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Gene expression profile in heat-shocked Holstein and Nelore oocytes and cumulus cells

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Abstract. The present study determined the transcriptome profile in Nelore and Holstein oocytes subjected to heat shock during IVM and the mRNA abundance of selected candidate genes in Nelore and Holstein heat-shocked oocytes and cumulus cells (CC). Holstein and Nelore cows were subjected to *in vivo* follicle aspiration. Cumulus–oocyte complexes were assigned to control (38.5°C, 22 h) or heat shock (41°C for 12 h, followed by 38.5°C for 10 h) treatment during IVM. Denuded oocytes were subjected to bovine microarray analysis. Transcriptome analysis demonstrated 127, nine and six genes were differentially expressed between breed, temperature and the breed × temperature interaction respectively. Selected differentially expressed genes were evaluated by real-time polymerase chain reaction in oocytes and respective CC. The molecular motor kinesin family member 3A (*KIF3A*) was upregulated in Holstein oocytes, whereas the pro-apoptotic gene death-associated protein (*DAP*) and the membrane trafficking gene DENN/MADD domain containing 3 (*DENND3*) were downregulated in Holstein oocytes. Nelore CC showed increased transcript abundance for tight junction claudin 11 (*CLDN11*), whereas Holstein CC showed increased transcript abundance for antioxidant metallothionein 1E (*MT1E*) . Moreover, heat shock downregulated antioxidant *MT1E* mRNA expression in CC. In conclusion, oocyte transcriptome analysis indicated a strong difference between breeds involving organisation and cell death. In CC, both breed and temperature affected mRNA abundance, involving cellular organisation and oxidative stress.

Additional keywords: *Bos taurus indicus*, *Bos taurus taurus*, mRNA, microarray, real-time polymerase chain reaction.

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Introduction

Genetic divergences have been observed in animal breeds that evolved under different environmental conditions. For example, *Bos taurus indicus* animals are less sensitive to the deleterious effects of heat stress than *Bos taurus taurus* (Rocha *et al.* 1998). High environmental temperatures during the hot months of the year reduce reproductive performance in cattle. Furthermore, the magnitude of the deleterious effects of heat stress on fertility is more pronounced in high-producing dairy cows because the high metabolic heat production associated with lactation can lead to hyperthermia (Al-Katanani *et al.* 1999). The resistance of *B. taurus indicus* to heat stress is related to their

thermoregulatory efficiency (Hansen 2004) and cellular thermotolerance (Paula-Lopes *et al.* 2003, 2013).

Heat stress promotes a series of physiological and cellular alterations in different tissues compromising follicular growth (Wolfenson *et al.* 1995), hormonal secretion (Roth *et al.* 2000), endometrial function (Malayer *et al.* 1988), uterine blood flow (Roman-Ponce *et al.* 1978) and preimplantation embryonic development (Ealy *et al.* 1993), as well as the function of oocytes (Al-Katanani *et al.* 2002) and cumulus cells (CCs; Rispoli *et al.* 2013). It has already been demonstrated that exposure of bovine oocytes to high temperatures reduces fertilisation and oocyte developmental competence (Roth and Hansen 2005).

Heat-induced damage in bovine oocytes causes cytoplasmic changes, such as a reduction in oocyte protein synthesis (Edwards and Hansen 1996), disorganisation of microfilaments and microtubules (Roth and Hansen 2005), changes in cortical granule redistribution (Maya-Soriano *et al.* 2013) and a reduction in mitochondrial activity (Paula-Lopes *et al.* 2013). Heat shock also affects nuclear events, reducing oocyte meiotic maturation (Roth and Hansen 2005) and inducing DNA fragmentation (Roth and Hansen 2004). Moreover, high temperature affects oocyte quality at the molecular level. Exposure of germinal vesicle (GV) oocytes to seasonal heat stress increased the abundance of heat shock protein 70 (*HSP70*) transcript; this heat-induced increase in *HSP70* mRNA was greater for Holstein than Gyr oocytes (Camargo *et al.* 2007). Seasonal heat stress also reduced oocyte relative abundance of transcripts involved in oocyte maturation and preimplantation embryonic development (Gendelman and Roth 2012). However, very little is known regarding the effects of heat stress during oocyte maturation on gene expression.

The bovine oocyte is surrounded by layers of CCs. Bidirectional communication between the oocyte and its CCs plays an important role in oocyte growth, metabolism (Haghighat and Van Winkle 1990) and maturation (Tanghe *et al.* 2002). Removal of CCs before IVM is detrimental to oocyte maturation in cattle (Chian and Sirard 1995). Similarly, a reduction in CC function may affect both the oocyte and CCs. Exposure of cumulus–oocyte complexes (COCs) to heat shock during IVM induced CC DNA fragmentation (Nabenishi *et al.* 2012) and altered the transcriptome profile in CCs (Rispoli *et al.* 2013).

Bovine preimplantation embryonic development is dependent on mRNA stored during oocyte growth (Trounson *et al.* 2001). Translational control pathways, such as translation activation through polyadenylation, transcript silencing and degradation through desadenylation, ensure mRNA storage required for oocyte competence and subsequent preimplantation embryonic development (Decker and Parker 1994; Brevini-Gandolfi and Gandolfi 2001). The bovine oocyte is transcriptionally quiescent during maturation. Indeed, oocyte transcriptional activity has been shown to be high until the oocyte reaches 110 µm (2- to 3-mm diameter follicle), decreases up to the GV stage and decreases markedly as the oocyte reaches the MII stage (Brevini-Gandolfi and Gandolfi 2001). For example, the amount of poly(A) RNA was reported to be highest in GV oocytes and to drop after 12 h IVM (Payton *et al.* 2011). Interestingly, exposure of bovine oocytes to 41°C heat shock during the first 12 h of IVM did not affect oocyte total RNA, RNA size distribution or poly(A) RNA (Payton *et al.* 2011). Hence the molecular mechanisms triggered by elevated temperature in bovine oocytes during maturation remain unknown, as do the determinant factors related to thermotolerance between *B. taurus taurus* and *B. taurus indicus*.

Thus, the aim of the present study was to evaluate the hypothesis that differences in thermotolerance between *B. taurus indicus* and *B. taurus taurus* oocytes are due to differential gene expression. Therefore, the global gene expression profile was determined in Nelore and Holstein oocytes subjected to heat shock during IVM. In addition, candidate genes selected from oocyte microarray were evaluated by real-time polymerase chain reaction (PCR) in Nelore and Holstein oocytes and their surrounding CCs. Even though the gene expression profile between oocytes and CCs can differ, the present study also evaluated how genes differentially expressed on oocyte microarray behave in CCs subjected to the same treatments.

Materials and methods

Unless stated otherwise, all reagents and media were purchased from Sigma-Aldrich. Animals were managed according to the School of Veterinary Medicine and Animal Sciences Bioethical Committee from University of São Paulo (São Paulo, Brazil).

Experimental design

The experiment was designed as a 2 × 2 factorial to evaluate the effects of breed (Holstein and Nelore) and temperature (38.5°C and 41°C) on differential gene expression in bovine oocytes (microarray and real-time PCR) and CCs (real-time PCR). Therefore, Holstein and Nelore COCs were randomly assigned to *in vitro* heat shock (41°C for 12 h, followed by 38.5°C for 10 h) and control (38.5°C, 22 h) treatments during IVM.

Animals

Non-lactating Holstein ($n = 13$) and Nelore ($n = 14$) cows were housed together at the central-east region of Sao Paulo State, Brazil (22°42′30″S, 47°38′30″W; 546 m above sea level) and maintained under the same management conditions, including maintenance diet. Animals were 4–8 years of age, with a mean (± s.e.m.) body condition score (on a scale of 1–5) of 3.1 ± 0.1 for Nelore and 2.8 ± 0.2 for Holstein and mean (± s.e.m.) weight of 508 ± 17 kg for Nelore and 575 ± 20 kg for Holstein.

One week before follicular aspiration, a data logger was installed in the free stall to monitor environmental temperature (ET; °C) and relative air humidity (RH; %) each hour of the day during the entire experiment. Average ET and RH were determined to calculate the temperature and humidity index (THI) (Kelly and Bond 1971) in order to characterise environmental conditions. According to Armstrong (1994), THI <72 indicates the absence of heat stress, THI 72–78 indicates mild heat stress, THI 79–88 indicates moderate heat stress and THI 89–98 indicates severe heat stress. Rectal temperature (RT; °C) and respiratory rate (RR; movements per minute (m.p.m.)) of each animal were also determined during follicular aspiration.

Oocyte collection

Ovum pick-up (OPU; $n = 12$) was performed during the winter and spring seasons (July–November). The device used was an Aloka SSD-500 ultrasound with a sector transducer of 5 MHz adapted to an 18-G needle connected to a sterile test tube and a vacuum line (85 mmHg). Recovered COCs were transferred to 50-mL conical tubes containing 10 mL phosphate-buffered saline (PBS) supplemented with 5 IU mL^{-1} heparin. COCs were transferred to 80 µL washing medium (TCM 199–HEPES supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO BRL), 2 µg mL^{-1} pyruvate and 78 µg mL^{-1} amikacin sulfate) and stored in cryotubes containing 400 µL transport medium (TCM 199–HEPES supplemented with 10% (v/v) FBS, 22 µg mL^{-1} pyruvate, 78 µg mL^{-1} amikacin sulfate, 1 µg mL^{-1} FSH (Folltropin; Bioniche) and 10 IU mL^{-1} LH (Lutropin-V; Bioniche)) under mineral oil. The cryotubes were transported to the laboratory at 38.5°C in a portable incubator.

