Environmental factors affecting interferon-τ expression and secretion by *in vitro* produced bovine blastocysts

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Dedicated to my family:

Iara and Roberto,
Fiona and Roger,
Tom and Mia,

who have been a source of inspiration and encouragement throughout this doctorate.

Declaration

I hereby declare that the thesis was composed by me, that the work is my own and that the work has not been submitted for any other degree or professional qualification except as specified.

Abstract

Interferon (IFN)τ is the luteotrophic signal in ruminants and is secreted by bovine blastocysts both *in vivo* and *in vitro*. IFNτ secretion is highly variable and its control is only partly understood. Most studies on the effects of environmental factors on IFNτ production have evaluated IFNτ production during the time of embryo elongation and attachment. There is less knowledge of how IFNτ production at the blastocyst stage is modulated. Therefore, the hypothesis of this thesis was that the amounts of IFNτ expressed and/or secreted by bovine blastocysts produced *in vitro* were modulated by environmental factors. In the first set of experiments, bovine embryos were incubated with a cytokine (granulocyte macrophage colony stimulating factor, GM-CSF). GM-CSF had been shown previously to promote embryo viability in a range of species and to modulate IFNτ secretion by ovine blastocysts and thus was classified as a beneficial environmental factor. Three experiments were conducted to test whether GM-CSF stimulated bovine blastocyst development and IFNτ secretion. Embryos were incubated with a range of different concentrations of GM-CSF (2, 5, 10 and 50 ng mL^{-1}) and at different stages of development (1 to 3 and 1 to 9 days post-insemination). Bovine embryos were unresponsive to GM-CSF in terms of IFNτ secretion, pyruvate oxidation, rate of development, blastocyst yield, morphological quality and apoptotic index, irrespective of timing of exposure and/or concentration of GM-CSF. In the second part of the thesis, bovine blastocysts were exposed to a mild heat treatment (42°C for four h) to determine whether heat stress affected IFNτ expression by bovine blastocysts. A novel multiplex reverse-transcription polymerase chain reaction methodology was validated to detect IFNτ and heat shock protein (HSP)70 mRNA in individual bovine embryos relative to an endogenous gene (YWHAZ) and an exogenous mRNA (α-globin) and results were expressed both in absolute terms and in relation to the endogenous control. Heat treatment upregulated IFNτ mRNA expression, suggesting that detrimental environmental factors may influence IFNτ expression. Heat treatment also caused an increase in HSP70 mRNA expression but did not affect blastocyst morphology, suggesting that the level of stress caused by the heat treatment was great enough to activate the cellular stress response, but mild enough not to cause a change in morphology. In addition, the positive correlation between HSP70 and IFNτ transcript levels and the higher IFNτ expression by embryos which showed signs of degeneration and collapse compared to those which progressed in development suggested that IFNτ expression may be indicative of stress. The relationship between IFNτ expression and secretion *in vitro* with morphology, pyruvate metabolism, apoptotic index and cell number was inconsistent, suggesting that IFNτ production did not correlate with 'quality' (defined as an index of viability). Blastocyst yield, day of blastulation and change in morphology index did account for at least part of the variation in IFNτ production, suggesting that some intrinsic factors may regulate IFNτ secretion. These intrinsic factors, however, did not explain all the variation in IFNτ secretion between blastocysts. Therefore, the amount of IFNτ secreted by bovine blastocysts is modulated by both intrinsic and environmental factors. A model was proposed where different levels of stress affect survivability to different extents, and the ability to respond to mild levels of stress may be indicative of improved survivability.

Keywords: embryology; bovine; interferon-tau; maternal recognition of pregnancy; GM-CSF; heat stress; RT-PCR; IVF

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The idea of engaging in research, contributing to academic knowledge and gaining ownership of your thoughts and ideas meant that a doctorate was an intellectual challenge that just had to be taken on.

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The technical aspects to this thesis were definetly a challenge. Several frustrating months were spent on trying to determine why an ELISA for IFNτ detection no longer worked. Trying to determine why bovine embryos were not responding to GM-CSF also lead to the feeling that we were going down a number of paths with dead ends. The move from Aberdeen to Edinburgh involved extensive periods of lab closures. From the embryology point of view, the shortage of ovaries lead to further limitations to achieving the objectives initially set out for this thesis. But from the problems rose solutions. The ELISA was replaced with the cell cytopathic assay, and the limited ovaries lead to molecular biology techniques, in an attempt to maximise information from each blastocyst. There were numerous glorious moments when things would just come together. I would like to thank my supervisor, Dr John Rooke, who guided me through the day to day challenges for this thesis and trusted me in exploring the then unfamiliar molecular techniques.

The first version of the thesis was finished in March 2008, and it took a further 12 months to develop the critical analytical thought and get the confidence necessary to publish the thesis. The moments of inspiration came at the randomest of times with bursts of inspiration on how to go about framing the thesis. One thing I learned is how important it is to seize these bursts of inspiration, and how easily grand ideas can dissolve into nothing. The practicalities of writing the thesis alongside a full time job and raising a child meant that it was hard to keep continuity going in my thoughts and a considerable amount of time was spent trying to catch up with where I was when I left off. This thesis involved a period of profound change, challenge and obstacles. I would like to express my gratitude and appreciation to my supervisors, Dr John Rooke and Professor Cheryl Ashworth, for their guidance, helpful suggestions and help with overcoming the difficulties in writing this thesis.

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LIST OF PUBLICATIONS

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ABBREVIATIONS

A	adenine
A	absent
ANOVA	analysis of variance
AP1	activator protein site of a gene, target binding to PKC
b	bovine
BAX	apoptosis regulator box-2
Bcl-2	B-cell lymphoma-2
BSA	bovine serum albumin
Bq	becquerel
BRU	blastocyst RNA unit
C	cytosine
cAMP	adenosine 3′,5′-cyclic monophosphate
cDNA	complementary DNA: single-stranded DNA that is synthesized from a messenger RNA template.
CAT	chloramphenicol acetyltransferase
CCD	charge coupled device
CDX2	caudal-related homeobox protein 2
CHO	chinese hamster ovary cells: cell line often used in studies of genetics, toxicity screening, nutrition and gene expression.
Cl^-	chloride
CO_2	carbon dioxide
COCs	cumulus oocyte complexes
COX2	cyclooxygenase-2
CR1	Charles Rosenkrans medium, as reported in Rosenkrans et al. (1993)
CSF	colony stimulating factor
CV	coefficient of variation, the standard deviation divided by the mean
Cx	connexin
DLX3	homeobox distal-less 3
DMEM	dulbecco's modified embryo medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpi	days post-insemination
dpm	disintegrations per minute
dpo	days post-oestrus
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic
ELISA	enzyme-linked immunosorbent assay
ERα	oestrogen receptor alpha
ETS	E26 transforming sequence
FBS	foetal bovine serum
FCS	foetal calf serum
FGF	fibroblast growth factor
g	gram
G	guanine
Glut	glucose transporter
GLMM	generalised linear mixed model
GM-CSF	granulocyte macrophage colony stimulating factor
GOI	gene of interest
h	hour(s)
H^+	hydrogen ion
HCO_3^-	bicarbonate ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, an organic chemical buffering agent
hpi	h post-insemination

HSE	heat shock element
HSC	heat shock cognate protein
HSF	heat shock transcription factor
HSP	heat stress protein or heat shock protein
IFN	IFN
IFNτ	the antiluteolytic protein secreted by a ruminant embryo during maternal recognition of pregnancy. Also known as **trophoblastin**, **ovine trophoblast protein-1**, and **bovine trophoblast protein-1**.
IFNs	Interferons: cytokines induced by a viral infection and have antiviral and proliferative activities
IGF	Insulin-like growth factor, IGF I, IGF II
IL	interleukin: a type of cytokine produced by leukocytes that influences a variety of cells. IL 1, IL 2, IL 3, IL 5.
IRF2	IFN regulatory factor 2
ISG	IFNτ stimulated gene(s)
IU	international units. According to Hernendez-Ledezma et al. (1992), 1 IU is equivalent to about 0.1 ng of bIFNτ.
IVC	*In vitro* Culture
IVF	*In vitro* Fertilisation
IVM	*In vitro* Maturation
IVP	*In vitro* Produced
JAr	human choriocarcinoma cell line
JAK/STAT	janus-activated kinase/signal transducers and activators of transcription
λ_{ex}	wavelength (excitation)
λ_{em}	wavelength (emmission)
LIF	leukemia inhibitory factor
LMM	linear mixed model
LUA	laboratory unit of activity
m	milli
M	molar
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MDBK	Madin-Darby bovine kidney
mL	millilitre
Mn-SOD	manganese superoxide dismutase
mOsmo	measure of osmolarity
mRNA	messenger RNA
Mx	mini-exon
Mx/CAT	plasmid containing a human MxA promoter driving a CAT reporter gene
n	nano
Na^+	sodium ion
NK cells	natural killer cells
o	ovine, such as oIFNτ, oGM-CSF
p	pico
P	present
P	probability
PBS	phosphate buffer saline
PDGF	platelet-derived growth factor: a protein that regulates cell growth and division
PG	prostaglandin
PGF2α	prostaglandin F2α
pi	post-insemination
PI3K	phosphatidyl-inositol 3-kinases
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
po	post-oestrus
PVA	polyvinyl alcohol: a water soluble polymer
PVP	polyvinylpyrrolidone
r	recombinant: produced by genetic engineering

R	receptor, such as IFNτR, progesterone receptor (PR), oxytocin receptor (OR)
Ras/MAPK	Pathway involving the Ras subfamily of proteins and the MAPK signalling pathway, involved with cell proliferation
REML	restricted maximum likelihood
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RPA	RNase protection assay
RT-PCR	Real Time Polymerase Chain Reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOF	synthetic oviductal fluid
SOX	sarcoside oxidase
T	thymine
TBE	Tris/Borate/EDTA
TCA	tricarboxylic acid
TCM	tissue culture medium
TdT	Terminal Deoxynucleotidyl Transferase
TGF	transforming growth factor: growth factors involved in tissue development, cell differentiation and embryonic development.
TNF	tumor necrosis factor
TUNEL	Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling
v:v	volume per volume
µ	micro
°C	degrees Celsius
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
3'OH	hydroxide terminal at the 3' end of DNA

1 LITERATURE REVIEW

1.1 Maternal recognition of pregnancy in cattle

1.1.1 Oestrous cycle

During a normal oestrous cycle in the bovine, the hypothalamus secretes gonadotropin-releasing hormone which acts on the cells of the anterior pituitary gland, which in turn secrete gonadotropins (follicle stimulating hormone and luteinizing hormone). Under the influence of gonadotropins and growth factors, follicles containing oocytes develop in the ovary through the different stages of folliculogenesis: primordial, pre-antral, antral and Graafian follicles. Graafian follicles are able to undergo ovulation and the cells within the ovulatory follicle change into luteal cells which form the corpus luteum. When fertilisation does not occur, new follicles continue to develop in the ovaries and secrete increasing levels of oestrogen. Oestrogen acts on the uterine endometrium to promote oestrogen receptor synthesis, which, in turn, stimulates oxytocin receptorsynthesis (Spencer et al., 2008). Oxytocin produced by the posterior pituitary gland binds to oxytocin receptors in the uterine endometrium, causing the uterine endometrium to secrete prostaglandin (PG) F2α (Asselin et al., 1997). PGF2α acts on the corpus luteum causing its regression (Peters et al., 1985). Since the corpus luteum is the source of progesterone, its regression leads to a decrease in progesterone concentration (Peters et al., 1985), allowing another Graafian follicle to mature and ovulate and another oestrous cycle then begins, 21 days after the previous oestrus.

1.1.2 Progesterone: the hormone of pregnancy

Progesterone, secreted by the corpus luteum, has a negative feedback effect on gonadotropin secretion by the hypothalamus, thus, preventing development of another Graafian follicle and initiation of the next oestrous cycle. Moreover, progesterone binds to receptors in the uterine endometrium changing the pattern of protein secretion by endometrial cells (the progestational stage) to maintain the endometrium in an optimal state for embryonic survival and development (Mann et al., 1999, Mann and Lamming, 2001). Therefore, progesterone is the hormone of pregnancy through its actions on the hypothalamus and the uterine endometrium.

Removal of the corpus luteum by ovariectomy in the first 210 days of gestation terminates pregnancy in cattle (Johnson, 1981), emphasizing the importance of the corpus luteum in maintaining pregnancy in cattle. Therefore, the prevention of luteolysis is necessary for maintenance of pregnancy.

In eutherian mammals where the length of pregnancy exceeds that of the oestrous cycle, a signal is required to prevent luteolysis. Northey and French (1980) prolonged luteal lifespan by infusing homogenates of embryos (17 to 18 days post-insemination, dpi) into the uteri of cyclic cows between 14 and 18 days post-oestrus (dpo), suggesting that the embryo may be the source of the antiluteolytic signal. The physiological process in which the embryo signals its presence to the maternal system to prolong the lifespan of the corpus luteum and prevents the continuation of the oestrous cycle was first termed maternal recognition of pregnancy by Short (1969).

Bartol et al. (1985) first identified a prominent, but transient, protein that was secreted by embryos from 16 to 24 dpi. The transient nature of production coincident with the timing of establishment of pregnancy suggested a role for this protein in maternal recognition of pregnancy. Since this protein was immunologically related to ovine trophoblast protein-1 (the maternal recognition of pregnancy signal in sheep), this protein was later termed bovine trophoblast protein-1 by Helmer et al. (1987). Trophoblast protein-1 was then classified as an IFN because of its antiviral activity (Pontzer et al., 1988) and its effectiveness against a range of viruses (Roberts et al., 1989) and named interferon (IFN)τ. Therefore, maternal recognition of pregnancy in ungulate ruminants requires that the embryo secretes IFNτ.

