Alterations in follicular dynamics and steroidogenic abilities induced by heat stress during follicular recruitment in goats

M Ozawa, D Tabayashi, T A Latief, T Shimizu, I Oshima and Y Kanai

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305-8572, Japan

Correspondence should be addressed Y Kanai, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305-8572, Japan; Email: kanaiy@sakura.cc.tsukuba.ac.jp

M Ozawa is currently at the Genetic Diversity Department, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba-shi, Ibaraki 305-8602, Japan

Abstract

We investigated the changes in follicular dynamics and steroidogenic activity during heat stress in goats. Adult female goats were exposed to heat stress at 36 °C and 70% relative humidity for 48 h and then injected with prostaglandin (PG) F_{2α} (the time of PGF_{2α} injection was designated as 0 h). In experiment 1, every follicle greater than 2 mm in diameter was monitored by ultrasonography to investigate the follicular dynamics, and plasma concentrations of FSH, LH, progesterone, and oestradiol were measured from –48 h to 120 h. In experiment 2, the follicles were recovered from the goats at 48 h, and the concentration of oestradiol, the aromatase activity, and the LH receptor level in the follicles were determined. In control (non-heat-stressed) goats, ovulatory follicles were mainly recruited from –24 h to 0 h, whereas no follicles recruited during that period were ovulated in the heat-stressed goats. The timing of the recruitment of ovulatory follicles was delayed by heat stress by approximately 24 h. The plasma concentration of oestradiol in the heat-stressed goats was significantly lower from 36 to 54 h compared with the controls, although the concentrations of FSH and progesterone did not differ between the treatments. In addition, the concentration of oestradiol, the aromatase activity, and the LH receptor level in the follicles from heat-stressed goats were significantly lower compared with the controls. These results indicate that heat stress during follicular recruitment suppresses subsequent growth to ovulation, accompanied by decreased LH receptor level and oestradiol synthesis activity in the follicles.

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Introduction

Low fertility is widely observed in domestic animals during the summer (Ingraham *et al.* 1974, Gwazdauskas *et al.* 1975). This problem has recently been exacerbated by increases in livestock productivity and the concurrent rise in metabolic heat production. In lactating cows, hyperthermia can occur at ambient temperatures as low as 27 °C (Berman *et al.* 1985) and causes a decreased rate of conception (Udompraset & Williamson 1987). Although low summer fertility syndrome is attributed to multiple mechanisms (reviewed by Hansen *et al.* 2001), alteration of follicular dynamics, including steroidogenic ability, is a major factor in the development of the syndrome (Badinga *et al.* 1993, Trout *et al.* 1998, Wolfenson *et al.* 2000).

During the 1990s, ultrasonographic techniques were developed using the intrarectal approach to visualize ovaries (Fortune *et al.* 1991, Griffin & Ginther 1992), providing a direct and noninvasive means of monitoring heat-stress-induced follicular changes. It has been reported that heat stress suppresses the functions of the largest follicle,

i.e. the dominant follicle. In lactating cows under heat stress, the size of the dominant follicle is reduced during the first and second follicular waves (Badinga *et al.* 1993, Wilson *et al.* 1998a,b), and the number of follicles of the next largest size is increased (Wolfenson *et al.* 1995). In contrast, discrepancies still exist between previous studies concerning follicular recruitment under heat stress; it was found that heat stress caused either a decrease (Wolfenson *et al.* 1995) or an increase (Trout *et al.* 1998) in the emergence of small follicles (2–5 mm in diameter). In addition, although it was reported that heat stress caused a decrease in the oestradiol concentration of the fluid of the dominant follicle (Wolfenson *et al.* 1997), the steroidogenic activity in small and medium follicles under heat stress has been poorly investigated.

Circulating concentrations of gonadotrophin, which plays an important role in regulating follicular dynamics, are also altered by heat stress. A previous study found that heat stress caused an increase in the plasma follicle-stimulating hormone (FSH) concentration in cows (Roth *et al.*

2000). On the other hand, conflicting findings concerning the effect of heat stress on plasma luteinizing hormone (LH) have been reported; heat stress has been reported to cause the plasma LH concentration in cows to decrease (Madan & Johnson 1973, Miller & Alliston 1974), remain unchanged (Gwazdauskas *et al.* 1981, Rosenberg *et al.* 1982), or increase (Roman-Ponce *et al.* 1981). More recently, Kanai *et al.* (1995) found that heat stress did not change the pituitary LH secretion in long-day-treated anoestrous goats that were injected with exogenous gonadotrophin-releasing hormone (GnRH) to induce artificial LH pulses, whereas it reduced the follicular responsiveness to LH. Follicular LH responsiveness is attributed to the expression of LH receptors on the follicles (Webb & England 1982). Therefore, it appears probable that heat stress induces alterations in the follicular LH receptors. In the present study, we investigated the follicular alterations induced by heat stress during follicular recruitment by monitoring (i) follicular dynamics using ultrasonography; (ii) plasma concentrations of LH, FSH, oestradiol, and progesterone; and (iii) follicular steroidogenic activity and LH receptor expression in heat-stressed and control goats.

Materials and methods

Animals

The 3–5-year-old female Shiba goats (*Capra hircus*) used in this study were housed in an environmental chamber at 25°C and 50% relative humidity (RH) with a 12-h light/12-h dark photoperiod (lights on at 0800 h) and were fed alfalfa-hay cubes (25 g/kg body weight per day). Water and mineralized licks (Koen; Nippon Zenyaku Kogyo Co., Hukushima, Japan) were available *ad libitum*. All experimental protocols and animal handling procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba.

