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Effects of superovulated heifer diet type and quantity on relative mRNA abundances and pyruvate metabolism in recovered embryos

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This study investigated the effects of quantity and type of diet fed to superovulated donor heifers on molecular and metabolic indices of embryonic development. These effects included the relative abundances of mRNAs for the α1 subunit of Na/K-ATPase and the antioxidant enzyme Cu/Zn-SOD, as well as pyruvate utilization in bovine morulae and blastocysts developed *in vivo*. Heifers were fed a daily ration of either grass silage and a citrus–beet pulp-based concentrate or grass silage and a barley-based concentrate for 116 days, both at 3 kg per day or *ad libitum*. In embryos derived from heifers fed the pulp-based diets, the relative abundances of the transcripts were not affected by either day of collection or quantity of diet. In embryos derived from heifers fed the barley-based diets, the relative abundances of the Na/K-ATPase transcripts were also not changed by these main effects, while the relative abundances of the Cu/Zn-SOD transcripts were affected by day of collection and by the quantity of diet. Pyruvate metabolism was affected by day of collection, and was significantly increased in day 8 embryos compared with day 7 and day 6 embryos. Diet quantity did not affect pyruvate utilization, whereas diet type did increase pyruvate metabolism in the barley group when compared with the pulp group. The results of this study show for the first time that molecular and metabolic variations may exist in embryos derived *in vivo* and developed in donor heifers on nutritional regimens differing in type and quantity. Differences in embryos collected on different developmental days may be attributed to varying cell numbers. Alterations in the relative abundances of the Cu/Zn-SOD transcripts and pyruvate metabolism caused by the quantity of diet fed to the donor animal were likely to have been due to alterations in metabolic end products that accumulate in reproductive tract fluids, whereas differences in embryonic metabolism caused by type of diet are related to the composition of the diet. These findings characterize embryos produced *in vivo* at the molecular level, indicating that the molecular markers used in the present study can differentiate between populations of embryos produced under different nutritional regimens and determine conditions conducive to the production of good quality embryos.

Introduction

Superovulation of donors provides multiple embryos for the embryo transfer industry (Boland *et al*., 1991; Armstrong, 1993). Despite major research initiatives to enhance and standardize the superovulatory response to exogenous gonadotrophins, animal to animal variation in number and quality of embryos remains a problem (Goulding *et al*., 1996; Kelly *et al*., 1997). Several factors, such as season, breed, type of gonadotrophin and the presence or absence of a dominant follicle, contribute to the variable number and quality of embryos produced (Armstrong, 1993; Bungartz and Niemann, 1994). In superovulated beef heifers, an increase in concentrate intake from 3 kg per day to *ad libitum* reduced the number of ovulations and provided fewer good quality embryos (Mantovani *et al*., 1993). The type of diet fed before superovulation also affects superovulatory response and embryo quality. Heifers fed a high concentrate–low fibre diet before superovulation had fewer high quality embryos

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(Yaakub *et al*., 1999). Similarly, rations of high rumen-degradable protein decreased fertilization rates and preimplantation development (Blanchard *et al*., 1990). Thus, diet type, either in terms of energy source or protein content, can alter embryo yields and survival. The quantity of the food can also affect other parameters of the reproductive response, such as follicle size and progesterone concentrations (Murphy *et al*., 1991; Nolan *et al*., 1998).

The main energy substrates used by ruminants are volatile fatty acids (VFA) which are produced in the rumen. The type of concentrate fed affects rumen fermentation, with VFA profiles differing between cattle fed on starch-based or fibre-based concentrate (Moloney *et al*., 1994). The relative amounts of each VFA produced are diet-dependent, but rapidly fermented concentrate supplements tend to favour the production of propionate, and the more slowly fermented fibre-based diets favour acetate production (Sloan *et al*., 1988). Propionate is the main precursor of glucose synthesis in the liver. Glucose, amino acids or nutrient-related metabolites, such as insulin, growth hormone, and the insulin-like growth factors and their binding proteins, have been postulated to operate at the ovarian level and to support the growth of the gonadotrophin-dependent follicles by reducing the amount of FSH required for follicle growth (Downing and Scaramuzzi, 1991). Bovine embryos are sensitive to glucose, as high concentrations in culture media inhibit embryo development *in vitro* (Takahashi and First, 1992).

The gene transcripts analysed in the present study are known to play essential roles during preimplantation development. Na/K-ATPase is a key enzyme for blastocyst development, mediating fluid transfer across the outer blastomeres to form a fluid-filled cavity (Watson, 1992, Kidder, 1993). In bovine embryos produced *in vitro*, transcripts encoding multiple isoforms of both the α and β subunits of Na/K-ATPase are expressed throughout early development (Betts *et al*., 1997). A similar transcription pattern was detected for the antioxidant enzyme, Cu/Zn-SOD (Harvey *et al*., 1995), which is involved in protection from oxidative stress.

Another accepted marker of embryo quality is energy metabolism (Rieger, 1984, 1992; Overström, 1996; Gardner, 1998). Temporal, stage-specific pathways of energy metabolism have been investigated and metabolic activity may provide an objective means to assess the viability of embryos (Rieger, 1992). The patterns of uptake and metabolic utilization of energy substrates, such as pyruvate, lactate, glucose and glutamine, have been determined in bovine embryos (Thompson *et al*., 1996).

