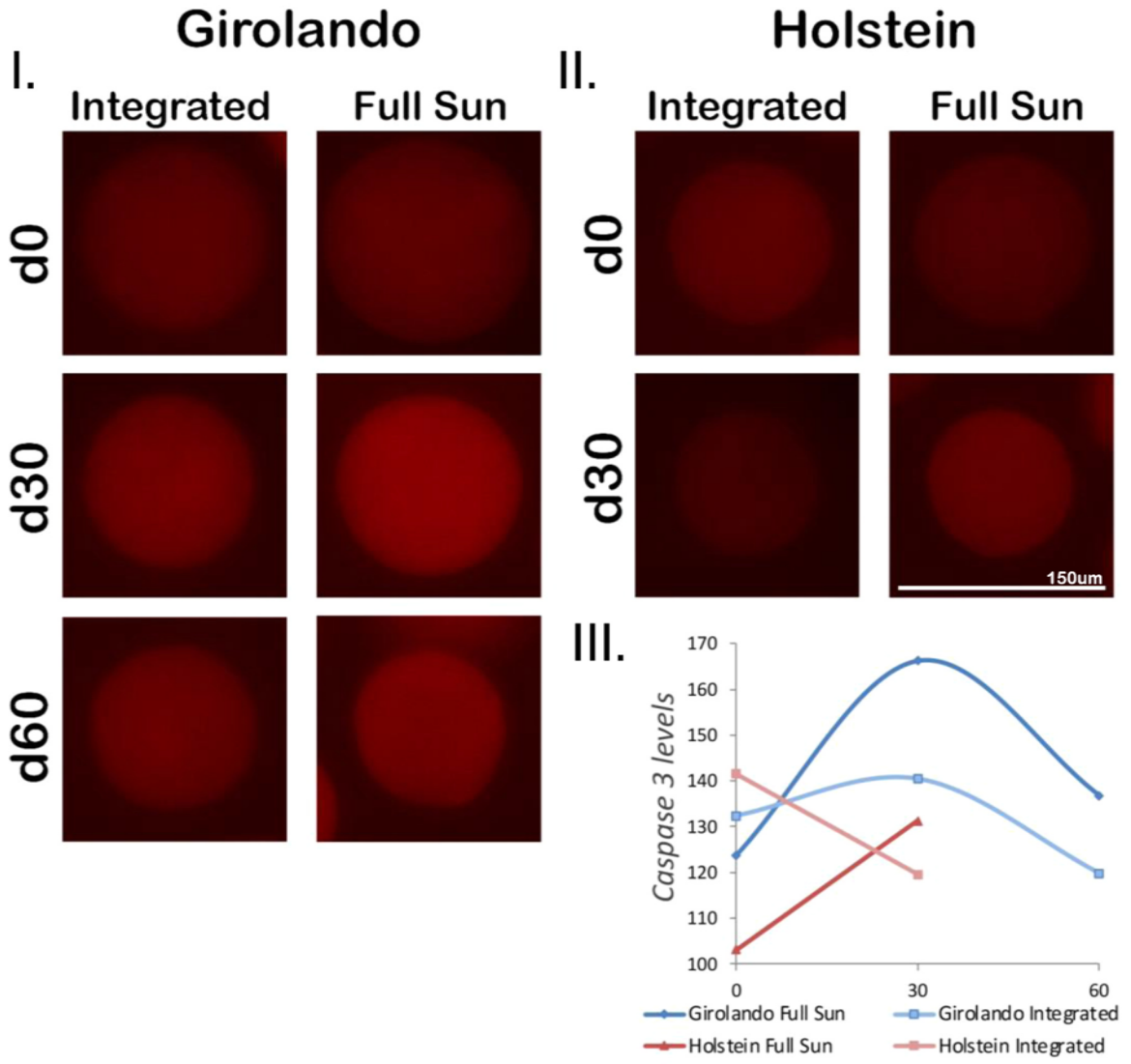

Fig. 4. Caspase 3 oocyte levels during summer days (0, 30 and 60) in heifers kept at Integrated Livestock-Forest (Integrated LF) or Full Sun pasture. Images of Girolando (I) and Holstein (II) caspase 3 immunostained oocytes are shown. Graph shows Girolando and Holstein caspase 3 levels inside system and day factors (III).

