

Genetic divergence in cellular resistance to heat shock in cattle: differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures

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The detrimental effects of heat stress on fertility in cattle are less pronounced in heat-tolerant breeds. Although these genetic differences reflect differences in thermoregulation, cells from heat-tolerant breeds are less adversely compromised by increased temperature (that is, heat shock) than cells from heat-sensitive breeds. Experiments were performed to test the hypothesis that cells and tissues from two thermotolerant breeds (Brahman and Senepol) are better able to survive and function after exposure to increased temperature than cells and tissues from two thermosensitive breeds (Holstein and Angus). Exposure of embryos at > eight-cell stage at day 5 after insemination to heat shock of 41.0°C for 6 h decreased development to the blastocyst stage and the number of cells per embryo. However, the deleterious effect of heat shock on blastocyst formation and the number of cells per embryo was less pronounced for Brahman than for Holstein and Angus breeds. Embryos from Senepol cows had very low

development and it was not possible to determine heat shock effects in this breed. In contrast to the sensitivity of embryos to heat shock, there was no effect of a 41.0°C heat shock on [³H]leucine incorporation into proteins secreted by oviductal or endometrial explants. Lymphocytes from Brahman and Senepol cows were more resistant to heat-induced apoptosis than lymphocytes from other breeds. Heat shock reduced lymphocyte glutathione content but the magnitude of the decrease was not affected by breed. In conclusion, embryos from Brahman cows are more resistant to heat shock than embryos from Holstein or Angus cows. Genetic differences are also present in thermotolerance for apoptosis response in lymphocytes, with Brahman and Senepol cattle being more resistant to heat shock than Angus and Holstein breeds. It is likely that the evolutionary forces that led to the Brahman and Senepol breeds being adapted to hot climates resulted in the selection of genes controlling resistance to cellular heat shock.

Introduction

Mammalian species have evolved to be adapted for different ecosystems. Among other characteristics, domestic cattle can be distinguished on the basis of

adaptation for life in hot climates. Breeds of cattle of *Bos indicus* origin such as Brahman as well as some *B. taurus* breeds (for example, Senepol) are more resistant to tropical conditions such as increased temperature and humidity than breeds that evolved in Europe such as Angus and Holstein. Most of this adaptation to increased temperature is attributable to superior ability of thermotolerant breeds to regulate body temperature (Adeyemo *et al.*, 1979; Bennett *et al.*, 1985; Hammond *et al.*, 1996; Gaughan *et al.*, 1999). There are also indications that breed differences in thermal resistance extend to the cellular level. For example, DNA synthesis by cultured endometrium is reduced by heat shock to a greater extent for Holstein than for Brahman breeds (Malayer and Hansen, 1990). In addition, the decrease

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in lymphocyte viability caused by heat shock of 42 or 45°C was greater for lymphocytes from Angus cows as compared with lymphocytes collected from Brahman or Senepol cattle (Kamwanja *et al.*, 1994).

Failure of cows to maintain homeothermy when exposed to increased ambient temperature can compromise fertility. Heat-stress induced infertility is a multifactorial problem as hyperthermia can affect cellular function in different tissues of the female reproductive tract (Wolfenson *et al.*, 2000; Hansen *et al.*, 2001). Embryos in particular are very susceptible to increased temperature. Exposure of cows to heat stress during early pregnancy reduces embryonic survival (Ealy *et al.*, 1993) and culture of embryos at increased temperatures reduces development to the blastocyst stage (Edwards and Hansen, 1997; Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002).

The hypothesis of this experiment was that cells and tissues from Brahman and Senepol cattle (thermotolerant breeds) are better able to survive and function after exposure to increased temperature than cells and tissues from Holstein and Angus cattle (thermosensitive breeds). Analyses were performed to determine: (1) ability of embryos to develop to the blastocyst stage after exposure to increased temperature; (2) the magnitude of heat-shock induced changes in protein secretion by cultured endometrium and oviduct; (3) apoptosis responses in lymphocytes; and (4) depletion of the antioxidant glutathione in lymphocytes. Apoptosis responses were evaluated because heat shock can induce apoptosis in many types of cell (Mosser *et al.*, 1997; Sodja *et al.*, 1998; Paula-Lopes and Hansen, 2002). Glutathione was evaluated because it is an antioxidant that has been implicated in preventing the deleterious effects of heat shock in preimplantation embryos (Aréchiga *et al.*, 1995). Thus, lymphocytes were used as a model to test whether genetic control of cellular resistance to heat shock involved differences in apoptosis or antioxidant status. The temperature used to evaluate heat shock effects was 41°C. This temperature is near the high range of rectal temperatures of lactating cows exposed to heat stress in Florida (Rivera and Hansen, 2001).

Materials and Methods

Materials

Materials for *in vitro* maturation, fertilization and culture were purchased as described by Paula-Lopes and Hansen (2002). Oocyte collection medium was TCM-199 with Hank's salts without phenol red and supplemented with 2% (v/v) bovine steer serum (containing 2 U heparin ml⁻¹), 100 U penicillin-G ml⁻¹, 0.1 mg streptomycin ml⁻¹ and 1 mmol glutamine l⁻¹. Oocyte maturation medium was TCM-199 with Earle's salts supplemented with 10% (v/v) bovine steer serum, 22 µg sodium pyruvate ml⁻¹, 20 µg FSH ml⁻¹ (Folltropin-V;

Vetrepharm, Ontario), 2 µg oestradiol ml⁻¹, 50 µg gentamicin ml⁻¹ and an additional 1 mmol glutamine l⁻¹. Frozen semen from various bulls was purchased from the American Breeders Service (Madison, WI) or was obtained from a repository at USDA-ARS Subtropical Agricultural Research Station (Brooksville, FL). Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallette, NJ) to prepare Hepes-Tyrode's Albumin Lactate Pyruvate (TALP), IVF-TALP and Sp-TALP (Parrish *et al.*, 1986). Potassium Simplex Optimized Medium (KSOM) was also obtained from Cell and Molecular Technologies. The KSOM, which contains 1 mg BSA ml⁻¹, was modified on the day of use by adding an additional 2 mg essentially fatty acid free BSA ml⁻¹, 2.5 µg gentamicin ml⁻¹, essential amino acids (Basal Medium Eagle) and non-essential amino acids (Minimum Essential Medium) purchased from Sigma (St Louis, MO).

