Embryonic genotype and inbreeding affect preimplantation development in cattle

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Abstract

Infertility in cattle herds is a growing problem with multifactorial causes. Embryonic genotype and level of inbreeding are among the many factors that can play a role on reproductive efficiency. To investigate this issue, we produced purebred and crossbred bovine embryos by *in vitro* techniques from Holstein oocytes and Holstein or Brown Swiss semen and analyzed several cellular and molecular features. In the first experiment, purebred and crossbred embryos, obtained from abattoir oocytes, were analyzed for cleavage, development to morula/blastocyst stages, amino acid metabolism and gene expression of developmentally important genes. The results indicated significant differences in the percentage of compacted morulae, in the expression of three genes at the blastocyst stage (*MNSOD*, *GP130* and *FGF4*) and in the utilization of serine, asparagine, methionine and tryptophan in day 6 embryos. In the second experiment, bovine oocytes were collected by ovum pick up from ten Holstein donors and fertilized with the semen of the respective Holstein sires or with Brown Swiss semen. The derived embryos were grown *in vitro* up to day 7, and were then transferred to synchronized recipients and recovered on day 12. We found that purebred/inbred embryos had lower blastocyst rate on days 7–8, were smaller on day 12 and had lower expression of the trophoblast gene *PLAC8*. Overall, these results indicate reduced and delayed development of purebred embryos compared with crossbred embryos. In conclusion, this study provides evidence that embryo genotype and high inbreeding can affect amino acid metabolism, gene expression, preimplantation development and therefore fertility in cattle.

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Introduction

Several investigators have shown a clear decline in reproductive efficiency in dairy cattle over the last 50 years. Butler (1998), in particular, has reported that the first service conception rate has decreased from ~65% in 1951 to 40% in 1996 for dairy cattle of New York state. Equivalent declines in reproductive efficiency have occurred in other countries and specific studies have been published for Ireland (Roche *et al.* 2000), UK (Royal *et al.* 2000), Spain (Lopez-Gatius 2003) and Australia (Macmillan *et al.* 1996). Several authors have attributed this decline to the selection for increasing milk production (Butler 1998, Olori *et al.* 2002, Wall *et al.* 2005) and published large datasets show a clear inverse relationship between milk production and reproduction efficiency in dairy cattle (Dematawewa & Berger 1998, Hansen 2000). Other changes, besides increased milk production, have occurred in the dairy cattle industry over the last 50 years. For instance, the decline in the

number of dairy herds and the concentration of the dairy industry in larger farms has brought about new challenges in reproductive management. Larger herds require more time for heat detection, record keeping, sorting and insemination and also different strategies from visual heat detection, such as timed artificial insemination. Such strategies can offer management advantages although conception rate is lower (Nebel & Jobst 1998). By contrast, in herds with poor estrus detection and low fertility, this choice can improve the interval to first service and overall pregnancy rate (Schmitt *et al.* 1996, Pursley *et al.* 1997), thus achieving a positive effect on reproductive management. Therefore, the size of the herd and its reproductive management can play an important role in reproductive efficiency. Finally, other factors such as season of calving, *post partum* diseases (Grohn & Rajala-Schultz 2000) and negative energy balance in high-producing cows are also involved in the genesis of infertility

although to variable degree according to numerous published reports (Staples *et al.* 1990, Lucy *et al.* 1992).

In addition to all these factors, the rising inbreeding in dairy herds has also been included in the multifactorial causes of low reproductive efficiency. The coefficient of inbreeding measures the percentage of genes of an individual that are identical by descent and, over the last 30 years, this coefficient has increased from about 1 to ~5% in the Holstein breed. Estimates of the effects of inbreeding on reproduction indicate a detrimental effect on reproductive parameters such that every 1% increase in inbreeding led to 0.17 increase in service/conception, 2 days increase in the period from calving to conception (days open) and 3.3 percentage-unit decrease in conception rate (Hermas *et al.* 1987). As a strategy to counterbalance the rise in inbreeding in dairy breeds, crossbreeding has been proposed as a possibility to increase productive and reproductive efficiency (McAllister 2002). The economical benefits of crossbreeding have been reported in a large study in the USA, (VanRaden & Sanders 2003) and interestingly, a recent *in vitro* study (Boediono *et al.* 2003) demonstrated that crossbred embryos develop faster and at higher rate than purebred embryos, suggesting that genotype and possibly inbreeding can influence developmental competence.

In light of all these published reports, the aim of this study was to further investigate the effect of inbreeding on reproductive efficiency by focusing on a number of cellular and molecular parameters of preimplantation development in embryos of different genotype and level of inbreeding.

Results

Embryo development: experiment 1

A total number of 2274 oocytes were collected and matured *in vitro*, 1135 were fertilized with six different Holstein bulls and 1139 with six different Brown Swiss bulls. Oocytes collected from each batch of ovaries were divided and assigned to the crossbred or purebred group to reduce the effect of the oocyte quality. We found no difference in cleavage rate at 30 h (28.78 vs 26.41%) and in total cleavage rate (78.79 vs 74.35%), although we observed a significant difference in favor of crossbred embryos in the compaction rate on day 6 (26.5 vs 30.03%; $P=0.01$) and a tendency for higher

blastocyst development on day 7 (18.93 vs 20.89%) and 8 (29.18 vs 33.18%; $P=0.07$, χ^2 test). Results are summarized in Table 1.