3.5. IGFBP2 levels from D0 to D60

The protein expression of IGFBP2 is depicted in Fig. 5. The data has been transformed by taking logarithm of the values. Significant effects were observed for the breed ($P<0.001$), the system ($P<0.001$) and the day ($P<0.001$) factors. Furthermore, a significant ($P<0.001$) interaction among these variables was identified. Additional details from the orthogonal decomposition analysis can be found in Table 3.

Holstein oocytes exhibited lower IGFBP2 levels compared to Girolando oocytes (4.88 vs 5.05, $P<0.001$), irrespective of the system. Within the Girolando breed, Integrated LF oocytes exhibited decreased IGFBP2 levels in comparison to Full Sun (5.00 vs 5.11, $P<0.001$). However, this difference was not detected in Holstein oocytes (4.88 vs 4.89, $P>0.05$).

For Holstein oocytes, IGFBP2 levels increased from D0 to D30 in the Full Sun system (4.81 vs 4.95, $P<0.001$) but decreased in the ILF (4.93 vs 4.84, $P<0.001$) system. At D30, Holstein oocytes exhibited lower ($P<0.001$) IGFBP2 levels In the ILF compared to the Full Sun system (4.84 vs 4.95, $P<0.001$).

Girolando oocytes followed a quadratic pattern for IGFBP2 levels. These levels increased from D0 to D30 and then decreased from D30 to D60 (ILF: $y = 4.99+0.003x-0.00006x^2$; FS: $y = 5.05+0.006x-0.0001x^2$). At D60, IGFBP2 levels were lower in the ILF system compared to Full Sun (4.96 vs 5.09, $P<0.001$).