Given the reported effects of diet type and quantity on embryo quality after superovulation and the effects of diet type and quantity on energy metabolism in cattle, the purpose of this experiment was to evaluate the effects of quantity and type of diet on the relative abundance of two different gene transcripts and pyruvate metabolism in cattle embryos recovered at the morula and blastocyst stages after superovulatory treatment. While *in vitro* culture conditions can have profound effects on the expression of important genes in developing rodent and bovine embryos (Ho *et al*., 1994, 1995; Morita *et al*., 1994; Shim *et al*., 1996; Wrenzycki *et al*., 1996, 1998a, 1999; Koerber *et al*., 1998), the effects of nutrition regimens on gene expression and metabolism in bovine embryos collected from superovulated donors have not yet been reported. The nutritional model used was designed to provide differing amounts (3 kg per day versus *ad libitum*) and types (starch (barley)-based versus sugar (citrus–beet pulp)-based) of concentrate supplement to alter rumen metabolism. The heifers were superovulated to produce adequate embryos from the nutritionally treated heifers.

Materials and Methods

Animals and treatments

The nutritional and superovulatory treatments were described by Yaakub *et al*. (1999). Briefly, seventy-six continental crossbred heifers of approximately 500 kg live weight were offered grass silage supplemented with either barley concentrate at 3 kg per day ($n = 20$) or *ad libitum* ($n = 19$), or a citrus–beet pulp concentrate at 3 kg per day ($n = 18$), or *ad libitum* ($n = 19$) for 116 days. Both concentrates were formulated to contain 14% crude protein. The major ingredients of the barley concentrate consisted of 80% barley, 12% soybean meal, 5% molasses, and citrus–beet pulp concentrate consisted of 31.9% citrus pulp, 31.9% molassed beet pulp, 18% maize gluten and 12% soybean meal. Grass silage contained 12.1% crude protein, with a pH of 4.4 and 18.2% dry matter. Silage intake for heifers offered 3 kg concentrates per day was *ad libitum*, but for heifers offered *ad libitum* concentrate silage intake was limited to approximately 1 kg dry matter per day**.** The diets were chosen because the carbohydrate metabolism for starch (barley)-based and sugar–fibre (citrus–beet pulp)-based energy sources would vary significantly in the rumen, resulting in different fatty acid production profiles.

Synchronization of oestrus, superovulation and embryo recovery

Heifers were treated for 7 days with an intravaginal progesterone-releasing device containing 1.9 g progesterone (CIDR-B, InterAg, NZ) after 100 days on the treatment diets. Heifers received a total of 265 mg NIH-FSH-P1 equivalent (pFSH, Folltropin-V, Vetrepharm Canada, London, Ontario) administered over 8 injections at 12 h intervals. The last two injections were given at 12 and 24 h after CIDR withdrawal. Heifers received a prostaglandin $F_{2\alpha}$ analogue (15 mg luprostiol, PG, Prosolvin; Intervet, Boxmeer) injection with the fifth injection of pFSH. Heifers were artificially inseminated twice, at 56 and 72 h after CIDR withdrawal, without reference to oestrus, using frozen–thawed semen from a single bull with proven fertility. Embryos were recovered from excised reproductive tracts according to the procedures reported by Goulding *et al*. (1994) on day 6, 7 or 8 after first insemination into PBS (Oxoid Ltd, Basingstoke) containing 5% fetal calf serum (FCS, Gibco, Grand Island, NY) and transferred into CR-2 collection medium (114.7 mmol NaCl l^{-1} 1 mmol KCl l^{-1}, 26.2 mmol $NaHCO_3$, 5.5 mmol

hemi-calcium lactate l^{-1}, 0.4 mmol pyruvate l^{-1}, 500 mmol glutamine l^{-1}, 1% BSA, and 0.1% (w/v) gentamicin (Gibco-BRL, Gaithersburg, MD). Embryos were graded on the basis of morphology using a scale of 1 to 5 (Boland *et al*., 1978), washed twice with PBS containing 0.1% polyvinylalcohol (PVA), and then transferred, as pools of two or three embryos, in a minimal volume (< 1 µl) to the bottom of a 0.5 ml tube. All embryo pools were lysed in 10 µl GITC lysis buffer (4 mol guanidinium thiocyanate l^{-1}, 0.1 mol Tris l^{-1} (pH 7.4), 1 mol beta-mercaptoethanol l^{-1}). Samples were quick frozen in liquid nitrogen and transported on dry ice to the laboratory for analysis, where they were stored at –70°C. Only embryos from morphological grades 1 and 2 were used, and these were balanced across treatments. Details on embryo yields have been reported by Yaakub *et al*. (1999).

Semi-quantitative RT–PCR

Relative changes in the abundance of mRNA transcripts in embryo samples were determined using a semi-quantitative (SQ) RT–PCR assay, using exogenous added globin mRNA as an internal standard (Temeles *et al*., 1994). In this assay, the abundance of a given gene transcript in an embryo sample is determined by expressing the abundance of a gene-specific amplification product as a fraction of the α-globin amplification product. This assay can be used to compare the relative abundance of one mRNA among different samples, but not the absolute amount of one mRNA compared with that of another (Temeles *et al*., 1994; Ho *et al*., 1994, 1995; Latham *et al*., 1995).

Since embryos isolated from heifers fed pulp- or barley-based diets were analysed in two different laboratories, using methods for measuring the relative abundance of mRNAs with minor differences, direct comparisons among diet types cannot be made and only general trends can be evaluated.

