Seasonal effects on gene expression, cleavage timing, and developmental competence of bovine preimplantation embryos

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Abstract

We examined the association between season and expression of genes involved in early embryonic development with an emphasis on cleavage rate and timing of the first embryonic cleavage. In Exp. 1, oocytes were aspirated during the cold (Dec–Apr) and hot (May–Nov) seasons. Matured oocytes were chemically activated and cultured *in vitro*. The developmental peak to the two- and four-cell stages occurred earlier, with a higher proportion of first-cleaved embryos, during the cold season relative to the hot season ($P < 0.01$). In Exp. 2, a time-lapse system was employed to characterize the delayed cleavage noted for the hot season. Cleavage to the two-cell stage occurred in two distinct waves: early cleavage occurred between 18 and 25 h post activation, and late cleavage occurred between 27 and 40 h post activation. In Exp. 3, oocytes were aspirated during the cold and hot seasons, matured *in vitro*, fertilized, and cultured for 8 days. In each season, early- and late-cleaved two-cell stage embryos were collected. Total RNA was isolated, and semi-quantitative and real-time PCRs were carried out with primers for *GDF9*, *POU5F1*, and *GAPDH* using *18S rRNA* as the reference gene. In both seasons, the expression of all examined genes was higher ($P < 0.05$) in early- versus late-cleaved embryos. *POU5F1* expression was higher ($P < 0.05$) in early-cleaved embryos developed in the cold season versus the hot season counterparts. The findings suggest a deleterious seasonal effect on oocyte developmental competence with delayed cleavage and variation in gene expression.

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Introduction

Reduced reproductive performance of lactating cows during the summer has been well documented and mostly attributed to the sensitivity of the ovarian tissues to elevated temperature (Wolfenson *et al.* 2000, Roth *et al.* 2008). Hyperthermia can directly disrupt follicular function (Roth *et al.* 2000) as expressed by altered follicular development, depressed dominance, and impaired follicular steroidogenesis and gonadotropin secretion (Wolfenson *et al.* 2000). Alternatively, perturbation in the physiology of the follicle-enclosed oocyte can potentially lead to its reduced competence for fertilization and subsequent development.

Mammalian oocytes are arrested in the prophase stage of the first meiotic division, and acquire their meiotic competence and fertilization potential in a stepwise manner (Masui 2001). As a result, they are potentially exposed to various environmental stressors during follicular development. Oocytes harvested from cows during the summer exhibit reduced embryonic developmental competence following IVF (Rocha *et al.* 1998, Al-Katanani *et al.* 2002) or chemical activation (Zeron *et al.* 2001). A seasonal study, performed from late

summer to early winter, indicated that a period of two to three estrous cycles is required for recovery from heat damage and appearance of competent oocytes (Roth *et al.* 2001). Similarly, induction of maternal hyperthermia in mice carried over through three pregnancy cycles (Aroyo *et al.* 2007), suggesting that not only the individual ovulated oocyte, but also the ovarian pool of oocytes can be damaged during heat exposure. Nevertheless, the mechanism underlying the disruption in oocyte developmental competence is not entirely clear, and therefore, the changes that occur at the molecular and cellular levels in the oocytes in response to heat stress need to be investigated further.

Changes in gene expression are an integral part of the cellular response to heat shock. While the genes encoding heat shock proteins (HSPs) have been best studied, it has recently become apparent that thermal stress also affects a substantial number of genes which are not directly associated with HSPs (Sonnen *et al.* 2002). Therefore, the current study examines the association between seasons and the expression of genes playing an essential role during oogenesis, folliculogenesis, or early embryonic development. Among these genes is growth differentiation factor 9 (*GDF9*), a germ-cell marker and

member of the large transforming growth factor β superfamily (TGFB; McPherron & Lee 1993). *GDF9* plays a pivotal role in folliculogenesis since homozygous knockout female mice are sterile due to blockage of follicles at the primary stage (Dong *et al.* 1996). It also regulates cumulus cell function in the preovulatory period (Gui & Joyce 2005), and therefore, it might be involved in oocyte maturation. Another examined gene is *POU5F1* (also known as *OCT4*), a member of the POU family of transcriptional activators which contain the DNA-binding POU domain (Ryan & Rosenfeld 1997). The *POU5F1* gene is essential for the maintenance of totipotency/pluripotency in embryonic stem cells and primordial germ cells: a progressive loss of *POU5F1* has been associated with loss of pluripotency (Okamoto *et al.* 1990, Yeom *et al.* 1996). The current study examines the association between elevated temperature and expression of genes involved in oocyte maturation early in embryonic development, with an emphasis on cleavage timing of the two first embryonic developmental deviations.