Amino acid analysis

Overall, purebred and crossbred embryos displayed a similar amino acid profile but there was a significant difference in the utilization of serine ($P=0.0079$), asparagine ($P=0.012$), methionine ($P=0.000$) and tryptophan ($P=0.034$) between the two groups (Fig. 1). Purebred embryos contained an average of 105.87 ± 3.12 cells ($n=45$), which was not significantly different from 108.56 ± 3.06 cells ($n=44$) obtained for crossbred embryos. There was also no difference in total amino acid depletion, appearance or turnover between the two groups (Fig. 2).

Embryo development: experiment 2

A total of 39 and 40 ovum pick ups (OPUs) were performed on ten Holstein heifers to generate inbred and crossbred embryos respectively. Six hundred and eighty oocytes were fertilized with the Holstein sire of each donor heifer and 688 with four different Brown Swiss bulls chosen from those used in experiment 1. Results indicate a significantly higher cleavage rate (74.3 vs 63.4%, see Table 2) for crossbred embryos compared with inbred embryos. In addition, we found that the rate of morula compaction, calculated on the total number of oocytes (22.7 vs 25.4%, see Table 2), the blastocyst rate on day 7, calculated both on the oocytes and on the cleaved embryos (11.6 and 18.3 vs 18.5 and 24.8%), and the blastocyst rate on day 8, calculated on the oocytes (16.2 vs 22.5%), were significantly higher for crossbred embryos (Table 2).

The higher developmental rate observed on day 7 was further investigated by transferring the embryos in synchronized recipients and recovering them on day 12 (Fig. 3). A total of 43 inbred embryos and 70 crossbred embryos were transferred on day 7 in eight recipient heifers in total, four recipients for each group. Overall, 29 embryos (67.4%) and 48 embryos (68.6%) respectively were recovered on day 12 (Table 3). Although the recovery rate was similar in the two groups, we found a significantly higher rate of ovoid embryos (56.3 vs 20.6%) in the crossbred group, while in

Table 1 Experiment 1: effect of genotype on embryo development.

Type of embryo	Number of oocytes	Number of cleaved (%)	Number of compacted morulae, day + 6 (% of cleaved)	Number of blastocysts, day + 7 (% of cleaved)	Number of blastocysts, day + 8 (% of cleaved)
Purebred	1135	898 (78.79)	238 (26.5) ^a	170 (18.93)	262 (29.18)
Crossbred	1139	847 (74.35)	257 (30.03) ^b	177 (20.89)	281 (33.18)

χ^2 test. Values within columns with different letters are statistically different ($P<0.05$).

Figure 1 Amino acid depletion/appearance by purebred and crossbred bovine embryos. Inset is an enlargement of those amino acids which have a value between 2 and -2 fmol/cell per hour. Bars with the same superscript are significantly different. a, $P=0.0079$; b, $P=0.012$; c, $P=0.000$; d, $P=0.034$.

the inbred group, most of the embryos were spherical (79.4 vs 43.7%), indicating that also in the second week of development crossbred embryos developed faster and at higher rate. This observation was confirmed also by the difference in the average size of the recovered embryos: crossbred embryos mean size was $0.48 \pm 0.12 \times 0.40 \pm 0.10$ mm (length $\times$ width) compared with $0.35 \pm 0.18 \times 0.28 \pm 0.09$ mm (length $\times$ width) of inbred embryos.

Gene expression analysis: experiment 1

Statistical analysis using a Student's *t*-test indicated that, in day 7 blastocysts, the relative expression of three genes was significantly higher in the crossbred group: *MNSOD*, a mitochondrial Mn-superoxide dismutase, *GPI30*, a glycoprotein subunit of the LIF receptor and fibroblast growth factor 4 (*FGF4*; Fig. 4A). Also *PED*, the preimplantation embryo development gene, and *GJA1* (also known as *CX43*), a connexin implicated in cellular communication, were more expressed in the crossbred group but without reaching a statistical difference. By contrast, transcripts of stress- and apoptosis-related genes, *G6PDH* and *BAX*, a glucose-6-phosphate dehydrogenase and the apoptosis regulator box-a had a tendency for higher expression in the purebred group. *SLC2A5*, a glucose transporter (also known as *GLUT5*), insulin-like growth factor 1 receptor (*IGF1R*) and interferon tau (*IFNT*) showed a similar expression in both groups.

Gene expression analysis: experiment 2

In day 12 embryos, we found that the genes responsible for embryo elongation, trophoblast development and fetus-maternal interaction, *IFNT*, *CDX2* and *PTGS2* (also known as *COX2*), had a tendency for increased expression in crossbred groups, but only *PLAC8*

displayed statistical significance using a Student's *t*-test (Fig. 4B). On the contrary, genes involved in programmed cell death such as *MSX1* and *BAX* were slightly more expressed in inbred embryos as previously observed in day 7 blastocyst. Expression of *HSP70* was linked more to the season in which the flushing was performed than to the class of embryos: in both groups, inbred and crossbred, the expression of *HSP70* was twice as high and statistically different in the summer season from that in the spring (data not shown).