Synchronization of oestrous cycles and temperature treatment

The plasma progesterone concentrations of the goats were assayed to determine the phase of the oestrous cycle, and the oestrous cycles were subsequently synchronized using a single intramuscular injection of 0.5 ml prostaglandin (PG) F_{2α} (Panacelan-Hi; Fine Chemical Technology Co., Toyama, Japan) administered 8–10 days following the detection of oestrus. The time of the PGF_{2α} injection was designated 0 h, and the goats were heat stressed in an environmental chamber at 36°C and 70% RH from –48 to 0 h. The control goats remained at 25°C and 50% RH.

Monitoring of rectal temperature, respiration and heart rates, and water and feed intake

The rectal temperature of each goat was measured at 6-h intervals from –72 to 24 h with a Thermistor instrument (D611; Takara Thermistor Co., Tokyo, Japan). Respiration

and heart rate were counted once daily during the experimental period. The water and feed were weighed each day, and daily intakes were calculated. All measurements were made inside the environmental chamber.

Ultrasound evaluation of follicular dynamics

Follicular dynamics were observed once a day from –48 to 120 h by imaging using a B-mode scanner (SSD-630; Aloka Co., Tokyo, Japan) with a 7.5 MHz Transrectum Electronic Linear Probe (UST-660-7.5; Aloka Co.). Every observed follicle of ≥ 2 mm in diameter was recorded. We preliminarily observed that the mean diameter of the ovulatory follicle was 5.1 ± 0.1 mm (data not shown). In this study, therefore, the follicles were classified by diameter as small (2–3.4 mm), medium (3.5–4.9 mm), or large (≥ 5 mm), according to our preliminary examination and a previous report on sheep by Carson *et al.* (1979). On each examination, the relative location of follicles and their diameters were recorded to determine the patterns of recruitment, growth, and regression of individual follicles. Follicular recruitment was identified as the day of emergence of new small follicles (Ravindra *et al.* 1994). Ovulation was determined using three criteria: (i) the disappearance of a large follicle, (ii) the subsequent development of a corpus luteum within 3 days, and (iii) an increase of the plasma progesterone concentration above 1 ng/ml.

Sampling of blood and follicles

Blood samples (3–8 ml) were collected from the jugular vein once daily from –48 to 0 h and at 6-h intervals from 0 to 144 h to determine plasma progesterone, oestradiol, FSH, and LH levels. For monitoring of pulsatile LH secretions, blood samples were collected at a 6-min interval from 36 to 40 h. The samples were centrifuged, and the supernatants were stored at –20°C until assayed for each hormone ($n = 6$ in each group). In addition, another six ovariectomized (OVX) goats were monitored for LH pulses according to the method described by Tanaka *et al.* (1994), with some modifications. In brief, OVX goats were implanted subcutaneously with capsules (3.35 mm inner diameter, 4.65 mm outer diameter, 4.0 mm length; Dow Corning Toray Silicone Co., Tokyo, Japan) containing 0.1 g oestradiol for 5 days, or implanted with capsules made of Silastic sheet (50 × 75 mm; Dow Corning Toray Silicone Co.) containing 1.0 g progesterone additionally with oestradiol capsules for 5 days. Blood samples were recovered 1 day before steroid implantation (without steroids), 5 days after oestradiol implantation (E implantation), or 5 days after oestradiol and progesterone implantation (E + P implantation), to monitor the pulsatile LH secretions. The pulses were identified by the method as described by Merriam & Wachter (1982).

The ovaries were surgically recovered at 48 h from eight other goats ($n = 4$ each in the control and heat-stress

groups). Every follicle that was recruited from -24 to 0 h and that had a diameter of 2 mm or larger was collected from the ovaries and classified by size. The follicles, grouped by size, were minced with ophthalmic scissors in 1 ml PBS in plastic centrifuge tubes held on ice and then centrifuged at 1000 *g* for 15 min. The supernatants containing the follicular fluids were assayed for hormone levels. The precipitates containing the cumulus and theca cells were washed with M199 medium (Sigma Chemical Co., St Louis, MO, USA) and assayed for aromatase activity and LH receptor content.

Plasma and follicular fluid hormone assays

The plasma concentrations of FSH and LH were assayed by double-antibody RIA as described by Araki *et al.* (2000) for FSH and Mori & Kano (1984) for LH. Rabbit anti-sheep FSH serum (NIAMDD-anti-oFSH-1) and rabbit anti-sheep LH serum (YM-18) were used as the primary antibodies. Purified sheep FSH (NIAMDD-oFSH-RP-1) and LH (NIAMDD-oLH-24) were used as reference standards and as iodinated ligands. The radioactivity was counted on a gamma counter (ARC-380; Aloka Co.). The sensitivities of the assays were 0.05 ng/ml, and the inter-assay coefficients of variation were 12.0% for FSH and 11.3% for LH.