IVM

Oocytes classified as Grade I, II and III containing one or more layers of compact CCs and homogeneous cytoplasm were used in the experiment (Leibfried and First 1979). COCs were transferred to 50-µL microdrops of IVM medium (TCM 199–bicarbonate containing 10% (v/v) FBS, 22 µg mL^{-1} pyruvate, 75 µg mL^{-1} gentamycin, 1 µg mL^{-1} FSH and 10 IU mL^{-1} LH) under mineral oil. At each OPU ($n = 12$), Holstein and Nelore COCs were randomly distributed into control (38.5°C, 22 h) and heat shock (41°C for 12 h, followed by 38.5°C for 10 h) treatment groups during IVM under an atmosphere of 5% of CO_2 in humidified air. The total number of COCs collected for each treatment was as follows: Nelore control, 302 COCs; Nelore heat shock, 316 COCs; Holstein control, 102 COCs; and Holstein heat shock, 106 COCs. A separate group of Nelore slaughterhouse-derived COCs was matured *in vitro*, fertilised and cultured. These COCs were used as an IVF laboratory control. The percentage of oocytes that cleaved at Day 2 and reached the blastocyst stage at Day 7 after insemination was $84.5 \pm 2.3\%$ and $39.5 \pm 2.5\%$ respectively. Both IVF and values of cleaved and reached blastocyst were obtained by Silva *et al.* (2013).

Removal and storage of CCs

After maturation, COCs were initially denuded by repeated pipetting in 100-µL drops of Ca^{2+}- and Mg^{2+}-free PBS. Oocytes were removed from the drop and CCs ($n = 5$ pools in 100 µL per experimental group) were stored at −80°C until real-time PCR analysis. Partially denuded oocytes were transferred to 50-µL microdrops containing 10 000 IU mL^{-1} hyaluronidase at 38°C for 3–7 min, followed by incubation in 50-µL microdrops containing 5 mg mL^{-1} protease for 5–10 s. Denuded oocytes were washed in 50-µL microdrops of Ca^{2+}- and Mg^{2+}-free PBS containing 5% (v/v) FBS for protease inactivation and washed three times in 50-µL microdrops of Ca^{2+}- and Mg^{2+}-free PBS. Oocytes ($n = 3$ replicates of 25 putative MII oocytes per experimental group) were stored at −80°C until microarray and real-time PCR analysis.

RNA extraction, amplification and concentration

Total RNA was extracted from oocytes using the RNeasy Mini Kit (Qiagen) as recommended by the manufacturer. Total RNA quality and yield were determined using a Bioanalyser 2100 (Agilent Technologies) with an RNA 6000 Pico LabChip Kit (Agilent Technologies) and spectrophotometer (NanoDrop ND-2000 UV-Vis Spectrophotometer; NanoDrop Technologies). To obtain 100 ng RNA from oocyte pools, samples were amplified using a MessageAmp II aRNA Amplification Kit (Ambion) according to the manufacturer's instructions. Briefly, samples were subjected to the reverse transcription process, double-stranded cDNA synthesis and *in vitro* transcription for amplified RNA (aRNA) synthesis. Samples that did not reach the desired concentration were further concentrated (Concentrator 5301; Eppendorf) for short intervals of 5 and 10 min over a period of 40 min, at room temperature. aRNA was quantified in a NanoDrop spectrophotometer and stored at −80°C.

Total RNA was extracted from CCs using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was quantified in a NanoDrop spectrophotometer and stored at −80°C.

Oocyte microarray analysis

Total RNA samples (100 ng) were subjected to reverse transcription reaction for cDNA formation. cDNA was used as template for the *in vitro* transcription reaction resulting in biotin-labelled aRNA. Then, aRNA was fragmented using a 3′IVT Express Kit (Affymetrix) and subjected to hybridisation using a GeneChip Bovine Array (Affymetrix) according to the manufacturer's instructions. GeneChips were scanned using a GeneChip scanner (Model 3000 7G; Affymetrix). In all, 75 oocytes per treatment ($n = 3$ replicates of 25 putative MII oocytes per experimental group) were subjected to microarray analysis. DAVID Bioinformatics Database software (www.david.abcc.ncifcrf.gov, accessed 15 February 2013; Huang *et al.* 2009) and Ingenuity pathways analysis (IPA; Ingenuity Systems; www.ingenuity.com, accessed 15 February 2013) were used to identify functions and relationships that were most significant between genes.

Oocyte microarray validation by real-time PCR

Following microarray, genes associated with cellular changes triggered by heat shock were selected for validation by real-time PCR (Table 1). Primers for kinesin family member 3A (*KIF3A*), claudin 11 (*CLDN11*), baculoviral IAP repeat-containing 3 (*BIRC3*), death-associated protein (*DAP*), metallothionein 1E (*MT1E*), chaperonin containing TCP1, subunit 4 (*CCT4*), acyl-coenzyme A oxidase 1, palmitoyl (*ACOX1*), dicer 1, ribonuclease type III (*DICER1*) and DENN/MADD domain containing 3 (*DENND3*) were designed from sequences available in the GenBank database. Selected primers were synthesised by Integrated DNA Technologies (IDT) Inc. Nucleotide sequences and the annealing temperature of selected forward and reverse primers are presented in Table 2. Oocyte RNA samples were pretreated with DNAse I (amplification grade; Invitrogen) before real-time PCR in order to avoid contamination with genomic DNA. aRNA (100 ng per sample) from

Table 1. Selected genes for validation by real time–polymerase chain reaction

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
Breed				
Upregulated in Holstein oocytes				
CB167318	Kinesin family member 3	*KIF3A*	10.83	0.0032
CB442788	Claudin 11	*CLDN11*	4.01	0.0286
CB448293	Baculoviral IAP repeat-containing 3	*BIRC3*	3.65	0.0037
Downregulated in Holstein oocytes				
CK975976	Death-associated protein	*DAP*	1.53	0.0382
Temperature				
Downregulated in heat-shocked oocytes				
BE685559	Metallothionein 1E	*MT1E*	−1.52	0.0436
Breed × temperature interaction				
CK946415	Acyl-CoA oxidase 1, palmitoyl	*ACOX1*	−1.58	0.0359
CB451598	Chaperonin containing TCP1, subunit 4	*CCT4*	−1.61	0.0259
CB446568	DENN/MADD domain containing 3	*DENND3*	−1.68	0.0318
NM_203359.1	Dicer 1, ribonuclease type III	*DICER1*	−1.77	0.0400

Table 2. Primers sequences and amplicon size used for real time–polymerase chain reaction

KIF3A, kinesin family member 3A; *CLDN11*, claudin 11; *BIRC3*, baculoviral IAP repeat-containing 3; *DAP*, death-associated protein; *MT1E*, metallothionein 1E; *ACOX1*, acyl-coenzyme A oxidase 1, palmitoyl; *CCT4*, chaperonin containing TCP1, subunit 4; *DENND3*, DENN/MADD domain containing 3; *DICER1*, dicer 1, ribonuclease type III; *ACTB*, β-actin; *RPL15*, ribosomal protein L15; *RPL30*, ribosomal protein L30; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *PPIA*, peptidylprolyl isomerase A

Gene	Primer sequence	Size (bp)	Annealing temperature (°C)
KIF3A	Forward: 5′-GCAGACTCCAGTTCCAGATAAA-3′ Reverse: 5′-CGAAGGCTTTCCTCAGTATAGG-3′	90	60
CLDN11	Forward: 5′-TCTCAGCTCCAAGGTCACTTA-3′ Reverse: 5′-CCTCACCTTACAAGAGGGAAA-3′	126	60
BIRC3	Forward: 5′-GACTGAGGTGTTGGGAATCTG-3′ Reverse: 5′-ACTGGCTTGAACTCGACTAATG-3′	119	60
DAP	Forward: 5′-CACCAGAGAAGAGAAGGACAAG-3′ Reverse: 5′-CGATGACTCCGGAGATAAACAC-3′	86	60
MT1E	Forward: 5′-CTCTACTTTGCCACTTGCTTTG-3′ Reverse: 5′-CTTCTTGCAGGAGGGACATC-3′	137	60
ACOX1	Forward: 5′-GACTCGAGATAATTGGCACCTAC-3′ Reverse: 5′-CCTGGGTTTCAGGGTCATAAG-3′	99	60
CCT4	Forward 5′-GCTGAGGAGGTCAGCTTAAAT-3′ Reverse: 5′-CACAGAGAGCATCATGGATAGAG-3′	148	60
DENND3	Forward: 5′-GTGGGAACATCACCTACTCTAAC-3′ Reverse: 5′-CTC TGG ACC CTC TGA ATG AAC-3′	91	60
DICER1	Forward: 5′-CGTTTACTCATCTGGCTCCTAAA-3′ Reverse: 5′-CAACAATGGAGGCTCGAAGA-3′	108	60
ACTB	Forward: 5′-GCGTGGCTACAGCTTCACC-3′ Reverse: 5′-TTGATGTCACGGACGATTTC-3′	55	60
RPL15	Forward: 5′-CTCATCGTTGGTGCCAATGCAAGT-3′ Reverse: 5′-TCACATCCACCCTGGGAAACAGAA-3′	192	60
RPL30	Forward: 5′-TGGTGTCCATCACTACAGTGGCAA-3′ Reverse: 5′-ACCAGTCTGTTCTGGCATGCTTCT-3′	133	60
GAPDH	Forward: 5′-GGCGTGAACCACGAGAAGTATAA-3′ Reverse: 5′-CCCTCCACGATGCCAAAG-3′	119	60
PPIA	Forward: 5′-GCCATGGAGCGTTTTGG-3′ Reverse: 5′-CCACAGTCAGCAATGGTGATCT-3′	65	60

oocyte samples subjected to microarray was reverse transcribed using Oligo-dT primer and SuperScript III (Invitrogen). Expression of target genes was investigated using a Power SybrGreen detection system (Applied Biosystems) and a Step One Plus Real-Time PCR System (Applied Biosystems). Initial real-time PCR analysis investigated genes affected by temperature, breed and the breed × temperature interaction using 75 oocytes per treatment ($n = 3$ replicates of 25 putative MII oocytes per experimental group). The RefFinder program (http://www.leonxie.com, accessed 7 October 2013) was used to determine the best reference gene for oocytes. For these samples β-actin (*ACTB*) was chosen as the most appropriate reference gene rather than the ribosomal protein L15 (*RPL15*), ribosomal protein L30 (*RPL30*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes.