Eagle's Minimum Essential Medium (MEM) was purchased from Sigma. Pig MEM (a modification of MEM) was prepared as described by Malayer *et al.* (1988). L-[4,5-³H]leucine (specific activity: 173 Ci mmol⁻¹) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Prostaglandin F_{2α} (Lutalyse®) was obtained from Pharmacia & Upjohn (Kalamazoo, MI) and GnRH (Cystorelin) was from Sanofi Animal Health (Overland Park, KS). The medium for lymphocyte culture was TCM-199 supplemented with 5% (v/v) horse serum, 200 U penicillin-G ml⁻¹, 0.2 mg streptomycin ml⁻¹, 1 mmol glutamine l⁻¹ and 1 × 10⁻⁵ mol β-mercaptoethanol l⁻¹. Glutathione reductase, β-nicotinamide adenine dinucleotide phosphatase reduced form (NADPH) and 5,5-dithiobis(2-nitrobenzoic acid) (DNTB) were obtained from Sigma. *In situ* cell death detection kit (fluorescein) and propidium iodide were obtained from Roche Diagnostics Corporation (Indianapolis, IN) and Sigma, respectively. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak Company (Rochester, NY). Prolong Antifade® kit was obtained from Molecular Probes (Eugene, OR); RQ1 RNA-free DNase was obtained from Promega (Madison, WI) and RNase A was from Qiagen (Valencia, CA). Fico-Lite LymphoH® was obtained from Atlanta Biologicals (Norcross, GA) and red blood cell lysis buffer was from Sigma. Other reagents were purchased from Fisher (Pittsburgh, PA) or Sigma.

Animals

The experiment was performed using non-lactating cows: 20 Brahman, 11 Senepol, 20 Holstein and 20 Angus. Cows were moved to a common facility at the USDA-STARS and housed together for 20 weeks to ensure they had been exposed to similar environmental conditions. For each of 10 replicates (one replicate per week), the oestrous cycles of eight cows (two per breed) were synchronized with the administration of GnRH (100 µg, i.m.) on day 0, PGF_{2α} (12.5 mg, i.m.) on day 7,

PGF_{2α} (12.5 mg, i.m.) on day 8 and GnRH (100 μg, i.m.) on day 9. A 40 ml blood sample was collected from the jugular vein on day 0 and transported to the laboratory. Cows were slaughtered on day 12 after the first GnRH injection (about day 2 after oestrus). Ovaries were obtained after slaughter and morphological structures on each ovary (corpus luteum, corpus hemorrhagicum, corpus albicans and large preovulatory follicle) were recorded to determine whether ovulation had occurred. Research was conducted after approval by the local institutional Animal Care and Use Committee.

In vitro production of embryos

After slaughter and examination for ovarian structures, ovaries were sorted by breed, placed into buckets containing sterile saline (0.9% (w/v) NaCl solution) supplemented with 100 U penicillin-G ml⁻¹ and 0.1 mg streptomycin ml⁻¹, and transported to the laboratory at ambient temperature. Ovaries were washed several times with prewarmed sterile saline at 25–30°C to remove blood and debris. For each replicate, cumulus-oocyte complexes (COCs) were pooled from a pair of cows from each breed. COCs were obtained by slicing 2–10 mm follicles on the surface of the ovary into a beaker containing oocyte collection medium. COCs with at least one layer of compact cumulus cells were washed twice and matured in groups of 10 in 50 μl drops of oocyte maturation medium overlaid with mineral oil for 22 h at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. Groups of 30 COCs were transferred to four-well plates containing 600 μl IVF-TALP per well for fertilization. Frozen-thawed spermatozoa from each breed (three straws; a separate bull was used for each replicate to avoid confounding breed effects with bull) were purified by centrifugation on a Percoll gradient and resuspended in IVF-TALP to give an approximate concentration of 40 × 10⁶ spermatozoa ml⁻¹. Oocytes were fertilized with semen from the same breed by adding 25 μl sperm suspension and 25 μl PHE (0.5 mmol penicillamine l⁻¹, 0.25 mmol hypotaurine l⁻¹ and 25 μmol epinephrine l⁻¹ in 0.9% (w/v) NaCl) to each well. Presumptive zygotes were removed from fertilization wells, denuded of cumulus cells, washed two or three times in Hepes-TALP to remove remaining cumulus cells and associated spermatozoa, and placed in groups of 15–20 presumptive zygotes in 50 μl drops of modified KSOM overlaid with mineral oil at 38.5°C and 5% CO₂ (v/v) in humidified air.