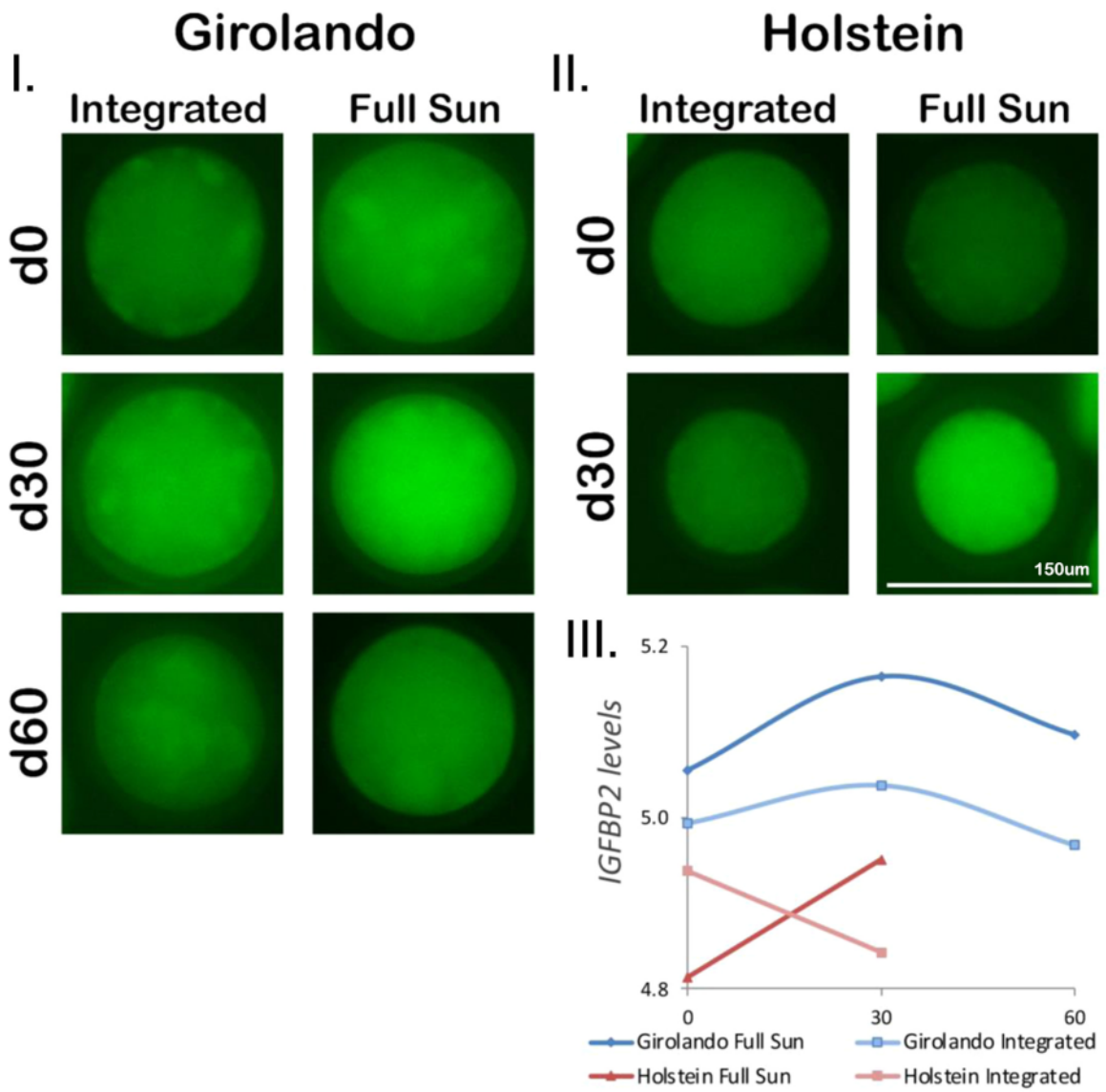

Fig. 5. IGFBP2 oocyte levels during summer days (0, 30 and 60) in heifers kept at Integrated Livestock-Forest (Integrated LF) or Full Sun pasture. Images of Girolando (I) and Holstein (II) IGFBP2 immunostained oocytes are shown. Graph shows Girolando and Holstein caspase 3 levels inside system and day factors (III).

4. Discussion

In the present study, we investigated the effectiveness of an integrated Livestock-Forest (ILF) system as a potential alternative to the conventional full sun pasture system, with the objective of mitigating the adverse effects of heat stress on reproductive function. It is important to note that this study was conducted using a prototype with a small number of animals. Therefore, our conclusions suggest several avenues for future research aimed at expanding upon the data presented here. Our findings, which encompassed both Holstein and Girolando breeds, revealed that the ILF system played a crucial role in ameliorating maternal hyperthermia, particularly during the initial weeks of summer. Moreover, it appeared to offer some protection against the decline in oocyte quality that was observed in the full sun system. This encompassed the prevention of oocyte size reduction, apoptosis as indicated by caspase 3 levels, and modulation of the IGF system, particularly IGFBP2.

This study was performed under tropical climate conditions, during summer, and revealed periods of intense heat stress. The presence of a Temperature-humidity index (THI) exceeding 78 throughout the experiment is known to result in elevated body temperatures, leading to a notable decline in conception rates by approximately 20–30 % (Collier et al., 2017; Wolfenson and Roth, 2019) and a reduction in milk production (Armstrong, 1994; Damasceno et al., 1998) in bovine. The upper limit of normal body temperature for adult cattle is 39.1 °C, and animals above this threshold experience hyperthermia and its negative effects on fertility and production

(Gwazdauskas, 1985; Polsky et al., 2017; Wheelock et al., 2010). In our study, the vaginal temperature was higher than 39.1 °C during the warmest period of the days, and reached 41 °C in Holstein animals, which were more susceptible to environmental temperature increase than Girolando animals.

This difference in sensitivity can be attributed to the genetic characteristics inherent to the Gir and Holstein breeds. *Bos indicus* breeds are less affected by heat stress than *Bos taurus* breeds (Hansen, 2007). Under tropical conditions, Gir oocytes are more likely to develop after IVF than Holstein oocytes (Camargo et al., 2007), and Gir cows exposed to heat stress do not show immediate effects on reproductive function (Torres-Júnior et al., 2008). A marked decline in oocyte quality is reported for Bos taurus oocytes in high temperature and humidity seasons, but Bos indicus oocytes exposed to similar conditions display normal competence and morphology regardless of season (Rocha et al., 1998).