Discussion

In this study, we have investigated the effect of genotype on embryo development, metabolic activity and gene expression. First of all, we monitored embryo development by examining morphological parameters. In experiment 1, we observed that crossbred embryos derived from oocytes of slaughtered donors had a tendency to cleave faster than purebred embryos. Interestingly, several studies performed on different species have demonstrated that the time of first cleavage is positively correlated with the developmental competence of the embryos (van Soom *et al.* 1997, Fenwick *et al.* 2002) and it was demonstrated that bovine fast-cleaving embryos have a different gene expression that reflects their higher quality compared with late-cleaving embryos (Gutierrez-Adan *et al.* 2004). Another determining step for embryo development before blastocyst formation is the compaction of the morula on day 6. In experiment 1, we found that crossbred embryos have a significantly higher rate of compaction compared with purebred and inbred embryos, strongly indicating a greater competence/quality of this group. This finding was confirmed in experiment 2 in which we observed higher blastocyst development of crossbred embryos. Remarkably, this trend was confirmed by the data on embryo development at day 12, where we demonstrated that crossbred embryos undergo elongation at a higher rate compared with inbred embryos. Elongation is a crucial step for bovine embryo development since most embryonic losses occur between blastulation and elongation (Diskin & Sreenan 1980) and embryos more advanced on day 12 establish pregnancies at significantly higher rate than smaller embryos (Lazzari *et al.* 2002). Therefore, our observations, starting from the

Figure 2 Total amino acid depletion, appearance and turnover for purebred and crossbred bovine embryos.

Table 2 Experiment 2: effect of genotype on embryo development.

Type of embryo	Number of OPUs	Number of oocytes	Number of cleaved (%)	Number of compacted morulae, day + 6 (% of oocytes) (% of cleaved)	Number of blastocysts, day + 7 (% of oocytes) (% of cleaved)	Number of blastocysts, day + 8 (% of oocytes) (% of cleaved)
Inbred	39	680	431 (63.4) ^a	98 (14.4) ^a (22.7) ^A	79 (11.6) ^a (18.3) ^A	110 (16.2) ^a (25.5) ^A
Crossbred	40	688	511 (74.3) ^b	138 (20.1) ^b (25.4) ^A	127 (18.5) ^b (24.8) ^B	155 (22.5) ^b (30.3) ^A

χ² test. Values within columns with different letters are statistically different (*P* < 0.05).

early cleavage stage to elongation, indicate a higher developmental capacity and most likely a higher ability to establish pregnancies for crossbred embryos. This detrimental effect of inbreeding on embryo development reminds, to some extent, the fact that in the mouse, most outbred stocks and some inbred strains display a compromised *in vitro* embryo development that is normally not observed in hybrids (Scott & Whittingham 1996). All together, these findings further confirm that embryo genotype can play a significant role during early embryo development and also highlight the importance of the preservation of rare and endangered breeds to maintain the species gene pool as wide as possible.

The level of amino acids utilized by purebred and crossbred embryos were found to be similar to that previously observed in bovine blastocysts (Gopichandran & Leese 2003). Although the overall depletion, appearance and turnover of amino acids were not significantly different between purebred and crossbred embryos, there was a significant difference in the utilization of serine, asparagine, methionine and tryptophan. This suggests that purebred embryos have a different amino acid requirement from crossbred embryos. To the best of our knowledge, this is the first report of genotype affecting the amino acid utilization of bovine embryos.

Figure 3 Ovoid and spherical day 12 embryos transplanted at day 7 in synchronized recipients and recovered after 5 days. Scale bar corresponds to 200 μM.

Regarding gene expression, we examined a panel of genes indicative of embryo quality, developmental competence, metabolism and stress reactivity. Among them, *MNSOD* is a mitochondrial Mn-superoxide dismutase that indicates mitochondrial activity and also has a role in detoxification of reactive oxygen species, cellular differentiation and embryo compaction. As recently demonstrated (Lonergan *et al.* 2003), the level of *MNSOD* expression is higher in *in vivo*-produced embryos in respect to those produced *in vitro*. Therefore, the higher level of expression found in the crossbred group suggests greater mitochondrial activity and improved quality compared with the purebred group. Two other genes significantly up-regulated in the crossbred group are *GP130* and *FGF4*. In bovine embryos, both genes start to be expressed at the time of morula compaction and the expression is maintained during blastulation. In the mouse, *Fgf4* is also expressed at the morula to blastocyst stage and is required for normal development since *Fgf4* null embryos undergo implantation but do not develop substantially thereafter (Feldman *et al.* 1995). Published bovine data indicate that embryos with low developmental potential, such as nuclear transfer embryos, have lower or absent *FGF4* expression, therefore suggesting an important role for this gene. (Daniels *et al.* 2000). *GP130* is a LIF and interleukin 6 receptor subunit, and even if it is not clear whether the LIF–LIF receptor system has, in ruminant embryos, the same role in implantation known in the mouse, it was demonstrated that addition of LIF to the culture of ovine blastocysts significantly improves pregnancy rate after transfer in recipients ewes (Fry *et al.* 1992). In summary, a higher expression of *FGF4* and *GP130* can indicate, as mentioned above for *MNSOD*, a higher developmental and implantation competence of the crossbred group. Another examined gene was *PED*. Interestingly, *in vivo*-cultured embryos and early-cleaving embryos show high expression of *PED* (Fair *et al.* 2004), and these observations applied to our results suggest that crossbred group have both higher quality and greater developmental potential. *GJA1* also displayed an increased expression in the crossbred group, and like *PED*, a higher level of expression has