1.1.3 IFNτ signalling mechanism

Using bovine blastocysts, IFNτ was immunolocalised to the Golgi complex and associated clear vesicles (Morgan et al., 1993), where the protein product is concentrated and packaged for export, suggesting that IFNτ is a secretory protein. Although exocytosis at the cell surface was not observed by Morgan et al. (1993), Johnson et al. (2007) captured an IFNτ positive signal inside a vesicle approaching the apical surface of the cell for exocytosis, suggesting that IFNτ is a secretory protein which exits the cell by exocytosis. Moreover, Morgan et al. (1993) observed that the highest level of immunolabelling of the maternal endometrium of pregnant cows occurred during the time of maximum IFNτ production (between 19 and 21 dpi), indicating that the transfer of IFNτ from the embryo to the uterus occurred in a paracrine manner. The paracrine action of IFNτ on the maternal endometrium is thought to occur through binding to the Type I IFN receptor (IFNR, Knickerbocker et al., 1986), which, through a tyrosine kinase gene transcription pathway, instigates a change in gene expression and a subsequent change in the pattern of proteins and hormones secreted by the endometrium (Gross et al., 1988).

1.1.4 IFNτ stimulated genes

Genes stimulated by IFNτ are denominated IFNτ stimulated genes (ISG, Austin et al., 2004). Spencer et al. (2008) outlined endometrial genes whose expression was enhanced during pregnancy in cows and sheep, and found that the expression of 19% (51/264) of these genes were stimulated by IFNτ. The high proportion of ISGs of genes whose expression is stimulated by pregnancy highlighted the importance of IFNτ in establishment of pregnancy.

ISGs are thought to promote pregnancy by reducing PGF2α secretion and subsequent luteolysis either (1) by ligating and modifying proteins involved with the release of PGF2α or (2) by inhibiting transcription of oestrogen receptor by maternal endometrial cells, therefore causing a decrease in oxytocin receptor levels, preventing oxytocin from binding to oxytocin receptor and reducing PGF2α secretion (Mann and Lamming, 2001). The preserved corpus luteum then maintains a high concentration of progesterone in the maternal blood. Therefore, by modulating ISG expression, IFNτ is able to activate a range of endometrial anti-luteolytic mechanisms. Failure of the endometrium to respond to the IFNτ signal has been implicated as a cause for early pregnancy failure in domestic ruminants (Winkelman et al., 1999).

1.1.5 Pre-attachment embryo development in cattle is modulated by genetic and environmental factors

During the pre-attachment phase of development, the embryo undergoes dramatic changes in its physiology in terms of structure, function and size (Figure 1, page 24). Ovulation occurs 10 to 12 hours (h) after the onset of heat and fertilisation occurs between 24 and 30 h after the end of heat. After fertilisation (day 0), the viable zygote must undergo cleavage division (two-cell by day 1, four-cell by day 2, eight-cell by day 3, 16-cell by day 4) and embryonic genome activation (between the two- and eight-cell stage) in the oviduct. Compaction (process where the cells bind tightly to each other, forming a compact sphere) is necessary for the formation of the morula as the embryo enters the uterus and cavitation (process where the trophoblast secretes water into the morula forming a cavity) and cell differentiation into inner cell mass and trophectoderm cells are necessary for the formation of the blastocyst (blastocyst development is reviewed by Watson et al., 1992). The blastocyst then undergoes expansion and hatching. After elongation, the embryo is ready to attach to the uterus, between 30 and 35 dpi (Gordon, 2003). The viability of the embryo is dependent on the genetic material of the gametes from which the embryo was derived as well as the environment meeting the changing requirements of the embryo, so that the varied nutritional composition of the fluids of the oviduct and the uterus should mirror the changing requirements of the embryo (Gardner and Schoolcraft, 1998).

Little is known about the regulation of IFNτ secretion (Martal et al., 1998, Kubisch et al., 2001a, Neira et al., 2007). The relative role of the genetic factors inherent in the embryo and the environmental factors and the interaction between the two in influencing timing and amount of IFNτ secretion have been investigated. This review will examine the properties of IFNτ and the methods available for IFNτ detection, the variation in IFNτ secretion by bovine embryos and how IFNτ secretion patterns may be regulated by genetic and environmental factors to produce discrete patterns of IFNτ secretion during pre-implantation development.

1.2 IFNτ detection

Protein synthesis involves a multi-step process in which the information coded within a sequence of a gene is transcribed into mRNA which in turn is translated into proteins. IFNτ has been detected in bovine embryos at the mRNA and protein levels. In this section, the techniques used to detect IFNτ mRNA expression and protein synthesis will be reviewed.

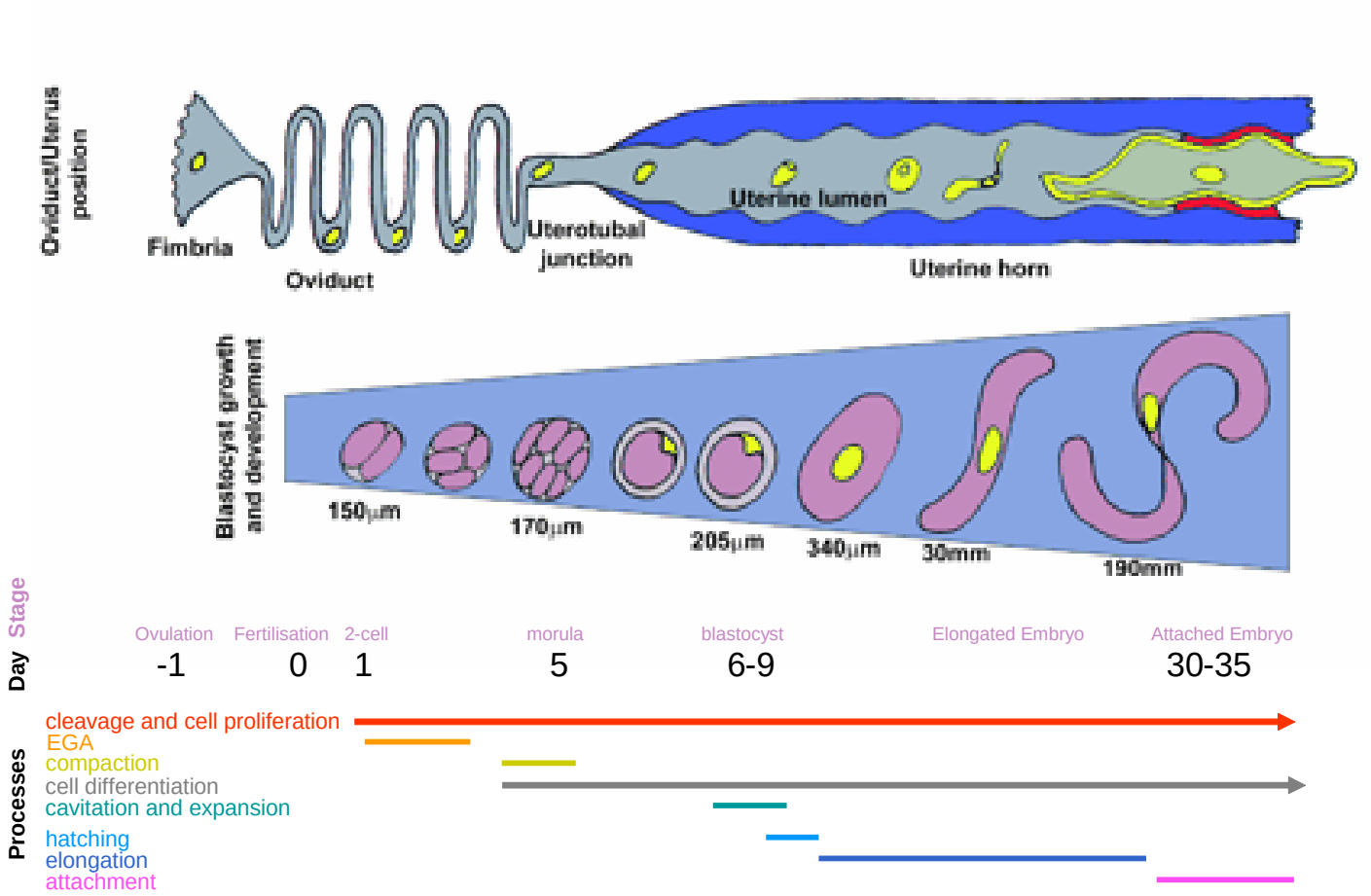
1.2.1 Detection of IFNτ mRNA

1.2.1.1 Properties of embryo mRNA

On average, a single mammalian cell contains 10 pg of total RNA, of which around 80% is ribosomal RNA, 15 to 20% low molecular weight species such as transfer RNA, and only 1 to 5% mRNA (100 to 500 fg, Wrenzycki et al., 2004). A bovine morula, which contains around 100 cells, contains around 700 pg of total RNA (Bilodeau-Goessels and Schultz, 1997a), so that the amount of total RNA per blastomere is in the same order of magnitude as in other mammalian cells. However, the total number of cells available for analysis with embryos is limited (around 100 cells at the morula to blastocyst stage) compared to cell cultures and tissues (generally in excess of 10^6 cells). Therefore, the limited quantity of total RNA in embryos is one of the main difficulties associated with determining mRNA levels in pre-attachment embryos.

Murine oocytes contain between 0.35 ng (Piko et al., 1982) and 0.47 ng (Olszanska et al., 1993) of total RNA, whilst bovine oocytes, which are larger in diameter, contain between 0.98ng (Olszanska et al., 1993) and 2.4 ng (Bilodeau-Goessels et al., 1997a) of total RNA. Therefore, the amount of total RNA in oocytes varies between species. Between species variations may be due to variations in oocyte size, so that it is possible that variation in embryo size may also be a factor affecting total RNA per embryo.

Figure 1 Events associated with pre-attachment embryo development in cattle (modified and supplemented from Spencer and Bazer, 2004).



The amount of total RNA in an embryo also varies with stage of development. Before ovulation, the oocyte is in a state of active gene expression, synthesizing maternal RNAs and proteins required for early embryonic development. The competent ooplasm has all the mRNA needed for embryonic development up to the eight-cell stage (Barnes and First, 1991). Total RNA content of bovine oocytes produced by IVM procedures averages 2.4 ng (Bilodeau-Goessels and Schultz, 1997a). Fertilisation initiates a complex series of events resulting in fundamental alterations in expression of developmentally important genes in a time- and stage-dependent manner. These events include the first cleavage division, embryonic genome activation, compaction, blastocoele formation and cell differentiation into inner cell mass and trophectoderm cells. After fertilisation, maternal mRNA and protein reserves in the embryo decline with time. There is a 30% decrease in total RNA content by the one- to two-cell stage and a 70% decrease by the morula stage (Table 1, page 26). Subsequent development becomes dependent on the embryonic genome becoming transcriptionally active. Early studies indicated that the maternal-embryo transition in bovine zygotes began at the four- to eight-cell stage, when major changes were shown to occur in the pattern of protein synthesis (Frei et al., 1989), when [^{3}H]uridine incorporation into RNA was first detected indicating increased transcriptional activity (Camous et al., 1986), and when electron-dense nucleolus precursors became transformed into an active fibrillo-granular nucleolus during the functional organization of the nucleolus (Kopecny et al., 1989). A more recent study employing more sensitive autoradiography techniques suggested that bovine zygotes were transcriptionally and translationally active as early as the two-cell stage (Viuff et al., 1996). Currently, it is accepted that the major activation of the bovine embryonic genome occurs around the eight-cell stage whereas minor activation occurs at the two-cell stage (Memilli and First, 2000). Therefore, at the eight-cell stage, there is little change in the level of RNA since the rate of embryonic RNA synthesis is similar to the rate of decay of maternal RNA (Bilodeau-Goessels and Schultz, 1997b). As cell numbers increase during cavitation, blastocyst growth and blastocyst expansion, the rate of RNA synthesis exceeds the rate of decay, leading to a seven-fold increase in RNA in blastocysts relative to morulas (Table 1, page 26). Therefore, other than the limiting levels of mRNA in single embryos, the variation in total transcription levels during development further hamper experimental design for studies assessing mRNA levels in embryos.

Therefore, the method used to detect mRNA levels in embryos must account for the pg levels of mRNA in pre-attachment embryos (35 to 265 pg) and the plasticity of the embryonic transcriptome.

Table 1 Amounts of total RNA in bovine embryos at different stages of development (Bilodeau-Goessels and Schultz, 1997a). Estimated amount of mRNA is calculated as 5% of total RNA.