Results

Experiment 1: seasonal effects on cleavage timing and developmental competence of bovine oocytes following chemical activation

Oocytes were collected during the cold ($n=2218$) and hot ($n=3198$) seasons. The number of follicles (3–8 mm in diameter) per ovary collected during the cold season was higher than that collected in the hot

Figure 1 Cleavage timing of bovine embryos into the two- and four-cell stages. Oocytes were aspirated and activated during cold (Dec–Apr; squares) and hot (May–Nov; stars) seasons. The proportion of embryos that developed to the two-cell stage (A) and that developed to the four-cell stage (B) was recorded at 17, 22, 27, 40, 46, 52, 64, and 72 h post activation. Results are presented as means $\pm$ S.E.M.; seasonal effect, $P<0.01$.

season (29.0 ± 1.0 vs 24.0 ± 1.0 respectively; $P<0.03$). The average number of oocytes aspirated in the cold season was higher than that aspirated in the hot season (8.5 ± 0.5 vs 6.0 ± 0.5 respectively; $P<0.01$). The proportion of parthenotes that cleaved to the two- to four-cell stage was higher in the cold season than in the hot season (72 ± 0.9 vs $46 \pm 0.8\%$ respectively; $P<0.01$). The developmental peak of the two-cell stage embryo was higher (39 vs 21%; $P<0.01$) and occurred earlier (22–27 vs 40 h post activation; Fig. 1A) in the cold versus hot season respectively. Similarly, the developmental peak of the four-cell stage was higher (33 vs 21%; $P<0.01$) and occurred earlier (46–52 vs 52–70 h post activation; Fig. 1B) in the cold versus hot season respectively.

Experiment 2: using time-lapse system to evaluate the accurate cleavage timing

This experiment was performed during two consecutive hot seasons using a time-lapse system. In the first year, 137 bovine oocytes, in two replicates, were examined (Fig. 2A). In the second year, 171 oocytes, in four replicates, were examined (Fig. 2B). Time-lapse examination of the first embryonic division revealed that during the hot season, embryos cleave in two distinct waves. The first cleavage wave occurred between 18 and 25 h post activation and the second cleavage wave occurred between 26 and 42 h post activation.

Figure 2 First cleavage division into the two-cell stage of bovine parthenotes during two consecutive hot seasons (A – two replicates, $n=137$ oocytes, and B – four replicates, $n=171$ oocytes respectively). Oocytes were activated and observed by EmbryoGuard time-lapse system that was pre-programmed to take photos at 1-h intervals. Embryos cleaved in two distinct waves: the first wave occurred between 18 and 25 h post activation, and the second wave occurred between 26 and 42 h post activation. Results are presented as means $\pm$ S.E.M.

Experiment 3: seasonal variation in gene expression in two-cell stage embryos

The percentage of embryos developed to the blastocyst stage was higher in the cold season than in the hot season (23.0 ± 2.3 vs $9.0 \pm 3.5\%$ respectively; $P < 0.05$). The percentage of embryos that cleaved to the two- to four-cell stage (42 h post fertilization) did not differ between seasons (80.0 ± 4.5 vs $73.3 \pm 3.2\%$ respectively; Fig. 3). However, the proportion of two-cell stage embryos recorded in the hot season was twofold higher than that of the four-cell stage embryos, suggesting a delay in the second embryonic division (Fig. 4). In addition, the proportion of embryos developed to the four-cell stage (42 h post fertilization) was higher in the cold season than in the hot season (Fig. 4).

Seasonal effect on gene expression in two-cell stage embryos

Examination of gene expression in both early (27 h post fertilization) and late (42 h post fertilization) first-cleaved embryos (i.e. two-cell stage embryos) revealed higher expression of *GDF9* and *POU5F1* in the early- versus late-cleaved embryos in both the cold and hot seasons (Figs 5A and B and 6A and B). With respect to *GAPDH*, while semi-quantitative PCR did not reveal any differences in its expression between early- and late-cleaved embryos (Figs 5A and 6A), real-time PCR, which is a more sensitive method, demonstrated a significant difference (Figs 5B and 6B). Therefore, *GAPDH* was not used as a reference gene for PCR analysis. Instead, the *18S rRNA* gene was used as an internal reference since it was expressed at similar levels in both early- and late-cleaved embryos, in both seasons. A comparison of gene expression in early-cleaved embryos revealed higher expression of *POU5F1* in the cold versus hot season, without any effect on *GDF9* or *GAPDH* expressions (Fig. 7). No seasonal variations in gene expression were noted for late-cleaved embryos (data not shown).