CC real-time PCR

CC real-time PCR was conducted for the same genes and primers described above for oocyte real-time PCR (Table 2). CC RNA samples were pretreated with DNAse I (amplification grade; Invitrogen) before real-time PCR. CC total RNA (100 ng per sample) was reverse transcribed using Oligo-dT primer and SuperScript III (Invitrogen). Expression of target genes was investigated using a Power SybrGreen detection system (Applied Biosystems) and Step One Plus Real-Time PCR System (Applied Biosystems). The RefFinder program was used to determine the best reference gene for CCs. For these samples, *RPL30* was chosen as the most appropriate reference gene rather than *RPL15*, *ACTB* and peptidylprolyl isomerase A (*PPIA*).

Statistical analysis

Environmental temperature, RH, THI, RR and RT were analysed by least-squares analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS (SAS for Windows, version 9.0; SAS Institute). The significance of differences between individual means was further analysed by complete pair-wise comparisons (probability of difference analysis; SAS Institute).

Microarray data were analysed using FlexArray 1.6.1.1 (Blazejczyk *et al.* 2007). Data were subjected to a simple background correction, normalised within and between each array and analysed statistically through the use of linear models for microarray data analysis (LIMMA; Smyth 2005). Further analyses were performed using the *affy* program package developed in the R language (Gautier *et al.* 2004). Probeset intensity row data for each probe was determined, followed by background subtraction, normalisation between arrays and mean probeset intensity from each transcript representing its final intensity. Genes with fold-changes of at least 1.5 and $P < 0.05$ were considered differentially expressed in both analyses.

Pearson's correlation was calculated between oocyte microarray and real-time PCR fold-changes using GraphPad Prism 6 (GraphPad Software). For oocyte and CC real-time PCR analysis, the relative expression of each target gene was calculated using the $^{\Delta\Delta}C_T$ method. Amplification efficiency correction was determined using the equation described by Pfaffl (2001). Average efficiency values for each gene were calculated using LinRegPCR software (Ramakers *et al.* 2003). Oocyte data were subjected to ANOVA using JMP version 7.0 (SAS Institute), whereas CC data were subjected to ANOVA using the GLM procedure of SAS for Windows, version 9.0 (SAS Institute). Logarithmic or square root transformation was used to obtain a normal distribution whenever necessary. The model included the main effects of breed and temperature and the breed × temperature interaction.

Results

Environmental parameters

ET and RH were measured every hour during the OPU period to determine animal comfort (Fig. 1). ET varied from a maximum of 28.05 ± 0.29°C to a minimum of 16.88 ± 0.29°C. ET was lower in the morning than in the evening (19.21 ± 0.73 vs 25.00 ± 0.73°C respectively; $P < 0.0001$). RH varied from a maximum of 77.36 ± 1.30% to a minimum of 44.79 ± 1.30% and was lower in the evening than in the morning (52.77 ± 2.10% vs 71.09 ± 2.10% respectively; $P < 0.0001$).

Critical temperatures for *B. taurus taurus* and *B. taurus indicus* are 27°C and 35°C respectively (Curtis 1981). Thus, hyperthermia in *B. taurus taurus* cows can occur at temperatures above 27°C (Berman *et al.* 1985). Despite the fact that in the present study maximum air temperature was borderline, there was an inverse relationship between ET and RH promoting evaporative heat loss, thermoregulation and maintenance of normothermia.

The THI ranged from 58.43 ± 0.72 to 76.11 ± 0.72 with a mean of 68.06 ± 0.40 across the days of the study (OPU). According to Armstrong (1994), these THI values indicate that the animals were not subjected to heat stress for most of the study. Mild environmental stress was observed on 13 random days in September (Fig. 2). It has been reported that THI >72 is indicative of mild stress for lactating *B. taurus taurus* cows (Armstrong 1994). Females used in the present study were not lactating, which reduces endogenous heat production, leading to thermal comfort (Sartori *et al.* 2004).

Physiological parameters

RT and RR differed between breeds. RT was higher for Nelore than Holstein cows (38.37 ± 0.04°C vs 38.23 ± 0.05°C

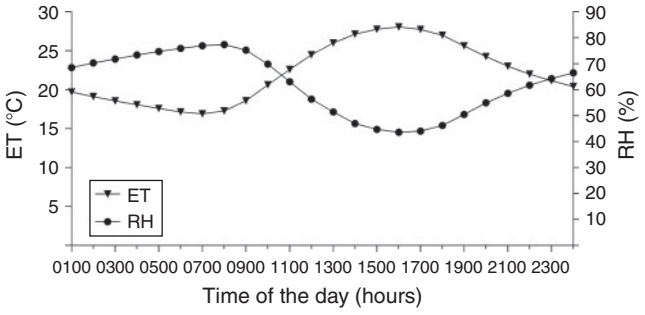

Fig. 1. Hourly environmental temperature (ET) and relative humidity (RH) readings in free stalls during the ovum pick-up period. Data are the least-squares mean ± s.e.m.

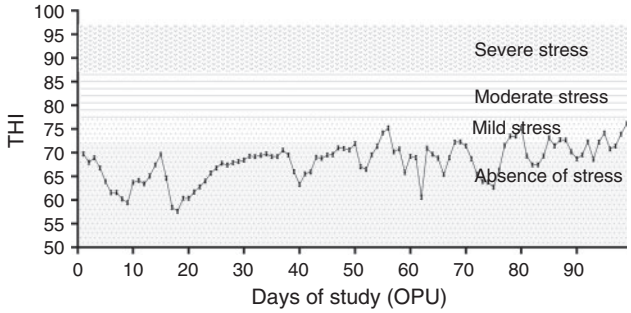

Fig. 2. Characterisation of animal comfort and heat stress according to the temperature and humidity index (THI), calculated using environmental temperature and relative humidity values, during the experimental period. Data are the least-squares mean ± s.e.m. OPU, ovum pick-up.

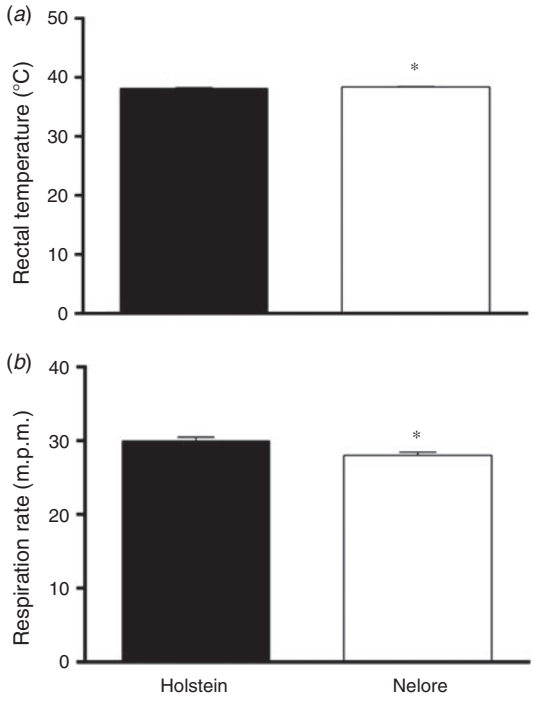

Fig. 3. (*a*) Rectal temperature and (*b*) respiration rate in Holstein and Nelore animals measured during ovum pick-up. Data are the least-squares mean ± s.e.m. *$P < 0.05$ compared with Holstein animals. m.p.m., movements per minute.

respectively; $P < 0.05$; Fig. 3*a*), but RR was higher for Holstein than Nelore cows (29.99 ± 0.9 vs 28.05 ± 0.44 m.p.m. respectively; $P < 0.05$; Fig. 3*b*). The RR and RT of the animals were typical of thermoneutrality (Rhoads *et al.* 2009).