Analysis of mRNA expression in embryos collected from heifers fed the pulp-based diets

RNA isolation, RT–PCR, and quantification of amplification product on embryos from the pulp-based diets was performed as described by De Sousa *et al*. (1998a). At the time of thawing, 0.1 pg rabbit globin RNA (Gibco-BRL, Burlington) were added per embryo equivalent, lysed in a sample and mixed by pipetting. For each sample, a 2 mm × 2 mm square of Hybond™-messenger affinity paper (mAP; Amersham International, Little Chalfont), wetted with 0.5 mol NaCl l^{-1}, was soaked in the lysed sample for 2–3 h at room temperature to allow for binding of poly(A)$^+$ RNA. Unabsorbed lysates were pipetted onto respective mAP squares supported on Whatman 1 Filter paper (Whatman International Ltd, Springfield Mill) on parafilm. mAP squares were then transferred individually into separate 0.5 ml tubes and washed by gentle inversion with 200 µl of 0.5 mol NaCl l^{-1}, 0.1 mol Tris l^{-1} (3×), 0.5 mol NaCl l^{-1} (3×), and 70% ethanol (2×). Poly(A)$^+$ RNA was eluted from each mAP square in fresh tubes in 11 µl sterile H_2O containing 0.5 µg oligo(dT)$_{12–18}$ (Gibco-BRL) by incubation at 70°C for 10 min, followed by cooling on ice for 5 min. RNA isolated by this procedure has been shown to be free of any contaminating genomic DNA (De Sousa *et al*., 1998a).

Reverse transcription reactions took place in a final volume of 20 µl buffer, consisting of 50 mmol Tris–HCl l^{-1} (pH 8.3), 75 mmol KCl l^{-1}, 3 mmol $MgCl_2$ l^{-1}, 10 mmol dithiothreitol l^{-1}, 750 µmol dNTPs l^{-1}, and 300 iu Superscript™ RNase H$^-$ (Gibco-BRL) for 90 min at 43°C. Reactions were terminated at 5 min at 95°C and then placed on ice. Reverse transcribed cDNA was either used directly for PCR or stored at –20°C. As a negative control for RNA isolation and reverse transcription, a blank mAP square was carried along with the samples during the procedure.

Oligonucleotide primers for the amplification of Na/K-ATPase α1, Cu/Zn-SOD and α-globin were designed using known sequence information (Table 1). PCRs were performed in 25 µl of 1× GeneAmp PCR Buffer II (10 mmol Tris–HCl l^{-1}, pH 8.3, 50 mmol KCl l^{-1}; Perkin Elmer, Vaterstetten) containing 200 µmol dNTPs l^{-1}, 2.5 iu AmpliTaq Gold (Perkin Elmer), 1 µmol l^{-1} of each of the appropriate 3′ and 5′ gene specific primers, 1 mmol $MgCl_2$ l^{-1} (Na/K-ATPase α1, and Cu/Zn-SOD) or 1.25 mmol $MgCl_2$ l^{-1} (α-globin), and a volume of the reverse transcription reaction corresponding to 0.1–0.2 embryo equivalents. The basic programme for amplification of gene transcripts consisted of a 10 min soak at 94°C, followed by a cycle programme of 1 min at 94°C, a transcript-specific annealing temperature (58, 60 and 55°C, for Na/K-ATPase α1, Cu/Zn-SOD, and α-globin, respectively) for 30 s, and 1 min at 72°C. The number of

Table 1. Primers used for RT–PCR and the size of diagnostic amplification products

Gene primers[a]		Primer sequence	Product size (bp)
Na/K-ATPase α1	5′	5′-ACCTGTTGGGCATCCGAGAGAC-3′	336
	3′	5′-AGGGGAAGGCACAGAACCACCA-3′	
Cu/Zn-SOD	5′	5′-AAGGCCGTGTGCGTGCTGAA-3′	246
	3′	5′-CAGGTCTCCAACATGCCTCT-3′	
α-globin	5′	5′-GCAGCCACGGTGGCGAGTAT-3′	257
	3′	5′-GTGGGACAGGAGCTTGAAAT-3′	

[a]Na/K-ATPase α1 primers were based on regions of shared homology between the rat and horse sequences (Shull *et al*., 1986; Kano *et al*., 1989) and amplified a product from bovine cDNA that was 90.1% identical to the corresponding cDNA sequence in rats (Betts *et al*., 1997). Cu/Zn-SOD primers were based on the rat sequence (Ho and Crapo, 1987), and have been shown to amplify a product of the correct size, with the anticipated diagnostic restriction enzyme site, from bovine cDNA (Harvey *et al*., 1995). For α-globin, the 5′ and 3′ primers correspond to bp 241–260 and 555–657, respectively, in the rabbit α-globin genomic clone (Cheng *et al*., 1986).

cycles were 35 (Na/K-ATPase α1), 38 (Cu/Zn-SOD), and 31 (α-globin).

RT–PCR products were visualized by separation for 45 min at 100 V on 2% agarose gels in 1 × TAE buffer (40 mmol Tris–acetate l^{-1}, 1 mmol EDTA l^{-1}) containing 0.5 μg ml^{-1} ethidium bromide. RT–PCR products were quantified by capillary electrophoresis (De Sousa *et al*., 1998a), using a Beckman P/ACE System 2100 in conjunction with a laser-induced fluorescence (LIF) detector operating with an argon ion 488 nm laser and a 530 nm emission filter.

Analysis of mRNA expression in embryos collected from heifers fed the barley-based diets

Poly(A)$^+$RNA was isolated using a Dynabeads mRNA DIRECT Kit (Dynal®, Oslo) according to the manufacturer´s instructions, with minor modifications as described by Wrenzycki *et al*. (1998b, 1999). Briefly, embryos were lysed by adding 150 μl lysis-binding buffer (100 mmol Tris–HCl l^{-1}, pH 8.0, 500 mmol LiCl l^{-1}, 10 mmol EDTA l^{-1}, 1% (w/v) LiDS (SDS), 5 mmol dithiothreitol l^{-1}). Rabbit globin mRNA (0.1 pg; BRL, Gaithersburg, MD) per oocyte or embryo was added to each tube as an internal standard. After vortexing for 10 s, brief centrifugation at 12 000 ***g*** for 5 s and incubation at room temperature for 10 min, 10 μl prewashed Dynabeads®Oligo $(dT)_{25}$ was pipetted into the fluid. After 5 min incubation at room temperature for binding poly(A)$^+$RNAs to oligo (dT) Dynabeads, the beads were separated employing a Dynal MPC-E-1 magnetic separator, washed once using 100 μl washing buffer 1 (10 mmol Tris–HCl l^{-1}, pH 8.0, 0,15 mol LiCl l^{-1}, 1 mmol EDTA l^{-1}, 0,1% (w/v) LiDS) and three times with 100 μl washing buffer 2 (10 mmol Tris–HCl, pH 8.0, 0,15 mol LiCl l^{-1}, 1 mmol EDTA l^{-1}). Poly(A)$^+$RNAs were then eluted from the beads by incubation in 11 μl sterile water at 65°C for 2 min, and aliquots were used immediately for reverse transcription.