Figure 3 Seasonal effects on oocyte developmental competence. The proportion of oocytes cleaved to the two- to four-cell stage, 42 h post fertilization, and the proportion of embryos that developed to the blastocyst stage 8 days post fertilization are presented. Data are presented as means $\pm$ S.E.M.; ^{a,b}different superscripts indicate seasonal effect within embryonic stages, $P < 0.05$.

Figure 4 Seasonal variation in embryonic-stage distribution. The proportion of oocytes that were fertilized and cleaved to two- and four-cell stages at 42 h post fertilization is presented. Data are presented as means $\pm$ S.E.M.; ^{a,b}different superscripts indicate seasonal effect between and within embryonic stages, $P < 0.05$.

Discussion

Season-induced disruption in the developmental competence of bovine oocytes appears to involve more than one mechanism. The current study indicates that the adverse effect of summer thermal stress on embryonic development is associated with seasonal variation in the expression of genes involved in oocyte maturation and early embryonic development. These alterations were also associated with delayed cleavage of the two first

Figure 5 Transcript levels of *GDF9*, *POU5F1*, and *GAPDH* in both early- (27 h post fertilization (PF)) and late-cleaved (42 h PF) embryos to the two-cell stage in the cold season. Data from semi-quantitative PCR (A) and real-time PCR (B) are presented as means $\pm$ S.E.M.; ^{a,b}different superscripts indicate cleavage timing effect within genes, $P < 0.05$.

Figure 6 Transcript levels of *GDF9*, *POU5F1*, and *GAPDH* in both early- (27 h post fertilization (PF)) and late-cleaved (42 h PF) embryos to the two-cell stage in the hot season. Data from semi-quantitative PCR (A) and real-time PCR (B) are presented as means $\pm$ S.E.M.; ^{a,b} different superscripts indicate cleavage timing effect within genes, $P < 0.05$.

embryonic deviations and decreased embryonic development to the two- and four-cell stage followed by a reduced proportion of embryos developing to the blastocyst stage.

Early cleavage and cleavage timing

Findings of the present study indicated delayed cleavage timing for both bovine parthenotes (Exp. 1) and *in vitro* derived embryos (Exp. 3) which developed from oocytes collected during the hot season. In the cold season, the developmental peak to the two-cell stage was higher and occurred about 13 h earlier than that in the hot season. A similar delay was found for the second deviation to the four-cell stage, as expressed by the twofold higher proportion of two-cell stage embryos versus four-cell stage embryos at 42 h post fertilization. Early stages of embryonic development are key parameters in predicting which embryos will have high developmental competence. Early cleavage and the time of the first mitotic division are considered to be indicative of embryo quality, in particular of whether the embryo is transferable (Hasler *et al.* 1995, Shoukir *et al.* 1997, Sakkas *et al.* 1998) or competent to survive cryopreservation (Hasler *et al.* 1995, Van Montfoort *et al.* 2004). Indeed, the timing of the first divisions (Shoukir *et al.* 1997, Sakkas *et al.* 1998) and of the formation of an

eight-cell embryo (Edwards *et al.* 1984) or blastocyst (Shoukir *et al.* 1998, Schwarzler *et al.* 2004) are used as valuable means of narrowing the number of embryos selected for transfer or cryopreservation. It has been reported that embryo evaluation based on sequential assessment, which takes several development stages into account, appears to be superior to the selection of the best embryos at a specific time point (Gardner & Sakkas 2003, Neuber *et al.* 2003). Therefore, the delayed cleavage timing noted in the current study throughout the hot season might suggest a seasonal reduction in oocyte developmental competence.

A recent study in which the time-lapse system EmbryoGuard was used to determine the accurate cleavage timing of mouse embryos suggested that the time of the first embryonic cleavage to the two-cell stage, rather than further embryonic divisions, predicts developmental competence of embryos (Arav *et al.* 2008). The time-lapse system facilitates continuous noninvasive monitoring of embryos while maintaining them under optimal culture conditions in the incubator. Use of this system in the current study made it possible to collect accurate data on cleavage timing of the entire embryo population without examining embryos at subjectively selected time points, as performed in Exp. 1. *In vitro* studies have shown a clear relationship between early cleavage and developmental competence of bovine embryos (Lonergan *et al.* 1999). It has also been demonstrated that early-cleaved embryos have significantly higher cell numbers and better embryo morphology (van Soom *et al.* 1997, Rieger *et al.* 1999). Use of the EmbryoGuard system enabled us to accurately define the time at which bovine oocytes are cleaved. Cleavage to the two-cell stage was found to occur in two distinct waves: early cleavage occurred between 18 and 25 h post activation and late cleavage occurred between 26 and 42 h post activation. Although the experiment was performed only during the hot season, the

