Introduction

Heat stress alters the follicular development pattern in cattle. Exposure of cows to heat stress led to a reduction in the size of the dominant follicles of the first and second follicular wave of the oestrus cycle (Badinga et al., 1993; Wilson et al., 1998a,b). Depression of follicular dominance by heat stress was indicated by: the absence of a decrease in medium sized follicles during the first follicular wave or during the follicular phase of the oestrous cycle; a large size and a slow decrease in the size of the second largest follicle; an increase in the number of large follicles during the first follicular wave; and an early emergence of the preovulatory follicle (Badinga et al., 1993; Wolfenson et al., 1995; Wilson et al., 1998b). These studies examined the responses to heat exposure during days 1–8 of the oestrous cycle (Badinga et al., 1993), during days 11–21 of the oestrous cycle (Trout et al., 1998; Wilson et al., 1998a,b) or during a hormonally programmed oestrous cycle (Wolfenson et al., 1995). However, the effect of heat stress on follicular dynamics during a complete unprogrammed oestrous cycle, in particular, the turnover of the first and the second follicular waves has not been investigated. Furthermore, FSH secretion and its association with follicular dynamics in heat-stressed
cattle has not been documented, even though FSH plays a major role in emergence of the follicular wave (Adams et al., 1992, 1993).

Conception rates of lactating cows decrease from about 50% in the winter to less than 20% in the summer. However, fertility in the autumn (30%) is lower than that in winter, although ambient temperatures decrease and animals are no longer exposed to thermal stress (Ron et al., 1984; Cavestany et al., 1985). A seasonal study (Badinga et al., 1994) indicated variations in follicular dynamics during autumn, which were related to summer heat stress. A delayed effect of summer heat stress on follicular steroidogenic capacity in the autumn was suggested by Wolfenson et al. (1997). The possibility of a delayed effect of heat stress on follicular development is supported by the fact that small antral follicles take about 40–50 days to develop into large preovulatory follicles (Lussier et al., 1987). Thus, exposure to heat stress during the early stages of follicular development in the summer may subsequently impair preovulatory follicular function (and the fertility of the cow) in the autumn.

The objectives of the present study were: (i) to determine the effect of heat stress on follicular growth during a complete unprogrammed oestrous cycle; (ii) to examine a possible delayed effect of heat stress on follicular development; and (iii) to characterize changes in plasma FSH and inhibin concentrations, and their involvement in the alteration of follicular dynamics.

Materials and Methods

Animals

The experiment was carried out during the summer on 13 cyclic multiparous Holstein cows in the early stages of lactation (average of 90 days post partum), yielding an average of 35 kg milk per day. Cows were kept under an open shade structure and were fed a complete mixed ration containing 16.5% protein and 1.7 Mcal dry matter kg−1. Daily mean maximum and minimum air temperatures and relative humidity were 30.5°C and 19°C, and 89% and 51%, respectively. Oestrous cycles were synchronized by progesterone and PGF2α. A controlled intravaginal drug releasing device (CIDR) (Eazi Breed, Hamilton) was inserted for 9 days and 500 μg Cloprostenol, a PGF2α analogue (Estrumate, Coopers, Berkhamsted), was injected i.m. 7 days after insertion of the CIDR device. Cows were checked for oestrus manifestation three times each day for 45 min; in addition, oestrus was confirmed three times each day before milking, by a computerized pedometric system (Afimilk, Afikim, Israel). Cows expressing oestrous behaviour within 48 h after the removal of the CIDR device were included in the experiment. Ovulation was confirmed by ultrasonography. The experiment was approved by the local ethics committee.