	Amount of total RNA (pg)	Estimated amount of mRNA (pg)
Oocyte	2400	120
two-cell	1700	85
Morula	700	35
Blastocyst	5300	265

1.2.1.2 Techniques available for detection of IFNτ mRNA

Techniques used to detect IFNτ transcript levels have included northern blotting (Stewart et al., 1989, Imakawa et al., 1989, Cross et al., 1991), RNase Protection Assays (RPA, Mann et al., 1999, Ealy et al., 1998a) and *in situ* hybridisation (Farin et al., 1989, 1990, Guillomot et al., 1990). These qualitative techniques lacked the sensitivity necessary to detect low RNA levels so that large numbers of embryos were pooled or larger embryos (older, 13 dpi onwards) were used to give sufficient RNA for analysis. Reverse transcription polymerase chain reaction (RT-PCR) is a technique that allows conversion of small quantities of single-stranded mRNA to double-stranded cDNA by RT and then amplification of a known sequence of interest by enzymatically replicating DNA (Bustin et al., 2002). As a result of its high sensitivity and specificity and the need for only small amounts of material to generate data, real time RT-PCR is currently the optimal method for identifying and verifying changes at the RNA level in bovine embryos. Due to the limited quantity of mRNA in embryos, early RT-PCR methods (de Sousa et al., 1998a,c, Kawarsky and King et al., 2002, Wrenzycki et al., 1999) required pooling of large numbers of embryos to give sufficient RNA for analysis. Large pools, however, are expensive and time consuming to collect (Bilodeau-Goessels and Schultz, 1997). Moreover, significant variation in total RNA has subsequently been demonstrated between blastocysts, both due to natural biological variation due to unique set of maternal or paternal DNA, and to the rate of development, so that poolings of embryos obscured variation in mRNA expression between individual embryos (Wrenzycki et al., 2004). Therefore, it is desirable to estimate mRNA levels at the single embryo level as variation between embryos is accounted for and quantified. Improvements to RT-PCR, such as the emergence of real-time PCR, highly specific probes and improvements in nucleotide extraction methods, have enabled qualitative analysis of IFNτ in individual bovine embryos (Wrenzycki et al., 2001a, Bertolini et al., 2002), so that methodologies are no longer limited to pools of embryos.

1.2.1.3 Problems with current RT-PCR methodologies used to assess IFNτ mRNA levels

Quantitative RT-PCR assumes that "the amount of amplification of a target gene transcript is related to its starting abundance and can be predicted if the number of cycles of amplification

is known" (Lechniak, 2002). In practice, however, many variables (Table 2, page 28) influence the efficiency of RNA extraction, RT processes and PCR amplification, so that the amount of product obtained from a sample following amplification may not necessarily reflect the initial target concentration. Therefore, the method by which data are quantified and the inherent assumptions can confound experimental results. Therefore, the variation caused by each step of the gene-transcription analysis needs to be controlled to detect true differences in transcription levels of a particular gene. Sources of variations that need to be controlled include differences between samples in overall transcriptional activity (differences in number of cells or total RNA amount), amount of starting material extracted (some RNA may remain trapped inside undisrupted cells, or on the silica gel when using column based RNA purification methods), varied efficiency of the reverse transcriptase and DNA polymerase (Thellin et al., 1999), varied annealing efficiencies of primer to target sequences, or polymerase errors (which occurs in approximately 1 in 10,000 bases, so that, if an error occurs in the earlier PCR cycles, it can lead to an alteration of large proportions of the final product, Wrenzycki et al., 2004). The RT reaction should be as efficient as possible in order to produce maximum good quality cDNA yield, leading to maximum PCR sensitivity. However, several factors affect RT efficiency, including RNA quality (Bustin et al., 2004), priming strategy (Stahlberg et al., 2004), enzyme efficiency (Brooks et al., 1995), the duration of an RT reaction, the annealing temperature, the length of the target sequences (if using gene specific primers), all of which can result in a cDNA population that does not reflect the original mRNA population in terms of transcript abundance and complexity (Battaglia et al., 1998). Therefore, for meaningful quantification of mRNA levels in bovine embryos, there is a need for a method that controls for variations caused by gene-transcriptions analysis and variations in overall transcriptional activity among samples.

Another disadvantage of the use of RT-PCR to detect IFNτ mRNA is that the lack of an IFNτ mRNA standard has meant that there are currently no data available on absolute IFNτ expression from individual embryos. Also, the use of non-specific DNA binding dyes to detect IFNτ amplicons has meant that PCR artefacts cannot be discriminated from IFNτ amplicons, incurring inaccuracies on the current PCR data available for IFNτ. Another problem with current techniques for detection of IFNτ expression is that further developments in the RT-PCR technology, such as use of sequence specific probes, have not been applied to IFNτ expression analysis. Therefore, there is a need for improved methods for detection of IFNτ mRNA levels.

Table 2 Endogenous (End), exogenous (Exo) and reference dye controls normalize data for different sources of variation during the RT-PCR process. GOI: gene of interest

Type of well-to-well variation	Source of variation	Ref	End	Exo	Multiplex GOI, Exo, Ref
Variation in expression of target gene per blastocyst/total RNA	Environmental or genetic factors	x	x	x	x
Variation in total RNA per blastocyst	Number of cells, rate of expression and degree of degradation	x	√	x	√
Variation in RNA quality and RNA yield	Variable RNA extraction efficiency	x	√[1]	√[1]	√[1]
Variation in cDNA yield	Variable RT efficiency	x	√[2]	√[2]	√[2]
	Pipetting error when loading into PCR well	√	x	x	√
Variation in amplicon yield	Variable PCR efficiency	x	x	x	√[3]
Variation in non-PCR related fluorescence signal	Variation in amount of light reaching each well, optical properties of each well and concentration or volume (due to evaporation)	√	x	x	√

[1]Assuming similar RNA extraction between GOI and control

[2]Assuming similar RT efficiency between GOI and control

[3]Assuming similar PCR efficiency between GOI and control

1.2.1.4 Post-transcriptional modulation of IFNτ

Roberts et al. (1991) reported 18 distinct polymorphic ovine and 12 different bovine IFNτ cDNA or genomic sequences, whilst Ealy et al. (2001) later identified 24 bovine IFNτ cDNA sequences and Alexenko et al. (2000) reported 43 sequences representing both genes and cDNA for IFNτ, and 33 proteins unique in primary sequence. Searching through the NCBI database, 78 unique IFNτ proteins are currently registered (Figure 2, page 29). All cDNAs identified had single non-synonymous base changes. These isoforms have been described and categorised by Ealy et al. (2001), Alexenko et al. (2000) and Roberts et al. (1991) based on predicted amino acid sequence. None of the IFNτ cDNA identified by Ealy et al. (2001)

differed by more than 1.8% in nucleotide sequence, although the differences in inferred amino-acid sequence were greater than nucleotide sequence (3.5%). Although many distinct transcripts and genes for IFNτ have been detected in cDNA cloning studies (Imakawa et al., 1987, 1989, Stewart et al., 1989), not all are transcribed (Demmers et al., 2001). Moreover, each protein isoform is likely to be translated by more than one species of mRNA (Anthony et al., 1988). In fact, Roberts et al. (1991) demonstrated by predicting the protein sequences from the nucleotide sequences of different IFNτ isoform cDNA that these isoelectric variants are products of different genes. However, because sequences for cDNA clones for the ovine are derived from mRNA, it is difficult to determine whether different variants represent different genes, or allelic forms of the same gene. Given the similarity between different IFNτ mRNA isoforms, one of the difficulties of detecting IFNτ mRNA relates to designing primers that will detect all the multiple mRNA isoforms or specific isoforms depending on the requirements of the study for total IFNτ expression or a specific IFNτ isoform expression.

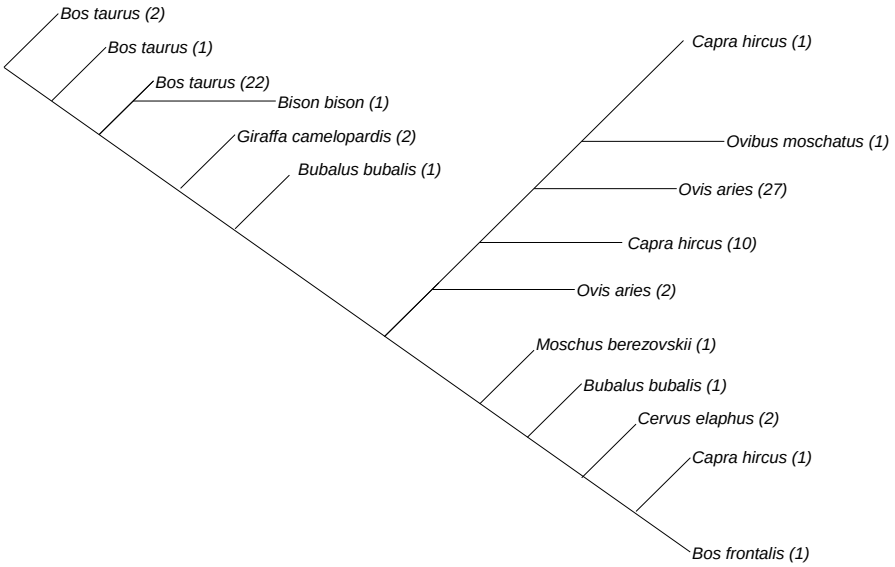


Figure 2 A phylogenic tree based on amino acid sequence of IFNτ. Amino acid sequences were inferred from gene and cDNA nucleotide sequences in the NCBI database and aligned using the Multiple Alignment Viewer available on www.ncbi.nlm.nih.gov/sutils/alnview.cgi (as of 2 April, 2008). The tree for protein sequences was constructed using the Phylogenic Tree option on the same website, and then simplified based on species. 100 total proteins were identified, of which 22 were 'similar to IFNτ' and therefore excluded. Of the 78 remaining IFNτ isoforms, 25 are bovine, 29 ovine, 12 are caprine and 12 are other ruminants.

The genetic control from different isoforms of IFNτ mRNA being transcribed (Demmers et al., 2001) coupled with possible post-transcriptional regulation (such as alternative RNA splicing, modification, editing, localisation, translation and stability, expression of non-coding RNA, antisense RNA and microRNAs, Niemann et al., 2007) and translational regulation (affecting efficiency of translation) may lead to discrepancies between total IFNτ expression and secretion levels, as reported by Robinson et al. (2006), so that the assumption that IFNτ mRNA levels reflect IFNτ protein levels (as suggested by Wrenzycki et al., 2004) may not always be true. Since the known role of IFNτ as the maternal recognition of pregnancy signal occurs at the protein level, there is a need to assess IFNτ protein levels as well as mRNA levels.

1.2.2 Methods for detection of IFNτ protein synthesis

IFNτ protein may be detected within the embryo and outside the embryo. Since IFNτ is a secretory protein, intracellular IFNτ protein is present at a very low concentration, so that only qualitative immunological methods (within the embryo by immunohistochemistry or within the blastomeres by immunocytochemistry) have been used to assess intracellular IFNτ protein. Most studies assessing IFNτ protein use methods to assess its concentration in culture medium (*in vitro*) or uterine flushings (*in vivo*) which rely on its molecular weight, isoelectric, antiviral and immunological properties.

1.2.2.1 Techniques measuring IFNτ based on molecular weight and isoelectric properties

Bartol et al. (1985) first purified IFNτ secreted by *in vivo* produced bovine embryos (16 to 24 dpi) into culture medium using ion exchange chromatography and observed that IFNτ was more acidic (had a higher net charge) than other proteins secreted by the conceptus. Bartol et al. (1985) then separated the proteins secreted by the embryo using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and confirmed that IFNτ was an acidic polypeptide with a low molecular weight (molecular weight/isoelectric point, Mr/pI: 22000-26000/6.5-5.6).

Later, using the 2D-PAGE technique, Anthony et al. (1988) determined that bovine IFNτ had variants differing in molecular mass (between 22000 and 24000 kDa) as well as pI (Anthony et al., 1988). The different sizes are due to differential glycosylation with N-linked oligosaccharides (Godkin et al., 1988) through a single potential site for glycosylation: asparagine at position 78 (Bartol et al., 1985, Anthony et al., 1988, Helmer et al., 1988). IFNτ

was also shown to have 3 or 4 isoelectric variants using 2D-PAGE (Godkin et al., 1982, Imakawa et al., 1987) due to changes in isoelectric properties of different amino acids substituted in different IFNτ isoforms (Roberts et al., 1991).

2D-PAGE has the advantage of being able to differentiate between different isoforms of IFNτ which will have varied size and isoelectric points. However, 2D-PAGE has the disadvantages of being qualitative and insensitive, as this technique has only been reported to assess IFNτ secretion in elongating embryos (10 dpi) which secrete around 10 ng of IFNτ per day (Bartol., 1985). Therefore, methods quantifying the amount of IFNτ protein based on antiviral activity or immunological properties are the preferred methods of measuring IFNτ protein.

1.2.2.2 Techniques measuring IFNτ based on antiviral activity

One of the characteristics of IFNs is that they have antiviral activity. Therefore, in 1981, Familletti et al. first suggested a cell cytopathic assay for detection of IFNs. The cell cytopathic assay determines the concentration of IFN in the medium as assessed by the ability of IFNs to protect cells from viral exposure (Alexenko et al. 1997).

Pontzer et al. (1988) first demonstrated that ovine IFNτ purified from conceptus culture media (12 to 16 dpi) had a high specific antiviral activity of 2 to 3 x 10^8 international units (IU) milligram $(mg)^{-1}$ protein, and was therefore as potent as any known IFN. Recombinant (r) bovine IFNτ had a similar antiviral activity to ovine, 1 x 10^8 IU mg^{-1} protein (Klemann et al., 1990). Cross et al. (1991) first used the cell cytopathic assay to detect IFNτ secretion by bovine embryos and the cell cytopathic assay has continued to be the commonest technique for measuring IFNτ secretion by ruminant embryos (Roberts et al., 1989, Hernandez-Ledezma et al., 1992, 1993, Stojkovic et al., 1995, 1999, de Moraes et al., 1997, Demmers et al., 2001, Neira et al., 2007), probably due to the sensitivity of the method (0.12 IU mL^{-1}, Hernandez-Ledezma et al., 1992, equivalent to about 1 pg mL^{-1}, assuming 1x10^8 IU mg^{-1} specific activity, Pontzer et al., 1988), the ease of reproducing the method in different laboratories and its ability to distinguish between different variants of IFNτ with similar immunological properties but varied antiviral activity. However, antiviral activity measured using the cell cytopathic assay may not correspond to biological activity of IFNτ in maternal recognition of pregnancy. Given that the role of IFNτ *in vivo* occurs through receptor binding and not though its antiviral activity, use of a technique based on the ability of IFNτ to bind to its receptor may be more biologically relevant.