Table 3 Development and size of day 12 inbred and crossbred embryos after *in vivo* culture.

	Number of embryos transferred on day +7	Number of embryos recovered on day +12	Recovery rate (%)	Number of ovoid embryos	Percentage of recovered	Number of spheric embryos	Percentage of recovered
Inbred	43	29	67.4	7	20.6 ^a	27	79.4 ^a
Crossbred	70	48	68.6	27	56.3 ^b	21	43.7 ^b

χ^2 test. Values within columns with different letters are statistically different ($P < 0.05$).

been found in faster-developing embryos (Gutierrez-Adan *et al.* 2004), indicating that this gene is associated with embryo quality. Remarkably, *GJA1*, which is involved in gap junctional intracellular communication and compaction, was higher in crossbred embryos which displayed an increased rate of compaction compared with purebred embryos. The genes related to stress and apoptosis tended to display increased expression in the purebred embryos, although the difference was not significant. *BAX* is a proapoptotic gene previously suggested to be a marker of blastocyst quality with the level of expression being lower in good-quality embryos. *G6PDH* was defined as sentinel for oxidative stress (Lonergan *et al.* 2003), and in this study, the expression is higher in the inbred group. *G6PDH* is an enzyme indispensable for maintaining the cellular redox state and the high level suggests a stressful condition for the embryo.

The same higher developmental competence in terms of gene expression was also observed comparing ovoid day 12 crossbred and inbred embryos. The expression of *IFNT*, a trophoblastic-specific gene (Degrelle *et al.* 2005) strictly involved in embryo–endometrial interaction and pregnancy signalling (Spencer *et al.* 2008) and also other elongation- and implantation-related genes, such as *PLAC8*, *CDX2* and *PTGS2*, displayed increased expression in crossbred ovoid embryos. Recently, it was demonstrated that these three latter genes are more expressed in biopsies of blastocysts, resulting in the delivery of a calf, compared with those resulting in resorption (El-Sayed *et al.* 2006). In the same work, it was observed that genes linked to programmed cell death such as *MSX1* were up-regulated in embryos, resulting in no pregnancy. Our data indicated that *MSX1* was more expressed in the inbred group and, as previously observed in day 7 embryos, *BAX* expression was also slightly higher. Interestingly, we found that *HSP70* expression was significantly higher in both groups in the summer season compared with the spring, indicating a high level of stress induced by environmental conditions. This casual observation demonstrates that the temperature to which the embryos are exposed plays an important role in the expression of stress-related genes and should be always taken into account in an experimental design.

A final particular aspect to be considered about experiment 2 is that the data refer to embryos with extreme inbreeding levels, inbred–purebred, that are not

commonly found in bovine herds. Therefore, a more accurate evaluation of the embryonic effects of different levels of inbreeding, within the Holstein breed for example, is needed to identify the threshold level that can negatively affect the developmental characteristics of preimplantation bovine embryos. These data, when available, could be integrated with recent findings on candidate genes and pathways affecting fertilization rate and early embryo development (Khatib *et al.* 2009, Wang *et al.* 2009). Ultimately, all this information would be of practical use in the design of selection breeding programmes.

In conclusion, this study demonstrates, using a panel of cellular, metabolic and molecular parameters, that embryo genotype and high inbreeding affect embryonic development in the first 2 weeks after fertilization, a period that represents the crucial developmental window bringing to the establishment of pregnancy in cattle.

Finally, this study was conducted on purebred Holstein Friesian and crossbred Holstein Friesian–Brown Swiss embryos; therefore, more research is needed to further investigate the embryonic consequences of inbreeding and crossbreeding in other cattle breeds.

Figure 4 (A) Relative expression of *FGF4* in purebred and crossbred blastocysts in five different experimental groups. (B) Relative expression of *PLAC8* and *IFNT* in inbred and crossbred day 12 embryos.