Experimental protocol

On day 2 of the oestrous cycle (day 0 = day of oestrus), cows were randomly assigned to either cooled (n = 7) or heat-stressed (n = 6) treatment groups. During the treated oestrous cycle, cows in the cooling group were cooled by a sprinkler and ventilation system (Berman and Wolfenson, 1992) from 06:00 h to 18:00 h. Cows in the heat stress group were exposed to direct solar radiation from 08:00 h to 15:00 h each day and, thereafter, moved into a shade structure without a cooling system, until the next morning. Body (rectal) temperatures were monitored every 2 h during week 1 of heat exposure. Thereafter, during the treated oestrous cycle, the heat-exposed cows were monitored four times each day (morning, noon, at the end of heat exposure and 4 h later) with an electronic digital thermometer (Toshiba, Japan), to ensure the desired levels of hyperthermia in heat-stressed cows and of normothermia in cooled cows were maintained. Heat exposure was terminated when cows manifested oestrus. The day after oestrus, both heat-stressed and cooled cows were grouped together and cooled during the first 10 days of the subsequent cycle, and their body temperatures were recorded as described above. This experimental design enables characterization of the immediate effect of heat stress on follicular dynamics during the treated oestrous cycle and its possible delayed effect during the first half of the subsequent oestrous cycle.

Follicular development was monitored on days 2, 5 and 8, and then each day from day 10 of the treated oestrous cycle to day 10 of the subsequent oestrous cycle, by means of a real-time ultrasound instrument (Aloka, model SSD-210DXII, Tokyo), equipped with a 7.5 MHz transrectal linear transducer. The size of the large follicles (> 10 mm) in each follicular wave, the size of the corpus luteum, and the numbers of small (3–5 mm), medium (6–9 mm) and large follicles (> 10 mm) were recorded. Dominant and second largest follicles were determined according to Ginther et al. (1989). Blood samples were collected on the day of ultrasonography and centrifuged (2000 g for 20 min) and plasma was stored at −20°C pending hormonal determination.

Hormone analyses

Plasma samples were extracted with diethyl ether as described by Badinga et al. (1992). The concentration of progesterone in extracted plasma was determined by a validated radioimmunoassay (Meidan et al., 1990) using specific antibodies (donated by F. Kohen, Department of Hormone Research, Weizmann Institute, Rehovot). Assay sensitivity was 0.04 ng ml−1, and intra- and interassay coefficients of variation were 8.6% and 9.9%, respectively. Extracted plasma samples were analysed for oestradiol concentrations by means of a single antibody radioimmunoassay (Badinga et al., 1992). The assay had been validated in this laboratory (Shaham-Albalancy et al., 1997). The antibody (Diagnostic Products, Los Angeles, CA) did not crossreact with testosterone or progesterone, and had low crossreactivity with oestradiol (0.2%) and 17a-oestradiol (0.01%). Assay sensitivity was 0.5 pg ml−1, and the intra- and interassay coefficients of variation were 3% and 5%, respectively. The plasma concentration of immunoreactive inhibin was measured by radioimmunoassay supplied by
The range of the standard curve was 0.1–25.0 ng ml$^{-1}$, the iodination USDA-bFSH-I$_2$ was used (Iodo-gen method) and first antibody was NIH-anti-oFSH-1 (dilution 1:80 000); for validated in this laboratory (Braw-Tal was determined by a homologous radioimmunoassay 5% and 14%, respectively. The plasma concentration of FSH respectively.

interassay coefficients of variation were 3% and 10%, respectively.

The sensitivity of the assay was 0.1 ng ml$^{-1}$, the antiserum showed high crossreactivity (288%) with the pro-$\alpha$ inhibin subunit. The sensitivity of the assay was 0.1 ng ml$^{-1}$, and the intra- and interassay coefficients of variation were 3% and 10%, respectively.