Since the cell cytopathic assay measures antiviral activity, it has the disadvantage of not being specific to IFNτ and will identify other cytokines IFNs. Moreover, the cell cytopathic assay is time-consuming. Once confluent Madin Darby bovine kidney (MDBK) cell cultures are established, the assay takes 3 days (day 1: exposing cells to sample, day 2: exposing cells to virus, day 3: assessing cell survival). Therefore, despite the cell cytopathic assay being time consuming and non-specific, its high sensitivity has led to its common use in quantifying IFNτ secretion.

In 2001, Fray et al. developed a reporter gene assay which uses MDBK cells transfected with a plasmid, containing a human mini-exon (Mx) promoter driving a chloramphenicol acetyltransferase (CAT) complementary (c) deoxyribonucleic acid (DNA). Mx gene transfection is dependent on Type I IFNs and CAT expression allows for quantification in an enzyme linked immunosorbent assay (ELISA). Compared to conventional cell cytopathic assay, the Mx/CAT reporter gene assay has the advantage of being specific to Type I IFNs, since only Type I IFNs are able to induce gene transcription and synthesis of Mx proteins (Ellinwood et al., 1998, Simon et al., 1991). Moreover, Mx/CAT relies on objective quantitation of cell survival, making it more reliable than previous cell cytopathic assays which rely on subjective observation of cell survival. However, the sensitivity of the Mx/CAT reporter assay is lower (0.5 IU mL^{-1}, equivalent to 5 pg mL^{-1} , Mann et al., 2002) than that of the conventional assay (0.12 IU mL^{-1}), which may explain why only one lab has reported to use the Mx/CAT method of IFNτ detection (Fray et al., 2001, Mann et al., 2002).

1.2.2.3 Techniques measuring IFNτ based on immunological properties

In 1988, Vallet et al. developed a radioimmunoassay (RIA) to detect ovine IFNτ and in 2005, Takahashi developed a RIA specific to bovine IFNτ. RIAs have the advantage of being faster (2 days) than other cell cytopathic assays (3 days) and simpler since RIAs do not require maintenance of a permissive cell line or maintenance of viral stocks as required with antiviral assays. Nevertheless, RIAs have the disadvantages of requiring use of radioisotopes and not making a distinction between biologically active and inactive IFNτ.

In 1996, Zhu et al. developed an antigen-competitive ELISA to detect ovine IFNτ with the advantages of being specific to IFNτ, less time-consuming than previous methods (4 to 5 h), and having a lower detection limit (0.005 ng mL^{-1}, Zhu et al., 1996 to 0.1 ng mL^{-1}, Rooke et al., 2005) than RIA (2 ng mL^{-1}, Vallet et al., 1988 to 6 ng mL^{-1}, Takahashi et al., 2005). This method has been used to detect IFNτ secreted by bovine blastocysts (J.A. Rooke, personal communication). However, the ELISA has the major disadvantage that it is difficult to

reproduce in different laboratories due to variation in stability between different batches of the rIFNτ used as an antigen in the ELISA (J.A. Rooke, personal communication). This may explain why the ELISA has not become the current method of choice for IFNτ protein detection and why no studies have been published using the ELISA to detect bovine IFNτ.

1.2.2.4 Techniques measuring intracellular IFNτ synthesis based on immunological properties

Immunohistochemistry and immunocytochemistry detect the localisation of proteins in tissues and cells through the principle of antibodies binding specifically to antigens. There is some discrepancy as to the localisation of IFNτ in bovine embryos. Neira et al. (2007) observed that IFNτ was detected in both the inner cell mass and trophectoderm. However, Johnson et al. (2006) found IFNτ protein only in the trophectoderm. However, in an earlier study Johnson et al. (2004) detected IFNτ in cumulus cell masses of *in vitro* matured oocytes and in morulae (5 to 6 dpi), but not earlier, although it is possible that this was due to non-specific staining.

In 1989, Lifsey et al. observed that IFNτ was more prominent in mononuclear cells, whilst binucleate cells stained to a lesser degree. However, Morgan et al. (1993) observed localisation of IFNτ being specific to uninucleate trophoblast cells. Despite discrepancies between different studies, the general consensus is that the ability to transcribe and translate IFNτ is unique to the uninucleate cells of the trophectoderm of ruminant embryos (Roberts et al., 2007).

IFNτ was immunolocalised to the cytoplasm of trophectoderm cells from bovine conceptus tissue (20 dpi), and was not detected in the nuclei or the endoderm (Lifsey et al., 1989). Johnson et al. (2006) observed that IFNτ was immunolocalised to the cytoplasm and IFNτ was not detected in the vacuole, mitochondria or villi. Morgan et al. (1993) observed that the labelling for IFNτ was consistently located over the Golgi complex and associated clear vesicles in the apical third of cell, with no significant labelling over the nucleus, mitochondria, endoplasmic reticulum, multivesicular bodies, lysosomes or cytoplasmic matrix. Despite earlier studies (Lifsey et al., 1989, Morgan et al., 1993) failing to note nuclear localisation of IFNτ, Johnson et al. (2006) did localise IFNτ to the nuclei of trophoblast cells of IVP blastocysts (7 dpi). However, earlier studies detected IFNτ in tissues from embryos much later in development (18-23 dpi, Lifsey et al., 1989, Morgan et al., 1993), and used *in vivo* derived embryos, as opposed to Johnson et al. (2006) who used earlier embryos (7 dpi) produced *in vitro*. Therefore, localisation of IFNτ within a cell is variable,

and may be controlled by factors associated with *in vitro* culture and /or stage of development.

1.2.2.5 Problems with detecting IFNτ protein

1.2.2.5.1 Similarity to other IFNs

Intramuscular injections or infusions of recombinant bovine (rb) IFNα, a Type I IFN, into the uteri of cyclic cows between 15 and 21 dpo extended luteal lifespan, so that rIFNα mimicked some of the biological effects of IFNτ. In sheep, IFNα was also able to compete with IFNτ for binding to endometrial receptors (Stewart et al., 1989; Hansen et al., 1989) and IFNα elicited similar effects to IFNτ on endometrial protein and PGF2α biosynthesis (Thatcher et al., 1989). Similar biological activities between IFNα and IFNτ suggested that IFNτ had a similar structure to Type I IFNs, which include IFNα, β, δ and ω. The similarity between IFNτ and other Type I IFNs was further substantiated by nucleotide substitution analysis, which determined that the IFNτ gene originated from a single duplication of the IFNω gene around 36 million years ago (Roberts et al., 1997, Figure 3, page 35), when the pecoran ruminants originated, which is why IFNτ is specific to ruminant ungulates, which include cattle, sheep, giraffes, antelopes and goats. The nucleotide sequence differences between cDNA for IFNτ, IFNω and IFNα are outlined in Table 3 (page 35) IFNτ is similar to other IFNs in terms of cDNA sequence (85% similar to IFNω, and 55-65% similar to α), amino acid sequence (70% homology with IFNω, 47% homology with IFNα, Roberts et al., 1991) and length of polypeptide (172 residues, as opposed to 166 in IFNα). The predicted hydrophilicity profiles for IFNτ, IFNω and IFNα suggest that these molecules have approximately the same shapes and dimensions despite minor differences in their architecture (Roberts et al., 1990). The secondary structure of IFNτ possesses a considerable amount of α-helical structure (Radhakrishnnan et al., 1996), as with other IFNs which are α-helical cytokines (Zoom and Wetzel, 1984). Moreover, IFNτ adopts the Type I IFN five-helix bundle topology, as opposed to four-helix structure observed in non-Type I IFNs (Sprang and Bazan, 1993). Therefore, IFNτ has a similar structure to other IFNs, and is particularly similar to Type I IFNs.

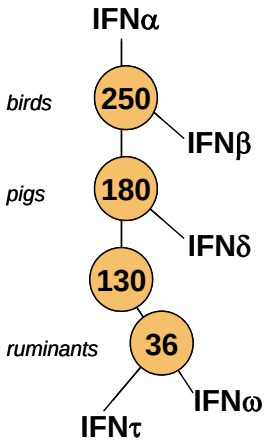


Figure 3 Evolutionary relationship between the Type I IFNs based on nucleotide substitution analysis, as reported by Roberts, et al. (1997). Numbers refer to timing of the origin of the gene (million years ago) which determined the species that different IFNs are specific to. For instance, IFNβ is only found in birds, IFNδ is specific to pigs and IFNτ is specific to ruminant ungulates.

Table 3 Comparison of the percentage homology of the base sequences within the coding regions of cDNA for ovine IFNτ, bovine IFNω and bovine IFNα relative to bovine IFNτ (Imakawa et al.,1987, 1989, Roberts et al., 1991, Yamaguchi et al., 2001).

	5' Non-coding	Coding	3' Non-Coding	Predicted amino-acid sequence identity
Ovine IFNτ	76	90	92	80
Bovine IFNω	72	85	70	70
Bovine IFNα	53	55-65	53	47

Despite the similarity between IFNτ and other IFNs, secretion of IFNτ in cattle in the absence of a known viral stimulus and the magnitude of its synthesis by an epithelium rather than blood cells is different to other IFNs (Roberts et al., 1989). The primary function of IFNτ is related to reproduction (Roberts et al., 1992), whilst other IFNs provide resistance to pathogens (Johnson et al., 1994). Therefore, there is a need to detect IFNτ separate from other IFNs. However, techniques available to detect IFNτ tend to lack specificity due to the similarity in biological and chemical properties between IFNτ and other Type I IFNs (Table 4, page 37). The conventional antiviral activity will not differentiate between IFNτ and other IFNs (Hernandez-Ledezma et al., 1992), whilst the more specific Mx/CAT gene reporter assay will not differentiate between IFNτ and other Type I IFNs (Fray et al., 2001). The ELISA uses a specific antibody for IFNτ (Rooke et al., 2005) whilst the antibody used for the RIA was shown to cross-react with IFNα (Takahashi et al., 2005). Differences in the specificity of different methodologies may explain some of the discrepancies between different studies.

1.2.2.5.2 Isoforms

Due to the high homology between IFNτ isoforms (more than 85% homologous, Imakawa et al., 1989), the only technique available which will differentiate between different isoforms is the 2D-PAGE. However, the 2D-PAGE will not distinguish biological activity. The cell cytopathic assays and the Mx/CAT reporter gene assay measure antiviral activity, whilst the RIA, ELISA, immunocytochemistry and 2D-PAGE measure amount of protein. Different IFNτ isoforms vary in antiviral activity (Alexenko et al., 1997), despite having similar amino acid sequences (Ealy et al., 1998a; 2001; Winkelman et al., 1999), so that the antiviral activity may not correlate to amount of protein. Therefore, there is no one method available that will account for the variation in antiviral activity, sequence, molecular weight and isoelectric point between different isoforms of IFNτ. Therefore, a method which integrated both a protein-specific method and an antiviral-specific method should ideally be used to assess embryonic IFNτ secretion to account for differences in biological activity of different isoforms of IFNτ.

1.2.2.5.3 Sensitivity

Occasionally, the level of secretion of IFNτ by early blastocysts is similar to or even below the detection limit of the currently available techniques for measuring IFNτ protein levels (0.001 to 6 ng mL^{-1},Table 4, page 37), so that it is impossible to distinguish between IFNτ not being present or being below the detection limit of the assay. Therefore, there is a need to develop techniques with improved sensitivity in order to assess IFNτ synthesis during the pre-blastocyst and post-attachment stages of development, when IFNτ secretion is not detectable (detectable levels of IFNτ at different stages of development are discussed in 1.3.2, page 39).

1.2.2.5.4 Accuracy

One of the problems with the cell cytopathic assay is that it is based on 1:3 serial dilutions of the embryo-derived culture medium. Due to this considerable serial dilution, Kubisch et al. (1998) suggested that this method is not accurate, leading to results with large standard deviations. How much of the variation in IFNτ between different blastocysts is accounted for by inaccuracies from the cell cytopathic assay is unclear.

Methods available for detecting IFNτ secretion by embryos are summarised in Table 4 (page 37), and can be categorised into methods that measure either antiviral activity or amount of IFNτ secreted. The ideal method of detection would be specific, rapid, sensitive, quantitative, safe and easy to reproduce in different laboratories. However, because of the nature of IFNτ (multiple isoforms which are similar to other IFNs), no one method has all these qualities,

reducing the confidence in the accuracy and reliability of these techniques. Therefore, there is a need to improve current methods of detection of IFNτ synthesis.

The confidence of techniques assessing IFNτ mRNA levels is higher than that of techniques assessing protein levels, as primers can be designed to be specific to IFNτ, whilst antibodies are more likely to cross-react with other Type I IFNs. Moreover, the primers may be designed to be specific to certain IFNτ isoform mRNAs (Ealy et al., 1998a), although non-specific binding with other isoforms may occur due to the high homology between different isoforms (over 85%). Nevertheless, whether at the protein or at the mRNA level, there is a need to develop fast, specific, simple, accurate, precise and sensitive IFNτ detection systems to assess IFNτ production by the pre-implantation bovine embryo.

Table 4 (next page) Techniques available for detecting IFNτ secretion. √: Yes, X: No, NA: Data not available from the literature, ≈: approximation, assuming a specific activity of 1 x 10^8 mg mL^{-1} (Pontzer et al., 1988).