The progesterone and oestradiol concentrations in the plasma and follicular fluid samples were measured by single-antibody RIA in duplicate. Rabbit anti-progesterone-11 α -BSA serum or rabbit anti-oestradiol-6-CMO-BSA serum was used as antibody, and [1,2,6,7-³H]progesterone (103 Ci/mmol) or [2,4,6,7,16,17-³H]oestradiol (151 Ci/mmol; both from Amersham International) were used as the radiolabeled hormone. For the progesterone assay, 300 μ l of each sample was added to 3 ml hexane, mixed, and centrifuged. The samples were separated into 1 ml aliquots and dried under N₂ gas. Then 100 μ l rabbit anti-progesterone serum diluted 1:6000 in 0.01 M PBS containing 0.1% (w/v) gelatin (PBS-gel), 100 μ l radiolabeled progesterone (20 000 d.p.m. in PBS), and 500 μ l PBS-gel were added to each sample tube and incubated at 4°C for 24 h. After incubation, 200 μ l dextran-coated charcoal solution (prepared by mixing equivalent volumes of 0.5% (w/v) Norit A and 0.05% (w/v) dextran solutions; Sigma Chemical Co.) was added and reacted for 15 min. The samples were centrifuged at 1000 *g* for 15 min at 4°C; 500 μ l of each supernatant was then transferred to a vial containing 4.5 ml scintillation fluid and counted on a scintillation counter (LSC-6000; Aloka Co.). For the oestradiol assay, 2 ml of each sample was added to 10 ml benzene and dried under N₂ gas. The residues were redissolved in 1.2 ml PBS-gel and separated into 500 μ l aliquots. Rabbit anti-oestradiol antibody (diluted 1:44 000 in PBS-gel; 100 μ l) and 100 μ l labeling hormone (20 000 d.p.m. in PBS) were added, and the procedure described above for the progesterone assay was followed to determine the oestradiol concentration. The sensitivities of the progesterone

and oestradiol assays were 1 and 4 pg/ml. The inter- and intra-assay coefficients of variation for progesterone were 12.3 and 7.1%, and for oestradiol were 11.1 and 7.9%.

Aromatase activity assay and LH receptor assay

The aromatase activity in the follicles was assayed using the methods described by Robert *et al.* (1980) and Thatcher *et al.* (1991). In brief, minced follicle samples were added to 4 ml M199 medium containing 9.3 mM [1 β ,2 β -³H]testosterone (0.5 μ Ci/mmol; New England Nuclear, Boston, MA, USA) and incubated at 38.5°C in 5% (v/v) CO₂ for 3 h. After incubation, sample tubes were centrifuged at 1000 *g* for 15 min at 4°C, and 1 ml of the supernatants were transferred into another tube containing 200 μ l 25% (w/v) Norit SX-II solution (Wako Pure Chemical Industries, Osaka, Japan) and incubated at 4°C for 2 h. Subsequently, tubes were centrifuged at 1000 *g* for 15 min at 4°C, and 500 μ l of supernatants were transferred into vials with 4 ml emulsion scintillator, and the scintillations were measured using a scintillation counter (Aloka Co.). After the measurement of the aromatase activity, the samples were washed in 3 ml PBS containing 1% (w/v) BSA (PBS/BSA) and centrifuged at 1000 *g* for 15 min at 4°C. The precipitates were used to determine the LH receptor levels using the method described by Kawate *et al.* (1989). In brief, 1 ml PBS/BSA was added to tubes containing sample precipitates, and homogenized on ice. Each homogenate was transferred to another tube with 100 μ l ¹²⁵I-labelled human chorionic gonadotrophin (300 000 d.p.m.) and incubated at 25°C for 24 h. Then, 2 ml assay buffer (PBS/BSA containing 0.5% (w/v) silica gel) were added into tubes, and centrifuged at 1000 *g* for 30 min at 4°C and the supernatants were removed. Then another 2 ml assay buffer was added and centrifuged again. Supernatants were removed and the radioactivities in the precipitates were counted using a gamma counter (Aloka Co.). The inter- and intra-assay coefficients of variation for aromatase were 7.3 and 6.7%, and for LH receptor were 11.5 and 7.6%, respectively.

Statistical analysis

The data are expressed as means $\pm$ S.E.M. The number of recruited follicles, rectal temperature, respiration and heart rates, water and feed intake, aromatase activity, LH receptor levels, and concentrations of oestradiol and progesterone in the follicular fluid were analyzed using Student's *t*-test. Changes in plasma oestradiol, progesterone, LH, and FSH concentrations were analyzed using ANOVA for repeated measures (Gill & Hafs 1971). When a significant effect was detected with ANOVA, the significance of the difference between means was determined by Duncan's multiple range test. The percentages of follicles that eventually ovulated were arc-sine transformed and then analyzed using Student's *t*-test.

Results

General response to heat stress

The general responses to heat stress in the goats are shown in Table 1. The average rectal temperature of the goats in the heat-stress group was significantly increased compared with that of the controls ($P < 0.001$). The respiration rate, water and feed intakes ($P < 0.001$), and heart rate ($P < 0.01$) were significantly different between the control and heat-stress groups. During the treatment period, no parameters differed significantly among the groups. In addition, none of the parameters differed before and after the heat exposure compared with the control (data not shown).

Effects of heat stress on follicular dynamics and ovulation

The numbers of recruited follicles and the percentages of follicles that eventually ovulated observed during the experiment are presented in Table 2. Follicles were recruited throughout the experimental period in both the control and heat-stress groups. The number of recruited follicles was increased ($P < 0.05$) at 24 h and decreased ($P < 0.01$) at 120 h in the heat-stressed animals compared with the controls. In the control group, the follicles that eventually ovulated were recruited from -48 to 24 h, with most observed from -24 to 0 h. In contrast, no follicles that eventually ovulated were recruited from -24 to 0 h in the heat-stress group, and this difference was significant at 0 h ($P < 0.05$). The ratio of follicles that eventually ovulated at 24 h was significantly higher ($P < 0.05$) in the heat-stress group compared with the controls. Ovulation

Table 1 Rectal temperature, respiration rate, heart rate, water intake, and feed intake in goats under control and heat-stress conditions. Data are expressed as the mean $\pm$ S.E.M. of six replicates.