Figure 7 Transcript levels of *GDF9*, *POU5F1*, and *GAPDH* in early-cleaved embryos. Data from real-time PCR of two-cell stage embryos collected 27 h post fertilization in both cold (Dec–Apr) and hot (Jun–Sep) seasons are presented as means $\pm$ S.E.M.; ^{a,b} different superscripts indicate seasonal effect within genes, $P < 0.05$.

information was highly valuable in collecting early- and late-cleaved embryos to examine seasonal effects on gene expression (Exp. 3).

Our results provided evidence of higher gene expression (*GAPDH*, *GDF9*, and *POU5F1*) in early- versus late-cleaved embryos, in both cold and hot seasons, suggesting a differential pattern of maternal gene expression which is tightly associated with cleavage timing. *GAPDH* is one of the most common 'housekeeping' genes, widely used to normalize gene data in various procedures, including quantitative and real-time PCR. Nevertheless, it has recently been reported that the transcription levels of *GAPDH* vary among cell types (Barber *et al.* 2005), developmental stages (Robert *et al.* 2002), and experimental conditions (Ito *et al.* 1996, Zhong & Simons 1999), emphasizing the need to validate housekeeping genes for each examination. In the current study, two genes were considered for use as the internal control gene, *GAPDH*, and *18S rRNA*. Our findings indicated that whereas the expression of *18S rRNA* was stable, that of *GAPDH* differed between early- and late-cleaved embryos, with a lower level in the latter. This was true for both hot and cold seasons. Similarly, Mamo *et al.* (2007) showed higher expression of *GAPDH* in early- versus late-cleaved *in vivo* derived mouse embryos. However, Dode *et al.* (2006) reported constant expression of *GAPDH* in early- and late-cleaved bovine embryos. *GAPDH* plays a key role in energy metabolism. It is one of the enzymes that catalyze reactions in the glycolytic pathway, in particular converting glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Sirover 1999). *GAPDH* also plays a role in cellular functions which are not related to glycolysis. These include membrane fusion (Robbins *et al.* 1995, Hessler *et al.* 1998), DNA repair (Baxi & Vishwanatha 1995), nuclear RNA export (Zhang & Snyder 1992, Singh & Green 1993), cytoskeletal organization (Launay *et al.* 1989, Walsh *et al.* 1989), phosphotransferase activity (Kawamoto & Caswell 1986, Engel *et al.* 1998), and apoptosis (Ishitani & Chuang 1996, Ishitani *et al.* 1997, Tajima *et al.* 1999). Therefore, reduced *GAPDH* expression or, alternatively, alterations in one or more of these *GAPDH* activities might underlie the reduced developmental competence of late-cleaved embryos. This, might explain, at least in part, the reduced developmental competence of bovine oocytes during the hot season.

Reduced expression of *GDF9* was also noted for late-cleaved two-cell stage embryos in both hot and cold seasons. Studies of mutant mice (Dong *et al.* 1996, Carabatsos *et al.* 1998) and *GDF9*-modified sheep (Hanrahan *et al.* 2004) have indicated that *GDF9* plays an important role in the stimulation of early follicular growth. Female mice lacking a functional *GDF9* gene were infertile and characterized by arrested follicular growth at the primary stage (Dong *et al.* 1996). In fact, *GDF9* transcript is highly expressed in immature oocytes

while they are enclosed in antral follicles, but decreases throughout early embryonic development, with only trace levels at the eight-cell stage (Pennetier *et al.* 2004). Therefore, it is suggested that seasonally induced alterations in *GDF9* expression are involved in the reduced developmental competence noted for oocytes collected in the hot season. *GDF9* is involved in the regulation of both oocyte and granulosa cell function at a very early stage of follicular development in rats (Vitt *et al.* 2000). In cattle and sheep, *GDF9* mRNA is expressed in the primordial and subsequent stages of developing follicles (Bodensteiner *et al.* 1999). *GDF9* has also been defined as a mitogenic factor since it stimulates proliferation of theca cells derived from small follicles of bovine (Spicer *et al.* 2008). Thus, it is possible that the seasonally induced alteration in *GDF9* expression in the ovarian pool of follicles and/or their enclosed oocytes, as observed here, underlies the immediate and carry-over effect of summer thermal stress on follicular function and oocyte developmental competence recently reported for bovine (Roth *et al.* 2008). Likewise, maturation of mouse oocytes with exogenous *GDF9* improves further embryonic development and fetal viability (Yeo *et al.* 2007). While not examined here, impairment in the cooperative functioning of TGF β superfamily proteins, bone morphogenetic protein 15, with *GDF9* to maintain cumulus-oocyte complexes (COCs) integrity cannot be ruled out (Su *et al.* 2004).