Transcriptome analysis

The Venn diagram in Fig. 4 illustrates global gene expression profile in Holstein and Nelore oocytes subjected to heat shock. Oocyte microarray revealed 127 genes affected by breed (Table 3), nine genes affected by temperature (Table 4) and six genes affected by the breed × temperature interaction (Table 5;

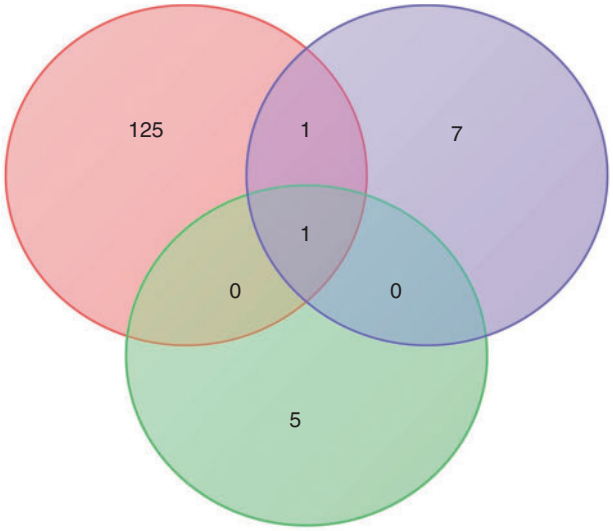

Fig. 4. Venn diagram illustrating the number of differentially expressed genes affected by breed (red; $n = 127$ genes), temperature (blue; $n = 9$ genes) and the breed × temperature interaction (green; $n = 6$ genes). Intersecting areas indicate common genes.

fold-change ≥1.5; $P < 0.05$). Among these genes, 82 were upregulated and 45 were downregulated in Holstein compared with Nelore oocytes (Table 3). Moreover, three genes were upregulated and six were downregulated in heat-shocked oocytes compared with control (Table 4). The *CCT4* gene was upregulated in heat-shocked compared with control Holstein oocytes, the *DICER1* gene was upregulated in Holstein heat-shocked compared with Nelore heat-shocked oocytes and the *ACOX1* gene was upregulated in Holstein heat-shocked compared with both Holstein control and Nelore heat-shocked oocytes (Table 6). The oncostatin M receptor (*OSMR*) gene was upregulated in Nelore heat-shocked compared with Holstein heat-shocked oocytes, whereas *DENND3* was upregulated in Nelore control compared with Holstein control oocytes (Table 6).

Functional classification

Functional analysis was performed using ingenuity pathways analysis (IPA; Ingenuity Systems; www.ingenuity.com, accessed 15 February 2013) and DAVID Bioinformatics Database software (www.david.abcc.ncifcrf.gov, accessed 15 February 2013). Genes that were differently expressed ($P < 0.05$) between Holstein and Nelore oocytes were involved in important biological functions, such as tissue morphology (*CLDN11*, claudin 23 (*CLDN23*) and ATPase Ca++ transporting type 2C member 1 (*ATP2C1*), embryonic development (*KIF3A* and Bos taurus A kinase (PRKA) anchor protein 12 (*AKAP12*), organism development (ectonucleotidepyrophosphatase/phosphodiesterase 2 (*ENPP2*) and excision repair cross-complementing rodent repair deficiency, complementation group 6 (*ERCC6*), tissue development (sirtuin 1 (*SIRT1*) and inosine triphosphatase (*ITPA*), cellular development (*DICER1* and *PPIA*) and cell death and survival (*BIRC3* and *DAP*; Fig. 5). The differently expressed ($P < 0.05$) genes between control and heat-shocked

Table 3. Differentially expressed genes in Holstein and Nelore oocytes (breed effect)

GPCR, G-protein-coupled receptor; ETBR-LP-1, endothelin B receptor-like protein 1; PAELR, Parkin-associated endothelin receptor-like receptor; AHA1, Activator of Heat Shock 90kDa Protein ATPase Homolog 1; IAP, Inhibitors of apoptosis proteins; HAUS, subunit human Augmin; Vpr, Viral Protein R; UPF3, Up-Frameshift Suppressor 3; LSM1, like Sm 1; PHD, plant homeodomain; ZMYM6, Zinc Finger MYM-Type Containing 6; CD3, cluster of designation 3; SCO, synthesis of cytochrome c oxidase; GPCR, G protein–coupled receptors; ArfGAP2, ADP Ribosylation Factor GTPase Activating Protein 2

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
Upregulated in Holstein oocytes				
CB167318	Kinesin family member 3A	*KIF3A*	10.83	0.0032
AV606331	Cynaptotagmin IV	*SYT4*	5.47	0.0404
CK972321	4102775 BARC 9BOV *Bos taurus* cDNA clone 9BOV24_C22 3′, mRNA sequence	–	5.43	0.0334
CK776415	AHA1 activator of heat shock 90 kDa protein ATPase homologue 1 (yeast)	*AHSA1*	5.29	0.0185
AW325978	–	–	5.28	0.0018
BE749242	C0009505I05.Q1KM13R KN510 *Bos taurus* cDNA clone C0009505I05 3′, mRNA sequence	–	4.75	0.0105
CB455872	713054 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	4.21	0.0344
CB442788	Claudin 11	*CLDN11*	4.01	0.0286
CK772462	Mitochondrial ribosomal protein L39	*MRPL39*	3.81	0.0278
BE750213	Claudin 23	*CLDN23*	3.67	0.0086
CB448293	Baculoviral IAP repeat-containing 3	*BIRC3*	3.65	0.0037
BP106440	Roundabout axon guidance receptor homologue 1 (*Drosophila*)	*ROBO1*	3.41	0.0215
BF775460	Arginyltransferase 1	*ATE1*	3.27	0.0247
CB453664	Zinc finger protein 398	*ZNF398*	3.27	0.0426
CK846085	Sirtuin (silent mating type information regulation 2 homologue) 1 (*Saccharomyces cerevisiae*)	*SIRT1*	3.26	0.0271
CK972862	*Bos taurus* HAUS augmin-like complex subunit 8	*HAUS8*	3.12	0.0443
BI848481	Male-specific lethal 3 homologue (*Drosophila*)	*MSL3*	3.09	0.0378
CK847223	Vpr (HIV-1) binding protein	*VPRBP*	3.05	0.0331
CK968849	RAD17 homologue (*Schizosaccharomyces pombe*)	*RAD17*	3.02	0.0294
BM251601	F-box and leucine-rich repeat protein 3	*FBXL3*	2.99	0.0319
CK848518	Ankyrin repeat and SOCS box-containing 3	*ASB3*	2.96	0.0092
BI774268	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 2	*PLEKHA2*	2.95	0.0101
CB428813	Activating transcription factor 1	*ATF1*	2.82	0.0360
CK777897	v-yes-1 Yamaguchi sarcoma viral oncogene homologue 1	*YES1*	2.78	0.0331
CB537961	ADP-ribosylation factor-like 1	*ARL1*	2.78	0.0102
CB460407	Coiled-coil domain containing 112	*CCDC112*	2.77	0.0226
BM107454	Similar to RAB18, member RAS oncogene family, RAB18, member RAS oncogene family	LOC616304 /// RAB18	2.56	0.0224
CB440290	–	–	2.56	0.0299
CK777386	Neural precursor cell expressed, developmentally downregulated 1	*NEDD1*	2.55	0.0163
CK970460	Kelch-like 2, Mayven (*Drosophila*)	*KLHL2*	2.50	0.0332
BI849511	Uncharacterised LOC100852378	LOC100852378	2.48	0.0363
CK727557	Bend_0A01-008-d12 Day 8 Uterus bend *Bos taurus* cDNA 3′, mRNA sequence	–	2.45	0.0466
CB445505	–	–	2.39	0.0450
CB421648	594684 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	2.37	0.0458
CK964049	4078547 BARC 9BOV *Bos taurus* cDNA clone 9BOV13_B12 5′, mRNA sequence	–	2.27	0.0256
CB464732	*Bos taurus* GTP binding protein 4	*GTPBP4*	2.25	0.0235
CB451120	DNA topoisomerase II, β isozyme; topoisomerase (DNA) II β 180 kDa	LOC787143 /// TOP2B	2.21	0.375
CB165419	Transmembrane protein 181	*TMEM181*	2.18	0.0497
CK770689	958892 MARC 1BOV *Bos taurus* cDNA 3′, mRNA sequence	–	2.18	0.0101
CK979673	Uncharacterised LOC100847840	LOC100847840	2.16	0.0171
CK967025	Excision repair cross-complementing rodent repair deficiency, complementation group 6	*ERCC6*	2.15	0.0327
CK846762	Similar to chromosome X open reading frame 57	LOC511907	2.15	0.0075
CK946910	Coagulation factor II (thrombin) receptor-like 1	*F2RL1*	2.11	0.0430
AW654046	ATP synthase mitochondrial F1 complex assembly factor 1	*ATPAF1*	2.10	0.0061
AW668954	Small nuclear ribonucleoprotein 48 kDa (U11/U12)	*SNRNP48*	2.05	0.0125
CK946184	Retinitis pigmentosa 2 (X-linked recessive)	*RP2*	2.04	0.0429

(*Continued*)

Table 3. (Continued)