Statistical analysis

The diameter of the dominant and subordinate follicles, the number of follicles classified by size and the concentration of the hormones were analysed according to the general linear model procedure of the Statistical Analysis System (SAS, 1987). The statistical model included effects of: treatment (cooled versus heat-stressed groups), cow (within treatment), day of the oestrous cycle and treatment by day interaction. Data were analysed separately for the treated and the subsequent oestrous cycles. Analysis was performed for the treated oestrous cycle (plasma inhibin, oestradiol and progesterone concentrations, number of small follicles and diameter of the corpus luteum), or separately for the first and the second follicular waves (plasma FSH concentrations, diameters of dominant and second largest follicles, medium and large follicles). In the treated oestrous cycle, data on plasma FSH concentrations were normalized to the average day of the cycle in which plasma FSH increase preceding the emergence of the second follicular wave was noted. Such an approach facilitated a proper synchronization of FSH data during follicular turnover. In the subsequent oestrous cycle, data of plasma FSH concentrations, as well as data of all other variables (hormone concentrations and follicular characteristics), were normalized to the day of oestrus of that cycle, enabling synchronization of events occurring during the follicular phase, such as oestradiol increase, preovulatory FSH surge and postovulatory FSH increase. For further characterization of the day of increase and decrease of plasma FSH, or of emergence and decrease of follicular wave dynamics, the contrast $t$ tests procedure was used, and relevant data were compared among days. Data are presented as mean $\pm$ SE.

Results

Exposure of cows in the heat-stressed group to solar radiation during the treated oestrous cycle led to an increase in body temperature compared with cows that were cooled ($P < 0.01$); the mean maximum body temperature during the treated oestrous cycle was $40.3 \pm 0.2^\circ$C (hyperthermia commonly experienced by lactating cows during the summer). After approximately 4 h in the shade, heat-stressed cows were still hyperthermic; the average rectal temperature was $39.6 \pm 0.1^\circ$C, reflecting slow body cooling after exposure to solar radiation. During the treated oestrous cycle, cooled cows maintained normothermia ($38.7 \pm 0.1^\circ$C). During the subsequent cycle (days 2–10), both cooled and heat-stressed cows maintained normothermia as expected in lactating cows during the cool seasons. The average body condition score was $2.5 \pm 0.25$ and it did not differ between the groups during the experiment.

Hormonal concentrations in plasma

During the treated oestrous cycle, the plasma FSH increase preceding emergence of the second follicular wave was significantly greater in duration, lasting 4 more days in heat-stressed cows than in cooled cows (days 8–13 of the cycle; Fig. 1; $P < 0.05$). In addition, the peak FSH amplitude was higher in heat-stressed cows than in cooled cows ($0.44 \pm 0.05$ and $0.32 \pm 0.11$ ng ml$^{-1}$, respectively; $P < 0.03$). The plasma concentration of the preovulatory FSH surge was also higher in heat-stressed cows than in cooled cows ($0.74 \pm 0.25$ and $0.29 \pm 0.08$, respectively; $P < 0.01$). In the subsequent oestrous cycle, the concentration of the postovulatory FSH increase (day 2) was higher in heat-stressed cows than in cooled cows ($0.77 \pm 0.14$ and $0.31 \pm 0.03$, respectively; $P < 0.01$; Fig. 1), and the FSH increase preceding emergence of the second follicular wave (days 8–10) tended to be higher in heat-stressed cows than in cooled cows, but this result was not significant. The plasma concentration of immunoreactive inhibin during the luteal phase of the treated oestrous cycle (days 5–18) was higher in cooled cows than in heat-stressed cows.
cows (0.39 ± 0.11 and 0.17 ± 0.11, respectively; P < 0.02; Fig. 2). The plasma concentration of oestradiol did not differ between heat-stressed cows and cooled cows during the luteal phase of the treated oestrous cycle (Fig. 3a). However, during the follicular phase, the plasma concentration of oestradiol peaked 1 day earlier and tended to be lower in heat-stressed cows than in cooled cows, but this result was not significant (10.2 ± 2.6 versus 14.5 ± 1.8, respectively). The plasma concentration of progesterone during the treated oestrous cycle did not differ between heat-stressed cows and cooled cows (Fig. 3b), and during the subsequent oestrous cycle the plasma concentrations of oestradiol (Fig. 3a) and progesterone (Fig. 3b) did not differ between previously heat-stressed cows and cooled cows.

Follicular development

The duration of the oestrous cycle did not differ significantly between the heat-stressed and cooled groups (21.5 ± 0.5 and 22 ± 0.5 days, respectively). Most oestrous cycles (77%) in both groups comprised two follicular waves. Only one of six heat-stressed cows and two of seven cooled cows had three follicular waves. Therefore, the statistical analysis was applied to the first and the second follicular waves.