Interestingly, while the expressions of *GAPDH* and *GDF9* did not differ among the subpopulation of early-cleaved embryos, those that developed during the hot season expressed lower levels of *POU5F1*. *POU5F1* is known to activate or repress the transcription of various target genes, such as *FGF4*, *ZPF42 (REX-1)*, *SPP1 (OPN)*, *UTF1*, *hCG*, and *IFNT*, during early embryonic development (Curatola & Basilico 1990, Liu & Roberts 1996, Liu *et al.* 1997, Ben-Shushan *et al.* 1998, Botquin *et al.* 1998, Nishimoto *et al.* 1999, Ezashi *et al.* 2001). Up- or downregulation of *POU5F1* can induce changes in developmental programs (Niwa *et al.* 2000). In mice, ablation of *POU5F1* causes early embryonic lethality, since blastomeres are differentiated to trophectoderm rather than to inner cell mass (ICM; Nichols *et al.* 1998), indicating involvement of *POU5F1* in the establishment of pluripotency. *POU5F1* also plays a role in maintaining mammalian germline viability since primordial germ cells lacking *POU5F1* expression have been shown to undergo apoptosis instead of differentiation (Kehler *et al.* 2004). A critical amount of *POU5F1* protein is required to sustain embryonic stem cells: increasing its expression by less than twofold causes differentiation into primitive endoderm and mesoderm. Repression of *POU5F1* induces loss of pluripotency and dedifferentiation to trophectoderm (Niwa *et al.* 2000). In *in vitro* derived bovine embryos, *POU5F1* transcription is expressed throughout the early developmental stages of pre-implantation embryos with high expression in immature

oocytes up to the four-cell stage, followed by down-regulation from the eight-cell stage to the morula, and relatively higher transcript at the blastocyst stage (Nganvongpanit *et al.* 2006). In contrast to mice (Palmieri *et al.* 1994, Kircof *et al.* 2000) and humans (Hansis *et al.* 2000), in bovine and porcine embryos, *POU5F1* is not restricted to the ICM: it is present in both the ICM and the trophectoderm (Kircof *et al.* 2000). Taken together, low expression of *POU5F1* in late-cleaved embryos supports the assumption that these embryos are of inferior quality. Furthermore, seasonal alteration in *POU5F1* in early-cleaved embryos might explain, in part, the decrease in blastocyst development observed in the hot season (current study) and the concomitant reduced reproductive performance of dairy cows (Al-Katanani *et al.* 2002). In addition, *POU5F1* plays a role as an anti-apoptotic factor (Guo *et al.* 2007), and therefore, a reduction in *POU5F1* expression might impair the balance between pro- and anti-apoptotic factors, which in turn might lead to increased apoptotic cells upon heat stress, as previously reported for bovine oocytes and pre-implantation embryos (Roth & Hansen 2004a, 2004b).

In summary, delayed embryo cleavage was prominent during the hot season, indicating that during the summer, a high proportion of oocytes are of low developmental competence. Development and growth of embryos depend upon maternal mRNA and proteins stored in the oocyte. Thus, it is reasonable to assume that heat-induced alteration in the ovarian pool of oocytes affects the expression pattern of genes involved in early embryonic development, which in turn leads to a high proportion of late-cleaved embryos and reduced blastocyst formation.

Materials and Methods

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich. FSH and the ovine pituitary extract Ovagen were purchased from ICP Bio (Auckland, New Zealand). TriZol reagent, superscript II, Dynabeads mRNA DIRECT kit and nonessential amino acids (NEAAs) were purchased from Invitrogen. RNasin oligo(dT)₁₂₋₁₈, moloney murine leukemia virus (M-MLV) RT, dNTPs, and RQ1 RNase-free DNase were purchased from Promega. 5× TBE buffer (53 g of Tris base, 27.5 g of boric acid, and 20 ml of 0.5 M EDTA (pH 8.0) and diethylpyrocarbonate (DEPC)-treated water were purchased from Biological Industries (Beit-Haemek, Israel). DyNAmo qPCR kit was purchased from Zotal (Espoo, Finland). Double distilled water was purchased from Merck, and the culture media HEPES-Tyrodé's lactate (TL), SP-TL, and IVF-TL were prepared in our laboratory: HEPES-TL was supplemented with 0.3% (w/v) BSA, 0.2 mM sodium pyruvate, and 0.75 mg/ml gentamicin (HEPES-TALP); SP-TL was supplemented with 0.6% BSA, 1 mM sodium pyruvate, and 0.2 mg/ml gentamicin (SP-TALP); IVF-TL was supplemented with 0.6% essential fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.05 mg/ml gentamicin, and 0.01 mg/ml heparin (IVF-TALP);