Materials and Methods

In this study, we compared groups of *in vitro*-produced embryos of two different origins: purebred embryos produced by fertilizing Holstein oocytes with Holstein semen and crossbred embryos obtained by fertilizing Holstein oocytes with Brown Swiss semen. The study was carried out in two separate experiments using either oocytes collected at the abattoir from slaughtered donors (experiment 1) or oocytes recovered by OPU from live donors (experiment 2). Different Holstein and Brown Swiss bulls were used for each replicate. For experiment 2, the bull sire of each heifer was chosen for IVF in order to produce highly inbred embryos. All the derived embryos were observed during *in vitro* development, evaluating the time of first cleavage, compaction at day 6 and blastocyst rate at days 7 and 8. In addition, part of the blastocysts derived from experiment 1 were analyzed for the pattern of expression of transcripts implicated in pre- and post-implantation periods, embryo metabolism, apoptosis and oxidative stress and the others were used for the evaluation of amino acid turnover. Embryo viability during the second week of development was investigated by transferring blastocysts derived from experiment 2, inbred and crossbred, in synchronous recipients on day 7 of the cycle. These embryos were non-surgically recovered 5 days later, on day 12, to evaluate their size, gene expression pattern and stage of development.

Experiment 1

Oocyte collection, maturation, fertilization and embryo culture.

All chemicals were purchased from Sigma–Aldrich, unless otherwise indicated.

Ovaries of Holstein cows were collected immediately after slaughter and transported to the laboratory in Dulbecco's PBS with antibiotics. Oocytes recovered by aspiration were selected for uniform, non-expanded and non-atretic cumulus morphology, and were matured for 20–24 h at 38.5 °C in 5% CO₂ and 5% O₂ in TCM199 supplemented with 10% FCS, gonadotropins (FSH and LH, 0.05 IU each; Pergovet, Serono), insulin, transferrin, sodium selenite (ITS), Long-IGF1 (100 ng/ml) and Long-EGF (50 ng/ml).

After maturation, oocytes were assigned to one of the two following groups: 1) purebred, 2) crossbred. Group 1 oocytes were fertilized with semen of Holstein bulls and group 2 oocytes were fertilized with semen of Brown Swiss bulls for a total of six replicates. After thawing, the semen was separated on a Percoll gradient and resuspended in synthetic oviduct fluid (SOF) medium (Tervit *et al.* 1972), HEPES buffered and supplemented with fatty acid-free BSA (6 mg/ml), modified Eagle medium (MEM) amino acids, 1 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine and 10 µM hypotaurine. Matured oocytes were coincubated with the semen for 18–20 h at 38.5 °C in 5% CO₂ and 5% O₂.

Zygotes were cultured in groups of 50–80 embryos in 400 µl of m-SOF (modified SOF) medium supplemented with MEM amino acids and BSA (4 mg/ml) until day 8, performing 50% changes of medium at day 4 and 6 at the same atmosphere as above. Each group was evaluated 30 h post fertilization to assess the first embryonic cleavage. Cleavage rate, compaction at day 6 and blastocyst rate at days 7 and 8 were recorded.

Amino acid analysis

Forty-five purebred and 43 crossbred, day 6, compacted morula were washed twice in SOF medium containing a complete mixture of amino acids (Tay *et al.* 1997) and cultured individually in 6 µl drops of this medium for 16 h. Embryo-free control drops of medium were also included in each dish to allow for any non-specific amino acid degradation. After incubation, the embryos were removed from the drops, fixed for 24 h in acetic acid: ethanol (1:3) mixture and stained with Lacmoid to count the number of cells. The spent medium was stored at –80 °C prior to amino acid analysis. The concentration of amino acids was determined by reverse-phase HPLC using a modification of a previously described method (Stokes *et al.* 2007). The spent medium (2 µl) was diluted with 23 µl HPLC-grade water and amino acid analysis was performed on an automated Agilent 1100 HPLC fitted with a Gemini 3 µm 50×4.6 mm C18 column (Phenomenex, Macclesfield, UK) and an Agilent fluorescence detector. Equal volumes of diluted samples and o-phthaldialdehyde were mixed and 5 µl of the solution was loaded onto the column. An elution gradient was established at a flow rate of 2.5 ml/min. Solvent A consisted of 80% 83 mM sodium acetate (pH 5.9), 18.5% methanol and 1.5% tetrahydrofuran. Solvent B consisted of 80% methanol and 20% sodium acetate (pH 5.9). Using this method, it was not possible to detect cysteine and proline.

Experiment 2

Oocyte recovery by OPU, maturation, fertilization and embryo culture.

Ten Holstein heifers were used as oocyte donors by OPU. Oocyte collection was performed by transvaginal ultrasound-guided follicular aspiration (Galli *et al.* 2001). Briefly, all ovarian follicles ranging from 3 to 15 mm size were aspirated using a 17 G, 55 cm long, single-lumen cannula with a 19 G long spinal needle mounted at the tip connected to an aspiration pump. Collected oocytes were *in vitro* matured as previously described in experiment 1. Each oocyte batch was fertilized either with the semen of the heifer's sire (inbred group) or with Brown Swiss semen (crossbred group), giving rise to purebred–inbred embryos or crossbred embryos. Development was assessed observing cleavage rate, compaction at day 6 and blastocysts formation at days 7 and 8 as in experiment 1.