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
CK846775	969322 MARC 4BOV *Bos taurus* cDNA 3′, mRNA sequence	–	1.98	0.0129
CK776519	967837 MARC 4BOV *Bos taurus* cDNA 3′, mRNA sequence	–	1.96	0.0138
BE668935	UPF3 regulator of nonsense transcripts homologue A (yeast)	*UPF3A*	1.95	0.0037
NM_178320.2	Peptidylprolyl isomerase A (cyclophilin A)	*PPIA*	1.95	0.0433
CK959025	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa)	*HNRNPD*	1.93	0.0474
AW336381	RAB27A, member RAS oncogene family	*RAB27A*	1.89	0.0360
CB451166	705913 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	1.87	0.0454
CK950528	LSM1 homologue, U6 small nuclear RNA associated (*S. cerevisiae*)	*LSM1*	1.87	0.0130
NM_203359.1	Dicer 1, ribonuclease type III	*DICER1*	1.87	0.0284
BP101546	Hypothetical protein LOC613274	LOC613274	1.86	0.0223
CB428865	F-box protein 22	*FBXO22*	1.86	0.0306
BP100422	BP100422 ORCS bovine liver cDNA *Bos taurus* cDNA clone ORCS20428 3′, mRNA sequence	–	1.85	0.0295
CB169241	Fumarylacetoacetate hydrolase domain containing 1	*FAHD1*	1.83	0.0334
CB166510	splicing factor 3b, subunit 2. 145 kDa	*SF3B2*	1.80	0.0449
BM363690	PHD finger protein 11	*PHF11*	1.74	0.0330
CK772848	Similar to chromosome 17 open reading frame 63	LOC506074	1.74	0.0227
CK775501	Calmodulin-like 4	*CALML4*	1.74	0.0440
CK729309	Cortactin-binding protein 2	LOC530341	1.71	0.0284
BP103418	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NADH-coenzyme Q reductase)	*NDUFS1*	1.71	0.0470
BI899164	Transmembrane protein 64	*TMEM64*	1.69	0.0475
CK770586	Nidogen 2 (osteonidogen)	*NID2*	1.69	0.0480
BP102089	Family with sequence similarity 122B	*FAM122B*	1.68	0.0355
CK946415	Acyl-CoA oxidase 1, palmitoyl	*ACOX1*	1.67	0.0226
CK955814	Ectonucleotide pyrophosphatase/phosphodiesterase 2	*ENPP2*	1.64	0.0154
BE722703	Translocase of inner mitochondrial membrane 13 homologue (yeast)	*TIMM13*	1.64	0.0384
CK846596	Proline-rich Gla (G-carboxyglutamic acid) 1	*PRRG1*	1.58	0.0467
CB437963	–	–	1.57	0.0284
AV613409	Adrenergic β receptor kinase 2	*ADRBK2*	1.54	0.0481
CK947524	–	–	1.54	0.0038
NM_174767.2	Visual system homeobox 1	*VSX1*	1.54	0.0308
NM_175785.2	ATPase Ca^{2+} transporting, type 2C, member 1	*ATP2C1*	1.53	0.0148
AW631892	Hypothetical protein LOC100140164	LOC100140164	1.52	0.0130
AU275513	Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)	*ITPA*	1.52	0.0131
CK961806	4076044 BARC 9BOV *Bos taurus* cDNA clone 9BOV10_J05 3′, mRNA sequence	–	1.52	0.0439
CK964884	ZMYM6 protein	LOC618247	1.52	0.0312
CB461358	Nucleoporin 62	LOC516074	1.50	0.0327
Downregulated in Holstein oocytes				
BE757556	–	–	−1.51	0.0346
CK848917	Histidyl-tRNAsynthetase	*HARS*	−1.51	0.0249
BE483227	169309 BARC 5BOV *Bos taurus* cDNA 5′, mRNA sequence	–	−1.51	0.0138
CK845823	*Homo sapiens* polymerase (DNA directed), gamma	*POLG*	−1.52	0.0153
CK775030	Importin 9	*IPO9*	−1.53	0.0237
CK975976	Death-associated protein	*DAP*	−1.53	0.0382
CB167910	Pelota homologue (*Drosophila*)	*PELO*	−1.54	0.0409
CK976501	Signal peptidase complex subunit 3 homologue (*S. cerevisiae*)	*SPCS3*	−1.54	0.0001
CK776838	Phosphodiesterase 8B	*PDE8B*	−1.55	0.0172
BI535325	398771 MARC 4BOV *Bos taurus* cDNA 5′, mRNA sequence	–	−1.56	0.0271
CK975798	–	–	−1.59	0.0103
CB421951	CD3g molecule, gamma (CD3-TCR complex)	*CD3G*	−1.60	0.0467
CK773978	962683 MARC 2BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−1.60	0.0082
CK968887	Family with sequence similarity 46, member B	*FAM46B*	−1.62	0.0460
CK951562	MAX dimerisation protein 1	*MXD1*	−1.63	0.0130
CB468445	734266 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−1.64	0.0044
CK777507	Peroxisomal biogenesis factor 11[a]	*PEX11A*	−1.66	0.0499

(*Continued*)

Table 3. (Continued)

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
CK772584	SCO cytochrome oxidase deficient homologue 1 (yeast)	*SCO1*	−1.68	0.0068
CK846593	969102 MARC 4BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−1.68	0.0196
CB437808	Zinc finger protein 362	*ZNF362*	−1.69	0.0182
CB421497	594518 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−1.69	0.0051
CK772342	GPCR37 (endothelin receptor type B-like) /similar to probable GPCR37	*GPR37 LOC790388*	−1.73	0.0024
CK950060	Predicted: *Bos taurus* transmembrane protein 87B	*TMEM87B*	−1.73	0.0183
CK957150	4097464 BARC 10BOV *Bos taurus* cDNA clone 10BOV3_F15 3′, mRNA sequence	–	−1.75	0.00006
CK953377	Hypothetical LOC614796	*MGC157372*	−1.76	0.0215
CB426313	Paraneoplastic antigen MA2	*PNMA2*	−1.77	0.0451
CB430767	–	–	−1.78	0.0474
X61612.1	ATPase H^+ transporting, lysosomal 70 kDa, V1 subunit A	*ATP6V1A*	−1.79	0.0498
BI848836	Two pore segment channel 1	*TPCN1*	−1.80	0.0133
CB421732	GPCR155	*GPR155*	−1.87	0.0195
BF073634	Similar to stromal membrane-associated GTPase-activating protein 2; small ArfGAP2	LOC790140 /// SMAP2	−1.92	0.0317
CB459849	RNA binding motif protein 25	*RBM25*	−1.92	0.0280
CB443312	–	–	−1.95	0.0284
CB451835	706630 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−1.99	0.0045
CK952663	–	–	−2.00	0.0028
CK951211	–	–	−2.12	0.0084
CK774760	Nipsnap homologue 3A	*NIPSNAP3A*	−2.13	0.0013
AW656161	TRANSMEMBRANE protein 167B	*TMEM167B*	−2.15	0.0133
CK775789	Transmembrane protein 97	*TMEM97*	−2.22	0.0410
CK948712	Predicted: *Bos taurus* A kinase (PRKA) Anchor protein 12	*AKAP12*	−2.41	0.0085
BM445534	Uncharacterised LOC100849050	LOC100849050	−3.03	0.0364
CB461876	721905 MARC 6BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−3.14	0.0475
CK770657	958857 MARC 1BOV *Bos taurus* cDNA 3′, mRNA sequence	–	−3.16	0.0268
CK730220	*Homo sapiens* polypyrimidine tract binding protein 3	*PTBP3*	−3.27	0.0116
AW484451	60795 MARC 4BOV *Bos taurus* cDNA 5′, mRNA sequence	–	−3.74	0.0004

Table 4. Differentially expressed genes in control and heat-shocked oocytes (temperature effect)
UPF3, xxx; FBR-MuSV, Finkel–Biskis–Reilly murine sarcoma virus

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
Upregulated in heat-shocked oocytes				
CK946415	Acyl-CoA oxidase 1, palmitoyl	*ACOX1*	1.88	0.0084
CK950864	Gag-Pro-Pol-Env protein; similar to envelope glycoprotein; similar to envelope glycoprotein; tetratricopeptide repeat domain 8	LOC782627 / LOC786117 / LOC787886 / *TTC8*	1.62	0.0387
BE668935	UPF3 regulator of nonsense transcripts homologue A (yeast)	*UPF3A*	1.52	0.0356
Downregulated in heat-shocked oocytes				
BP100734	Ribosomal protein, large, P1	*RPLP1*	−1.51	0.0421
BE685559	Metallothionein 1E	*MT1E*	−1.52	0.0436
NM_174731.2	FBR-MuSV ubiquitously expressed	*FAU*	−1.61	0.0403
CK978935	Splicing factor 3b, subunit 4, 49 kDa	*SF3B4*	−1.62	0.0203
CB447642	Coronin 1C	*CORO1C*	−1.67	0.0457
CK776273	Chromosome 11 open reading frame 16	*C11ORF16*	−1.89	0.0360

Table 5. Differentially expressed genes affected by breed × temperature interaction in bovine oocytes
TCP1, T-Complex Polypeptide 1

Representative public ID	Gene name	Gene symbol	Fold-change	*P*-value
CK776338	Oncostatin M receptor	*OSMR*	1.64	0.0303
CK728156	UMC-bend_0A02–007-a09 Day 8 Uterus bend *Bos taurus* cDNA 3′, mRNA sequence	–	1.51	0.0482
CK946415	Acyl-CoA oxidase 1, palmitoyl	*ACOX1*	−1.58	0.0359
CB451598	Chaperonin containing TCP1, subunit 4	*CCT4*	−1.61	0.0259
CB446568	DENN/MADD domain containing 3	*DENND3*	−1.68	0.0318
NM_203359.1	Dicer 1, ribonuclease type III	*DICER1*	−1.77	0.0400