The stage of embryonic development and cleavage rate were determined at day 4 after insemination. Embryos at > eight-cell stage were harvested from culture and randomly distributed to 25 μl drops of modified KSOM that were cultured at either 38.5°C continuously or 41.0°C (7% CO₂) for 6 h followed by culture at 38.5°C (5% CO₂) thereafter. Embryos were examined at this stage as this represents a period when the embryonic

genome would be activated and breed differences would be likely. The duration of heat shock was chosen to reduce development while avoiding exposing embryos to conditions so severe that breed differences in resistance to heat shock would be obscured. Moreover, the period of 6 h is within the mean range of periods for which heat-stressed lactating cows in Florida experience rectal temperatures above 40°C (1–8.5 h depending upon coat colour; Rivera and Hansen, 2001). The number of embryos per 25 μl drop of modified KSOM was fixed within runs and ranged between five and ten. The proportion of embryos that developed to the blastocyst stage was recorded at days 8 and 9 after insemination. Day 9 embryos were then processed for determination of number of cells using propidium iodide staining.

For statistical analysis, oocyte recovery rate, cleavage rates and the percentage of embryos developing to the blastocyst stage were calculated for each replicate (that is, pair of cows within a breed; $n=10$ for all breeds except Senepol where $n=6$ (5 pairs and 1 single cow)). The total number of oocytes examined for cleavage rate was 466 (Angus), 432 (Holstein), 784 (Brahman) and 295 (Senepol). The numbers of embryos > eight cells that were cultured were 115 (Angus, 38.5°C), 115 (Angus, 41°C), 111 (Holstein, 38.5°C), 127 (Holstein, 41°C), 258 (Brahman, 38.5°C), 256 (Brahman, 41°C), 89 (Senepol, 38.5°C) and 83 (Senepol, 41°C).

Determination of number of cells per embryo

Embryos were washed twice in 70 μl drops of PBS (10 mmol potassium phosphate l⁻¹, 0.9% (w/v) NaCl, pH 7.4) containing 1 mg polyvinylpyrrolidone ml⁻¹ (PBS-PVP) by transferring the embryos from drop to drop. Embryos with intact zona pellucida were fixed in 70 μl drops of 4% (w/v) paraformaldehyde in 100 μmol phosphate l⁻¹ with 0.9% (w/v) NaCl for 1 h at room temperature, washed twice in PBS-PVP, transferred to a poly-L-lysine coated slide and allowed to dry for 24 h at room temperature. Embryos were washed twice in PBS-PVP, incubated with 50 μg RNase A ml⁻¹ for 1 h at room temperature, and then with 0.5 μg propidium iodide ml⁻¹ for 30 min at room temperature. Embryos were washed four times in PBS-PVP to remove excess propidium iodide and mounted with coverslips with 16 μl medium containing Prolong Antifade®. Propidium iodide labelling was observed using a Zeiss Axioplan 2 fluorescence microscope with Texas red filter. The numbers of embryos analysed for number of cells were 84 (Angus, 38.5°C), 62 (Angus, 41°C), 53 (Holstein, 38.5°C), 70 (Holstein, 41°C), 133 (Brahman, 38.5°C), 139 (Brahman, 41°C), 61 (Senepol, 38.5°C) and 40 (Senepol, 41°C).

Oviductal and endometrial tissue

For a subset of cows, oviducts (from 14 Angus, 14 Holstein, 15 Brahman and 10 Senepol cows) and

uteri (from 12 Angus, 12 Holstein, 13 Brahman and 7 Senepol cows) were obtained after exsanguination and transported to the laboratory at ambient temperature. The oviducts were trimmed free of connective or vascular tissue and wiped with 70% (v/v) ethanol to remove blood and debris. Explants of endosalpinx were collected by stripping the oviducts of each cow. The oviducts were squeezed from the isthmus using sterile forceps and tissue from both oviducts of single cows were pooled together. Oviductal explants from each cow were divided into two equal amounts of tissue and placed in 60 mm Petri dishes containing 5 ml of pig MEM with 0.1 × the normal leucine concentration. The tissue wet mass was recorded (about 250 mg per dish). Tissue samples of intercaruncular endometrium from both uterine horns were collected, cut into 2–5 mm³ explants, washed two or three times, divided into four 100 mm Petri dishes containing 15 ml pig MEM with 0.1 × the normal leucine concentration, and the tissue wet mass was recorded (about 500 mg per dish). Oviductal and endometrial cultures were supplemented with 10 μCi [³H]leucine. Oviductal explants were cultured at 38.5°C or 41.0°C for 9 h. Endometrial explants were cultured at 38.5 or 41.0°C for 3 or 9 h. Oviduct and endometrium were exposed to heat shock of a longer duration than for embryos because of the assumption that embryonic development is more sensitive to heat shock than protein secretion by endometrium; the aim was to apply a heat shock great enough to compromise tissue function so breed × temperature interactions could be tested. At the end of the culture period, medium was harvested, centrifuged (1000 g, 10 min, 4°C) and stored at –20°C. Incorporation of [³H]leucine into newly synthesized protein secreted into the medium was determined by trichloroacetic acid (TCA) precipitation.

TCA precipitation

One inch square pieces of filter paper were soaked in 20% (w/v) TCA and allowed to dry. Aliquots (50 μl) of endometrial or oviductal medium from explants cultures were pipetted in duplicate on the filter paper and air dried. Filter papers were then sequentially soaked in 20% (w/v) TCA for 10 min, 5% (w/v) TCA for 20 min, 5% (w/v) TCA for 10 min and 95% (v/v) ethanol for 10 min, and 95% (v/v) ethanol for 5 min. Filter papers were then dried and subjected to scintillation spectrometry.

Preparation of lymphocytes

Lymphocytes were cultured as described by Kamwanja *et al.* (1994) except that the medium used was chosen because it was optimal for support of lymphocyte proliferation in sheep (Gottshall and Hansen, 1994) and also supported mitogen-induced lymphocyte proliferation in cattle (S. Tekin and P. J. Hansen, unpublished).