To evaluate embryo development in the second week post fertilization, pools of day 7 *in vitro*-grown inbred or crossbred blastocysts were nonsurgically transferred in synchronized recipients on day 7 of the cycle (Bowen *et al.* 1978). On day 12, the embryos were collected with a Dissi catheter (Minitub, Tiefenbach, Germany) by flushing the uterine horns using Dulbecco PBS supplemented with 1% FCS. The fluid was recovered in glass bottles and the embryos were allowed to settle. The supernatant was aspirated, the embryos were collected from the bottom and placed in HEPES-buffered SOF. Length and width of all recovered embryos were measured under a stereomicroscope, with the aid of a calibrated ruler (Lazzari *et al.* 2002). After multiple washings in PBS supplemented with 1 mg/ml of polyvinyl alcohol, embryos were collected in RNAase DNAase free tubes and snap-frozen for PCR analysis.

Table 4 Primers for RT-PCR.

Genes	Primer	Fragment size (bp)
<i>MNSOD</i>	5'-CCCATGAAGCCTTCTAATCCTG	307
	3'-TTCAGAGGCGCTACTATTTCCTTC	
<i>PED</i>	5'-GCACTACGGCTACGAAGGCAGAG	350
	3'-TCTCCACAAGCTCCATGTCCTG	
<i>SLC2A5</i>	5'-CATCTCCATCATCGTCCTCA	531
	3'-GTAGATGGTGGTGAGGAGAC	
<i>GP130</i>	5'-TCGTGCTGTTTGCTTAGCATTCTATTGA	367
	3'-CTGCTAGAAATGCTTGGCCTAGAAGATGAC	
<i>FGF4</i>	5'-TTCTTCGTGGCCATGAGCAG	208
	3'-AGGAAGTGGTGACCTTCAT	
<i>IGF1R</i>	5'-TTGAAGTATGCGCCATGTGCTGG	210
	3'-TGCTTCTTGGCGCCCGTTCAT	
<i>IFNT</i>	5'-GCCCTGGTGTGCTGCTCAGCTA	564
	3'-CATCTTAGTCAGCCAGAGTC	
<i>GJA1</i>	5'-TGAATGCAAGAGAGGTTGAAAGAGG	293
	3'-AACACTCTCCAGAACACATGATCG	
<i>BAX</i>	5'-TGCAGAGGATGATCGCAGCTGTG	198
	3'-CCAATGTCAGCCATCATGGTC	
<i>G6PDH</i>	5'-CGCTGGGACGGGTGCCCTTCATC	347
	3'-CGCCAGGCTCCCGAGTTCATCA	

Gene expression analysis

Total RNA was extracted from pools of fifteen day 7 blastocysts from each purebred and crossbred group or from day 12 inbred and crossbred embryos (RNeasy mini kit, Qiagen). Day 12 embryos were analyzed in pools of two embryos and each embryo was considered equivalent to a group of fifteen day 7 embryos and cDNA was diluted in the same volume. The RT reaction was performed immediately after extraction (First strand cDNA synthesis kit; Fermentas, St Leon-Rot, Germany) for 10 min at 25 °C followed by a retro-transcription step of 1 h at 38 °C and a 70 °C step for 10 min. The PCR was performed in a total volume of 25 µl containing 2.5 µl of 10× PCR buffer with 1.5 mM of MgCl₂ (supplied with HotStarTaq DNA Polymerase, Qiagen), 250 µM of each dNTP, 1 µM of each specific primer, 0.625 IU of Hot Start Taq Polymerase (HotStarTaq DNA Polymerase, Qiagen) and a volume of cDNA equivalent at 0.5 or 1 embryo for day 7, as indicated in Table 4. The reaction was performed using an MJ Minicycler (Bio-Rad) with the following program: 15 min at 95 °C as initial step followed by 30 cycles (35 cycles were applied for *FGF4* and *GJA1*) of 30 s at 95 °C, 30 s at 57 °C and 45 s at 72 °C. The final step of the last cycle was prolonged to 5 min at 72 °C as final extension. For each sample, two negative controls were prepared, one in which reverse transcriptase was omitted during RT reaction and one in which cDNA was omitted during PCR reaction.

After RT-PCR reaction, the samples were cooled at 4 °C and electrophoresis was performed on a 2.5% agarose gel in 1× TAE buffer containing 0.5 µg/ml of ethidium bromide. Running buffer was the same 1× TAE buffer supplemented with ethidium bromide. The results of the electrophoresis were captured with a Gel Logic 100 Imaging System (Eastman Kodak Company) and images were analyzed with a Kodak Molecular Imaging Software. The intensity of each band was analyzed by densitometry and the results were normalized using Histone H2a as a standard gene. Genes analyzed for day 7 blastocysts were selected from the literature with the objective of providing

an indication of the effects of embryo genotype and level of inbreeding on embryo quality, developmental competence and response to stress: *MNSOD*, *SLC2A5*, *IGF1R*, *IFNT*, *G6PDH*, *BAX*, *GJA1* (Gutierrez-Adan *et al.* 2004), *GP130* (Eckert & Niemann 1998), *FGF4* (Daniels *et al.* 2000) and *PED* (Fair *et al.* 2004). Primers are listed in Table 4. Genes examined in day 12 embryos included trophoblast-specific genes and proapoptotic genes: *IFNT* and *BAX* (Gutierrez-Adan *et al.* 2004), *PTGS2*, *PLAC8*, *MSX1* (El-Sayed *et al.* 2006) and *CDX2* (Degrelle *et al.* 2005).