Table 6. Further analysis of differentially expressed genes modulated by breed × temperature interactions in bovine oocytes
ACOX1, acyl-coenzyme A oxidase 1, palmitoyl; *CCT4*, chaperonin containing TCP1, subunit 4; *DENND3*, DENN/MADD domain containing 3; *DICER1*, dicer 1, ribonuclease type III; *OSMR*, oncostatin M receptor; HS, heat shock

Gene symbol	Comparison	Fold-change	*P*-value	Upregulated
CCT4	Holstein control × Holstein HS	1.87	0020	Holstein HS
ACOX1	Holstein control × Holstein HS	2.50	0005	Holstein HS
	Nelore HS × Holstein HS	2.48	0007	Holstein HS
DICER1	Nelore HS × Holstein HS	3.81	0009	Holstein HS
OSMR	Nelore HS × Holstein HS	−2.32	0007	Nelore HS
DENND3	Nelore control × Holstein control	−1.89	0010	Nelore control

oocytes involved cellular compromise (*ACOX1* and *MT1E*), cell cycle (*MT1E*), developmental disorders (splicing factor 3b, subunit 4 (*SF3B4*) and *ACOX1*), lipid metabolism (*ACOX1*), molecular transport (*ACOX1* and *MT1E*), energy production (*ACOX1*), free radical scavenging (*MT1E*) and cellular assembly and organisation (*ACOX1* and coronin 1C (*CORO1C*); Fig. 5). The interaction of breed and temperature also indicated differently expressed ($P < 0.05$) genes involved in significant main biological processes ($P < 0.05$) such as cell cycle (*DICER1*), cell morphology (*OSMR*), cellular assembly, organisation and compromise (*ACOX1* and *DICER1*), cellular development (*OSMR* and *DICER1*), lipid metabolism (*ACOX1*), post-translational modification and protein folding (*CCT4*) and protein catabolism (*DENND3*; Fig. 5). Gene terminology is described in Tables 3–5.

Oocyte real-time PCR

Real-time PCR was conducted to validate genes affected by breed (*KIF3A*, *CLDN11*, *BIRC3* and *DAP*), temperature (*MT1E*) and the breed × temperature interaction (*CCT4*, *DICER1*, *DENND3* and *ACOX1*) in bovine oocytes. Initial analysis showed that microarray and real-time PCR fold-changes had a Pearson's correlation coefficient of $r = 0.77$ ($P = 0.01$; Fig. 6). This indicated that the differential level of expression for the transcripts *KIF3A*, *CLDN11*, *BIRC3*, *DAP*, *MT1E*, *CCT4*, *DENND3* and *ACOX1* detected in both microarray and real-time PCR analyses were highly correlated. However, real-time PCR validation confirmed statistically significant expression of 22% of selected genes in oocytes (2/9; Figs 7, 8). The relative mRNA abundance for the molecular motor *KIF3A* was higher ($P < 0.05$; Fig. 7*a*) for Holstein than Nelore oocytes. In contrast, the relative abundance of *DAP* ($P < 0.05$; Fig. 7*a*) and *DENND3* ($P < 0.001$; Fig. 9) mRNA was higher in Nelore than Holstein oocytes. Even though real-time PCR data showed that *DENND3* gene expression was affected by breed, this gene was not considered as validated because according to microarray data there was a breed × temperature interaction. For the other genes evaluated, there was no statistically significant difference for breed (*CLDN11* and *BIRC3*), temperature (*MTIE*) or the breed × temperature interaction (*CCT4*, *DICER1* and *DENND3*). The *ACOX1* gene was not detected by real-time PCR.

CC real-time PCR

Real-time PCR was conducted to determine how genes that were differentially expressed in the oocyte microarray behaved in CCs subjected to the same treatments. Expression of *CLDN11* in CCs was affected by breed: relative *CLDN11* mRNA abundance was higher for Nelore than Holstein CCs ($P = 0.06$; Fig. 10*a*). *MT1E* expression was affected by both breed ($P < 0.05$) and temperature ($P < 0.05$). Relative *MT1E* mRNA abundance was higher for Holstein than Nelore CCs ($P < 0.05$; Fig. 10*a*) and exposure of COCs to heat shock decreased CC *MT1E* mRNA expression ($P < 0.05$; Fig. 10*b*). There was no effect of breed, temperature of the breed × temperature interaction on the relative mRNA abundance of *KIF3A*, *BIRC3*, *DAP*, *CCT4* and *DICER1* in CCs (Fig. 10). *ACOX1* and *DENND3* mRNAs were not expressed in these CCs.

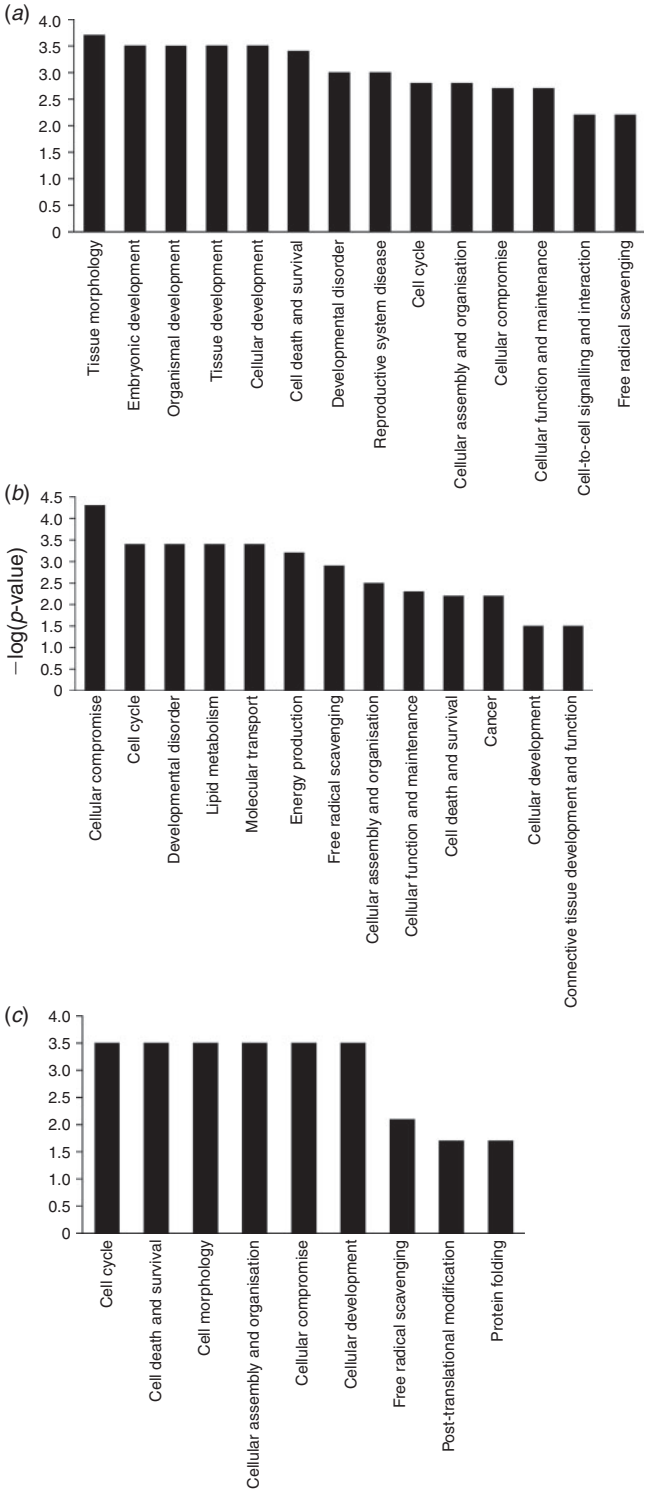

Fig. 5. Biological processes for differentially expressed genes in oocytes affected by (*a*) breed, (*b*) temperature and (*c*) the breed × temperature interaction. Data were obtained using ingenuity pathway analysis software (Ingenuity Systems; www.ingenuity.com, accessed 15 February 2013).

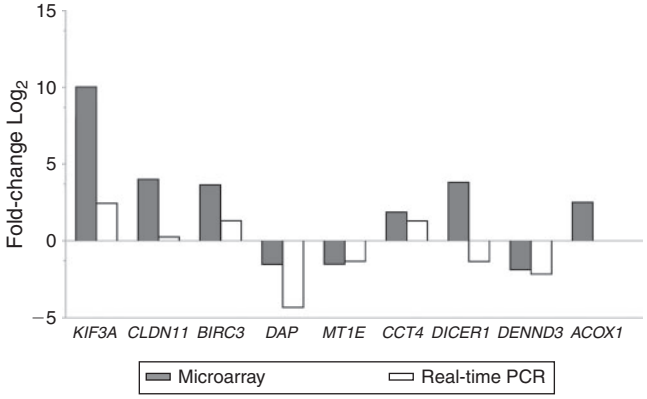

Fig. 6. Quantification (fold-change, $\log_2$) of the mRNA profile for selected genes in oocytes. Kinesin family member 3A (*KIF3A*), claudin 11 (*CLDN11*), baculoviral IAP repeat-containing 3 (*BIRC3*) and death-associated protein (*DAP*) were differentially expressed between breeds; metallothionein 1E (*MT1E*) was differentially expressed between temperature treatments (heat shock vs control); and chaperonin containing TCP1, subunit 4 (*CCT4*), acyl-coenzyme A oxidase 1, palmitoyl (*ACOX1*), dicer 1, ribonuclease type III (*DICER1*) and DENN/MADD domain containing 3 (*DENND3*) were affected by the breed × temperature interaction. Grey bars represent the differential level of expression of transcripts detected in the microarray study, whereas white bars represent the differential level of expression of the same transcripts obtained by real-time polymerase chain reaction (PCR; fold-change $= 2^{-\Delta\Delta CT}$). Pearson's correlation coefficient between PCR and microarray fold change values is $r = 0.7778$ ($P = 0.01$).