Assay	Cell cytopathic assay	Mx/CAT reporter gene assay	2D-PAGE	Western blotting	RIA	Antigen-competition ELISA
What it measures	Antiviral Activity	Antiviral Activity	Protein Size and isoelectric point	Protein sequence	Protein sequence	Protein sequence
Specificity	other IFNs	Type I IFN	IFNτ	IFNτ	IFNα and IFNτ	IFNτ
Rapidity	3 days	3 days	2 days	2 days	2 days	4-5hrs
Sensitivity	≈ 0.001 ng mL^{-1} [A]	≈ 0.003 ng mL^{-1} [B] ≈ 0.005 ng mL^{-1} [C]	NA	1 ng	2 ng mL^{-1} [D] 6 ng mL^{-1} [E]	0.005 ng mL^{-1} [F] 0.1 ng mL^{-1} [G]
Inter-assay variation	3.1%[J]	9.5%[I]	NA	NA	8.5%[E]	3.1%[G]
Distinguish Biological Activity	√	√	X	X	X	X
Distinguish between different Isoforms	X	X	√	X	Possible	Possible
Requires maintenance of a permissive cell line	√	√	X	X	X	X
Quantitative/Qualitative	Quantitative	Quantitative	Qualitative	Qualitative	Quantitative	Quantitative
Safety Implications	viral stocks	viral stocks	ethidium bromide	ethidium bromide	radioisotopes	X
Ease to reproduce in different Labs	√	NA	√	√	√	X

[A] Hernandez-Ledezma et al.,1992, [B] Fray et al., 2001, [C]Mann et al., 2002, [D] Vallet et al., 1988, [E] Takahashi et al. 2005, [F] Lo et al., 2002, [G]Rooke et al., 2005, [H] Girard & Fleischaker, 1984, [I]Robinson et al., 2006, [J] Michael et al., 2006a,b.

1.3 Variation in IFNτ production

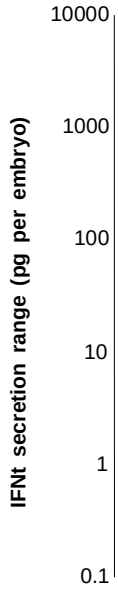
1.3.1 Variation in amount of IFNτ production

There is considerable variation in range of IFNτ detected between bovine studies (Figure 4, page 40), with Hernandez-Ledezma et al. (1992, 1993) and Neira et al. (2007) reporting lower IFNτ amounts secreted (two orders of magnitude) than Kimura et al. (2004a,b) and Kubisch et al. (1998, 2001a, 2007). All four studies used the cell-cytopathic assay to determine IFNτ secretion, and the difference in the magnitude of the amount of IFNτ detected may be due to differences in biological activity of the standards used in different studies. The specific activity of the rIFNτ standard used by Kubisch et al. (1998, 2001a,b, 2004) was 5.4 x 10^7 IU mg^{-1}, and by Kimura et al. (2004a), 7.5 x 10^7 IU mg^{-1}. Hernandez-Ledezma et al. (1992, 1993) used a human IFNα standard (GA-23-902-530) with specific activity ranging between 1 x 10^8 and 1 x 10^9 IU mg^{-1} which accounts for the 100 fold difference in amount of IFNτ detected in Hernandez-Ledezma et al. (1992, 1993) relative to Kubisch et al. (1998, 2001a,b, 2004) and Kimura et al. (2004a,b). Moreover, Neira et al. (2007) used a human IFNα standard with specific activity ranging between about 8.8 x 10^8 and 8.8 x 10^9 IU mg^{-1} (Neira et al., 2007), which accounts for the ten-fold difference in amount of IFNτ detected between Neira et al. (2007) and Hernandez-Ledezma et al. (1992, 1993). Therefore, differences in specific activity of the standards used in different studies account for the variation in IFNτ secretion reported between studies.

Within studies, IFNτ secretion by individual blastocysts was also highly variable, with values ranging ten-fold (Figure 4, page 40). Variation of IFNτ secretion could be caused by environmental conditions and/or by intrinsic factors within the embryo. Although some factors have been identified as modulators of IFNτ secretion, the nature of most of the natural variation in IFNτ secretion is still unknown. Moreover, unknown variables cause ample ranges in standard deviations, which may obscure experimental results and was one of the main obstacles in understanding IFNτ regulation.

1.3.2 Variation in timing of IFNτ production

Generally, IFNτ protein secretion by bovine embryos has been detected as early as day 8 pi (time of blastulation, Larson et al., 1991, Hernandez-Ledezma et al., 1992, de Moraes et al., 1997, Kubisch et al., 1998, 2001a, Chelmonska Soyta et al., 2001), but not before (day 1, 2, 3 and 6 pi Hernandez-Ledezma et al., 1992, days 1 to 7 pi Neira et al., 2007) and as late as day



References	1	2	3	4	5
Species	Bovine	Bovine	Bovine	Ovine	Bovine
Methodology	Cell cytopathic assay	Cell cytopathic assay	Cell cytopathic assay	ELISA	Cell cytopathic assay
rIFN standard	α	τ	τ	n/a	α
standard specific activity (IU)	10^8 to 10^9	10^7	10^7	n/a	10^8 to 10^9

Figure 4 The range (minimum and maximum) of amount of IFNτ (pg embryo^{-1} 24h^{-1}) detected in culture media as reported in different studies observing ruminant blastocysts. 1: Hernandez-Ledezma et al. (1992, 1993), 2: Kubisch et al. (1998, 2001a, 2004), 3: Kimura et al. (2004b), 4: Rooke et al. (2005), 5: Neira et al. (2007).

43 pi (timing of placentation, Stojkovic et al., 1995) but not after (day 69 pi Bartol et al., 1985, Godkin et al., 1988, Stojkovic et al., 1995), with maximal IFNτ secretion occurring between 16 and 19 dpi (time when luteolysis needs to be prevented by maternal recognition of pregnancy). IFNτ transcripts were detected in bovine embryos using RT-PCR from the eight-cell stage (3 dpi, Gutierrez-Adan et al., 2004a, Kato et al., 2007) to the elongation stage (24 dpi), with maximal IFNτ transcript levels occurring on 15 to 16 dpi (Farin et al., 1990), coinciding with the timing of maximal IFNτ secretion. IFNτ mRNA expression has not been reported after 24 dpi (Farin et al., 1990). The similar pattern in IFNτ mRNA expression and

protein secretion suggests that (1) changes in IFNτ mRNA expression explain, at least in part, changes in IFNτ protein secretion, and that (2) IFNτ production is modulated at the mRNA level. Also, the transient nature of IFNτ mRNA expression and protein synthesis during the time when luteolysis must be prevented (14 to 17 dpo) further substantiates the role of IFNτ as the maternal recognition of pregnancy signal.

Using 2D-PAGE, IFNτ secretion was detected in culture medium between 16 and 29 dpi (Bartol et al., 1985, Helmer et al., 1987, Godkin et al., 1988, Lifsey et al., 1989, Plante et al., 1990). Godkin et al. (1988) did not detect IFNτ secretion on days 32, or 38, whilst Morgan et al., 1993 did not detect intracellular IFNτ using immunocytochemistry on days 25, 30 and 37 pi. Meanwhile, Stojkovic et al. (1995) detected IFNτ protein secretion as late as day 43 pi using the more sensitive cell cytopathic assay, suggesting that some of the variation between studies in timing of cessation of IFNτ protein secretion is due to differences in detection limits between different methods of detecting IFNτ protein. Using the same method of detection of IFNτ (2D-PAGE), Bartol et al. (1985) did not detect any IFNτ on day 29 pi, whilst Godkin et al. (1988) did, suggesting that differences in detection limits of different methods of detection of IFNτ secretion explain only in part the variation between studies on the timing of cessation of IFNτ secretion.

Detection of IFNτ by *in vivo* produced and cultured embryos has not been reported prior to 14 dpi (Kazemi et al., 1988, Robinson et al., 2006), whilst IFNτ secretion was detected as early as day 7 and 8 pi in *in-vitro* (Johnson et al., 2006) and *ex-vivo (*de Moraes et al., 1997) produced embryos respectively. The low volume of fluid in the uterine cavity (around 0.2 mL, Casslen, 1986) means that direct aspiration of uterine fluid is difficult. Therefore, the uterus is flushed with about 2 mL of saline to collect uterine flushings. The dilution of the endometrial/conceptus secretions may lead to the concentration of IFNτ protein being below the detection limit of the techniques available for detecting IFNτ secretion. Therefore, differences between studies in the onset of IFNτ secretion may be due to differences in embryo production systems.

Culturing the embryo in smaller volumes *in vitro* (volume ranging from 0.02 and 0.05 mL, compared to 2 mL of saline required for uterine flushings, Hamilton et al., 1998) will lead to more concentrated levels of IFNτ in *in vitro* culture medium than in uterine flushings. Another advantage of detecting IFNτ in culture medium as opposed to uterine flushings is that uterine flushing methods result in uncertainty as to the proportion of the fluid retrieved

representing diluent or actual endometrial/conceptus secretion, which may compromise the determination of total protein secreted. Moreover, Hamilton et al. (1998) reported a high inter-individual variation in the volume of flushings retrieved. Despite the advantages of reduced variation and increased sensitivity of methods detecting IFNτ in culture medium as opposed to uterine flushings, the disadvantage is that the change in environment from *in vivo* to *in vitro* may affect the pattern of IFNτ secretion.

Nevertheless, the improved sensitivity of methods assessing IFNτ protein secretion in *in vitro* culture medium have led to detection of IFNτ protein earlier (as early as 7/8 dpi, Kubisch et al., 1998, 2001a) in embryo development than with methods dependent on uterine flushings (14 dpi, Robinson et al., 2006). It could be that bovine embryos are able to secrete IFNτ protein before 7 dpi, but at levels below the detection limit of the cell cytopathic assay (0.12 IU mL^{-1}, equivalent to about 1 pg mL^{-1}, assuming a specific activity of 1 x 10^8 IU mg^{-1}, Neira et al., 2007 and Hernandez-Ledezma et al., 1992, 1993) or isoforms with low antiviral activity. Using a sensitive ELISA, Rooke et al. (2005) detected IFNτ secretion by ovine IVP embryos as early as 3 dpi. The early detection of IFNτ protein using the ELISA suggests that the onset of IFNτ protein may occur earlier than is possible to detect IFNτ protein, given the detection limits of the current methods for detecting IFNτ protein. Therefore, it remains unclear when in development IFNτ mRNA and protein are first produced by bovine embryos and development of more sensitive techniques for detecting IFNτ mRNA and protein are necessary to determine the timing of the onset of IFNτ secretion by bovine embryos.

The sudden onset and cessation of IFNτ gene expression during embryo development has prompted several studies to identify the sequences responsible for controlling IFNτ expression in order to define the molecular mechanisms by which IFNτ gene transcription is regulated.

1.4 Molecular control of IFNτ gene expression

1.4.1 Molecular control of timing of IFNτ expression

Positive and negative regulatory domains have been identified in the promoter region of the ovine IFNτ gene. Given the homology between ovine and bovine isoforms in the non-coding regions of the gene (as discussed in section 1.2.2.5.1 and demonstrated inTable 3, page 35), the mechanisms of IFNτ gene regulation are expected to be similar in the bovine.

The duplication events that led to IFNτ from IFNω are thought to have disrupted the promoter element of the IFNτ gene so that the enhancer and promoter regions of IFNτ are very distinct, in contrast with the conserved 5'non-coding regions of other IFNs (Ezashi et al., 1998). The IFNτ gene contains a TATA box at base position -27 to -33 (Ezashi et al., 1998, 2001), which is the likely transcription start site (Fujita et al., 1987, Raj et al., 1989). The IFNτ gene also contains a conserved E26 transforming sequence (ETS) DNA binding domain at base position -78 to -70 (Ezashi et al., 1998, 2001), which is characterized by a specific enhancer sequence CAGGGAAGTG and is the binding site for transcription factor ETS2. Overexpression of ETS2 activated transcription of a luciferase reporter under the control of the IFNτ upstream regulatory region in the human choriocarcinoma cell line (JAr), suggests ETS2 is a principal regulator of IFNτ expression. Moreover, mutations of the ETS DNA-binding domain reduced ETS2 induction of IFNτ (Ezashi et al., 2001). Also, ovine IFNτ isoforms that are poorly expressed lack an ETS2 binding sequence in the proximal promoter region (Ezashi et al., 1998), whilst mutation of the promoter region to incorporate a 22 base pair active ETS2 binding site restores the full promoter activity of IFNτ (Matsuda-Minehata et al., 2005). These data collectively suggest that ETS2, through its interaction with the promoter region of the IFNτ gene, is the key regulator of IFNτ expression.

The promoter region of the IFNτ gene also contains two activator protein (AP1) binding sites between base pairs -77 and -69 (partially overlapping the ETS2 binding site, Ezashi et al., 1998, 2004) and -616 and -584 (Yamaguchi et al., 2000) upstream of the transcription start site of the IFNτ gene. AP1 site is the target of protein kinase C (PKC). The ETS2/AP1 site is mutated in certain ovine IFNτ genes that seem not to be expressed (Ezashi et al., 1998), suggesting that activation of the AP1 site is also required for the onset of IFNτ expression.

As well as enhancer regions, the 5' non-coding region of IFNτ also contains silencer regions. By the time the trophoblast is attached and haematopoiesis (the secretion and development of blood cells) begins, IFNτ secretion is undetectable (discussed in section 1.3.2). Therefore, physical association between the trophoblast and the uterine epithelium may play a role in inhibiting expression of the IFNτ gene. The decline of IFNτ production also coincides with initiation of secretion of Transforming Growth Factor (TGF) β, a cytokine regulating multiple post-attachment physiological events such as cell migration, growth and differentiation (Imakawa et al., 1998), suggesting that IFNτ may not be involved in embryo development post-attachment. Possible silencer elements have been identified in the promoter region of the ovine IFNτ gene (Yamaguchi et al., 1999, 2000, Guesdon et al., 1996) that may be involved

in the precise timed cessation of gene expression. The ovine IFNτ contains a nucleotide sequence identical to TGFβ inhibitory element (Yamaguchi et al., 2001) through which TGFβ is thought to negatively regulate various genes (Kerr et al., 1990). Therefore, the timed cessation of IFNτ production is thought to occur at the transcription level, possibly involving the binding of TGFβ onto its binding site on the promoter region of the IFNτ gene.