Poly(A)$^+$RNA was reverse transcribed into cDNA in a total volume of 20 μl, using 2.5 μmol random hexamers l^{-1} (Perkin-Elmer) to get the widest array of cDNAs. The reaction mixture consisted of 1× RT buffer (50 mmol KCl l^{-1}, 10 mmol Tris–HCl l^{-1}, pH 8.3, Perkin-Elmer, Vaterstetten), 5 mmol $MgCl_2$ l^{-1}, 1 mmol l^{-1} of each dNTP (Amersham, Brunswick), 20 iu RNase inhibitor (Perkin-Elmer) and 50 iu MuLV reverse transcriptase (Perkin-Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT reaction was carried out at 25°C for 10 min, 42°C for 1 h, followed by a denaturation step at 99°C for 5 min and flash cooling on ice.

PCR was performed with a volume of the RT reaction corresponding to 0.1 or 0.2 embryo equivalents and a volume of the RT reaction corresponding to 10 fg equivalent of globin RNA in a final volume of 50 μl consisting of 1 × PCR buffer (20 mmol Tris–HCl l^{-1}, pH 8.4, 50 mmol KCl l^{-1}, Gibco BRL, Eggenstein), 1.5 mmol $MgCl_2$ l^{-1}, 200 μmol l^{-1} of each dNTP, 1 μmol l^{-1} of each sequence specific primer (globin: 0.5 μmol l^{-1}) using a PTC-200 thermocycler (MJ Research, Watertown, MA). A 'hot start' PCR was performed to obtain specific amplification. During the hot start, 1 iu Taq DNA polymerase (Gibco, Paisley) was added at 72°C. The sequences and positions of the primers used, the fragment sizes and the sequence references of the expected PCR products are summarized (Table 1).

The PCR program used an initial step at 99°C for 5 min and 72°C for 2 min (hot start) followed by 28 cycles (globin: 30 cycles) of 15 s, each at 95°C for DNA denaturation, 15 s at different temperatures for annealing of primers, and 15 s at 72°C for primer extension. The last cycle was followed by a 5 min extension at 72°C and cooling to 4°C.

Generation of the diagnostic fragments was strictly dependent on the presence of RNA in the RT reaction, since omitting reverse transcriptase from the RT did not generate any amplified fragments (data not shown).

After addition of 5 μl of 10× loading buffer (0.25% (w/v) xylenecyanol and 25 mmol EDTA l^{-1} in 50% (v/v) glycerin), 10 μl of the RT–PCR products were subjected to electrophoresis on a 2% agarose gel in 1 × TBE buffer (90 mmol Tris l^{-1}, 90 mmol borate l^{-1}, 2 mmol EDTA l^{-1}, pH 8.3) containing 0.2 μg ethidium bromide (EtBr) ml^{-1} with a further addition of in the same concentration as in the running buffer. After running at 80 V for 45 min, the fragments were visualized on an 312 nm UV-transilluminator. The image of each gel was digitized using a CCD camera (Quantix, Photometrics, München) and the IP Lab spectrum (IP Lab Gel, Signal Analytics Corporation, Vienna, VA). The signal intensity of each band was quantitated by densitometric scanning using a computer-assisted image analysis system (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage. For each mRNA, experiments were repeated with at least three separate embryo batches.

Measurement of [^{14}C]pyruvate metabolism by individual embryos produced in vivo

Radiolabelled pyruvate ([2-^{14}C], 15.4 mCi $mmol^{-1}$; Dupont-NEN, Brussels) was reconstituted in CR1aa-Hepes medium (Rosenkrans and First, 1994) to a stock concentration of 0.05 μCi μl^{-1}, and sodium bicarbonate ([^{14}C], 0.1 mCi $mmol^{-1}$; Amersham International) was used at the concentration of 0.25 μCi μl^{-1}. Labelled reagents were stored at 4°C. The radiometric, hanging-drop method of Rieger *et al*. (1992a) was used to measure pyruvate metabolism. Individual embryos from each nutrition treatment were taken up in 2 μl of CR1aa-Hepes medium and transferred to the cap of a sterile 2.5 ml screw-cap mini-vial (Sarstedt, Newton, NC). Immediately, 2 μl CR1aa-Hepes, containing [2-^{14}C]pyruvate was added, resulting in a total culture volume of 4 μl, and the cap was carefully secured onto the 2.5 ml mini-vial. Each mini-vial contained 1.75 ml of 25 mmol $NaHCO_3$ l^{-1} that had been equilibrated for 2 h to a humidified atmosphere of 5% CO_2 in air at 39°C. Assay control mini-vials included sham preparations ($n = 5$) that contained all reagents but did not include an embryo (to account for non-specific counts), and background mini-vials containing 2 μl unlabelled CR1aa medium. Embryos were cultured for 3 h (39°C). The caps were removed, and the bicarbonate contained within the vial quickly poured into a 20 ml