Parrish *et al.* 1986). Oocyte maturation medium (OMM) was made up of TCM-199 with Earle's salts supplemented with 10% (v/v) heat-inactivated FCS (Bio-Lab), 0.2 mM sodium pyruvate, 50 µg/ml gentamicin, 1.32 µg/ml ovine FSH, and 2 µg/ml estradiol; potassium simplex optimized medium (KSOM) contained 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH₂PO₄, 0.2 mM MgSO₄·7H₂O, 0.8% (v/v) sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM D(+)-glucose, 25 mM NaHCO₃, 1 mM L-glutamine, 0.01 mM EDTA and 0.01 mM phenol red supplemented with 1.7 mM CaCl₂·2H₂O, 0.1 mg/ml polyvinyl alcohol, 10 µl/ml essential amino acids and 5 µl/ml NEAAs, 100 U/ml penicillin-G and 0.1 mg/ml streptomycin.

In vitro production of embryos

Oocyte recovery and in vitro maturation

Bovine ovaries were obtained from a local abattoir from multiparous Holstein cows during the hot and cold seasons, and transported to the laboratory in physiological saline solution (0.9% (w/v) NaCl at 37 °C) with 50 µg/ml penicillin-streptomycin. The ovaries were transferred to the laboratory within 60–90 min, washed with fresh saline, cut through the center, and placed over a transillumination stand, so that follicles could be easily visualized (Arav 2001). COCs were aspirated from 3- to 8-mm follicles with an 18-gauge needle attached to a 10-ml syringe. COCs with at least three layers of cumulus surrounding a homogeneous cytoplasm were selected for further processing. After selection, COCs were washed three times in HEPES-TALP, and groups of ten oocytes were transferred into 50-µl droplets of OMM overlaid with mineral oil. The droplets containing COCs were incubated in humidified air with 5% CO₂ for 22 h at 38.5 °C.

Chemical activation of oocytes

Activation was performed (Exp. 1 and Exp. 2) as previously described by Zeron *et al.* (2001). Matured oocytes were denuded from the cumulus cells and placed for 5 min in 2.47 µM ionomycin dissolved in TCM-199 supplemented with 25 mM HEPES, 10% (v/v) heat-inactivated FCS, 0.2 mM sodium pyruvate, and 5 µg/ml gentamicin. Oocytes were then transferred to 2.45 µM 6-dimethylaminopurine dissolved in TCM-199 for 4.5 h to prevent extrusion of the second polar body, thereby creating a diploid parthenogenetic embryo. Oocytes were washed three times in IVF cleavage medium (Cook, Sydney, Australia) and transferred in groups of 10 into 50-µl cleavage drops overlaid with mineral oil. Embryos were cultured at 38.5 °C, 5% CO₂, 5% O₂, and 95% humidity (ThermoForma Model 3110, Thermo Scientific, Marietta, OH, USA).

IVF

Matured COCs were washed three times in HEPES-TALP and transferred in groups of 30 oocytes to four-well plates containing 600 µl IVF-TALP and 25 µl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% (w/v) NaCl) per well and fertilized with ~1×10⁶ Percoll-purified spermatozoa from frozen-thawed semen. Spermatozoa were co-incubated with COCs for 18 h at 38.5 °C in a humidified atmosphere with 5% CO₂.

In vitro culture

After fertilization, putative zygotes were removed from the fertilization wells, denuded of cumulus cells by gentle vortexing in HEPES-TALP containing 1000 U/ml hyaluronidase, and randomly placed in groups of 10 in a 25- μ l droplet of KSOM. All embryo droplets were overlaid with mineral oil and cultured for 8 days at 38.5 °C in an atmosphere of humidified air with 5% CO₂ and 5% O₂.