Jugular venous blood (40 ml) was centrifuged at 2000 g for 30 min. The buffy coat was removed and mixed into 2 ml TCM-199. The cell suspension was transferred on top of 2 ml Fico-Lite LymphoH in a 13 ml tube (100 × 16 mm) and centrifuged at 2000 g for 30 min. Mononuclear cells (85–95% lymphocytes) were then collected from the Fico-Lite interface, transferred to 2 ml red cell lysis buffer for 20 s and diluted with 4 ml Dulbecco's PBS at twice the normal strength. Lymphocytes were centrifuged at 1000 g for 5 min and resuspended with modified TCM-199 (TCM-199 supplemented with 5% (v/v) horse serum, 200 U penicillin ml⁻¹, 0.2 mg streptomycin ml⁻¹, 1 mmol glutamine l⁻¹ and 10⁻⁵ mol β-mercaptoethanol l⁻¹). The number of lymphocytes was determined using a haemocytometer and adjusted to 1 × 10⁶ cells ml⁻¹. Lymphocytes were then assigned to treatments and cultured in modified TCM-199 to determine heat-induced apoptosis and intracellular glutathione.

Lymphocyte apoptosis

Effects of heat shock on apoptosis were evaluated for lymphocyte preparations from eight cows from each breed. For each cow, a total of 1–5 ml lymphocytes was cultured in 100 × 16 mm sterile tubes at 38.5°C for 24 h or at 41.0°C for 9 h followed by 15 h at 38.5°C. Lymphocytes were centrifuged for 10 min at 1000 g and the pellet resuspended in 5 ml PBS–PVP after culture. Lymphocytes were centrifuged again and the pellet was resuspended and incubated in 5 ml 4% (w/v) paraformaldehyde in 100 μmol phosphate l⁻¹ with 0.9% (w/v) NaCl for 1 h at room temperature. Lymphocytes were then washed in PBS–PVP by centrifugation at 1000 g for 10 min and resuspended in 1 ml PBS–PVP. A 100 μl aliquot of lymphocyte suspension was transferred to a poly-L-lysine coated slide and allowed to dry for at least 24 h at room temperature. Slides were stored at 4°C until TdT-mediated dUTP nick-end labelling (TUNEL) staining.

Slides were washed twice in PBS–PVP (2 min per wash) and cells were permeabilized with 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h at room temperature for TUNEL staining. Positive controls were incubated with RQ1 RNase-free DNase (50 U ml⁻¹) at 37°C for 1 h. Lymphocytes were washed in PBS–PVP and incubated with 50 μl TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Lymphocytes were then incubated with RNase A (50 μg ml⁻¹) for 1 h at room temperature, followed by 0.5 μg propidium iodide ml⁻¹ for 30 min at room temperature. Lymphocytes were washed four times in PBS–PVP to remove excess propidium iodide and coverslips were mounted with

medium containing Prolong Antifade® as recommended by the manufacturer. Slides were observed using a Zeiss Axioplan 2 fluorescence microscope with dual filter. The percentage of apoptotic lymphocytes was determined by counting the total number of nuclei and total number of TUNEL-labelled nuclei in 100 cells at a total of 10 different sites on the slide.

Lymphocyte glutathione content

Effects of heat shock on glutathione content were evaluated for lymphocyte preparations from 16 (Angus, Holstein, and Brahman) or seven (Senepol) cows from each breed. A total of 1–5 ml lymphocytes was cultured in 100 × 16 mm sterile tubes at 38.5°C or 41.0°C for 9 h. At the end of culture, lymphocytes were centrifuged at 1000 *g* for 10 min. The supernatant was discarded and cells were diluted with 1 ml deionized water. After 2 min, samples were centrifuged for 5 min at 1000 *g* and the supernatant was stored at –20°C until assay for glutathione.

Glutathione content was assayed in duplicate using a previously described assay (Baker *et al.*, 1990; Aréchiga *et al.*, 1995) based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid using glutathione. Fifty microlitres of sample or standards (1.6875–200 pmol per well) was pipetted into duplicate wells of a 96-well microtitre plate. This was followed by addition of 100 µl of a freshly prepared reaction mixture containing 5 ml 1 mmol DNTB l⁻¹, 5 ml 1 mmol NADPH l⁻¹, 5.75 ml 100 mmol NaPO₄ buffer l⁻¹, pH 7.5 with 1 mmol EDTA l⁻¹ and 0.1 ml 200 U glutathione reductase ml⁻¹. Absorbance at 405 nm of each well of the 96-well plate was determined at 2 min intervals using a microtitre plate reader (model EL 309; BIO-TEK Instruments Inc.).

Statistical analysis

Data were analysed by least-squares ANOVA using the mixed models procedure of SAS (SAS, 1989). Percentage data were transformed using arc sin transformation before analysis. Independent variables varied according to experimental design. The mathematical model included main effects and all interactions. For variables in which each cow was measured more than once, effect of cow nested within breed was considered random and all the other main effects were fixed. Tests of significance were conducted using appropriate error terms as determined by calculating expected mean squares. Effects of breed and breed × temperature were partitioned into individual orthogonal contrasts with the following breed comparisons: Brahman and Senepol versus Angus and Holstein; Brahman versus Senepol; and Angus versus Holstein. The percentage of embryos developing to the blastocyst stage was analysed with and without Senepol cows in the data set.