Parameter	Control	Heat-stressed
Rectal temperature ($^{\circ}\text{C}$)	39.0 ± 0.0	$40.6 \pm 0.2^{***}$
Respiration ratio (breaths/min)	33.8 ± 1.2	$190.3 \pm 3.6^{***}$
Heart ratio (beats/min)	74.5 ± 1.7	$64.2 \pm 2.3^{**}$
Water intake (ml/kg BW/day)	55.7 ± 3.3	$361.8 \pm 40.3^{***}$
Feed intake (ml/kg BW/day)	24.6 ± 0.4	$9.6 \pm 1.7^{***}$

, *Significantly different from control ($P < 0.01$ and $P < 0.001$, respectively) by Student's *t*-test. BW, Body weight.

Table 2 Numbers of recruited follicles and percentages of eventually ovulatory follicles recruited at each time during the experiment in goats under control and heat-stress conditions. Data are expressed as the mean $\pm$ S.E.M. of six replicates.

		Hours post PGF _{2α} injection							
		-48	-24	0	24	48	72	96	120
Number of recruited follicles	Control	5.2 ± 0.9	2.8 ± 0.7	1.8 ± 0.6	1.7 ± 0.6	1.5 ± 0.3	0.8 ± 0.4	2.7 ± 1.3	4.2 ± 0.5
	Heat	3.7 ± 0.6	2.5 ± 0.6	2.2 ± 0.7	$3.8 \pm 0.5^*$	2.3 ± 0.6	2.0 ± 0.4	1.8 ± 0.2	$1.3 \pm 0.6^{**}$
Percentage of eventually-ovulatory follicles	Control	26.5 ± 15.3	29.2 ± 16.4	61.1 ± 15.3	16.7 ± 16.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Heat	8.9 ± 5.9	0.0 ± 0.0	$0.0 \pm 0.0^*$	$61.7 \pm 13.7^*$	3.3 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

*, **Significantly different from control ($P < 0.05$ and $P < 0.01$, respectively). Numbers and arc-sine-transformed percentages were analysed by Student's *t*-test.

tended to be delayed in heat-stressed goats (128.0 ± 4.4 h) compared with that in controls (116.0 ± 2.7 h). The growth rates of follicles, which were recruited during -24 to 0 h, did not differ between treatments at small size (0.75 ± 0.25 mm/day in the control group versus 0.71 ± 0.06 mm/day in the heat-stress group) or middle size (0.74 ± 0.07 mm/day in the control group versus 0.76 ± 0.05 mm/day in the heat-stress group). The follicles did not grow into the large size in the heat-stress group (large-size follicles in the control group were 0.75 ± 0.03 mm/day). Mean follicle numbers in each size class during the experiment are shown in Fig. 1, and representative patterns of follicular growth from one of six control and heat-stressed goats are shown in Fig. 2.

Effects of heat stress on plasma hormone concentrations

The patterns of fluctuation of progesterone, oestradiol, LH, and FSH in the plasma are illustrated in Fig. 3. Progesterone concentrations rapidly decreased after the PGF_{2 α} injection and remained low throughout the remainder of the experimental period in both groups. Oestradiol concentrations increased after the PGF_{2 α} injection. The peak concentration of oestradiol was observed at 54 h in the control group and at 72 h in the heat-stress group. The patterns of the oestradiol levels differed significantly ($P < 0.05$) between the control and heat-stress groups, and the concentrations in the heat-stress group were significantly lower ($P < 0.05$) from 36 to 54 h compared with the control group. The peak value of the LH surge did not differ between the two groups ($P > 0.05$); however, the surge was significantly delayed by approximately 21 h in the heat-stress group compared with the controls (83.0 ± 2.5 and 62.0 ± 2.0 h, respectively; $P < 0.05$). No significant differences were detected in the patterns of the FSH concentrations between the control and the heat-stress group. The characteristics of pulsatile LH secretion are expressed in Table 3. The frequency of the LH pulse tended to decrease in the intact goat that was heat stressed compared with the control, whereas no significant difference was detected between heat stress and control in the OVX goat that had E implantation or E + P implantation,

Figure 1 Total follicle numbers of small size (2.0–3.4 mm; black bars), middle size (3.5–4.9 mm; shaded bars), and large size (>5 mm; white bars) in the control group and heat-stress group. The follicles were monitored during the experimental period using an ultrasonic scanner. Values are expressed as the means from six replicates.

or in the without-steroid group in each pulsatile characteristic ($P > 0.05$).

Effects of heat stress on LH receptor content, steroids levels, and aromatase activity in follicles

The levels of LH receptor, aromatase activity, oestradiol, and progesterone at 48 h in the follicles of each size are presented in Fig. 4. No follicles recruited from –24 to 0 h grew to large size in the heat-stress group, and no significant differences between the heat-stress and control groups were found for any parameter in the small follicles. In the medium follicles, however, a number of differences were found. Compared with the control condition, heat stress significantly ($P < 0.05$) reduced the LH receptor content (229.7 ± 33.8 versus 132.6 ± 24.0 fmol/follicle), aromatase activity (152.1 ± 25.0 versus 88.2 ± 17.6 pg oestradiol/follicle per 3 h), and oestradiol concentration (95.4 ± 13.7 versus 63.6 ± 0.7 ng/follicle). The progesterone concentration did not differ significantly between the two groups (7.9 ± 0.4 versus 8.6 ± 0.8 ng/follicle in the control and heat-stress groups, respectively).