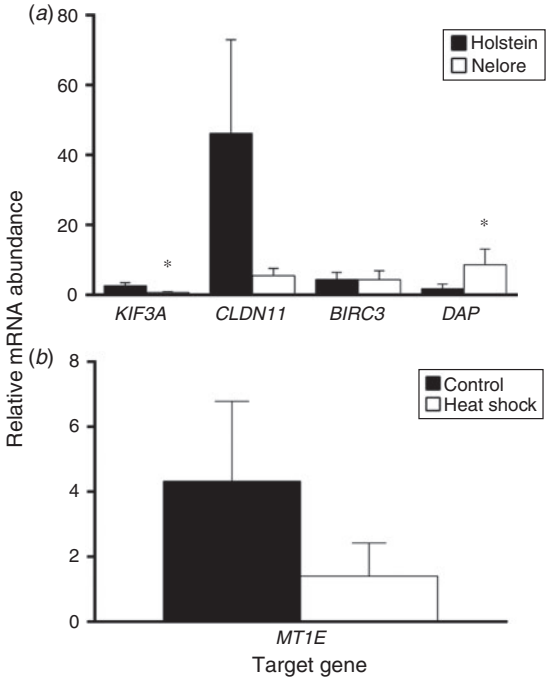

Fig. 7. Effects of (*a*) breed (Holstein vs Nelore) and (*b*) temperature (control vs heat shock) on mRNA abundance (relative to beta-actin gene (*ACTB*) of differentially expressed genes in oocytes. Data are the least-squares mean ± s.e.m. of three replicates using 75 oocytes per group. $^*P < 0.05$ compared with Holstein animals. *KIF3A*, kinesin family member 3A; *CLDN11*, claudin 11; *BIRC3*, baculoviral IAP repeat-containing 3; *DAP*, death-associated protein; *MT1E*, metallothionein 1E.

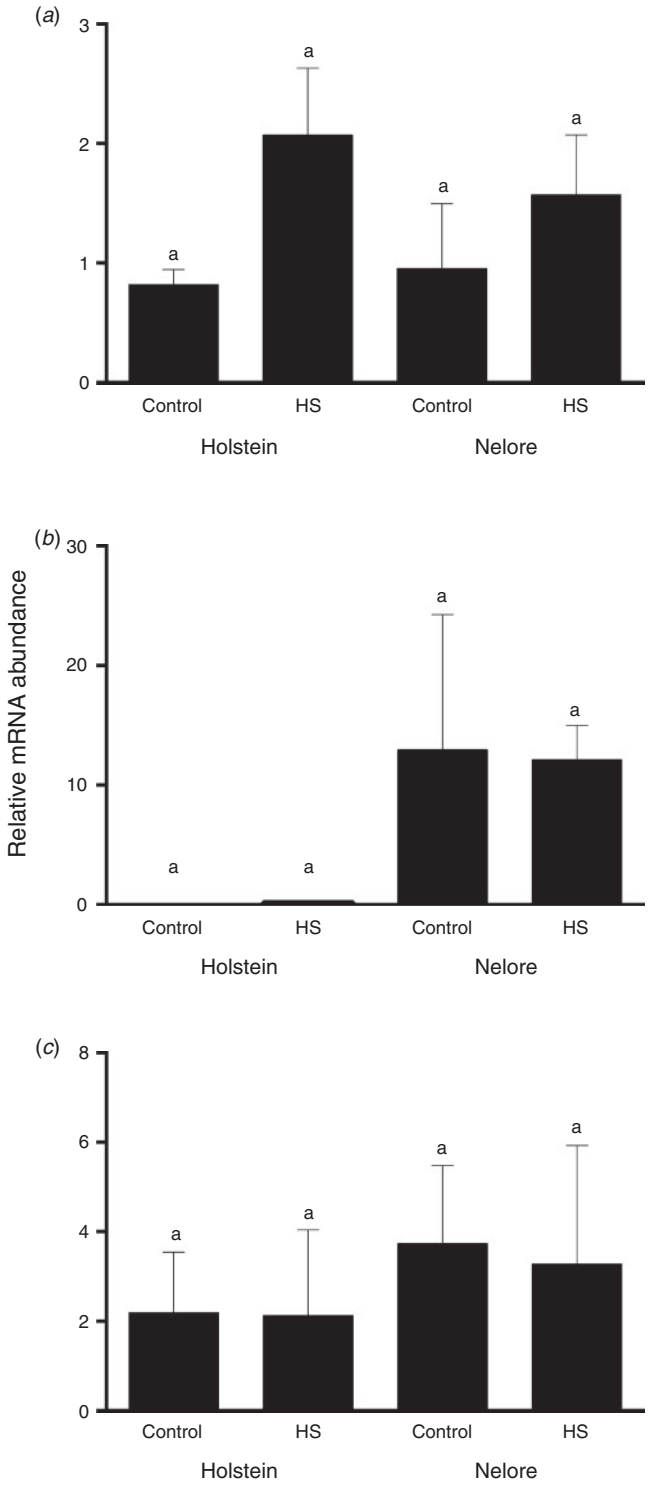

Fig. 8. Effects of the interaction of breed × temperature on mRNA abundance (relative to beta-actin gene (*ACTB*) of three differentially expressed genes in oocytes. (*a*) Chaperonin containing TCP1, subunit 4 (*CCT4*), (*b*) DENN/MADD domain containing 3 (*DENND3*) and (*c*) dicer 1, ribonuclease type III (*DICER1*). Results are the east-squares mean ± s.e.m. of three replicates using 75 oocytes per group. HS, heat shock.

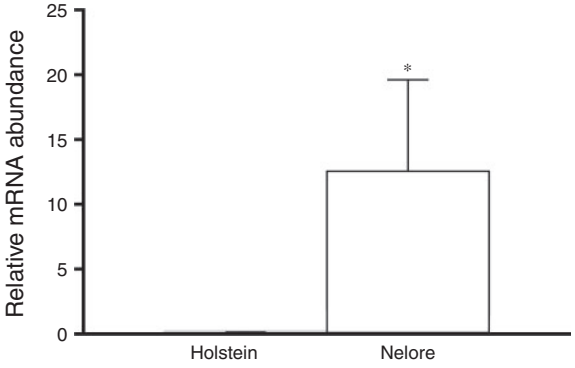

Fig. 9. Effects of breed (Holstein and Nelore) on mRNA abundance (relative to ribosomal protein L30 (*RPL30*)) of the DENN/MADD domain containing 3 (*DENND3*) gene in oocytes. Results are the least-square mean ± s.e.m. of three replicates using 75 oocytes per group. *$P < 0.001$ compared with Holstein animals.

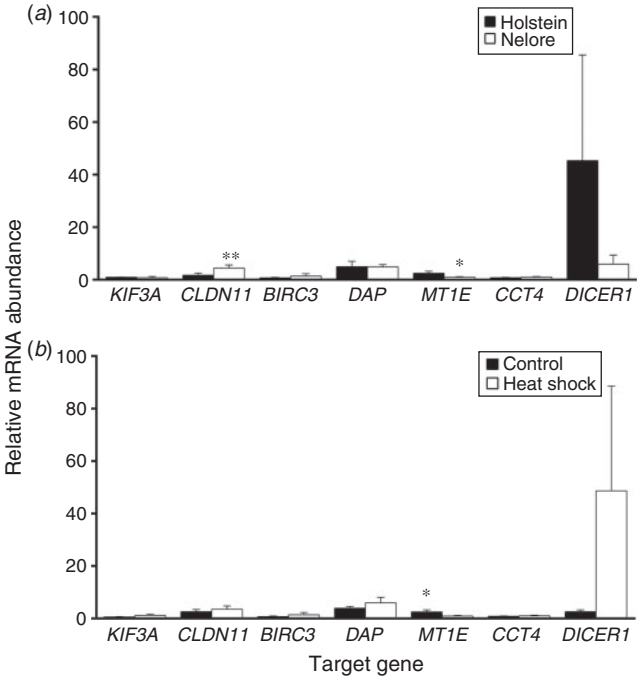

Fig. 10. Effects of (*a*) breed (Holstein vs Nelore) and (*b*) temperature (control vs heat shock) on mRNA abundance (relative to ribosomal protein L30 (*RPL30*)) of differentially expressed genes in cumulus cells. Results are the least-squares mean ± s.e.m. of five replicates. *$P < 0.05$, **$P = 0.06$ compared with Holstein animals (*a*) or control treatment (*b*). *KIF3A*, kinesin family member 3A; *CLDN11*, claudin 11; *BIRC3*, baculoviral IAP repeat-containing 3; *DAP*, death-associated protein; *MT1E*, metallothionein 1E; *CCT4*, chaperonin containing TCP1, subunit 4; *DICER1*, dicer 1, ribonuclease type III.