During the treated oestrous cycle, heat exposure did not alter the average size or the rates of growth and decrease of the first and second wave dominant follicles (Fig. 4a). The size of the second largest follicle of the first follicular wave decreased at a later time in heat-stressed cows than in cooled cows (treatment by day interaction; P < 0.02; Fig. 4b). Heat stress did not alter the size of the corpus luteum (data not shown) or the number of small follicles (3–5 mm; Fig. 5). During the subsequent oestrous cycle, there was no difference between treatment groups in the size of the dominant and second largest follicles, the diameter of the corpus luteum or the number of small follicles (Figs 4 and 5).

During the second follicular wave of the treated cycle, the number of medium follicles (6–9 mm) was greater in heat-stressed cows than in cooled cows (P < 0.04; Fig. 6). In terms of medium sized follicles, in heat-stressed cows the second follicular wave emerged 1 day (day 13) earlier and decreased 2 days later (day 17) than in cooled cows (P < 0.05). Further analysis indicated that disregarding the cows that showed three follicular waves did not alter the number or growth pattern of medium sized follicles during the second follicular wave. On day 4 of the subsequent oestrous cycle, the peak number of medium sized follicles decreased from 4.4 ± 0.6 in cooled cows to 2.9 ± 0.4 in previously heat-stressed cows (P < 0.05; Fig. 6). Regression analyses indicated that the rate of decrease in the number of medium sized follicles during days 4–10 tended to be slower in heat-stressed cows than in cooled cows, but this result was not significant (for heat-stressed cows: y = −0.40x + 4.5, r = 0.939; for cooled cows: y = −0.71x + 7.1, r = 0.968; where y is the number of medium follicles and x is the day of the cycle).

The overall number of large follicles (>10 mm) during the treated oestrous cycle did not differ between heat-stressed and cooled cows (Fig. 7a). However, analysis of the data from days 11–20 of the oestrous cycle showed a treatment by day interaction (P < 0.03). The difference between heat-stressed and cooled cows in the pattern of increase in the number of large follicles is unclear. Further analysis, in which the first

Fig. 2. Plasma concentration of inhibin during the treated oestrous cycle when cows were cooled (n = 7, △) or heat stressed (n = 6, ●). Pooled SEM for cooled and heat-stressed cows: 0.28 and 0.31, respectively.

Fig. 3. Plasma concentrations of (a) oestradiol and (b) progesterone during the treated oestrous cycle of cooled (n = 7, △) and heat-stressed (n = 6, ●) cows and during the subsequent oestrous cycle when both cooled (△) and previously heat-stressed (○) cows were cooled. Pooled SEM for progesterone and oestradiol: 0.20 and 0.24, respectively.

Fig. 4. Size of the second largest follicle of the first follicular wave (ng ml⁻¹).

Fig. 5. Corpus luteum size (ng ml⁻¹).

Fig. 6. Number of medium sized follicles (mg ml⁻¹).

Fig. 7. Number of large follicles (>10 mm) (mg ml⁻¹).
wave dominant (non-ovulatory) follicle was excluded from the data set, showed that heat-stressed cows tended to have a greater number of large follicles than cooled cows during the follicular phase, but this result was not significant ($1.4 \pm 0.1$ and $1.0 \pm 0.1$, respectively; Fig. 7b). During the subsequent oestrous cycle, there was no difference between the treatment groups in the number of large follicles.

**Discussion**

This study characterized immediate heat stress induced changes in plasma FSH and inhibin concentrations and the effect of heat stress on follicular dynamics. Moreover, it is the first study to show a delayed effect of heat stress on follicular growth.

Exposing cows to heat stress during a complete oestrous cycle altered the dynamics of the turnover of the first and second follicular waves. In terms of the number of medium sized follicles, the early emergence of the second follicular wave probably resulted from reduced dominance of the first wave dominant follicle. Similarly, attenuation of dominance of the second wave dominant follicle was reflected in a medium sized follicular wave that lasted for 2 days more in heat-stressed cows than in cooled cows. The number of medium sized follicles is a sensitive indicator of follicular dominance as the dominant follicle has an inhibitory influence on the growth of subordinate follicles (Ginther *et al.*, 1989; Fortune *et al.*, 1991; Ko *et al.*, 1991). Depression of dominance in heat-stressed cows was also reflected in the present study as a slower decrease in the size of the subordinate follicle during the first follicular wave, as also reported by Badinga *et al.* (1993) and Wilson *et al.* (1998b).