Discussion

In this study, we determined the changes in follicular dynamics and steroidogenic activity in goats under heat

stress. The results indicated that heat stress during follicular recruitment reduced the steroidogenic activity in follicles, as shown by the decreased concentration of oestradiol and low aromatase activity. Our data also indicated that all follicles that were exposed to heat stress during recruitment regressed before attaining a large size and were never ovulated, although the number of follicles recruited during heat exposure did not change compared with that in the control goats.

It has been shown that low summer fertility occurs in many mammalian species, especially in domestic animals because of their heightened productivity (Hansen *et al.* 2001, Rensis & Scaramuzzi 2003). Several causes of reduced summer fertility have been reported, including lowered oocyte competence (Al-Katanani *et al.* 2001, Zeron *et al.* 2001), reduced expression of oestrus (Hansen & Aréchiga 1999), and early embryonic death (Ealy *et al.* 1993, Ozawa *et al.* 2002). In addition, alteration of follicular functions during heat stress, including follicular dynamics and steroidogenic activity, has been described as a major factor in reduced summer fertility (Wolfenson *et al.* 1995, Wilson *et al.* 1998a,b). In the case of goats, although a number of studies have monitored ovarian dynamics using ultrasonography (Menchaca & Rubianes 2002, Medan *et al.* 2003), or analyzed the changes in ovarian steroids and gonadotrophin secretions during the

Figure 2 Representative growth patterns of individual follicles from goats in (A) the control group and (B) the heat-stress group. Female goats were either exposed to heat stress or not stressed (control group) for 48 h (–48 to 0 h) prior to intramuscular injection of PGF_{2α} at 0 h. Follicular growth was observed using an ultrasonic scanner. ■, Emergence of a recruited follicle; *, ovulation.

follicular phase under neutral temperature conditions (de Castro *et al.* 1998, Menchaca & Rubianes 2002), no reports have been published, to our knowledge, concerning follicular dynamics and steroidogenic activities under heat stress. Our present results, therefore, provide additional information on how to understand the effects of heat stress on follicular functions, not only in the goat as a domestic animal, but also as a ruminant experimental animal model.

Several studies have reported that plasma oestradiol concentrations are decreased by heat stress in cows (Wolfenson *et al.* 1995, 1997, Wilson *et al.* 1998a,b). The results in the present study also indicated that heat stress during follicular recruitment reduced the plasma oestradiol concentration during the 18-h period from 36 to 54 h. The decrease in plasma oestradiol could be related to the significant decrease in oestradiol concentration in the fluid of medium follicles caused by heat stress. Oestradiol production is regulated by aromatase activity in granulosa cells (Dorrington *et al.* 1975, Erickson & Hsufh 1978). In the cow, aromatase activity is drastically increased in medium-sized follicles (4–8 mm in diameter), accompanied by an increase in the oestradiol concentration in the follicles (McNatty *et al.* 1984). The results

obtained in the present study showed that the aromatase activity in the medium follicles was lower in the heat-stress group than in the control group. The reductions in oestradiol concentration and aromatase activity in the follicles are in agreement with a previous report showing that, in cows, the oestradiol concentration and aromatase activity in the dominant follicle are significantly lower in summer than in the same size follicle in autumn (Badinga *et al.* 1993). Thus, it is suggested that decreased aromatase activity under heat stress suppresses oestradiol synthesis in oestrogenic follicles. In contrast, the aromatase activity and fluid oestradiol concentration in the small follicles remained low and a significant difference was not detected between the heat stress and control groups. McNatty *et al.* (1984) reported that the aromatase activity in pre-oestrogenic follicles remains low and the oestradiol synthesis activity is insubstantial. Therefore, it is possible that the effect of heat stress on oestradiol synthesis activity might be less evident in small follicles, although the follicular size that is the boundary of the pre- to post-oestrogenic phases will be strongly required in the goat.

Aromatase activity in granulosa cells is highly regulated by FSH (Dorrington *et al.* 1975, Erickson & Hsufh 1978). Plasma FSH concentrations were not altered by heat stress in the present study. However, a recent study in immature rats indicated that heat stress suppressed equine chorionic gonadotrophin (eCG)-induced follicular growth and caused a significant reduction of FSH receptor content in the granulosa cells (Shimizu *et al.* 2000). These combined findings suggest that heat-stressed follicles lack sufficient FSH stimulation to enhance the aromatase activity owing to decreased FSH receptor expression, rather than to diminished FSH levels, and that this leads to the observed decrease in oestradiol concentrations.

Another possible mechanism that could explain the heat-induced reduction of oestradiol synthesis is suggested by the finding that androstenedione and oestradiol concentrations within follicular cells derived from the dominant follicle were reduced by heat stress in an *in vitro* study (Wolfenson *et al.* 1997). Previous studies had indicated that the concentration of androstenedione is a limiting factor in oestradiol synthesis and follicular growth (Badinga *et al.* 1992, Savio *et al.* 1993). It is thus possible that heat stress *in vivo* causes a similar reduction of androstenedione levels and may consequently limit oestradiol synthesis. Further study to determine the relationship between androstenedione and oestradiol in heat stressed follicles *in vivo* is required.

Another important finding in the present study was that every follicle exposed to heat stress during recruitment regressed before developing to large size and thus never ovulated, although the number of follicles recruited during the heat-stress period did not differ from the number in control goats (Table 2). This might directly account for the 1-day lag in the development of follicles that eventually ovulated and the LH surge in heat-stressed goats as

Figure 3 Plasma concentrations of (A) progesterone, (B) oestradiol, (C) LH, and (D) FSH in heat-stressed (●) and control (○) goats from –48 to 120 h. Plasma samples were collected at 24-h intervals from –48 to 0 h and at 6-h intervals from 0 to 120 h. Values are expressed as means $\pm$ S.E.M. from six replicates.

compared with controls. The timing of the ovulation, however, was not delayed significantly. We collected the blood sample every 6 h for the LH assay, and ovulation was observed in every 24-h interval. This may be the reason why the time lag of the ovulation could not be traced despite the significant delay of LH surge in this study.