The EmbryoGuard system

The EmbryoGuard system (IMT Ltd, Ness Ziona, Israel) is a robotic device enabling real-time monitoring of embryo dynamics without disturbing culture conditions, i.e. temperature, air composition, and humidity. It comprises an inverted microscope, charge-coupled device cameras, and a light source. The optical system is placed on a mobile optical table that can move along the X and Y axes as well as the Z axis for focusing of all lenses. Embryos can be observed at three different magnifications: 4 $\times$, 200 $\times$, and 1000 $\times$. Automatic time-lapse photographs can be taken at specific preset intervals, and are saved in a database for subsequent analysis. The camera optics enable both automatic continuous time-lapse photography and real-time microscopic observation of individual embryos (Arav *et al.* 2008). In the current study, the unit was programmed to collect images at 60-min intervals. Culture dishes were placed in the tissue-culture incubator maintained at 38.5 °C, 5% CO₂ in air and 95% humidity, in the EmbryoGuard unit. Embryos were observed for the occurrence of the first and second cleavages.

Gene quantification

Sample collection

Embryos were collected at specific cleavage times based on the EmbryoGuard data (Exp. 2): two-cell stage embryos collected at 27–28 h post fertilization were defined as early-cleaved embryos, while those collected at 42–43 h post fertilization were defined as late-cleaved embryos. For each group of embryos, four replicates were taken from different *in vitro* production runs, washed in PBS, snap frozen in liquid

nitrogen, and stored at –80 °C until RNA extraction. Each sample for RNA extraction contained ten embryos at the two-cell stage.

Semi-quantitative RT-PCR

Total RNA was extracted from each sample by TRIzol reagent according to the manufacturer's instructions (Invitrogen) with slight modifications. Briefly, 500 μ l of TRIzol reagent with 1 μ g glycogen as the carrier were added to each sample. After centrifugation with chloroform (100 μ l), RNA was recovered (upper aqueous phase), precipitated with isopropanol, washed twice with ethanol, and dried for 5 min. After extraction, the RNA pellet was resuspended in 8 μ l DEPC water and treated with DNase I according to the manufacturer's instructions (Promega) to remove any DNA contamination. Isolated RNA was reverse-transcribed into cDNA in a total volume of 20 μ l. The first step of the RT was incubation at 72 °C with 500 mg oligo(dT)_{12–18} for 10 min followed by incubation for 60 min with RT mix, containing M-MLV RT, 5 $\times$ M-MLV-RT buffer, 200 μ M dNTPs, 20 U of RNasin, and 3 μ l of DEPC water. This mixture was incubated at 40 °C for 1 h.

The cDNAs were used for separate PCR amplification with primer sets (10 μ M each) for *GDF9*, *POU5F1*, *GAPDH*, and *18S rRNA* in a 25- μ l reaction volume. The PCR program consisted of an initial denaturation step at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. Primer sequences, size of the amplified products, and GenBank accession numbers are provided in Table 1. The RT-PCR product (18 μ l) with 3 μ l of 6 $\times$ loading buffer (0.25% (w/v) xylene cyanol and 25 mmol EDTA/I in 50% (w/v) glycerin) was loaded onto a 1.5% (w/v) agarose gel in TBE buffer containing 0.2 μ g/ml ethidium bromide. After electrophoresis at 80 V for 60 min, the fragments were visualized on a 312-nm u.v. transilluminator. Scion-Image software (Frederick, MD, USA) was used to quantify the signal intensity of each band (data not shown).

Real-time PCR

Poly(A) RNA was isolated using Dynabeads mRNA DIRECT kit according to the manufacturer's instructions (Invitrogen). In brief, embryos were lysed by adding 100 μ l lysis-binding buffer. Prewashed oligo(dT)₂₅ Dynabeads (20 μ l) were added to each

Table 1 Primers used in this study for semi-quantitative and real-time PCR.

Genes	Primer	Accession number	Sequence (5' → 3')	Size (bp)
<i>GDF9</i>	Forward	NM_174681	TGGTCCTTGCTGAAGCATCTAGA	202
	Reverse		ACAGTGTGTAGAGGTGGCTTCT	
<i>GDF9</i> ^a	Forward	NM_174681	TGCACCTGTCTATGCCTTTG	157
	Reverse		AACATTGGCCATGAGGAAG	
<i>POU5F1</i>	Forward	AY490804	ATATACCCAGGCCGATGTGG	201
	Reverse		TGCACAAGGGTCTCTGCCTT	
<i>GAPDH</i>	Forward	U85042	CCAACGTGTCTGTTGTGGATCTGA	237
	Reverse		GACCTTGACAAAGTGGTCGTTGAG	
<i>18S rRNA</i>	Forward	AF176811	GAGAAACGGCTACCACATCC	337
	Reverse		GGACACTCAGCTAAGAGCATCG	
<i>18S rRNA</i> ^a	Forward	AF176811	GGAGAGGGAGCCTGAGAAAC	157
	Reverse		TCGCGGAAGGATTAAAGTG	

^aPrimers used for real-time PCR.

tube and mixed for 5 min at room temperature to allow binding of poly(A) to the beads. The samples were put into a magnetic separator to remove the lysis buffer while retaining the Dynabeads. The Dynabeads were washed twice with 100 μ l washing buffer A, twice with 100 μ l washing buffer B, and once with 100 μ l 10 mM Tris–HCl. After removal of Tris–HCl, 8 μ l of sterile DEPC water were added, and the samples were immediately subjected to RT.