scintillation vial containing 0.2 ml of 0.1 mol NaOH l^{-1} and capped. The scintillation vials were gently mixed and held at 4°C overnight to facilitate conversion of dissolved [^{14}C]O_2 and bicarbonate into carbonate. Scintillation fluid was added (15 ml), and disintegrations per minute (d.p.m.) determined by counting for 5 min using a Packard Tri-Carb scintillation counter programmed for automatic-quench correction. Two microlitres of labelled [^{14}C]pyruvate were mixed with 1.75 ml $NaHCO_3$ and 0.2 ml NaOH, and the radioactivity quantified in the same manner, to determine total substrate d.p.m. The amount of [^{14}C]pyruvate metabolized by individual embryos was calculated as described by Tiffin *et al.* (1991) and Rieger *et al.* (1992a,b). The mean d.p.m. for the sham preparations was subtracted from the d.p.m. value of each embryo, and the difference divided by the total [^{14}C]pyruvate d.p.m. and multiplied by the total quantity of substrate (labelled and unlabelled pyruvate) in 4 µl medium. This value was then multiplied by the product recovery correction factor of 1.04 (100/96.1) determined for the 3 h culture period (Rieger *et al*., 1992a). This calculation involved generating a standard percentage recovery curve over the 3 h incubation period to determine the plateau saturation value of NaH[^{14}C]O_3 at 3 h (data not shown).

Statistical analysis

Expression data were analysed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. Parametric analysis of differences in the means between two or more populations were tested using ANOVA with the main effects of day of collection and quantity of diet and their interactions followed by multiple pairwise comparisons using a Tukey's test. Pyruvate uptake data were analysed by the SAS software package, version 6.0 (SAS Institute Inc., 1989) using the generalized linear models (GLM) procedure, with the main effects of day of collection, quantity of diet and type of diet and their interactions. Differences of $P \leq 0.05$ were considered to be significant.

As the relative abundances of specific mRNAs in embryos from animals fed either the pulp- or barley-based diets were determined in two different laboratories, no comparisons can be made between these groups. However, pyruvate use was measured from both embryo types in the same lab, allowing comparisons among all groups.

Correlations between the relative abundance of each mRNA of embryos recovered from heifers fed the different diets and the corresponding pyruvate metabolism data were calculated with the Pearson product moment correlation.

Results

Superovulatory response (number of corpora lutea at slaughter) was greater ($P < 0.06$) when heifers were offered 3 kg per day (15.5 ± 1.6) than when they were offered *ad libitum* concentrates (12.3 ± 1.4). The superovulatory response for both citrus–beet pulp (14.4 ± 1.5) and barley (13.3 ± 1.5) was not different ($P > 0.05$). Heifers offered 3 kg concentrates per day produced greater numbers of transferable embryos (4.8 ± 0.07) compared with heifers offered *ad libitum* concentrates (2.8 ± 0.8; $P < 0.05$). Heifers offered citrus–beet pulp produced greater numbers of transferable embryos (4.8 ± 0.07) than heifers offered barley (2.9 ± 0.05; $P < 0.05$). Details have been reported by Yaakub *et al*. (1999).

Relative abundance of Na/K-ATPase α1 and Cu/Zn-SOD transcripts in embryos

For all primer pairs, the number of PCR cycles was kept within the linear range of amplification (De Sousa *et al*., 1998b; Wrenzycki *et al*., 1999). Globin RNA, added before RNA isolation as an internal standard, was effectively amplified at 30–31 PCR cycles (Fig. 1a), showing a linear increase in the amount of PCR products as a function of RNA input up to 160 fg (Fig. 1b).

Transcripts for Na/K-ATPase α1, Cu/Zn-SOD, and exogenously supplied α-globin were amplified from bovine embryos developed *in vivo* from heifers on 3 kg per day or *ad libitum* diets of citrus–beet pulp or barley are shown (Fig. 2a,b). In embryos derived from heifers fed the pulp-based diets, the relative abundances of both transcripts were not affected by either day of collection or quantity of diet (Fig. 3a). In embryos derived from heifers fed the barley-based diets, the relative abundances of the Na/K-ATPase transcripts were also not changed by these main effects, while the relative abundances of the Cu/Zn-SOD transcripts were affected by day of collection (significantly lower in day 6 than in day 7 embryos) and by the quantity of diet (Fig. 3b). No interactive effects were observed for day of collection and quantity of diets in the relative abundance of both transcripts.

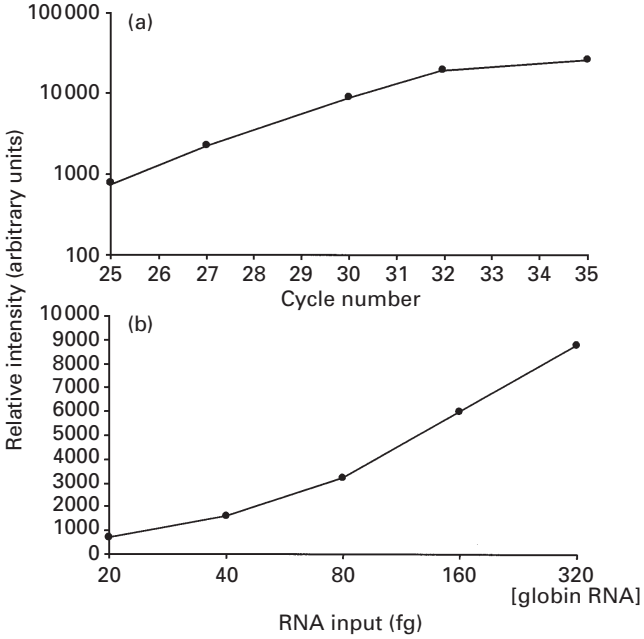

Fig. 1. Validation of the semi-quantitative RT–PCR assay in terms of the number of cycles using an amount of 20 fg globin RNA (a) or different amounts of globin RNA using 30 PCR cycles (b).