Follicular recruitment and differentiation are induced by exposure of FSH (Fortune 1994, Garverick *et al.* 2002), and then gonadotrophin dependent for further follicular growth is transferred from FSH to LH (Webb *et al.* 2003). LH regulates the progression of medium follicles to large follicles (Savio *et al.* 1993, Webb *et al.* 1994), as well as follicular dominance (Bodensteiner *et al.* 1996) in cows.

Table 3 Characteristics of pulsatile LH secretion in intact goat or steroid-implanted OVX goat under control and heat-stress conditions. Data are expressed as the mean $\pm$ S.E.M. of six replicates.

	Treatment	Intact goat	OVX goat		
			Without steroids	E implantation	E + P implantation
Frequency (pulse/4 h)	Control	3.50 $\pm$ 1.64	6.89 $\pm$ 0.78	3.89 $\pm$ 0.78	1.56 $\pm$ 0.44
	Heat stress	2.50 $\pm$ 0.29	6.44 $\pm$ 0.67	3.89 $\pm$ 0.56	1.78 $\pm$ 0.33
Amplitude (ng/ml)	Control	1.80 $\pm$ 0.38	1.67 $\pm$ 0.83	2.11 $\pm$ 0.22	1.44 $\pm$ 0.33
	Heat stress	1.53 $\pm$ 0.21	1.22 $\pm$ 0.19	1.80 $\pm$ 0.37	1.61 $\pm$ 0.13
Basal (ng/ml)	Control	1.61 $\pm$ 0.57	1.61 $\pm$ 0.57	0.72 $\pm$ 0.12	0.33 $\pm$ 0.18
	Heat stress	1.72 $\pm$ 0.28	1.72 $\pm$ 0.28	1.00 $\pm$ 0.12	0.28 $\pm$ 0.09

Figure 4 Effects of heat stress on (A) LH receptor, (B) aromatase activity, (C) oestradiol concentration, and (D) progesterone concentration in follicles of each size category from heat-stressed (■) and control (□) female goats. The follicular development was monitored during the experimental period, and ovaries were recovered from four goats in each group at 48 h post PGF_{2α} injection. Every follicle recruited from -24 to 0 h was collected from the ovaries, categorized by size (small, 2.0–3.4 mm; medium, 3.5–4.9 mm; large, >5 mm), and analyzed. Values are expressed as the means ± S.E.M. *Significantly different from follicles of the same size from the controls ($P < 0.05$). ND, no data due to heat-stressed follicles not reaching the large size (see text).

In addition, pulsatile LH secretions are known to drive follicular steroidogenesis directly since the follicle become oestrogenic (Baird 1983). Several stressors such as insulin-induced hypoglycemia (Medina *et al.* 1998) or endotoxin injection (Battaglia *et al.* 1997) are known to cause a decrease in LH pulse frequency. The present results also indicated that the number of LH pulses tended to decrease in intact goats due to heat stress (3.5 times in controls versus 2.5 times in heat stress). Therefore, the reduced stimulation of the growing follicle by LH, due to heat stress, could be a cause of follicular atresia. In contrast, our present data also indicated that no significant differences in the characteristics in LH pulse were detected between heat stress and control in OVX goats regardless of whether ovarian steroid(s) were implanted or not. These results suggest that the effect of heat stress in the present study was minimal to disturb the GnRH pulse generator. Previously, Kanai *et al.* (1995) demonstrated in long-day-treated anoestrous goats that heat stress prevented the increase in plasma oestradiol concentration and the LH surge induced by exogenous GnRH injection, although the frequency of LH pulses did not change due to heat stress, indicating that heat stress reduced follicular responsiveness to LH. In the present study we have shown direct evidence that the expression of LH receptor in heat-stressed follicles was decreased. Taken together, these

findings suggest that follicular responsiveness to LH is suppressed by heat stress and that heat-stressed follicles thus lack sufficient LH stimulation, leading to regression of the follicles before ovulation. Further studies investigating the mechanisms that block LH receptor expression during heat stress are required.

A significant reduction of feed intake during heat stress was also observed in this study. Prolonged energy imbalance is also an indirect factor that can alter follicular growth (Lucy *et al.* 1992, Butler 2001, Roche & Diskin 2001). However, the duration of the heat stress in this study was 48 h, and every parameter we monitored to determine the general response to heat stress did not differ between the two groups before and after the period of heat stress. Therefore, the indirect effects of energy imbalance were likely to be minimal compared with the direct influences of heat stress on follicular function. In conclusion, heat stress during recruitment severely compromises follicular growth from medium size to ovulation and these effects are associated with suppressed oestradiol and LH receptor levels.