1.4.2 Molecular control of amount of IFNτ expression

ETS2 is the key regulator of IFNτ expression through its interaction with the ETS binding site on the promoter region of the IFNτ gene (described in section 1.4). Factors affecting the level of IFNτ expression may be dependent on ETS2 binding to its binding site on the promoter of the IFNτ gene. For instance, activation of the IFNτ gene by protein kinase A (PKA) is dependent on the ETS binding site (Ezashi et al., 2008), and colony stimulating factor (CSF)1, a growth factor present in the uterus (Oshima et al., 2003) and shown to increase IFNτ production by ovine embryos (Martal et al., 1997), enhanced upregulation of IFNτ promoter through activation of ETS2 through the mitogen-activated protein kinase (MAPK) signal transduction pathway (Ezashi et al. 2004), suggesting that ETS2 mediates the action of cytokines on IFNτ through the MAPK pathway.

The promoter region of the IFNτ gene also contains two AP1 sites, which are thought to be the target of the PKC protein (described in section 1.4). Granulocyte macrophage colony stimulating factor (GM-CSF), a cytokine shown to modulate IFNτ expression (to be discussed in section 1.7.1.3) is thought to act via the proto-oncogene[1] c-jun[2] and an AP-1 site at -654 to -555 bp (Imakawa et al., 1993, Yamaguchi et al., 1999). Exposure of embryos to a PKC-inhibitor removed the GM-CSF effect on IFNτ mRNA (Yamaguchi et al., 1999). Phorbol 12-myristate 13-acetate (PMA) is a PKC activator. PMA was shown to affect ovine IFNτ transactivation in various cell lines (Yamaguchi et al., 1999). IFNτ transcription and enhancer regions of the IFNτ gene (-616 to -584 base pairs) confer PMA response (Yamaguchi et al., 2001). Exposure of PMA mimicked the enhancing effect of GM-CSF and interleukin (IL) 3 on IFNτ expression and secretion (Imakawa et al. 1995, 1997), suggesting that GM-CSF and IL 3 enhance upregulation of IFNτ promoter through a PKC mediated pathway.

[1] Proto-oncogene is a normal gene that can be altered.

[2] C-jun is a gene which, in combination with c-Fos, forms the AP-1 early response transcription factor. It is activated by the JNK pathway

Due to the close homology between IFNτ and other IFNs, the IFNτ gene does carry two putative viral response elements at base positions -69 to -74 and -88 to -93 (Pontzer et al., 1993). Unlike other IFNs, IFNτ is not induced by viruses (Guesdon et al., 1996), so that these viral response elements are likely to be inactive.

Recently, other transcription factors have been identified as modulators of IFNτ expression. The homeobox distal-less 3 (DLX3) is an established contributor to trophoblast function in mammals (Berghorn et al., 2005) and recognised a specific sequence with a central TAAT core motif (Feledy et al., 1999) located in the IFNτ promoter (-54 to -46, sequence GATAATGAG, Ezashi et al., 2008). Elimination of the DLX3 and ETS2 binding sites (in separate experiments) abolished the enhancing effect of DLX3 on IFNτ expression (Ezashi et al., 2008), suggesting that DLX3 associates with ETS2 to enhance expression of IFNτ. IFNτ is also regulated by a blastocyst specific transcription factor called caudal-related homeobox protein 2 (CDX2, Imakawa et al., 2006), although the location of its interaction on the IFNτ promoter region has not yet been established.

In summary, the IFNτ gene possesses a complex of regulatory sequences. The various binding sites identified on the promoter region of the IFNτ gene suggest that the IFNτ gene may require several sets of transcription factors for full transactivation and that more than a single cytokine and intracellular signalling pathway are involved. Thus, multiple genetic, intrinsic and environmental factors may modulate IFNτ expression.

1.5 Factors affecting onset and cessation of IFNτ production

1.5.1 Genetic factors affecting onset and cessation of IFNτ production

After fertilisation, the mRNA and protein required for early embryogenesis are derived solely from the maternal oocyte and the paternal genotype is not involved. Embryonic genome activation is the event where the embryonic genome becomes transcriptionally active, thus replacing transcription of the maternal genes. Subsequent embryo development is dependent on the timing and the correct activation of embryonic genes. Ruminant embryos undergo embryonic genome activation between the two- and eight-cell stage (Memilli and First, 2000). Therefore, genetic factors affecting IFNτ synthesis may be categorised as maternal, paternal and embryonic genotype.

Hernandez-Ledezma et al. (1992) suggested that the onset of IFNτ production is associated with the differentiation of blastomeres into trophectoderm cells, given that they only detected

IFNτ from the blastocyst stage onwards. Moreover, IFNτ protein (Johnson et al., 2006) and mRNA (Farin et al., 1990) have been localised specifically in the trophectoderm of bovine blastocysts (discussed in section 1.2.2.4). On the other hand, Neira et al. (2007) observed that IFNτ was constitutively synthesized in bovine expanding blastocysts, and Johnson et al., (2004) detected IFNτ in cumulus cell masses of *in vitro* matured oocytes and in morulae (5 to 6 dpi), although it is possible that these were due to non-specific staining. Therefore, the onset of IFNτ production may be associated with the emergence of a functional trophectoderm. However, the detection of IFNτ secretion at the morula and earlier stages (in the ovine, Rooke et al., 2005) suggests that some other event must be responsible for triggering the secretion of IFNτ. Since cell differentiation between the inner cell mass and the trophectoderm occurs after the embryonic genome activation, the embryonic genotype may have role in modulating the onset of IFNτ production.

Parthenotes are unfertilised oocytes that have been activated to initiate cell division. Blastocysts derived by parthenogenesis are capable of secreting IFNτ (Kubisch et al., 2003) suggesting that the onset of IFNτ secretion is largely dependent on maternal factors.

1.5.2 Environmental factors affecting onset and cessation of IFNτ production

In vivo embryos are fertilised and developed within the mother during pregnancy, with no contact with the *in vitro* environment. IVP embryos are fertilised and cultured *in vitro*, with no contact with the *in vivo* oviductal or uterine environment. *Ex-vivo* production of embryos involves fertilisation occurring *in vivo* followed by culture of the embryo in the *in vitro* environment. Since IFNτ is a secretory protein and detection of IFNτ in IVC medium is more sensitive and less variable than in uterine flushings (discussed in section 1.3.2), the *ex-vivo* culture system has been used in studies assessing IFNτ secretion by *in vivo* embryos (Helmer et al., 1987, Godkin et al., 1988, Plante et al., 1990, Stojkovic et al., 1999, Kubisch et al., 2001b, Kimura et al., 2004a, Neira et al., 2007). The *ex-vitro* production system involves fertilisation *in vitro* before transferring the embryo into the *in vivo* environment. IFNτ expression and secretion has been detected by embryos produced and cultured in these different embryo production systems.

IFNτ mRNA was detected using RT-PCR in *in vivo* blastocysts recovered at 7 dpi (Wrenzycki et al., 1998, Bertolini et al., 2002) and has not been reported in earlier stages in *in vivo* produced bovine embryos. In contrast, IVP bovine embryos expressed detectable amounts of IFNτ mRNA as early as 3 dpi (Lonergan et al., 2003, Gutierrez-Adan et al., 2004a). In the

same study, IVP bovine embryos transferred into the ewe oviduct 20 hpi and retrieved daily between 1 and 4 dpi (Gutierrez-Adan et al., 2004) did not produce detectable amounts of IFNτ mRNA before 4 dpi, suggesting that the *in vitro* environment advances the temporal pattern of transcription of the IFNτ gene compared to the *in vivo* environment.

Secretion of IFNτ protein by embryos produced in an *in vivo* production system has not been detected before 14 dpi, whilst embryos produced in an *ex-vivo* production system secreted IFNτ as early as day 8 (Kimura et al., 2004a), earlier than in *in vivo* developed embryos not exposed to the *in vitro* environment. Using IVP embryos, Kubisch et al. (1998, 2001a) and Hernandez-Ledezma et al. (1992) detected IFNτ on day 8 pi, thus IVP embryos secreted IFNτ earlier than embryos exposed to the *in vivo* environment. Rather than the *in vitro* environment advancing the onset of IFNτ secretion, it is more likely that the differences in the timing of first detection of IFNτ between different embryo production systems are due to differences in detection limits from the different methods used by different studies to detect IFNτ.

The similar timing of onset of IFNτ secretion (Figure 5, page 49) by IVP (Hernandez-Ledezma et al., 1992, Neira et al., 2007) and *ex-vivo* produced embryos (de Moraes et al., 1997) suggests that IFNτ secretion occurs at a specific chronological age. However, Hernandez-Ledezma et al. (1992) only detected IFNτ expression and secretion by blastocysts, irrespective of day of blastulation, and did not detect IFNτ expression or secretion by morulae, suggesting that the onset of IFNτ is developmentally regulated, as opposed to chronologically regulated.

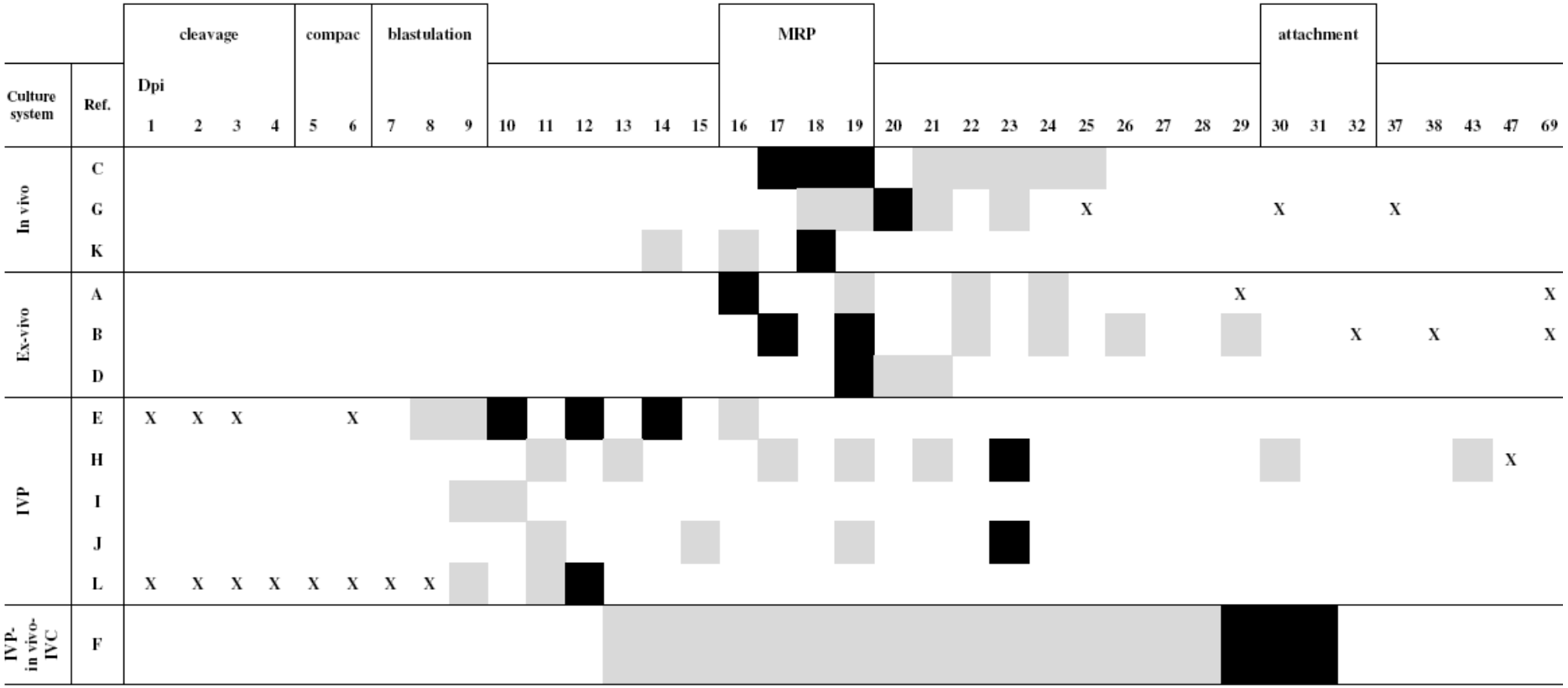
In vivo, Godkin et al. (1988) observed that although one variant of IFNτ was present up to 22 dpi, other isoforms were present beyond 24 dpi and up to 29 dpi, but not thereafter, coinciding with the time of attachment. *In vitro*, Hernandez-Ledezma et al. (1992) found that IVP embryos produced IFNτ from 8 to 16 dpi, after which the embryos did not survive. However, exposure of IVP embryos to the uterine environment *in vivo* between 8 to 12 dpi extended embryo survival to 31 dpi, and the embryos continued to secrete IFNτ until 31 dpi, after IFNτ secretion ceases *in vivo*, suggesting that signalling from the uterine environment is necessary to stop secretion of IFNτ.