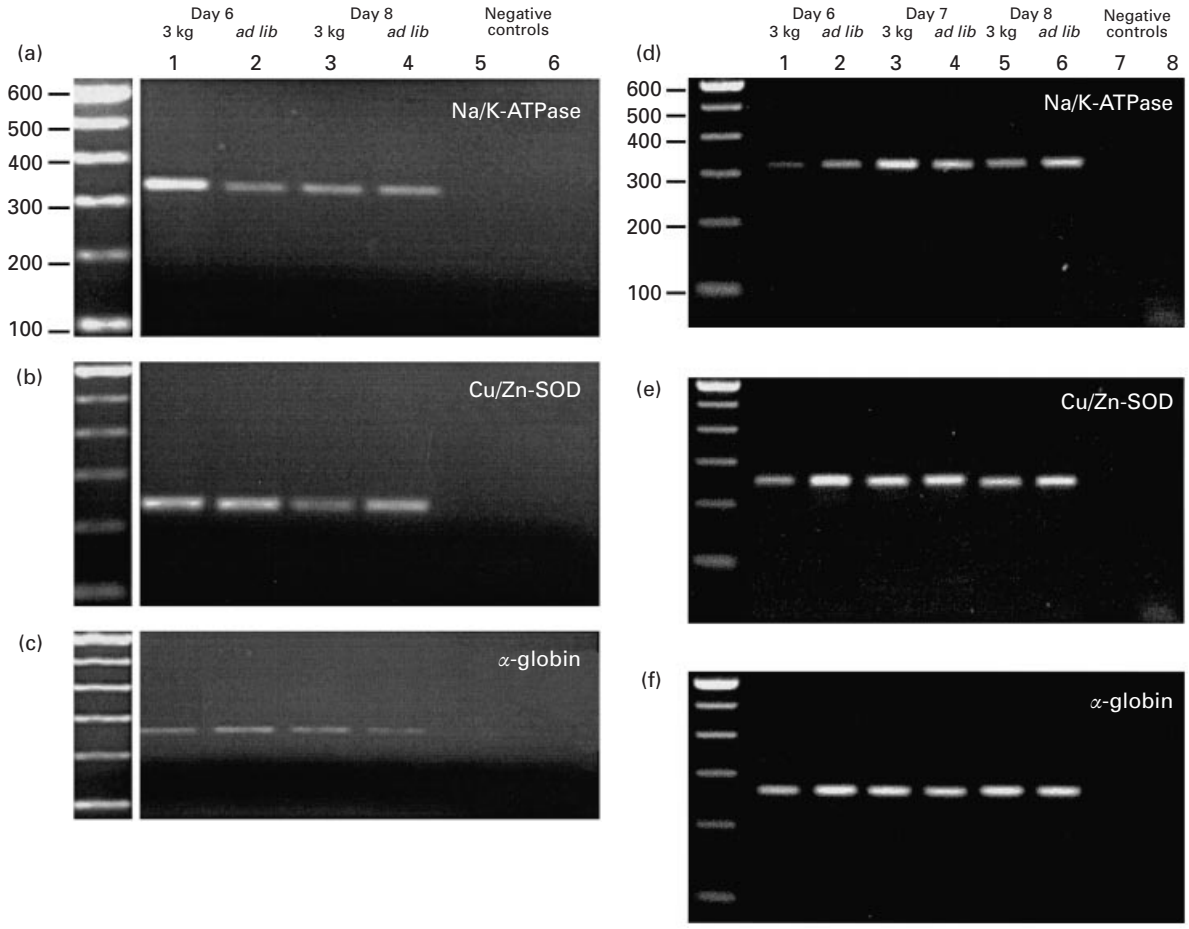

Fig. 2. Detection of genetranscripts in bovine morulae and blastocysts developed *in vivo* from heifers on 3 kg per day or *ad libitum* daily intakes of citrus–beet pulp (a–c) or barley (d–f). Representative ethidium bromide-stained gels showing amplification of a 336 bp product representing Na/K-ATPase α1 transcript (a,d), a 246 bp product representing the Cu/Zn-SOD transcript (b,e), and a 257 bp product representing the exogenously supplied α-globin transcript (c,f), amplified from day 6 (lanes 1 and 2), day 7 (d–f, lanes 3 and 4) and day 8 (a–c, lanes 3 and 4; d–f, lanes 5 and 6) from heifers on 3 kg per day (a–c, lanes 1 and 3; d–f, lanes 1, 3 and 5) or *ad libitum* (a–c, lanes 2 and 4; d–f, lanes 2, 4 and 6) diets. As negative controls, transcript-specific PCR was performed on aliquots of mock reverse transcriptions on blank mAP squares run through the RNA isolation procedure (a–c: lane 5) and water (a–c: lane 6), while RNA (d–f: lane 7) or reverse transcriptase (d–f: lane 8) was omitted during the RT reaction. *ad lib*: *ad libitum*.

[^{14}C]pyruvate metabolism by individual bovine embryos

The effects of day of collection (Fig. 4a), quantity of diets (Fig. 4b), and type of diets (Fig. 4c) on the utilization of [2-^{14}C]pyruvate by bovine embryos produced *in vivo* is shown. Pyruvate metabolism was affected by day of collection and showed a significant increase in day 8 embryos compared with day 7 and day 6 embryos. Diet quantity did not affect pyruvate utilization, whereas pyruvate metabolism was significantly increased in the barley diet group compared with the pulp diet group. No interactive effects were observed for day of collection, quantity of diets and type of diets in pyruvate metabolism.