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References

- Al-Katanani YM, Paula-Lopes FF & Hansen PJ 2001 Effect of season and exposure to heat stress on oocyte competence in Holstein cows. *Journal of Dairy Science* **85** 390–396.
- Araki K, Araki YK, Watanabe G & Taya K 2000 Involvement of inhibin in the regulation of follicle-stimulating hormones secretion in the young adult male Shiba goat. *Journal of Andrology* **21** 558–565.
- Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, De La Sota RL & Thatcher WW 1992 Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle. *Biology of Reproduction* **47** 871–883.
- Badinga L, Thatcher WW, Diaz T, Drost M & Wolfenson D 1993 Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. *Theriogenology* **39** 797–810.
- Baird DT 1983 Factors regulating the growth of the preovulatory follicle in the sheep and human. *Journal of Reproduction and Fertility* **69** 343–352.
- Battaglia DF, Bowen JM, Krasa HB, Thrun LA, Viguie C & Karsch FJ 1997 Endotoxin inhibits the reproductive neuroendocrine axis while stimulating adrenal steroids: a simultaneous view from hypophyseal portal and peripheral blood. *Endocrinology* **138** 4273–4281.
- Berman A, Folman Y, Kaim M, Mamen M, Herz Z, Wolfenson D, Arieli A & Graber Y 1985 Upper critical temperatures and forced ventilation effects for high-yielding dairy cows in a subtropical climate. *Journal of Dairy Science* **68** 1488–1495.
- Bodensteiner KJ, Wiltbank MC, Bergfelt DR & Ginther OJ 1996 Alternations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. *Theriogenology* **45** 499–512.
- Butler WR 2001 Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. In *Fertility in the High-Producing Dairy Cow*, vol. 26, pp 133–145. Ed. MG Diskin. Edinburgh: BSAS Occasional Publication.
- Carson RS, Findlay JK, Burger HG & Trounson AO 1979 Gonadotropin receptors of the ovine follicle during follicular growth and atresia. *Biology of Reproduction* **21** 75–87.
- de Castro T, Rubianes E, Menchaca A & Rivero A 1999 Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goat. *Theriogenology* **52** 399–411.
- Dorrington JH, Moon YS & Armstrong DT 1975 Estradiol-17 β biosynthesis in cultured granulosa cells from hypophysectomized immature rats: stimulation by follicle stimulating hormone. *Endocrinology* **97** 1328–1331.
- Ealy AD, Drost M & Hansen PJ 1993 Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *Journal of Dairy Science* **76** 2899–2905.
- Erickson GF & Hsufh AJW 1978 Stimulation of aromatase activity by follicle stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. *Endocrinology* **102** 1275–1282.
- Fortune JE 1994 Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50** 225–232.
- Fortune JE, Sirois J, Turzillo AM & Lavoie M 1991 Follicular selection in domestic ruminants. *Journal of Reproduction and Fertility* **43** (suppl) 187–198.
- Garverick HA, Baxter G, Gong J, Armstrong DG, Campbell BK, Gutierrez CG & Webb R 2002 Regulation of expression of ovarian mRNA encoding steroidogenic enzymes and gonadotropin receptors by FSH and GH in hypogonadotrophic cattle. *Reproduction* **123** 651–661.
- Gill JL & Hafs HD 1971 Analysis of repeated measurements of animals. *Journal of Animal Science* **33** 331–336.
- Griffin PG & Ginther OJ 1992 Research applications of ultrasonic imaging in reproductive biology. *Journal of Animal Science* **70** 953–972.
- Gwazdauskas FC, Wilcox CJ & Thatcher WW 1975 Environmental and management factors affecting conception rate in a subtropical climate. *Journal of Dairy Science* **58** 88–92.
- Gwazdauskas FC, Thatcher WW, Kiddy CA, Paape MJ & Wilcox CJ 1981 Hormonal pattern during heat stress following tam salt induced luteal regression in heifers. *Theriogenology* **16** 271–285.
- Hansen PJ & Aréchiga CF 1999 Strategies for managing reproduction in the heat-stressed dairy cow. *Journal of Dairy Science* **82** (suppl 2) 36–50.
- Hansen PJ, Drost M, Rivera RM, Paula-Lopes FF, Al-Katanani YM, Krininger CE III & Chase CC Jr 2001 Adverse impact of heat stress on embryo production: causes and strategies for mitigation. *Theriogenology* **55** 91–103.
- Ingraham RH, Gillette DD & Wagner WD 1974 Relationship of temperature and humidity to conception rate of Holstein cows in a subtropical climate. *Journal of Dairy Science* **57** 476–481.
- Kanai Y, Yagyu N & Shimizu T 1995 Hypogonadism in heat stressed goats: poor responsiveness of the ovary to the pulsatile LH stimulation induced by hourly injections of a small dose of GnRH. *Journal of Reproduction and Development* **41** 133–139.
- Kawate N, Inaba T & Mori J 1989 Number of gonadotropin receptors and concentrations of steroid hormones in bovine follicles. *Japanese Journal of Animal Reproductive Technology* **35** 106–111.
- Lucy MC, Savio JD, Badinga L, De La Sota RL & Thatcher WW 1992 Factors that affect ovarian follicular dynamics in cattle. *Journal of Animal Science* **70** 3615–3626.
- Madan ML & Johnson HD 1973 Environmental heat effects on bovine luteinizing hormone. *Journal of Dairy Science* **56** 1420–1423.
- McNatty KP, Heath DA, Henderson KM, Lun S, Hurst PR, Ellis LM, Montgomery GW, Morrison L & Thurley DC 1984 Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary. *Journal of Reproduction and Fertility* **72** 39–53.
- Medan MS, Watanabe G, Sasaki K, Sharawy S, Groome NP & Taya K 2003 Ovarian dynamics and their associations with peripheral concentrations of gonadotropins, ovarian steroids, and inhibin during the estrous cycle in goats. *Biology of Reproduction* **69** 57–63.
- Medina CL, Nagatani S, Darling TA, Bucholtz DC, Tsukamura H, Maeda KI & Foster DL 1998 Glucose availability modulates the timing of the luteinizing hormone surge in the ewe. *Journal of Neuroendocrinology* **10** 785–792.
- Menchaca A & Rubianes E 2002 Relation between progesterone concentrations during the early luteal phase and follicular dynamics in goats. *Theriogenology* **57** 1411–1419.
- Merriam GR & Wachtler KW 1982 Algorithms for the study of episodic hormone secretion. *American Journal of Physiology Endocrinology and Metabolism* **243** E310–E318.
- Miller HL & Alliston CW 1974 Influence of programmed circadian temperature changes upon levels of luteinizing hormone in the bovine. *Biology of Reproduction* **11** 187–190.
- Mori Y & Kano Y 1984 Changes in plasma concentrations of LH, progesterone and estradiol in relation to the occurrence of luteolysis, estrus and time of ovulation in Shiba goats (*Capra hircus*). *Journal of Reproduction and Fertility* **72** 223–230.
- Ozawa M, Hirabayashi M & Kanai Y 2002 Developmental competence and oxidative state of murine zygotes heat stressed maternally or *in vitro*. *Reproduction* **124** 683–689.