Statistical analysis

Embryos development data were analyzed with χ^2 test, while statistical differences in genes expression were determined with Student's *t*-test.

All amino acid data were analyzed to determine whether they were normally distributed using the Anderson–Darling normality test. Differences between amino acid appearance/depletion and turnover between purebred and crossbred embryos were determined using Student's *t*-test or Mann Whitney *U* test.

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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References

- Boediono A, Suzuki T & Godke RA 2003 Comparison of hybrid and purebred *in vitro*-derived cattle embryos during *in vitro* culture. *Animal Reproduction Science* **78** 1–11. (doi:10.1016/S0378-4320(03)00065-4)
- Bowen JM, Elsdon RP & Seidel GE Jr 1978 Non-surgical embryo transfer in the cow. *Theriogenology* **10** 89–95. (doi:10.1016/0093-691X(78)90083-3)
- Butler WR 1998 Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *Journal of Dairy Science* **81** 2533–2539. (doi:10.3168/jds.S0022-0302(98)70146-8)
- Daniels R, Hall V & Trounson AO 2000 Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biology of Reproduction* **63** 1034–1040. (doi:10.1095/biol-reprod63.4.1034)
- Degrelle SA, Campion E, Cabau C, Piumi F, Reinaud P, Richard C, Renard JP & Hue I 2005 Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. *Developmental Biology* **288** 448–460. (doi:10.1016/j.ydbio.2005.09.043)