The relative abundances of the Cu/Zn-SOD transcript and pyruvate metabolism showed a negative significant correlation ($r = -0.84$, $P \leq 0.05$) in embryos collected from heifers fed barley diets.

Discussion

The present study is the first to investigate the effects of quantity and type of diet on mRNA expression and energy metabolism in preimplantation bovine embryos. Alterations in the relative amounts of specific gene transcripts in bovine oocytes produced *in vitro* and embryos during preimplantation development have been determined using a sensitive semi-quantitative RT–PCR assay (Lequarre *et al*., 1997; De Sousa *et al*., 1998a; Wrenzycki *et al*., 1999). Furthermore, this technique has been used to determine the effects of different culture conditions on the amount of mRNA expression of various developmentally important genes (Wrenzycki *et al*., 1998b, 1999). Variations in the detected amounts of different mRNAs may be attributed to the mRNA structure or the intracellular environment affecting the stability and turnover rate of the mRNA (Ross,

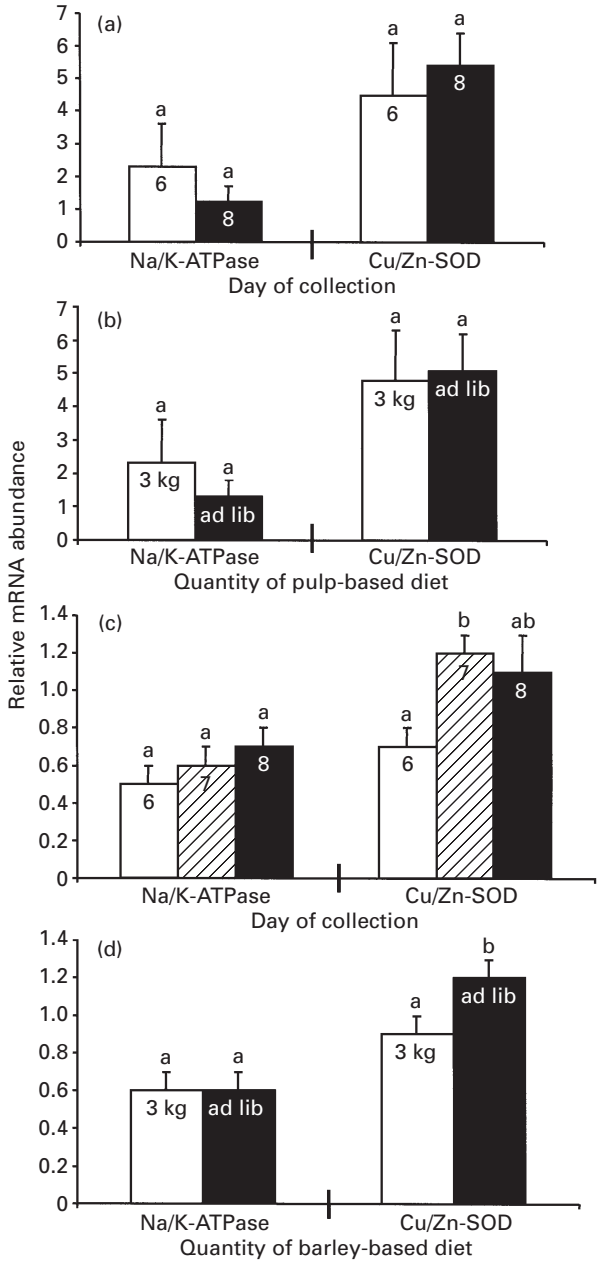

Fig. 3. Variations in the relative abundance of mRNA in bovine morulae and blastocysts developed in heifers on diets of citrus–beet pulp (a,b) or barley (c,d) affected by day of collection (a,c) or quantity of diets (b,d). The mean relative mRNA abundance (± SEM) was determined for Na/K-ATPase α1, and Cu/Zn-SOD from embryos in three independent RNA isolation RT–PCR experiments. Significant differences are denoted by different superscripts ($P \leq 0.05$). *ad lib*: *ad libitum*.

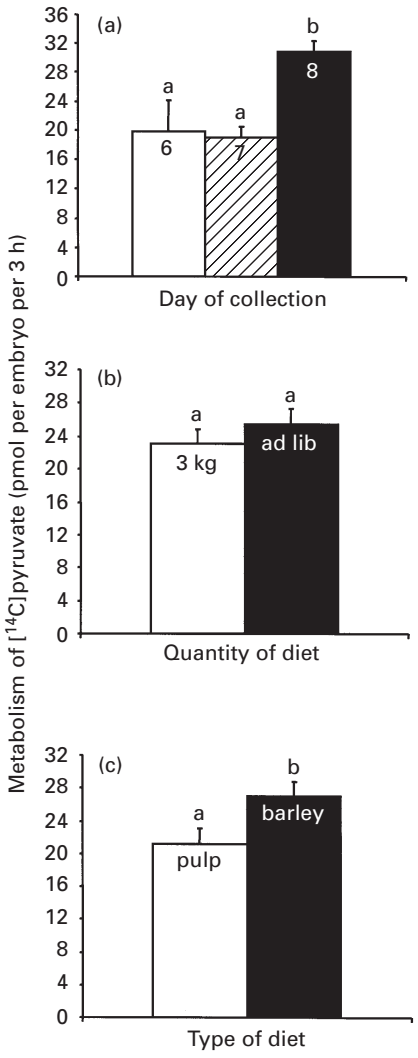

Fig. 4. Pyruvate metabolism by individual embryos produced *in vivo* from heifers on diets of citrus–beet pulp and barley affected by (a) day of collection, (b) quantity of diets or (c) type of diet.