- Ravindra JP, Rawlings NC, Evans ACO & Adams GP 1994 Ultrasonographic study of ovarian follicular dynamics in ewes during the oestrus cycle. *Journal of Reproduction and Fertility* **101** 501–509.
- Rensis FD & Scaramuzzi RJ 2003 Heat stress and seasonal effects on reproduction in the dairy cow—a review. *Theriogenology* **60** 1139–1151.
- Robert GC, Heather M & Dorrington J 1980 An alternative method for the study of follicle-stimulating hormone effects on aromatase activity in sertoli cell cultures. *Endocrinology* **107** 464–471.
- Roche JF & Diskin MG 2001 Resumption of reproductive activity in the early post-partum period of cows. In *Fertility in the High-Producing Dairy Cow*, vol. 26, pp 31–42. Ed. MG Diskin. Edinburgh: BSAS Occasional Publication.
- Roman-Ponce H, Thatcher WW & Wilcox CJ 1981 Hormonal interrelationships and physiological responses of lactating dairy cows to shade management system in a tropical environment. *Theriogenology* **16** 139–154.
- Rosenberg M, Folman Y, Herz Z, Flamenbaum I, Berman A & Kaim M 1982 Effect of climatic conditions on peripheral concentrations of LH, progesterone and estradiol-17 β in high milk-yielding cows. *Journal of Reproduction and Fertility* **66** 139–146.
- Roth Z, Meidan R, Braw-Tal R & Wolfenson D 2000 Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *Journal of Reproduction and Fertility* **120** 83–90.
- Savio JD, Thatcher WW, Badinga L, de la Sota RL & Wolfenson D 1993 Regulation of dominant follicle turnover during the oestrous cycle in cows. *Journal of Reproduction and Fertility* **97** 197–203.
- Shimizu T, Ohshima I, Tajima A & Kanai Y 2000 Effect of heat stress on follicular development in PMSG-treated immature rats. *Animal Science Journal* **71** 32–37.
- Tanaka T, Mori Y & Hoshino K 1994 Long-term recording of hypothalamic GnRH pulse generator activity during programmed administration of progesterone and estradiol in the ovariectomized goat. *Journal of Reproduction and Development* **40** 183–188.
- Thatcher WW, Driancourt DD, Terqui M & Badinga L 1991 Dynamics of ovarian follicular development in cattle following hysterectomy and during early pregnancy. *Domestic Animal Endocrinology* **8** 223–234.
- Trout JP, McDowell LR & Hansen PJ 1998 Characteristics of the estrous cycle and antioxidant status of lactation Holstein cows exposed to heat stress. *Journal of Dairy Science* **81** 1244–1250.
- Udompraset P & Williamson NB 1987 Seasonal influences on conception efficiency in Minnesota dairy herds. *Theriogenology* **28** 323–336.
- Webb R & England BG 1982 Relationship between LH receptor concentration in thecal and granulosa cells and *in vivo* and *in vitro* follicles during the preovulatory period. *Journal of Reproduction and Fertility* **66** 169–180.
- Webb R, Gong JG & Bramley TA 1994 Role of growth hormone and intrafollicular peptides in follicle development in cattle. *Theriogenology* **41** 25–30.
- Webb R, Nicholas B, Gong JG, Campbell BK, Gutierrez CG, Garverick HA & Armstrong DG 2003 Mechanisms regulating follicular development and selection of the dominant follicle. *Reproduction* **61** suppl 71–90.
- Wilson SJ, Kirby CJ, Koenigsfeld DH, Keisler DH & Lucy MC 1998a Effects of controlled heat stress on ovarian function of dairy cattle. 2. Heifers. *Journal of Dairy Science* **81** 2132–2138.
- Wilson SJ, Marion RS, Spain JN, Spiers DE, Keisler DH & Lucy MC 1998b Effects of controlled heat stress on ovarian function of dairy cattle. 1. Lactating cows. *Journal of Dairy Science* **81** 2124–2131.
- Wolfenson D, Thatcher WW, Badinga L, Sovio JD, Meidan R, Lew BJ, Braw-tal R & Berman A 1995 Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. *Biology of Reproduction* **52** 1106–1113.
- Wolfenson D, Lew BJ, Thatcher WW, Graber Y & Meidan R 1997 Seasonal and acute heat effects on steroid production by dominant follicles in cow. *Animal Reproduction Science* **47** 9–19.
- Wolfenson D, Roth Z & Meidan R 2000 Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Animal Reproduction Science* **60–61** 535–547.
- Zeron Y, Ocheretny A, Kedar O, Borochoy A, Sklan D & Arav A 2001 Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction* **121** 447–454.

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