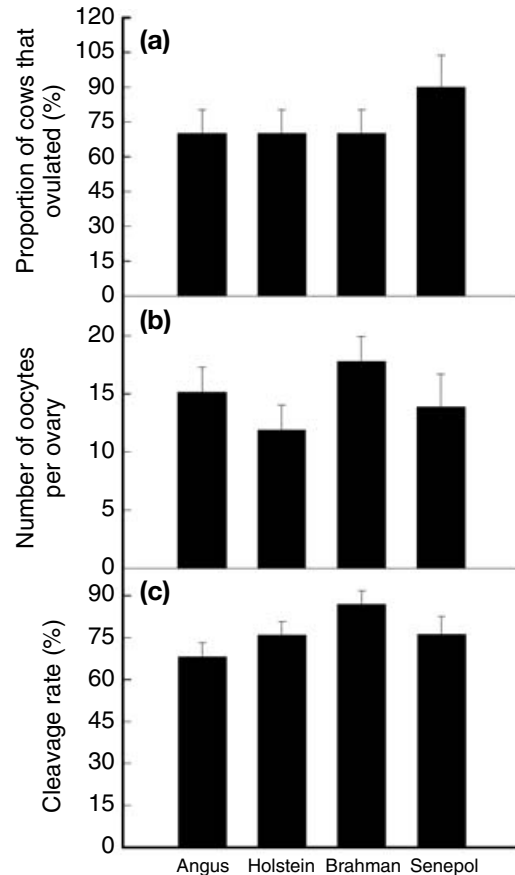


Fig. 1. Breed differences in the proportion of cows that ovulated after oestrous synchronization, oocyte recovery and cleavage rate after *in vitro* maturation and fertilization. Results are least-squares means ± SEM. There was no difference among breeds in (a) the proportion of cows that ovulated or (b) the number of oocytes recovered per ovary. However, there was a breed effect for cleavage rate (c) ($P < 0.05$). Separation of the breed effect into individual degree-of-freedom orthogonal comparisons revealed the following trends: Brahman and Senepol versus other breeds, $P = 0.07$; Brahman versus Senepol, $P = 0.07$.

Results

Breed differences in resistance of embryos to heat shock

There was no difference in the proportion of cows that ovulated after synchronization (Fig. 1a) or number of oocytes recovered per ovary among breeds (Fig. 1b). However, there was a breed effect for cleavage rate ($P < 0.05$). Cleavage rate tended ($P = 0.07$) to be higher for Brahman than for other breeds (Fig. 1c).

The stage of embryonic development for cleaved embryos was assessed visually for each replicate on day 4 after insemination. There was no difference among breeds on stage of embryonic development at day 4 after insemination (Brahman = 8.38 ± 0.50 cells; Holstein = 8.05 ± 0.91 cells; Angus = 7.68 ± 1.2 cells; and Senepol = 8.90 ± 1.01 cells). Therefore, embryos from

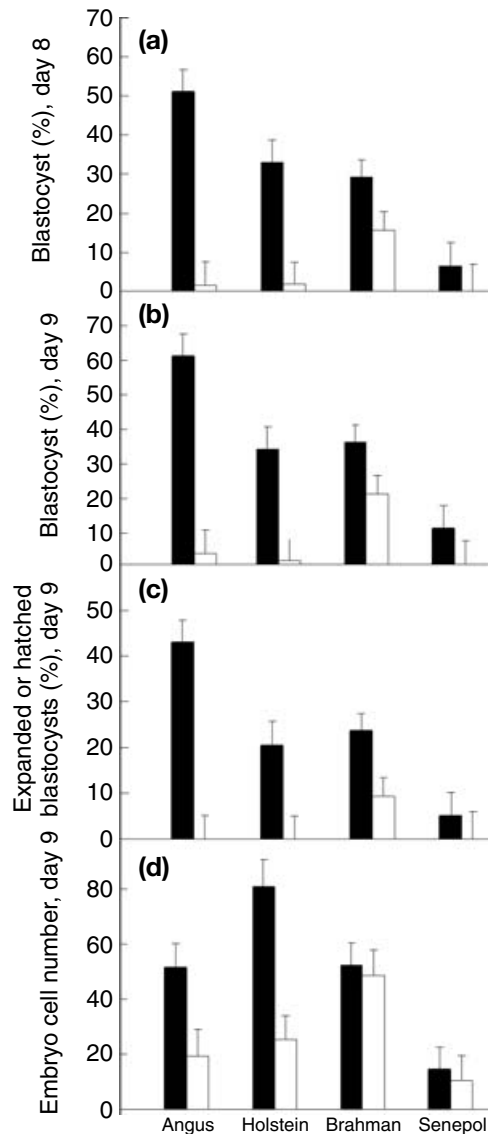


Fig. 2. Effect of breed on the action of heat shock (■: 38.5°C, □: 41.0°C) on embryonic development in cows. Results are least-squares means \pm SEM. Data were analysed excluding Senepol cows because of the low development of this breed. Heat shock of 41°C for 6 h reduced the percentage of embryos that developed to the blastocyst stage on (a) day 8 ($P < 0.001$) and (b) day 9 ($P < 0.001$) after insemination; (c) the proportion of embryos that became expanded or hatched blastocysts ($P < 0.001$); and (d) the total number of cells per embryo ($P < 0.01$). There was a breed \times temperature interaction for each of these variables ($P < 0.05$ for the percentage of blastocysts at days 8 and 9 and $P < 0.05$ for the percentage of embryos that were expanded or hatched blastocysts at day 9 and total number of cells). These interactions reflect the fact that the negative effect of heat shock was less pronounced for embryos from Brahman cows than for embryos from Angus and Holstein cows (Brahman versus others \times temperature; $P < 0.05$ for each variable) and because responses differed between Angus and Holstein breeds ((Angus versus Holstein) \times temperature; $P = 0.05$ for the percentage of blastocysts and the percentage of expanded or hatched blastocysts at day 9; total number of cells).

different breeds were at the same developmental stage when they were selected for treatments.