The endocrine milieu responsible for alterations in follicular dynamics and depression of dominance under heat stress is complex and not fully understood. The occurrence of
an increase in FSH preceding the emergence of the follicular wave, and the role it plays in follicular wave turnover, is well documented (Adams et al., 1992; Ginther et al., 1996). Gibbons et al. (1997) showed that the number of medium sized follicles that advanced into a follicular wave was increased by a greater increase in FSH. Thus, the increase in the number of medium sized follicles in heat-stressed cows is most likely a result of the higher plasma FSH increase that preceded the second follicular wave. The observation of an FSH increase in agreement with the finding of Armstrong et al. (1986) that serum FSH concentrations in primiparous sows were higher in the summer than in the winter. However, Gilad et al. (1993) showed a lower plasma FSH surge in heat-stressed cows. The latter findings were obtained by administration of a GnRH analogue, as opposed to the spontaneous FSH surge observed in the present study. The potential involvement of an alteration in pulsatile LH secretion in the increased growth of medium (> 8 mm) and large follicles can be excluded, because Wise et al. (1988) showed a decrease in pulsatile LH secretion in heat-stressed cows, and Gilad et al. (1993) showed a decrease in pulse amplitude in heat-stressed cows.

Inhibin is an important factor in the regulation of FSH secretion (Findlay, 1993; Kaneko et al., 1993). A clear inverse relationship between plasma FSH and immunoreactive inhibin concentrations was found throughout the oestrous cycle (Kaneko et al., 1995, 1997). Consistent with this concept, the increase of plasma FSH concentration during heat exposure in the present study corresponded well to a pronounced decrease in the plasma concentration of immunoreactive inhibin, resulting in the alterations in follicular dynamics described earlier. Granulosa cells of large follicles are known to be the main source of plasma inhibin (Findlay et al., 1993). In the present study, despite the increase in the number of medium and large follicles, the plasma inhibin concentration remained low. Decreased mRNA for inhibin subunits in the early stages of follicular atresia has been reported by Braw-Tal (1994), and Guilbault et al. (1993) demonstrated changes in the forms of the inhibin content in the follicular fluid during the growth and regression phases of dominant follicles. Therefore, it is reasonable to consider that the decrease in plasma inhibin concentration under heat stress resulted from alterations in granulosa cell function. A tendency for a decrease in plasma inhibin concentration in heat-stressed cows was reported by Wolfenson et al. (1995), and was also detected in buffalos in a seasonal study during the summer (Palta et al., 1997).

Inhibin and oestradiol secretion by the largest follicle within a wave is thought to mediate the inhibitory effect of the dominant follicle on FSH secretion (Kaneko et al., 1991; Findlay, 1993), and a synergistic effect of inhibin and oestradiol antiserum on FSH secretion during the follicular phase in cows has been reported (Kaneko et al., 1995). A decrease in plasma oestradiol concentration, as was found in heat-stressed cows during the follicular phase in the present study, was also reported by Gwazdauskas et al. (1981) and Wilson et al. (1998a,b). Thus, it seems that the high preovulatory FSH surge in heat-stressed cows was induced synergistically by low secretion of both oestradiol and inhibin. However, the decrease in plasma oestradiol and the increase in plasma FSH concentrations in heat-stressed cows during the follicular phase should be regarded with caution because of the low frequency of blood sampling.