Crossbred animals also display remarkable differences from purebred animals, regarding sensitivity to heat stress. Girolando F1 (Holstein ½, Gir ½) females shows heat stress signs when THI is higher than 78 (Fialho et al., 2018). In contrast, Holstein animals are already heat stressed when THI is above 72 (da Silva et al., 2002). Among crosses, increased Holstein composition means more sensitivity to heat stress. Comparing ½, ¾ and 7/8 Holstein proportions in Gir-Holstein crossbreds, upper critical levels of THI changed from 79 to 77 and 76 (de Azevedo et al., 2005). Indeed, the response of Girolando cows to heat stress is determined by the genetic composition, being cows with Holstein proportion higher than ¾ less able to thermoregulate (Carvalheira et al., 2021).

In our study, for both breeds, the Integrated LF system positively affected the control of reproductive tract temperatures, as a decrease in the number of hours vaginal temperature exceeded 39.1 °C and maximum temperatures was noticed for both breeds. A particularly intriguing finding in our study was the interaction between the system and the day of data collection. This interaction highlighted a noticeable contrast between the Integrated LF and the Full Sun systems: over time, there was a reduction in the number of hours during which vaginal temperature exceeded 39.1 °C (from D1 to D5), only for the Full Sun system. This observation suggests the presence of an adaptive mechanism for body temperature regulation, particularly during the onset of summer. It highlights the importance that the ILF system, along with other potential alternatives, can play in mitigating the adverse effects of heat stress. This adaptation mechanism offers valuable insights into how innovative farming practices can help maintain the well-being and performance of livestock, particularly during the challenging period of rising temperatures.

Adaptive responses following heat stress have been described in cows. A different response to acute and chronic exposure to heat stress was shown in Holstein, suggesting thermoregulatory responses (de Andrade Ferrazza et al., 2017), and HSP70 increases after mild heat stress can induce resistance to heat shock in Girolando embryos (Oliveira et al., 2021) and affect gene expression of thermoprotective players (Sakatani et al., 2013). Indeed, elevated temperatures can cause reversible or irreversible cellular damages, and such effects can trigger adaptive cellular responses instead of cellular death responses (Paula-Lopes et al., 2012).

In the second phase of our study, following the confirmation that the Integrated LF could offer some protection against heat stress in heifers, our focus turned to understanding how this would impact reproductive function, in high susceptible (Holstein) and median susceptible (Girolando) breeds. It is well-established that heat stress can negatively affect various processes related to fertility. Among these, oocyte quality was chosen as our key indicator for this study. Oocytes within follicles during the early antral phase and those in the early growth phase are known to be particularly sensitive to heat stress (De Rensis et al., 2021). Heat stress can lead to decreased fertilization rates (Hansen, 2007) and delayed cleavage of resulting zygotes (Khan et al., 2020).

The apoptosis pathway, which includes caspase activity, has been linked to heat-stressed oocytes (Paula-Lopes et al., 2012), and insulin-like growth factor-I is associated with resistance of bovine embryos to heat shock (Jousan and Hansen, 2007, 2004), for its autocrine, paracrine and endocrine actions in cellular metabolism, proliferation, growth and differentiation, also suggesting a thermoprotective role in GV-stage and maturing oocytes (Paula-Lopes et al., 2012). Therefore, in this study, we selected two assays to assessed oocyte quality under our experimental conditions: caspase 3, an effector enzyme involved in apoptosis, and IGFBP2, a binding protein related to insulin-like growth factors (IGFs). Caspase-3 and low molecular weight IGFBPs (-2, −4 and −5) levels have been found to be positively correlated in atresia, and lower IGFBP levels are associated with oocytes having higher developmental potential (Nicholas et al., 2005). In our previous studies, we also observed that higher caspase 3 and IGFBP2 oocyte levels were indicative of lower developmental competence of bovine oocytes (Silva et al., 2022).

It was expected that the disparities in vaginal temperature observed between the Integrated LF and Full Sun groups would manifest in the reproductive function. Fertility of animals under heat stress is highly compromised due to its deleterious effects on oocyte function (Khan et al., 2020), and benefits following integrated system allocation have been described for Gir lactating cows (Martins et al., 2021). In our study, we assessed morphological quality parameters, associated with oocyte competence, and markers associated with two pathways affected by heat stress (IGF system and apoptosis). Those features were affected by the summer heat stress in both breeds, but Integrated LF attenuated its effects.