RT was performed in a total volume of 20 μ l. The first step was incubation at 70 °C with 8 μ l RNA sample, 1 μ l oligo(dT)_{12–18} (500 μ g/ml), 1 μ l RNaseout, 1 μ l dNTPs (10 mM each), and 1 μ l (50 ng) random primer followed by 50-min incubation at 42 °C and 5 min at 70 °C with RT mix containing 4 μ l 5 $\times$ RT buffer, 200 U of superscript II RT, 2 μ l 0.1 M dithiothreitol, and DEPC water. The samples were transferred to –20 °C until use.

Real-time PCR was conducted on an Mx3000p cycler (Stratagene, La Jolla, CA, USA) using SYBR green in a final volume of 20 μ l containing 3 μ l cDNA (equivalent to ten embryos at the two-cell stage) and 500 nM of each primer. The amplification program included preincubation for *tag* polymerase activation at 95 °C for 7 min followed by 40 amplification cycles of denaturation at 95 °C for 10 s and annealing–elongation at 60 °C for 15 s. All samples were run in duplicate in 96-well plates. A melting curve analysis was recorded at the end of the amplification to confirm that there is no contamination by primer dimers. For each gene, standard curves were established using six serial dilutions of a reference cDNA. Fluorescence was acquired to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence rose above background. Genes were quantified and analyzed by MxPRO QPCR Software for Mx3000p and Mx3005p QPCR ver 3, and the $\Delta\Delta C_t$ method was used to calculate relative concentration of each gene which was expressed relative to *18S rRNA*.

Experimental design

A seasonal study was performed over three consecutive years. The first experiment was performed during the cold (Dec–Apr) and hot (May–Nov) seasons. Environmental data were obtained from the central meteorological station in Bet-Dagan, Israel. The maximum average air temperature and relative maximum humidity were 31.8 ± 1.4 °C and $84.3 \pm 3.8\%$ during the

summer, and 16.8 ± 2.3 °C and $46.0 \pm 15.0\%$ during the winter respectively. Oocytes were aspirated from ovaries collected at the local abattoir, matured, activated, and *in vitro* cultured (Fig. 8). Early embryonic development with an emphasis on the rate of the two first deviations was recorded on 17, 22, 27, 40, 46, 52, 64, and 70 h post activation. The time of developmental pick was defined as the time in which the highest proportion of developing embryos at a given stage was recorded. The second experiment was conducted during the hot season. Accurate timing of parthenos division was observed and documented with the EmbryoGuard system at 1-h intervals, to characterize the pattern of first cleavage of bovine embryos, as previously described by Arav *et al.* (2008). The third experiment was performed during both the cold (Dec–Apr) and hot (Jun–Sep) seasons. Oocytes were aspirated from ovaries collected at the local abattoir, matured, fertilized, and *in vitro* cultured as described above. The proportion of oocytes cleaved to the two- and four-cell stages and further developed to the blastocyst stage was assessed at 27, 42–44 h, and 8 days post fertilization respectively. Early- and late-two-cell stage embryos were collected in each season (four experimental replicates, ten embryos each). Total RNA was isolated, and semi-quantitative PCR and real-time PCR were carried out with primers for *GDF9*, *POU5F1*, and *GAPDH* using *18S rRNA* as the reference gene. The primers were derived from bovine sequences found in Genbank and designed using Primer Express software (Table 1).

Statistical analysis

Differences between treatments were subjected to one-way ANOVA (JMP-6; SAS Institute, Cary, NC, USA). χ^2 test or Student's *t*-test was used to compare data among the experimental groups. Data are presented as mean $\pm$ S.E.M. Relative gene expression was analyzed by one-way ANOVA followed by Tukey–Kramer test. Differences of $P < 0.05$ were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure 8 Schematic diagram of Exp. 1. The experiment was performed during the cold (Dec–Apr) and hot (May–Nov) seasons. Oocytes were *in vitro* matured (IVM), activated, and *in vitro* cultured (IVC). Embryonic development to the two- and four-cell stages was recorded from 17 to 70 h post activation.

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