Uncertainties remain as to what event triggers and stops IFNτ secretion during embryonic development. The fact that IFNτ was detected in IVP embryos suggests that initial induction

of IFNτ secretion does not require uterine factors, so that the onset of IFNτ expression is likely to be a genetically programmed event. Moreover, the capacity of parthenotes to secrete IFNτ (Kubisch et al., 2003) suggests that the onset of IFNτ is solely dependent on maternal factors. Alternatively, IFNτ was detected in IVP embryos at 43 dpi (Stojkovic et al., 1995), after the timing of cessation *in vivo* (25 dpi, Kazemi et al., 1988), suggesting that cessation of IFNτ is dependent on uterine factors. Therefore, although the onset of IFNτ secretion is likely to be genetically regulated, the cessation of IFNτ is likely to be environmentally regulated.

Figure 5 The effect of embryo origin and production system on timing (dpi: dpi, the expected stage of development) of IFNτ synthesis in bovine embryos. Maximum IFNτ secretion by *in vivo* embryos consistently occurs during the timing of maternal recognition of pregnancy (between 17 and 20 dpi). IFNτ secretion by *in vivo* embryos was not assessed before 14dpi, and was not detected during the period of attachment (30 and 37 dpi). The pattern of secretion by *ex-vivo* embryos followed a similar pattern of secretion than *in vivo* embryos, with maximum secretion between 16 and 19 dpi, reducing to minimal levels by 29 dpi, and not being detected on 32, 38 and 69 dpi. The pattern of secretion by *in vitro* produced (IVP) embryos reported by different studies is considerably more inconsistent than by *in vivo* or *ex vivo* embryos, possibly due to perturbations caused by the *in vitro* environment and/or lack of maternal signals. Exposure of IVP embryos to the in vivo environment between 8 to 16 dpi changed the pattern of secretion to a progressively increasing profile, with IFNτ secretion extending to 31 dpi, suggesting that the *in vivo* environment is necessary to reduce and stop IFNτ secretion after the timing of maternal recognition of pregnancy.

x: not detected, ▒: detected, ■: maximum level detected in the study, □ not assessed. Compac.: compaction, MRP: maternal recognition of pregnancy. Numbers refer to days post insemination (Dpi). References (Ref.): A. Bartol et al., 1985, B. Godkin et al.,1988, C. Kazemi et al., 1988, D. Lifsey et al., 1989, E. Hernandez-Ledezma et al., 1992, F. Hernandez-Ledezma et al., 1992, G. Morgan et al., 1993, H. Stojkovic et al., 1995, I. Kimura et al., 2004b, J. Stojkovic et al., 1999, K. Robinson et al., 2006, L. Neira et al., 2007.


cleavage
compac
blastulation
MRP
attachment
Culture system
Ref.
Dpi
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 37 38 43 47 69
In vivo
C
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Ex-vivo
A
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IVP
E
H
I
J
L
IVP-in vivo-IVC
F

1.6 Factors affecting the amount of IFNτ synthesis

1.6.1 Genetic factors affecting amount of IFNτ production

Ovine blastocysts expressed higher levels of IFNτ transcript than bovine blastocysts (Rizos et al., 2004), suggesting that genetics may play a role in the variation of IFNτ transcript levels.

1.6.1.1 Paternal genotype

Paternal breed differences was shown to significantly influence the ability of bovine oocytes to reach the blastocyst stage (Shamsuddin and Larson,1993), suggesting that the paternal genotype could be a modulator of IFNτ secretion. In fact, paternal genotype significantly influenced IFNτ secretion (Kubisch et al., 2001a), as blastocysts fertilised with Hereford semen secreted almost two-fold more IFNτ than those fertilised with Holstein or Angus semen. This difference was irrespective of differences in penetration given the cleavage rates were not affected (Kubisch et al., 2001a). However, parthenogenic embryos do not contain paternal genome and the similarity in the levels of IFNτ mRNA expressed between *in vitro* fertilised-embryos and parthenotes suggests that IFNτ expression is not affected by the lack of paternal genetic contributions (Kubisch et al., 2003). Embryo sex is a paternal factor. Although no sex bias was observed in IFNτ secretion by Kubisch et al. (2004), other studies have shown that female embryos expressed (Kimura et al., 2003, 2004a,b) and secreted (Larson et al., 2001, Kimura et al., 2004a,b) higher levels of IFNτ (two- to seven-fold) than male embryos. The reasons for discrepancies between studies are unknown, but suggest that at least in certain conditions, sex will modulate IFNτ expression and secretion, along with other paternal factors, such as genotype and breed.

1.6.1.2 Maternal genotype

Using fewer embryos (43 as opposed to 333 blastocysts used in the paternal genotype study) Kubisch et al. (2001a) also evaluated the effect of the maternal genotype on IFNτ production. Using the same batch of sperm from the same sire to artificially inseminate six cows of four different breeds (Santa Gertrudis, Angus, Simmental and Holstein cows), embryos were flushed after 7 dpi and 'maternal factor' was a significant determinant of IFNτ secretion ($P<0.01$). Unfortunately, this study did not report whether these differences were due to different cows or different breeds and the interaction between maternal and paternal factors were not evaluated.

The presence of GM-CSF between days 1 and 3 of *in vitro* culture (one-cell to eight-cell) of ovine IVP embryos stimulated IFNτ secretion at the blastocyst stage two- to three-fold,

suggesting that an event before or at activation of the embryonic genome activation was responsible for stimulating IFNτ secretion at the blastocyst stage (Rooke et al., 2005). Since the maternal genotype is solely responsible for embryo development before the embryonic genome activation, then it follows that that the maternal genotype is responsible for at least part of the variation in the levels of IFNτ secreted in this study.

In summary, maternal and paternal genotype may explain some of the individual variation in IFNτ synthesis between embryos cultured in similar conditions. Although the maternal genotype is likely to modulate the onset of IFNτ production, both the maternal and the paternal genotype may affect the amount of IFNτ synthesized.

1.6.2 Intrinsic factors affecting amount of IFNτ production

Intrinsic factors are defined as characteristics arising from the basic structure of the embryo which may have been modulated by both genetic and/or environmental factors, and include batch of ovaries, stage of development, rate of development, cell proliferation, morphology, size and relative transcript abundance. Intrinsic factors explain individual variation in IFNτ secretion between embryos exposed to similar conditions.

1.6.2.1 Batch of ovaries

Composition of ages, breeds, pregnancy status or stage of oestrous cycles of cows at slaughter vary between batches of ovaries, so that it is possible that variation in the proportion of these factors between batches may influence IFNτ secretion. Therefore, batch of ovaries is a determinant modulated by both genetic (such as maternal genotype) and environmental factors (such as hormones, temperature, cytokines). Batch of ovaries was a significant source of variation in the amount of IFNτ secreted (Kubisch et al., 2001a). Given that overall cleavage and development to the blastocyst stage was not affected by batches of ovaries suggests that the genetic and environmental factors associated with batches of ovaries are modulators of IFNτ synthesis.

1.6.2.2 Stage of development

Secretion of IFNτ protein increased with development from blastocyst to expanded blastocyst to hatching blastocyst (Kubisch et al., 1998, 2001a, Wrenzycki et al., 1999, Hernandez-Ledezma et al., 1992, 1993). IVP hatched blastocysts secreted 10-fold more IFNτ than expanding blastocysts (Hernandez-Ledezma et al., 1992). Similarly, Kimura et al. (2004b) observed a 10-fold increase in IFNτ secretion between 8 and 9 dpi compared to between 9 and 10 dpi, when hatching usually occurs. A linear increase in IFNτ secretion from three to ten ng embryo^{-1} day^{-1} was observed during the time of expansion, hatching and elongation in IVP

embryos (Neira et al., 2007). Stojkovic et al. (1999) also observed a six-fold linear increase in IFNτ secretion by IVP bovine embryos between days 11 and 23, when elongation and attachment occur *in vivo*. IFNτ secreted by IVP embryos exposed to the uterine environment *in vivo* between days 8 and 12 pi increased two fold from day 13 to 15 (when elongation occurs), three fold from day 13 to 27 pi (when elongation and attachment occur) and 10-fold by day 31 pi (when the chorionic membrane forms, Hernandez-Ledezma et al., 1992). Around the time of attachment (day 16 to 18 pi), Robinson et al. (2006) detected between 30 and 100 mg of IFNτ in uterine flushings from pregnant cows. Therefore, stage of development explains at least in part the variation in IFNτ secretion, irrespective of the embryo production system.

1.6.2.3 Speed of development

Speed of development determines the developmental stage reached at a certain time after insemination. Kubisch et al. (1998, 2001a) observed that the day at which blastocyst formation occurs affected the level of IFNτ secretion, with blastocysts forming on 10 dpi secreting six-fold more IFNτ than blastocysts forming on 7 dpi. In agreement, Larson et al. (2001) observed that blastocysts forming on day 10 pi secreted four-fold more IFNτ in the first 24 h after blastocyst formation than blastocysts forming on day 7 pi and two-fold more than blastocysts forming on day 8 pi. Similarly, earlier forming blastocysts consistently expressed less IFNτ than later forming ones (Wrenzycki et al 2003, Gutierrez-Adan et al., 2004a), suggesting that the modulation of IFNτ synthesis by timing of blastulation occurs at the transcriptional level.

Given that earlier developing blastocysts are more likely to give rise to pregnancies (Hasler et al., 1995), one possible explanation is that the higher IFNτ secretion by later forming blastocysts may, as suggested by Kubisch et al. (1998), be due to a breakdown of the genetic control over development caused by a reduction in viability, thus suggesting a negative relationship between IFNτ production and embryo viability.

1.6.2.4 Ability to hatch

Blastocysts which hatched by day 8 pi secreted significantly more IFNτ than non-hatched day 8 pi blastocysts (Stojkovic et al., 1995). Similarly, blastocysts which hatched by day 9 pi secreted significantly more IFNτ than non-hatched day 9 pi blastocysts (Neira et al., 2007) and blastocysts which hatched by day 10 pi secreted significantly more IFNτ than non-hatched day 10 pi blastocysts (Larson et al., 2001). Therefore, hatched and hatching blastocysts consistently produced more IFNτ than those that did not hatch. There are three

possible explanations for these findings. Firstly, hatching ability is an index of developmental competence (Rizos et al., 2004, George et al., 2006), and embryos which did not hatch may have had reduced viability. Secondly, embryos which did not hatch may be viable, but have a slower rate of development. This is unlikely to be the case given that increased rate of development decreases IFNτ expression and secretion levels (discussed in section 1.6.2.3). Thirdly, and most likely, IFNτ secretion is developmentally regulated, so that embryos later in development (hatched) secrete higher levels of IFNτ than embryos earlier in development (non-hatched), as discussed in section 1.6.2.2.

1.6.2.5 Cell proliferation

IFNτ transcript levels were higher in embryos cultured in tissue culture medium (TCM) with serum when compared with bovine serum albumin (BSA) supplemented TCM, and the increase in IFNτ expression was coupled with an increase in cell number (Russell et al., 2006), suggesting a positive relationship between IFNτ expression and cell count. Culturing embryos *in vitro* with polyvinyl alcohol (PVA)-supplemented medium led to fewer cells and more IFNτ secretion per embryo than with BSA supplemented media (Kubisch et al., 2001a), suggesting a negative relationship between IFNτ secretion and cell proliferation. However, these blastocysts also had a delay in development, which probably accounted for the fewer cells. However, other studies assessing IVP (Neira et al., 2007, Kubisch et al., 1998, Larson & Kubisch, 1999) or *ex-vivo* bovine embryos (Stojkovic et al., 1999) observed no significant relationship between IFNτ secretion and cell numbers. Therefore, there was no consistent relationship between IFNτ secretion and cell proliferation. One explanation is that IFNτ is specifically secreted by trophoblast cells (as discussed in section 1.2.2.4), and inner cell mass to trophectoderm ratios vary between blastocysts (Neira et al., 2007). Stojkovic et al. (1999) estimated trophoblast growth by calculating the surface areas of embryos from their diameters. Although this is only a crude estimation of trophoblast cell number since embryos may collapse and re-expand, there was a significant positive correlation between trophoblast area and IFNτ secretion. Lo and Summers (2001) also observed that embryo diameter was positively correlated with IFNτ secretion, suggesting that IFNτ secretion may be positively correlated to embryo size and number of cells secreting IFNτ.

1.6.2.6 Embryo morphology

Morphological parameters are used to grade embryos and include characteristics of the blastomeres (size, shape, colour and compaction), zona pellucida (rigidity and elasticity), perivitelline space (size and presence of cellular debris), presence of extruded cells or cell fragmentation. In human embryology, blastocyst morphology was positively associated with

attachment and pregnancy rate, with blastocysts with good morphological grade yielding higher attachment rates than blastocysts with poor morphological grade (Balaban et al., 2000).

Comparison of IFNτ secretion with embryo morphological grade has not been assessed in bovine embryos *in vivo*. However, in sheep, IFNτ activity was higher in uterine flushings (14 dpi) from embryos with good morphological grade compared to poor morphological grade (assessed on day 14pi), although low number of replicates meant that this difference was not significant (Abecia et al., 2001). Therefore, IFNτ secretion *in vivo* by ovine embryos may be positively correlated to morphological grade. Moreover, the same study reported that when the flushed embryos were cultured *in vitro*, embryos with good morphological grade secreted more (P=0.07) IFNτ than poor embryos, which could be due to higher numbers of degenerating cells in poor embryos. In agreement, Neira et al. (2007) reported that *ex-vivo* bovine embryos with good morphological grade secreted higher levels of IFNτ than poor embryos, suggesting that IFNτ secretion *in vitro* by *in vivo* derived bovine embryos was positively correlated to morphological grade.