Regarding oocyte morphology, our initial observation pertains to oocyte diameter. A noticeable reduction in oocyte size was observed in all groups, with the decline being more pronounced in the Full Sun groups as compared to the Integrated LF groups. Notably, the ILF groups exhibited larger oocyte diameters in the final assessment for both breeds. Bovine oocytes are supposed to acquire full developmental competence at a diameter of 120 microns (Otoi et al., 1997), and below 100 microns the expected maturational competence is below 20 % (Fair et al., 1995). For Girolando oocytes, diameters below 100 microns were only found in the Full Sun group. This result suggests an increased developmental competence for Girolando oocytes kept during summer at ILF system, evidencing ILF benefits over oocyte quality.

Holstein oocytes were below 100 microns regardless of pasture system already at D30, suggesting a decreased oocyte competence one month after summer onset. Furthermore, it is important to note that Holstein oocytes could not be properly recovered at D60. This could potentially be attributed to heightened sensitivity and susceptibility to damage resulting from vacuum pressure exposure during Ovum pick-up (OPU). It is worth mentioning that the follicular count remained consistent among groups and recoveries. Also, even

though the number and percentage of IVF-grade oocytes remained similar from the beginning to mid-summer in Girolando heifers, a marked decrease was detected for this parameter in Holstein breed. As expected, Holstein oocytes were more sensitive to tropical summer conditions compared to Girolando oocytes, regardless of the system.

In the final assessment, active caspase 3 levels were lower in the Integrated LF system compared to the Full Sun for both Girolando and Holstein breeds. This result reinforces the attenuation of heat stress effects by the Integrated LF system in oocytes. Caspase 3 is an effector protease that acts degrading structural proteins, including DNA repair enzymes and plays an extremely important role in apoptosis. The cumulative effects of caspase-3 cleavage events can disable homeostatic and repair processes, and even in situations where caspase-3 levels are below apoptosis threshold, can cause deleterious effects to cells, with the accumulation of aberrant processing events that culminates with an increased propensity to cell death. In other words, caspases effects below apoptosis threshold can escalate the propensity for apoptosis and exacerbate pathogenesis (Nicholson, 1999).

In addition to caspase 3, IGFBP2 levels were also gradually increased in Full Sun groups for Girolando and Holstein, and a decrease in final assessments for both Holstein and Girolando breeds was detected for Integrated LF groups compared to the Full Sun system. The IGF system regulates ovarian follicular development, growth, and steroidogenesis (van Dessel et al., 1996). The binding of IGF to its receptor is prevented from IGFBPs, regulating intrafollicular availability of free IGF. Preovulatory follicles have a lower concentration of IGFBP-2, which is much higher in subordinate follicles (Fortune et al., 2004). Thermal stress response involves IGF-1 axis and increases in IGF-1 bioavailability attenuate developmental inhibition and apoptosis following heat shock (Jousan and Hansen, 2007, 2004). As pointed out by our results, the Integrated LF system attenuated the increase in IGFBP2 oocyte levels during summer, which could reflect increased IGF-1 bioavailability and higher developmental competence in Integrated LF oocytes compared to Full Sun oocytes.

5. Conclusion

Here we showed that integrated livestock-forest systems can attenuate maternal hyperthermia during summer and its deleterious effects on oocytes. With these results, we confirmed the participation of caspase-3 and low molecular weight IGFBP in maternal hyperthermia response during summer in bovine oocytes, using an in vivo model. We also detected an adaptive response to heat stress, in animals kept at Full Sun system. These results shed light on the potential of integrated Livestock-Forest systems as a sustainable approach to enhance reproductive performance in dairy cattle, even in the challenging conditions posed by elevated environmental temperatures. Further research and exploration of the underlying mechanisms are warranted to fully understand and harness the benefits of such systems in cattle farming.

CRediT authorship contribution statement

Luiz Sergio de Almeida Camargo: Writing – review & editing, Validation, Resources, Project administration, Conceptualization. **Naiara Zoccal Saraiva:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Luiz Altamiro Garcia Nogueira:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Felipe Zandonadi Brandão:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. **Marcelo Dias Muller:** Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Conceptualization. **Viviane Luzia da Silva Feuchard:** Investigation, Formal analysis. **Anderson Mourão:** Investigation, Formal analysis. **Agostinho Jorge dos Reis Camargo:** Supervision, Methodology, Investigation. **Hugo Sabença Dias:** Writing – review & editing, Methodology, Investigation, Data curation. **Clara Oliveira:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rui da Silva Verneque:** Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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