- Dematawewa CM & Berger PJ** 1998 Genetic and phenotypic parameters for 305-day yield, fertility, and survival in Holsteins. *Journal of Dairy Science* **81** 2700–2709. (doi:10.3168/jds.S0022-0302(98)75827-8)
- Diskin MG & Sreenan JM** 1980 Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *Journal of Reproduction and Fertility* **59** 463–468. (doi:10.1530/jrf.0.0590463)
- Eckert J & Niemann H** 1998 mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-beta in bovine embryos derived *in vitro* or *in vivo*. *Molecular Human Reproduction* **4** 957–965. (doi:10.1093/molehr/4.10.957)
- El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K & Tesfaye D** 2006 Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological Genomics* **28** 84–96. (doi:10.1152/physiolgenomics.00111.2006)
- Fair T, Gutierrez-Adan A, Murphy M, Rizos D, Martin F, Boland MP & Lonergan P** 2004 Search for the bovine homolog of the murine *ped* gene and characterization of its messenger RNA expression during bovine preimplantation development. *Biology of Reproduction* **70** 488–494. (doi:10.1095/biolreprod.103.022137)
- Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM & Goldfarb M** 1995 Requirement of FGF-4 for postimplantation mouse development. *Science* **267** 246–249. (doi:10.1126/science.7809630)
- Fenwick J, Platteau P, Murdoch AP & Herbert M** 2002 Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. *Human Reproduction* **17** 407–412. (doi:10.1093/humrep/17.2.407)
- Fry RC, Batt PA, Fairclough RJ & Parr RA** 1992 Human leukemia inhibitory factor improves the viability of cultured ovine embryos. *Biology of Reproduction* **46** 470–474. (doi:10.1095/biolreprod.46.3.470)
- Galli C, Crotti G, Notari C, Turini P, Duchi R & Lazzari G** 2001 Embryo production by ovum pick up from live donors. *Theriogenology* **55** 1341–1357. (doi:10.1016/S0093-691X(01)00486-1)
- Gopichandran N & Leese HJ** 2003 Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid. *Reproduction* **126** 299–308. (doi:10.1530/rep.0.1260299)
- Grohn YT & Rajala-Schultz PJ** 2000 Epidemiology of reproductive performance in dairy cows. *Animal Reproduction Science* **60–61** 605–614. (doi:10.1016/S0378-4320(00)00085-3)
- Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, Boland MP & Lonergan P** 2004 Effect of speed of development on mRNA expression pattern in early bovine embryos cultured *in vivo* or *in vitro*. *Molecular Reproduction and Development* **68** 441–448. (doi:10.1002/mrd.20113)
- Hansen LB** 2000 Consequences of selection for milk yield from a geneticist's viewpoint. *Journal of Dairy Science* **83** 1145–1150. (doi:10.3168/jds.S0022-0302(00)74980-0)
- Hermas SA, Young CW & Rust JW** 1987 Effects of mild inbreeding on productive and reproductive performance of Guernsey cattle. *Journal of Dairy Science* **70** 712–715. (doi:10.3168/jds.S0022-0302(87)80062-0)
- Khatib H, Huang X, Tran AH, Bindrim AB, Schutzkus V, Monson RL & Yandell BS** 2009 Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. *Journal of Dairy Science* **92** 2238–2247. (doi:10.3168/jds.2008-1767)
- Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H & Galli C** 2002 Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biology of Reproduction* **67** 767–775. (doi:10.1095/biolreprod.102.004481)
- Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, de la Fuente J & Boland MP** 2003 Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or *in vivo*. *Biology of Reproduction* **69** 1424–1431. (doi:10.1095/biolreprod.103.018168)
- Lopez-Gatius F** 2003 Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology* **60** 89–99. (doi:10.1016/S0093-691X(02)01359-6)
- Lucy MC, Savio JD, Badinga L, De La Sota RL & Thatcher WW** 1992 Factors that affect ovarian follicular dynamics in cattle. *Journal of Animal Science* **70** 3615–3626.
- Macmillan KL, Lean IJ & Westwood CT** 1996 The effects of lactation on the fertility of dairy cows. *Australian Veterinary Journal* **73** 141–147. (doi:10.1111/j.1751-0813.1996.tb10007.x)
- McAllister AJ** 2002 Is crossbreeding the answer to questions of dairy breed utilization? *Journal of Dairy Science* **85** 2352–2357. (doi:10.3168/jds.S0022-0302(02)74315-4)
- Nebel RL & Jobst SM** 1998 Evaluation of systematic breeding programs for lactating dairy cows: a review. *Journal of Dairy Science* **81** 1169–1174. (doi:10.3168/jds.S0022-0302(98)75679-6)
- Olori VE, Meuwissen TH & Veerkamp RF** 2002 Calving interval and survival breeding values as measure of cow fertility in a pasture-based production system with seasonal calving. *Journal of Dairy Science* **85** 689–696. (doi:10.3168/jds.S0022-0302(02)74125-8)
- Pursley JR, Kosorok MR & Wiltbank MC** 1997 Reproductive management of lactating dairy cows using synchronization of ovulation. *Journal of Dairy Science* **80** 301–306. (doi:10.3168/jds.S0022-0302(97)75938-1)
- Roche JF, Mackey D & Diskin MD** 2000 Reproductive management of postpartum cows. *Animal Reproduction Science* **60–61** 703–712. (doi:10.1016/S0378-4320(00)00107-X)
- Royal M, Mann GE & Flint AP** 2000 Strategies for reversing the trend towards subfertility in dairy cattle. *Veterinary Journal* **160** 53–60. (doi:10.1053/tvj.1999.0450)
- Schmitt EJ, Diaz T, Drost M & Thatcher WW** 1996 Use of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin for timed insemination in cattle. *Journal of Animal Science* **74** 1084–1091.
- Scott L & Whittingham DG** 1996 Influence of genetic background and media components on the development of mouse embryos *in vitro*. *Molecular Reproduction and Development* **43** 336–346. (doi:10.1002/(SICI)1098-2795(199603)43:3<336::AID-MRD8>3.0.CO;2-R)
- van Soom A, Ysebaert MT & de Kruif A** 1997 Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in *in vitro*-produced bovine embryos. *Molecular Reproduction and Development* **47** 47–56. (doi:10.1002/(SICI)1098-2795(199705)47:1<47::AID-MRD7>3.0.CO;2-Q)
- Spencer TE, Sandra O & Wolf E** 2008 Genes involved in conceptus–endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. *Reproduction* **135** 165–179. (doi:10.1530/REP-07-0327)
- Staples CR, Thatcher WW & Clark JH** 1990 Relationship between ovarian activity and energy status during the early postpartum period of high producing dairy cows. *Journal of Dairy Science* **73** 938–947. (doi:10.3168/jds.S0022-0302(90)78750-4)
- Stokes PJ, Hawkhead JA, Fawthrop RK, Picton HM, Sharma V, Leese HJ & Houghton FD** 2007 Metabolism of human embryos following cryopreservation: implications for the safety and selection of embryos for transfer in clinical IVF. *Human Reproduction* **22** 829–835. (doi:10.1093/humrep/del447)
- Tay JI, Rutherford AJ, Killick SR, Maguiness SD, Patridge RJ & Leese HJ** 1997 Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Human Reproduction* **12** 2451–2456. (doi:10.1093/humrep.12.11.2451)
- Tervit HR, Whittingham DG & Rowson LE** 1972 Successful culture *in vitro* of sheep and cattle ova. *Journal of Reproduction and Fertility* **30** 493–497. (doi:10.1530/jrf.0.0300493)
- VanRaden PM & Sanders AH** 2003 Economic merit of crossbred and purebred US dairy cattle. *Journal of Dairy Science* **86** 1036–1044. (doi:10.3168/jds.S0022-0302(03)73687-X)
- Wall E, Brotherstone S, Kearney JF, Woolliams JA & Coffey MP** 2005 Impact of nonadditive genetic effects in the estimation of breeding values for fertility and correlated traits. *Journal of Dairy Science* **88** 376–385. (doi:10.3168/jds.S0022-0302(05)72697-7)
- Wang X, Schutzkus V, Huang W, Rosa GJ & Khatib H** 2009 Analysis of segregation distortion and association of the bovine FGF2 with fertilization rate and early embryonic survival. *Animal Genetics* **40** 722–728. (doi:10.1111/j.1365-2052.2009.01904.x)

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