Discussion

High ET have considerable economic impact on cattle production worldwide. The present study analysed global changes in gene expression in *B. taurus taurus* and *B. taurus indicus* oocytes subjected to heat shock and the relative abundance of

candidate genes in their CCs. The present is the first to evaluate the molecular mechanisms by which temperature and genotype affect oocyte and CCs.

Expression of the microtubule-dependent molecular motor *KIF3A* was higher in Holstein than Nelore oocytes. KIF3A protein has been localised to the spindle microtubules and around the cellular cortex of HeLa cells (Haraguchi *et al.* 2006). This member of the kinesins superfamily uses energy derived from ATP hydrolysis to promote unidirectional transport of organelles along the microtubules and completion of cytokinesis. For example, kinesins allow positioning of nuclei and proper segregation of genetic material (Shimizu *et al.* 1998). This implies an important role for *KIF3A* in the progression of meiosis in Holstein oocytes.

There is accumulating evidence that the mammalian cytoskeleton is susceptible to extremes of temperature. It has been shown that exposure of oocytes to heat shock (Roth and Hansen 2005) and cryopreservation (Wu *et al.* 2006) compromises cytoskeletal architecture, leading to spindle microtubule and actin filament disorganisation. In the present study, exposure of Nelore and Holstein oocytes to a physiologically relevant heat shock of 41°C for the first 12 h of IVM did not affect the expression of the microtubule-dependent molecular motor *KIF3A*. It is possible that such stress was not severe enough to induce changes in *KIF3A* transcript abundance during oocyte maturation. Indeed, more severe stress, such as cryopreservation, has been reported to downregulate *KIF3A* in human MII oocytes (Monzo *et al.* 2012). Even though the effects of high temperature on cytoskeletal organisation have been well characterised in bovine oocytes (Roth and Hansen 2004), little is know about the effects of high temperatures on the expression of cytoskeleton-associated transcripts.

The *DAP* transcript has not been previously described in bovine oocytes. In the present study, the abundance of the pro-apoptotic *DAP* transcript was higher in Nelore than Holstein oocytes. Although this higher abundance of *DAP* transcript may render the Nelore oocyte more prone to cell death, it may also make the Nelore embryo more capable of adapting to stress conditions. Apoptosis has been demonstrated to be an adaptive response in bovine preimplantation embryos that facilitates survival after heat shock (Paula-Lopes and Hansen 2002). This is supported by the finding that Brahman embryos are more resistant to elevated temperatures than Angus and Holstein embryos (Paula-Lopes *et al.* 2003). Moreover, as the embryo reaches the blastocyst stage, *DAP* transcript expression did not differ between Simmental (*B. taurus taurus*) and Nelore (*B. taurus indicus*) embryos (Sudano *et al.* 2013).

Nelore and Holstein oocyte transcriptome analysis demonstrated that the molecular motor *KIF3A* and the pro-apoptotic *DAP* genes were differentially expressed between breeds. Even though such breed difference occurred independent of temperature, these genes encode important proteins associated with cellular events highly susceptible to heat shock, such as oocyte nuclear maturation, cytoskeletal organisation (Roth and Hansen 2005) and apoptosis (Roth and Hansen 2004; Paula-Lopes *et al.* 2013). Interestingly, although Holstein oocytes are known to be susceptible to heat stress (Rocha *et al.* 1998), the *KIF3A* transcript, which favours meiotic division, was more abundant in Holstein than Nelore oocytes. It is possible that Holstein oocytes have a lower stress baseline so that exposure to the IVM system triggered earlier changes in transcript abundance compared with Nelore oocytes. Another possibility is that Holstein oocyte deficiency in cellular protection against heat stress occurs at the post-transcriptional or protein level rather than involving transcript regulation. Indeed, it has been demonstrated that exposure of bovine oocytes to heat shock during the first 12 h of IVM does not affect oocyte RNA integrity, total RNA and poly(A) RNA abundance or the abundance of specific transcripts (*HSP70*, growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), poly(A) polymerase (*PAP*), cyclin B1 (*CCNB1*), *28S* and *18S*) in bovine oocytes (Payton *et al.* 2011).

Another gene that was differentially expressed between Holstein and Nelore oocytes was *DENND3*. Even though *DENND3* encodes an evolutionarily conserved protein, little has been shown regarding its functional roles. DENN domains interact directly with members of the Rab family of small GTPases and function enzymatically as Rab-specific guanine nucleotide exchange factors (Yoshimura *et al.* 2010). Rab GTPases play a role in membrane trafficking, cell growth and differentiation (Schwartz *et al.* 2007). *DENND3* acts as regulator of Rab12 function (Matsui and Fukuda 2011), which controls transferrin receptor degradation and recycling (Matsui *et al.* 2011). Transferrin receptor, a regulator of intracellular iron homeostasis, has been demonstrated in baboon oocytes (Burch *et al.* 2009). Iron accumulation catalyses hydroxyl radical formation amplifying oxidative damage (Asano 2012). Moreover, it induces transferrin receptor degradation at lysosomes, a mechanism that is required for iron homeostasis in mammalian cells (Tachiyama *et al.* 2011). Therefore, high *DENND3* mRNA abundance suggests that Nelore oocytes are better able to control iron balance, avoiding oxidative stress and defects in nuclear events during IVM.

The positive correlation between fold-changes in oocyte microarray and real-time PCR validation indicated high similarity in the gene expression profile between these two techniques. Of the nine genes evaluated (*KIF3A*, *CLDN11*, *BIRC3*, *DAP*, *MT1E*, *CCT4*, *DICER1*, *DENND3* and *ACOX1*), the expression of seven was directly proportional between microarray and real-time PCR. Despite this high correlation, a more conservative analysis indicated that only two of the nine genes were statistically significant on real-time PCR validation. Although a higher number of repetitions lead to greater uniformity between samples, potentially increasing the proportion of validated genes, the low number of validated genes must not have occurred in this study due to the number of repetitions used in real time PCR (three biological replicates). It has been shown that an experiment should ideally include at least three independent biological replicates for each treatment (Udvardi *et al.* 2008). In addition, three biological replicates has been used as the default number to other microarray studies reported in the literature (Grado-Ahuir *et al.* 2011; Dias *et al.* 2013).

Considering the importance of CCs in oocyte competence, the present study broadened the investigation of genes differentially expressed in oocytes to the surrounding CCs. Tight junction claudin (*CLDN11*) expression was higher in Nelore

than Holstein CCs. In the bovine, several genes from the claudin family have been identified in granulosa cells of atretic follicles (*CLDN1*, *CLDN5*, *CLDN11*), suggesting that this protein family exerts a modulatory role on the apoptotic cascade in atretic follicles (Hatzirodos *et al.* 2014). Even though a modulatory action of claudins on the apoptotic cascade has not been demonstrated, there is evidence that *CLDN1* had an anti-apoptotic action in tumour cells (Akasaka *et al.* 2010).

Heat stress increases the production of reactive oxygen species (ROS) in bovine oocytes and the percentage of terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL)-positive CCs (Nabenishi *et al.* 2012). CCs have a critical role in protecting oocytes against oxidative stress-induced apoptosis during IVM (Tatemoto *et al.* 2000). Endogenous antioxidant networks of CCs acts through gap junction communication to reduce ROS toxicity (Cetica *et al.* 2001). In the present study, exposure of bovine COCs to heat shock during IVM downregulated *MT1E* mRNA in CCs. Similarly, this transcript was reduced in Nelore compared with Holstein CCs. The MT1E protein exhibits antioxidant activity (Chung *et al.* 2006), acting as a scavenger of the free radicals hydrogen peroxide, superoxide and hydroxyl radical (Babula *et al.* 2012). *MT1E* mRNA undergoes post-transcriptional regulation (Gurel *et al.* 2005) so that metallothionein protein levels often do not correlate with mRNA expression (Vasconcelos *et al.* 2002). Therefore, heat-induced reductions in *MT1E* mRNA expression in CCs in the present study may have been due to increased protein synthesis for the protection of both CCs and oocytes. Similarly, reduced *MT1E* abundance in heat-shocked oocytes suggests greater mRNA usage during heat shock.

Exposure of Holstein and Nelore COCs to 41°C during IVM did not affect the mRNA expression of *KIF3A*, *BIRC3*, *DAP*, *CCT4* and *DICER1* in CCs. This result is in agreement with the microarray study conducted by Rispoli *et al.* (2013), who found that the expression of these genes in CCs derived from Holstein COCs subjected to 41°C during IVM were not affected by temperature. There is evidence that mRNA expression of human *BIRC3* and *DAP* is downregulated in CCs from polycystic ovary patients (Haouzi *et al.* 2012).

In conclusion, global changes in the gene expression profile of Nelore and Holstein oocytes subjected to control and heat shock treatments during IVM indicate a strong difference between breeds. Such differences were not regulated by temperature. The genes regulated by oocyte breed were mostly involved in cellular organisation and cell death. In contrast, breed and temperature acted to alter CC expression of molecules involved in cellular organisation and oxidative stress. In addition, the results of the present study suggest that the deficiency in *B. taurus taurus* in cellular protection against heat stress is also regulated at the post-transcriptional and protein level.

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