Similar to the preovulatory FSH surge in the treated oestrous cycle, in the subsequent oestrous cycle, the postovulatory FSH increase was higher in previously heat-stressed cows than in cooled cows. The higher postovulatory FSH increase in heat-stressed cows implies a carryover effect of the low plasma inhibin concentration noted in the previous cycle. In agreement with this hypothesis, Kaneko et al. (1995) found that immunoneutralization of inhibin during the follicular phase in cows resulted in a prolonged increase (96 h) in plasma FSH concentration. In the present study, a delayed effect of heat stress was also reflected in a high plasma FSH increase preceding the second follicular wave of the subsequent oestrous cycle. This increase might be due to the impaired functioning of the first wave dominant follicle, but is most likely due to reduced inhibin secretion. Attenuation of dominance was also indicated by a slow decrease in the number of medium sized follicles during the first follicular wave.

In contrast to the large number of medium sized follicles in heat-stressed cows during heat exposure and despite the

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**Fig. 7.** (a) Numbers of large follicles (> 10 mm) of cooled (n = 7, △) and heat-stressed (n = 6, ●) cows during the treated oestrous cycle and during the subsequent oestrous cycle when both cooled (△) and previously heat-stressed (○) cows were cooled. (b) Number of large follicles, excluding the first dominant follicle, during the second half of the treated oestrous cycle. Pooled SEM for large follicles of cooled and heat-stressed groups: 0.10.
high postovulatory FSH increase, fewer medium sized follicles were recorded on day 4 of the first follicular wave of the subsequent oestrous cycle. This decrease occurred early in the oestrous cycle and, therefore, could not be related to the dominance function; it is more likely to reflect an earlier impairment of follicular development during heat exposure. These findings correspond to the study by Roth et al. (1997), which showed that cows that were heat stressed during days 2–6 of the oestrous cycle had a large number of atretic medium sized follicles on day 3 of the subsequent oestrous cycle. Although the present study focused on delayed effects in the period immediately after heat exposure, the hypothesis of a delayed effect of heat stress on follicular functioning is strengthened by the following findings: (i) steroid production by granulosa and theca cells obtained from ovaries of previously heat-stressed cows was lower than that by ovaries from control cows (Roth et al., 1997); (ii) a seasonal study showed lower androgen production by thecal cells obtained from dominant follicles in the autumn than from follicles collected in the winter (Wolfenson et al., 1997); and (iii) low quality oocytes and impaired embryo development were found in follicles aspirated in the autumn from previously heat-stressed cows (Roth et al., 1999). Collectively, these findings indicate that chronic heat stress during the summer can impair follicular function at a later time. Such delayed effects of heat stress may explain low fertility in cattle during the autumn.

Alterations in follicular dynamics during heat stress may have physiological significance. Firstly, early emergence of the second follicular wave during heat stress may induce early emergence of the second dominant follicle, as was reported by Wolfenson et al. (1995). Most of the cows in the present study ovulated the second wave dominant follicle, which indicates that heat stress results in ovulation of an aged dominant follicle. A negative relationship between duration of dominance of the preovulatory follicle and fertility has been shown in spontaneously cyclic cows (Bleach et al., 1998) and in experimentally induced persistent dominant follicles (Mihm et al., 1994; Austin et al., 1999). Secondly, it can be speculated that growth of more than one large follicle during the first follicular wave (Wolfenson et al., 1995) and during the follicular phase in the present study was a result of a high plasma FSH concentration in heat-stressed cows and was not a result of a high pulsatile LH secretion as suggested by Wise et al. (1988) and Gilad et al. (1993). This finding is supported by Adams et al. (1993) who showed that injection of exogenous FSH before the selection of the dominant follicle increased the number of ovarian follicles. This observation may explain the increase in double ovulations during the summer (Ryan and Boland, 1991).

In conclusion, this study characterizes immediate and delayed effects of heat stress on follicular development and function. The immediate effect of heat stress was manifested in depression of dominance. This effect was expressed by a pronounced decrease in plasma immunoreactive inhibin, and consequently increased FSH concentrations. This altered hormonal secretion led to early emergence of the second (preovulatory) follicular wave and an increased number of large follicles during the follicular phase. The delayed effect of heat stress was expressed by a marked increase in FSH secretion and a reduction in the number of medium sized follicles. Both the immediate and the delayed responses to heat stress could be involved in the low fertility of cattle during the summer and autumn.

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