Embryos derived from oocytes from Senepol cows had very low development to the blastocyst stage at 38.5°C and it was not possible to determine heat shock effects. Therefore, data from embryos from Senepol cows were removed from the final analysis. Exposure of embryos at > eight-cell stage to heat shock of 41.0°C for 6 h reduced development to the blastocyst stage on days 8 ($P < 0.001$; Fig. 2a) and 9 ($P < 0.001$; Fig. 2b) after insemination. However, the negative effect of heat shock on development of embryos to the blastocyst stage was less pronounced for embryos from Brahman cows than for embryos from Holstein or Angus cows on days 8 (breed \times temperature, $P < 0.05$; Fig. 2a) and 9 (breed \times temperature, $P < 0.05$; Fig. 2b). The proportion of embryos that became expanded or hatched blastocysts on day 9 after insemination was also reduced ($P < 0.001$) by heat shock and the deleterious effect of heat shock was less pronounced in embryos from Brahman cows than embryos from Holstein and Angus cows (breed \times temperature, $P < 0.05$; Fig. 2c). Moreover, heat shock reduced the total number of cells per embryo for Holstein and Angus cows but not for Brahman cows (breed \times temperature, $P < 0.05$; Fig. 2d).

Oviductal and endometrial explants

Culturing oviductal explants at 41.0°C for 9 h (Fig. 3) or endometrial explants at 41.0°C for 3 or 9 h (Fig. 4) had no effect on incorporation of [³H]leucine into newly synthesized proteins secreted into the medium. In general there were no breed effects except that oviductal protein secretion tended to be higher for Brahman than for other breeds (Brahman versus Senepol breeds; $P < 0.07$).

Lymphocyte apoptosis and glutathione content

Exposure of bovine lymphocytes to 41.0°C for 9 h increased ($P < 0.001$) the proportion of apoptotic cells (Fig. 5). However, there was a trend for a breed \times temperature interaction ($P = 0.07$; Fig. 5), although the effect was not significant. Further analysis revealed that the (Brahman plus Senepol breeds versus Angus plus Holstein breeds) \times temperature interaction was significant ($P < 0.05$) but that there were no interactions with temperature for Brahman versus Senepol breeds and Angus versus Holstein breeds. Thus, heat-induced apoptosis was less for Brahman and Senepol breeds than for Angus and Holstein breeds. Differences between Brahman and Senepol breeds in the effect of heat shock were difficult to interpret because the apoptosis at 38.5°C was more extensive for Senepol cows than Brahman cows. Accordingly, the effect of heat shock was also examined by calculating for each cow the absolute heat-induced increase in apoptosis (that is, percentage

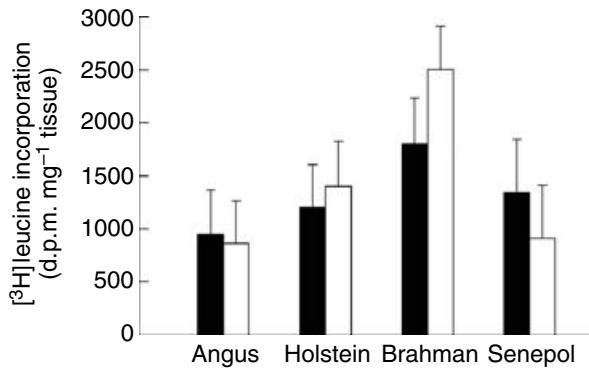


Fig. 3. Breed differences in heat shock (■: 38.5°C, □: 41.0°C)-induced changes in incorporation of [³H]leucine into trichloroacetic acid-precipitable protein secreted into the medium of oviductal explants from cows. There was no effect of temperature. There were also no breed effects except that secretion of oviductal protein tended to be higher for Brahman than for other breeds (Brahman versus Senepol breeds; $P < 0.07$).

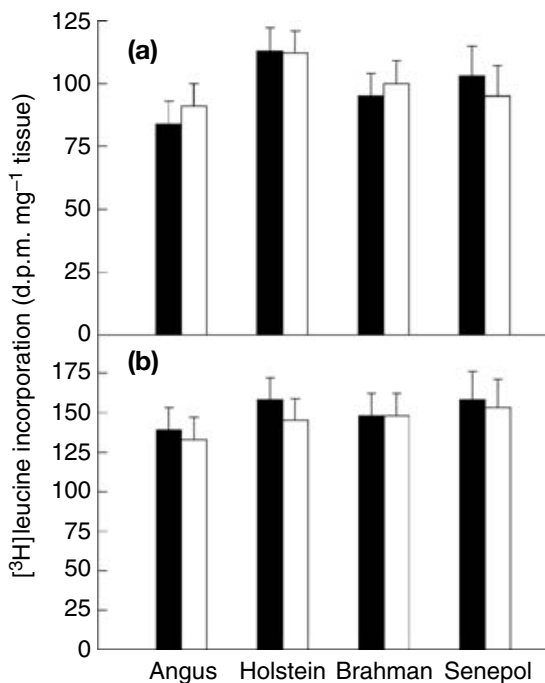


Fig. 4. Breed differences in heat shock (■: 38.5°C, □: 41.0°C)-induced changes in incorporation of [³H]leucine into trichloroacetic acid-precipitable protein into the medium of endometrial explants from cows after culture for (a) 3 h and (b) 9 h. Results are least-squares means \pm SEM. There was no effect of breed or temperature.