1996) as well as the methodology used to detect rare transcripts. Polyadenylation may increase the presence of a messenger after reverse transcription with an oligo-dT primer by increasing the probability that such a primer will anneal to the transcript (Moore *et al*., 1996; De Sousa *et al*., 1998b). However, the increase of most gene transcripts results from synthesis *de novo* after the major activation of the bovine embryonic genome at the 8–16-cell stage (Telford *et al*., 1990). Despite analysis by independent laboratories with slight modifications in methodology in the present study, the differences tend to be similar within the experimental subgroups.

During preimplantation development, the formation of the blastocyst is mediated by fluid transfer across the outer blastomeres through the activity of Na/K-ATPase. Na/K-ATPase is composed of two subunits, of which the α (catalytic) subunit represents the physiological role of the enzyme (Jorgensen, 1986), while the β (noncatalytic, glycolysated) subunit is thought to facilitate the processing and insertion of the α subunit into the plasma membrane (Geering, 1991). The functional expression of the α subunit is regulated after transcription during preimplantation

development (Kidder, 1992). As no differences in the relative abundance of the α1 transcripts were found in embryos from all treatment groups, this transcript seems to possess an enormous plasticity to various environments, or acts rather independent from environmental factors.

Free oxygen radicals (FOR) have been implicated in embryonic arrest and cell death (Johnson and Nasr-Esfahani, 1994). FOR production is a physiological process that occurs within cells when electrons leak to oxygen during electron transfer reactions. This has pronounced effects on DNA, RNA and protein synthesis, and pertubates cell membranes, increases intracellular pH and disturbs mitochondrial function. While there are numerous non-enzymatic antioxidant agents, specific antioxidant enzymes are able to detoxify O_2^-, H_2O_2, and organic peroxides (Johnson and Nasr-Esfahani, 1994), that is, Cu/Zn-SOD catalyses the dismutation reaction, removing O_2 species. The significant increase of Cu/Zn-SOD transcripts recovered from heifers fed barley-based diets, either *ad libitum* or restricted, may be caused by oxidative stress as a result of *ad libitum* feeding of the donor animals. In addition, it has been proposed that heat shock proteins, such as HSP 70, are induced by FOR (Donati *et al*., 1990). The transcription of this gene is induced by suboptimal culture conditions in mouse embryos (Christians *et al*., 1995) and bovine preimplantation embryos (Wrenzycki *et al*., 1998b, 1999). The differences between embryos collected on different developmental days may be attributed to varying cell numbers.

Evidence indicates that the rate of energy substrate utilization by preimplantation embryos is an appropriate predictive parameter for embryo viability (Rieger, 1984; Gardner and Leese, 1986, 1988; Rondeau *et al*., 1995). Radiolabelled energy substrates have been used extensively in studies of bovine embryo metabolism (Rieger, 1984, 1992; Rieger and Guay, 1988; Javed and Wright, 1991; Tiffin *et al*., 1991; Rieger *et al*., 1992a, b). Furthermore, peroxidative damage of mitochondrial lipids results in a shift in the cells from oxidative use of pyruvate in the Krebs cycle to metabolize succinate (Johnson and Nasr-Esfahani, 1994).

The effects of nutrition on preimplantation embryo development may reflect the general energy balance, while other effects may be attributed to those of specific nutrients, such as vitamins or minerals. When defining nutritional effects, the response to nutrition at one stage of the reproductive cycle may have profound responses at a later stage. Effects on the preovulatory development of the oocyte will carry over into the periovulatory period (Downing *et al*., 1995; Thomas *et al*., 1997) and these nutritional effects could continue to affect pre- and post-fertilization events within the oviduct (Rabiee *et al*., 1997). The uterine phase of development is affected by nutritional factors either indirectly, by effects on progesterone secretion, or directly at the uterus. In sheep, effects on circulating progesterone concentrations may be an important mechanism by which nutrition and metabolic state alter embryo survival (Parr *et al*., 1987, 1993). Furthermore, in superovulated ewes infused with glucose, a decrease in ovulation rate and embryo quality was found (Yaakub *et al*., 1997).

The correlation data demonstrate that feeding the barley-based diet *ad libitum* to the donor heifers led to a decreased pyruvate utilization and an increase in the relative abundance of the Cu/Zn-SOD transcript in day 8 embryos. The correlation indicates that the increased amounts of Cu/Zn-SOD mRNA are due to cellular stress in these embryos. This hypothesis is supported by the fact that, in embryos derived from heifers fed barley-based diets, pyruvate uptake is significantly increased compared with that in embryos from heifers fed pulp-based diets. Reduced development of bovine embryos grown *in vitro* under serum-free conditions is also associated with increased pyruvate uptake (Eckert *et al*., 1999). Furthermore, a significantly smaller number of transferable embryos was recovered from barley-fed compared with pulp-fed donor animals (Yaakub *et al*., 1999) indicating that there is a negative association between the barley diet and early embryonic development. In addition, oxidative stress caused by *ad libitum* feeding of the donor animals may also lead to a significantly decreased number of transferable embryos compared with donor animals fed restricted diets (Yaakub *et al*., 1999).

In conclusion, for the first time, differences in gene expression and metabolic substrate metabolism in *in vivo* derived embryos collected from heifers fed different quantities and types of diet were determined. These differences observed in embryos collected on the same day of development can be attributed to the composition and quantity of diet fed to the donor animal. The type of diet may also alter oocyte development and maturation and embryonic development by alteration of the microenvironment. As a result of these metabolic changes, transcription and translation of key developmental genes may be altered with effects on early development. The present findings characterize embryos produced *in vivo* at the molecular level, and indicate that these molecular markers can be used to differentiate between populations of embryos produced under different nutritional regimens, and to determine conditions conducive to the production of good quality embryos.

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