apoptotic at 41.0°C minus percentage apoptotic at 38.5°C). By this criterion, the increase in apoptosis caused by heat shock was greater ($P < 0.05$) for Angus ($6.4 \pm 1.3\%$) and Holstein ($4.1 \pm 1.4\%$) cows than for Brahman ($1.9 \pm 1.3\%$) and Senepol ($1.9 \pm 1.4\%$) cows

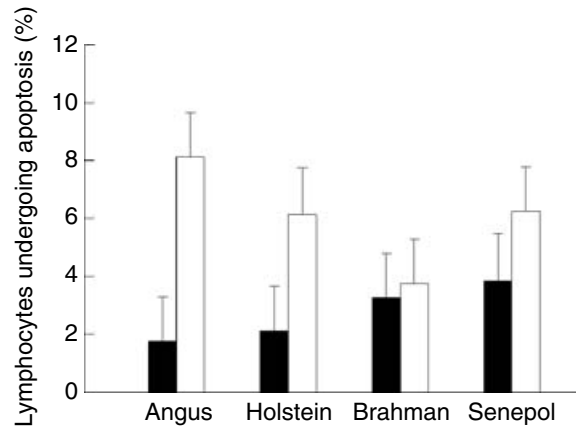


Fig. 5. Effect of breed on heat shock (■: 38.5°C, □: 41.0°C)-induced apoptosis in lymphocytes in cows. Results are least-squares means \pm SEM. Exposure of lymphocytes to 41°C for 9 h increased ($P < 0.001$) the proportion of cells undergoing apoptosis as determined by the TdT-mediated dUTP nick-end labelling procedure. There was a trend for a breed \times temperature interaction ($P = 0.07$). Separation of this interaction into individual degree-of-freedom, orthogonal contrasts revealed that Brahman and Senepol breeds responded to heat shock in a different way than Angus and Holstein breeds ((Brahman plus Senepol breeds versus Angus plus Holstein breeds) \times temperature; $P < 0.05$) but that responses were similar for Brahman versus Senepol breeds and Angus versus Holstein breeds (that is, interactions were not significant).

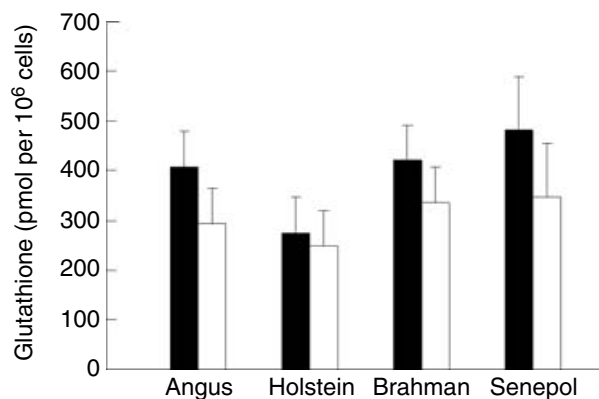


Fig. 6. Breed differences in heat shock (■: 38.5°C, □: 41.0°C)-induced changes in intracellular glutathione content of lymphocytes in cows. Results are least-squares means \pm SEM. Heat shock reduced ($P < 0.01$) intracellular glutathione in lymphocytes but there was no effect of breed or breed \times temperature.

but was similar for Brahman and Senepol cows and for Angus and Holstein cows.

In another experiment, heat shock of 41.0°C for 9 h reduced ($P < 0.01$) intracellular glutathione content in lymphocytes but there were no effects of breed or breed \times temperature (Fig. 6).

Discussion

Exposure to heat stress has a less deleterious effect on fertility of breeds of cattle adapted to hot climates than on that of breeds from temperate climates (Turner, 1982; Madalena *et al.*, 1990; Rocha *et al.*, 1998). This breed difference has been attributed to the superior ability of heat-tolerant cattle to regulate body temperature in the presence of heat stress (Adeyemo *et al.*, 1979; Bennett *et al.*, 1985; Hammond *et al.*, 1996; Gaughan *et al.*, 1999). The present study demonstrates that genetic adaptations are also present at the cellular level and that Brahman and Senepol cattle have acquired mechanisms to protect cells against the deleterious effect of high temperature. In particular, embryos from Brahman cattle are better able to survive exposure to increased temperature than embryos from Angus and Holstein, two heat-sensitive breeds of cattle. In addition, lymphocytes from Brahman and Senepol cattle experience less apoptosis after heat shock than do lymphocytes from other breeds. Thus, Brahman and Senepol cattle not only regulate body temperature in response to heat stress more effectively than breeds from temperate climates (Hammond *et al.*, 1996, 1998; Gaughan *et al.*, 1999), but also hyperthermia that results from heat stress is less likely to have severe effects on cellular function. Natural selection for traits that confer cellular resistance to high or low temperature has been described for ectothermic species (Johnston and Walesby, 1977; Crawford and Powers, 1989; Behan-Martin *et al.*, 1993; Somero *et al.*, 1996). To the authors' knowledge, the finding that cellular thermotolerance is different among breeds of cattle is the only case in which closely related homeotherms have been shown to have undergone such genetic divergence.

Differences in cellular thermotolerance between tissues from Brahman cattle versus Holstein and Angus cattle have been described for reproductive tract tissues (Malayer and Hansen, 1990) and lymphocytes (Kamwanja *et al.*, 1994). The present observation that this genetic difference is present in the preimplantation embryo makes this phenomenon physiologically relevant to embryonic mortality caused by heat stress. Although heat shock of 43°C can compromise oviductal and endometrial function (Malayer *et al.*, 1988; Putney *et al.*, 1988; Malayer and Hansen, 1990), this temperature is much higher than would be experienced by a heat-stressed cow (39.5–41.0°C). At more physiologically relevant temperatures, such as 41.0°C used in the present study, oviductal and endometrial tissue are not affected by heat shock. In contrast, as shown in the present study and elsewhere (Rivera and Hansen, 2001; Al-Katanani and Hansen, 2002; Paula-Lopes and Hansen, 2002), exposure of embryos to 41.0°C inhibits embryonic development. Identification of the genes controlling cellular thermotolerance in Brahman cattle may, therefore, lead to genetic strategies for improving embryonic survival during heat stress in breeds that are

not thermotolerant. It is important to determine the stage in development at which genetic differences in embryonic resistance to heat shock become evident. Bovine embryos are most sensitive to heat shock in the first few cleavage divisions (Edwards and Hansen, 1997; Krininger *et al.*, 2002). As this period is also one of limited gene expression (Memili and First, 2000), it is possible that genetic differences in thermotolerance will not be expressed until after embryonic genome activation is fully developed, that is, at the 8–16-cell stage in bovine embryos.

Like the Brahman breed, the Senepol breed is adapted for hot climates (Hammond *et al.*, 1996; Gaughan *et al.*, 1999) although it is *B. taurus* rather than *B. indicus*. In a previous study, lymphocytes from Senepol cows were more resistant to the lethal effects of exposure to 42 or 45°C than lymphocytes from Angus cows (Kamwanja *et al.*, 1994). The results of the present study are consistent with this finding as the effect of heat shock on the percentage of cells undergoing apoptosis was less for Brahman and Senepol cows than for Angus and Holstein cows. Moreover, the change in the percentage of apoptotic cells resulting from heat shock was similar for Senepol and Brahman cows. Unfortunately, the present study was not able to conclude whether embryos from Senepol cows are more resistant to heat shock than embryos from Angus and Holstein cows because the low development of embryos from Senepol cows at 38.5°C precluded a test of heat shock effects on development in this breed. The reason for low development in the Senepol breed is not known but could include conditions for freezing spermatozoa, oocyte maturation, or embryo culture that are inappropriate for that breed.

Little is known about the biochemical basis for cellular resistance to increased temperature in Brahman or Senepol cattle. Heat shock can cause increased free radical metabolism (Loven, 1998) but the lack of breed effect on the reduction in glutathione content caused by heat shock does not support the idea that the Brahman or Senepol breeds have a different antioxidant status than the other breeds tested. Additional studies on this topic are warranted, given the complexity of free radical biology. There is also no evidence for genetic differences in heat shock protein 70 responses among lymphocytes from Brahman, Angus and Senepol cows (Kamwanja *et al.*, 1994). The results of the present study indicate that lymphocytes from Brahman and Senepol cows are less likely to undergo apoptosis in response to heat shock than lymphocytes from Angus and Holstein cows. Perhaps the signal that leads to heat-induced apoptosis is dampened in Brahman and Senepol cows (for example, activation of membrane sphingomyelinase; Haimovitz-Friedman *et al.*, 1997; Pena *et al.*, 1997) or the ratio of pro-apoptotic to anti-apoptotic modulatory proteins is different in lymphocytes from Brahman and Senepol cows than in lymphocytes from Angus and Holstein cows. Whether cells from Brahman and Senepol cows are

more resistant to heat shock because they do not die through apoptosis or rather do not undergo apoptosis because cellular physiology is not compromised by heat shock needs to be established before the significance of breed differences in apoptosis can be understood.

In addition, it is necessary to determine whether breed differences in the induction of apoptosis in lymphocytes are relevant to other tissues as well. Preimplantation bovine embryos undergo apoptosis in response to heat shock (Paula-Lopes and Hansen, 2002). Perhaps one reason that embryos from Brahman cows are more resistant to heat shock is because the apoptotic pathway is less activated by heat shock for embryos of this breed than for embryos of Angus or Holstein cows.

One of the features of reproductive function in mammals is the large impact of the environment on reproductive success (Bronson, 1989; Thatcher and Hansen, 1993). Given this fact, and the crucial importance of reproduction in natural selection, it is not surprising that natural selection has resulted in divergence in the extent to which environmental stimuli can alter reproductive function. Such divergence has been demonstrated in rodents for control of reproduction by photoperiod (Dark *et al.*, 1983). The present study provides evidence that there is also genetic divergence in response of embryonic tissues to heat shock. The importance of this genetic adaptation for determining superior reproductive function of Brahman cattle during heat stress (Turner, 1982; Madalena *et al.*, 1990; Rocha *et al.*, 1998) probably depends upon the magnitude of heat stress. Certainly, the superior ability of the Brahman breed to maintain a constant body temperature under high environmental temperature (Hammond *et al.*, 1996; Hammond *et al.*, 1998; Gaughan *et al.*, 1999) is a major contributor to resistance of the Brahman breed to the deleterious effects of heat stress on reproduction. In addition, mild heat stress can result in some embryonic loss even when body temperatures are too low to block embryonic development directly (Dunlap and Vincent, 1971; Rivera and Hansen, 2001), and it is unlikely that genetic differences in embryonic resistance to heat shock would be of importance under such conditions. However, when severe heat stress occurs, such that core body temperatures reach temperatures such as 41°C that can directly block embryonic development, a greater ability of embryos from Brahman cows to function and develop after such heat shocks could contribute to the superior reproductive performance of the Brahman breed during heat stress.

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