Using IVP bovine embryos, Russell et al. (2006) observed that embryos cultured in TCM with serum showed greater expansion of the blastocoele and expressed higher levels of IFNτ mRNA than embryos cultured in other media (SOF, or BSA, supplemented TCM) which, in turn, were noticeably darker and had reduced expansion of blastocoelic cavity, suggesting that IFNτ secretion may be associated with morphological grade at the transcriptional level. Using IVP embryos, Hernandez-Ledezma et al. (1993) also reported that hatched blastocysts with good morphological grade secreted more IFNτ than hatched blastocysts with poor morphological grade, further suggesting that IFNτ secretion by *in vitro* derived bovine embryos was positively correlated to morphological grade. .

On the other hand, Kubisch et al. (1998) reported that IFNτ secretion was not affected by morphological grade and Larson et al. (2001) observed that female embryos, which showed poorer morphological grade compared to males, secreted more (two-fold) IFNτ than males. Also, ovine blastocysts that looked morphologically similar had different capabilities to secrete IFNτ and some viable day 10 embryos produced *in vitro* secreted low amounts of IFNτ (Lo and Summers, 2002), suggesting that other factors may modulate IFNτ secretion in sheep and cattle other than blastocyst morphological grade.

Different studies investigating the correlation between morphological grade and IFNτ secretion by blastocysts have used different criteria to categorise blastocysts. Hernandez-Ledezma et al. (1993) and Neira et al. (2007) used a criteria (established by Lindner and Wright, 1983) to grade embryos into 4 categories (Poor, Fair, Good, Excellent) based on embryo shape, size, cellular integrity and appearance of the cytoplasm and nucleus, whilst Kubisch et al. (1998, 2001a) graded embryos into 3 categories solely on embryo shape, cellular uniformity and integrity, extrusions and collapse, and Lo and Summers (2002) assessed viability based on survival in extended culture *in vitro* and morphology based on the presence of the inner cell mass, extent of blastocyst expansion and embryo size. Therefore, discrepancies between studies investigating the correlation between morphological grade and IFNτ secretion by blastocysts may be due to different studies using different criteria and different number of categories to segregate embryos according to morphology.

Morphological grade assessment evaluates an embryo according to its appearance at a specific time, without any account of the change in appearance during culture. Neira et al. (2007) used only blastocysts on day 7 pi with excellent morphological grade to assess IFNτ secretion for 24 hour individual culture, and then assessed the morphology of the blastocyst after individual culture, and observed a positive correlation between IFNτ secretion and embryo morphological grade. Meanwhile, Kubisch et al. (1998) used embryos with a range of morphological grades before individual culture for assessment of IFNτ secretion, and observed no relationship between IFNτ and morphological grade. Also, Hernandez-Ledezma et al. (1993) assessed the relationship between IFNτ and embryo morphology within each stage of blastocyst development, whilst Lo and Summers (2002) took no account of stage of development. Therefore, differences in homogeneity of the population of embryos may affect the relationship between IFNτ and morphological grade.

Therefore, the relationship between IFNτ secretion and embryo morphology was inconsistent due to variation between studies in criteria used to evaluate embryos according to morphology, homogeneity of the population of embryos assessed, and due to the subjective nature of morphological grading, so that it remains inconclusive whether IFNτ secretion is associated with embryo morphological grade assessed as morphology.

1.6.2.7 Viability

Hernandez-Ledezma et al. (1992) observed a reduction in IFNτ secretion from an IVP bovine embryo from 15 to 16 dpi, when the embryo died, suggesting that the reduction in IFNτ secretion may have been due to a breakdown in the molecular control of gene expression and

the ability of the embryo to synthesize IFNτ as the embryo died. Although these observations are on a single embryo, they suggest that embryo viability may be an intrinsic factor affecting amount of IFNτ produced, with embryo death being a cause for cessation of IFNτ production.

1.6.3 Environmental factors affecting amount of IFNτ production

Extrinsic factors are environmental determinants that are not transmitted genetically, and include the embryo production system, medium composition, embryo density, ambient temperature, oxygen, cytokine and hormone levels and embryo manipulation. Environmental factors observed to affect IFNτ production may be categorised as those which promote embryo development (discussed in section 1.7), and those which are detrimental to embryo development (discussed in section 1.8).

1.7 Beneficial environmental factors

The embryo has certain environmental requirements that must be met for it to develop. These include nutritional needs (pyruvate, glucose, amino-acids, albumin) necessary to maintain the embryos metabolism, temperature (around 38.5°C), osmolarity (around 290 mOsmo) and pH (around 7.2). Other factors are not essential for embryonic development, but have been shown to improve embryo viability (measured in terms of blastocyst rate, pregnancy rate, live birth rate). Beneficial environmental factors shown to affect IFNτ secretion include hormones and cytokines.

Embryos flushed from cows in which the luteal phase had been advanced by administration of progesterone between days 1 and 4 in the cycle produced more IFNτ than controls (Garrett et al., 1988, Mann et al., 2006). Similarly, ewes with a history of an early luteal phase gave rise to embryos that secreted more IFNτ than ewes with delayed luteal phases (Nephew et al., 1991). Therefore, IFNτ secretion is modulated by the timing of the maternal oestrous cycle, suggesting that IFNτ may be modulated by hormones. *In vivo*, follicle stimulating hormone was shown to affect translocation of IFNτ into the nucleus, suggesting a role for hormones in localisation of IFNτ *in vivo* (Johnson et al., 2004).

Bovine embryos cultured in groups secreted more IFNτ per embryo than embryos cultured individually (Larson and Kubsich, 1999), suggesting that compounds secreted by other embryos may modulate IFNτ secretion. Embryos secrete cytokines which act as intercellular communication signals essential for controlling immunological adaptation and tissue remodelling processes necessary for successful development. Therefore, cytokines may be among the factors responsible for enhanced IFNτ secretion in group culture compared to

individual culture. Culturing bovine embryos in the presence of supernatants of leukocytes activated with a pathogen caused an increase in IFNτ production (Chelmonska-Soyta et al., 2001), suggesting that cytokines secreted by leukocytes are able to modulate IFNτ secretion. *In vivo* studies on oviductal and uterine tissue from pregnant cows have demonstrated expression and translation of cytokines in these tissues, confirming that as the pre-attachment embryo moves through the female reproductive tract, it is exposed to a range of cytokines (Table 5, page 58). mRNA expression of interleukins (IL 1α; Leung et al., 2000), growth factors (leukaemia inhibitory factor; Oshima et al., 2003; insulin-like growth factor (IGF 1), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), TGF, Watson et al., 1992) and CSFs (GM-CSF, macrophage-CSF (M-CSF), CSF1) have been detected in the oviduct and the uterine endometrium of pregnant cows. Given that the timing and amount of IFNτ expression and secretion differ in *in vitro* relative to *in vivo* (Gutierrez-Adan et al., 2004a, Neira et al., 2007) this implies that cytokines may be involved in IFNτ production (as discussed in section 1.7.1, page 59).

Table 5 Cytokines implicated or detected in establishment and maintenance of pregnancy in cattle and their suggested roles. IL: interleukin; GM: granulocyte macrophage; CSF: colony stimulating factor; LIF: Leukaemia Inhibitory Factor; IGF: insulin growth factor; PDGF: platelet derived growth factor; TGF: transforming growth factor; FGF: fibroblast growth factor; q: quantitative; RT-PCR: reverse transcription polymerase chain reaction; ELISA: enzyme linked immunosorbent assay, pi: post-insemination, po: post-oestrus. References (Ref): 1. Roberts et al., 1992, 2. Watson et al., 1992, 3. Mathialagan et al., 1992, 4. Davidson, 1994, 5. de Moraes et al., 1999, 6. Leung et al., 2000, 7. Oshima et al., 2003, 8. Lee et al., 2003, 9. Michael et al., 2006a.

Cytokine	Tissue Source	Timing of Secretion	Detection Method	Implications in Pregnancy	Ref
IFNτ	Embryo (Trophoblast)	~Day 7-25 pi, Peak during luteolysis (days 16-19 pi)	Cell cytopathic assay	Maternal recognition of pregnancy	1
IL 6	Embryo	At time of embryo elongation/attachment	RT-PCR	Unknown	3
IL 1αα	Endometrium	Day 11-17 po in cyclic and pregnant cows Not detected in pregnant cows at days 25-30 pi	ELISA	Stimulate prostaglandin synthesis, prepare endometrium for attachment	4
IL 1	Endometrium	Day 16 po	qRT-PCR		6
GM-CSF	Endometrium (Uterus) Embryo	Days 14- 17 pi in pregnant cows	Immuno-histochemistry	Embryogenesis	5
M-CSF	Endometrium	Day 16-17, 20-21, 30-36, 48-49, 74-140. Higher during days 74-140.	qRT-PCR	Regulate invasion during attachment	7
CSF 1	Endometrium (Uterus) Embryo (Trophoblast)	Day 14-70 pi	*In situ* hybridisation	Embryogenesis, Placenta formation.	8
LIF	Endometrium	Day 16-140 pi	qRT-PCR	Attachment	7
IGF 1, FGF, PDGF, TGF	Endometrium (Oviduct) Embryo	Day 1-10 pi	qRT-PCR and southern blotting		2
FGF 2	Endometrium (Uterus)	Day 17-18 po	ELISA	Embryogenesis	9

Supplementation of bovine embryo culture medium with leukaemia inhibitory factor (LIF) improved development to the expanded blastocyst stage (Fukui et al., 1994), increased numbers of cells per blastocyst (Funstun et al., 1997) and improved survival rates after thawing of cryopreserved bovine embryos (Han et al., 1995). FGF2 was shown to increase blastocyst yield (Lim and Hansel, 2000). Using cell lines, FGF2 has also been shown to enhance synthesis and secretion of IFNτ by a trophoblast cell line (Michael et al., 2006 a) whilst PGF2α synthesis in endometrial cells was stimulated by IL 1 (Davidson et al., 1995). Of these cytokines, only GM-CSF was shown to modulate IFNτ secretion by bovine embryos cultured *in vitro* (Emond et al., 2004), whilst IL 3 (Imakawa et al., 1995, 1997), IGF-I and IGF II (Ko et al. 1991) and GM-CSF (Imakawa et al. 1995) significantly increased IFNτ secretion by ovine embryos cultured *in vitro*. Therefore, cytokines present in the oviductal fluid may be vital for the processes of oocyte maturation, fertilisation and early embryonic development before the embryo reaches the uterus.

1.7.1 GM-CSF

GM-CSF is a major CSF[3] which belongs to the 'short chain 4-helical bundle family' of cytokines, consisting of a monomeric protein with a compact globular shape and secondary structures containing both α-helix and β-sheet structures (Wingfield et al., 1988). GM-CSF stimulates stem cells to produce granulocytes and macrophages, from which its name was derived (Burgess and Metcalf, 1980). In mammals, GM-CSF is produced by immune cells (dendritic cells, T cells and macrophages), endothelial cells and fibroblasts (Haig, 1992) and modulates proliferation, differentiation and survival of *in vitro* cell cultures of various lineages (such as granulocytes and macrophages). GM-CSF is also implicated in enhancing immune functions of mature cells (Metcalf, 1984) by upregulating Class II major histocompatibility antigens (Fischer et al., 1988), increasing the bactericidal activity of macrophages (Morissey et al., 1989) and activating neutrophils and macrophages to enhance phagocytosis (Clark and Kamen, 1987). Moreover, GM-CSF was shown to induce production of other cytokines, such as IL 1 and tumour necrosis factor (TNF)α by cells such as monocytes and macrophages (Ihle, 1990).

GM-CSF exerts its autocrine and paracrine effects on target cells by binding to high affinity heterodimeric receptor complexes with α and β subunits (Miyajima et al., 1992) which are members of the cytokine receptor superfamily, so that only cells expressing GM-CSF

[3] Colony stimulating factors are a family of glycoprotein cytokines which were named after their stimulatory effect on cultures of hemopoietic stem cells (Metcalf, 1984).

receptors are able to exhibit biological responsiveness to GM-CSF. The α subunit is unique to GM-CSF, whereas the β subunit is shared by other cytokines (IL 3 and IL 5 receptors, Groot et al., 1998). The α subunit is able to bind to GM-CSF on its own with low affinity. Although the β subunit cannot bind ligand by itself, when in a receptor complex with the α subunit, the interaction between the ligand and its receptor becomes high affinity (Hayashida et al. 1990). Binding of GM-CSF to its receptor leads to the activation of signal transduction pathways which eventually lead to changes in the gene expression of the target cell. Most research on GM-CSF signalling has focused on the regulation of proliferation, differentiation and cellular survival by GM-CSF through the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT), MAPK and phosphatidyl-inositol (PI3K) pathways (reviewed in Groot et al., 1998) rather than on its roles in improving immune function. More work is therefore required to understand the cytoplasmic signalling route used by GM-CSF to exert its immune effects on the target cell.

Although not assessed in bovine embryos, human and murine embryos express GM-CSF receptor (GM-CSFR) (human: Loke et al., 1992, Jokhi et al., 1994a, Sjoblom et al., 2002, murine: Robertson et al., 2001), and in murine embryos, GMCSFR mRNA was detected from fertilisation, and GMCSFR protein from the two cell stage (Robertson et al., 2001), suggesting that the embryo is able to respond physiologically to the presence of GM-CSF through binding to its receptor from the two-cell stage. This was demonstrated in *in vitro* studies where supplementation of culture medium with GM-CSF was shown to promote IFNτ secretion and embryo development.

Although not assessed in the bovine ovary, GM-CSF mRNA expression and protein synthesis in the human ovary (Table 6, page 61) has been detected mainly in small and large luteal cells and the theca interna of large follicles, which are associated with the later stages of follicular development (Zhao et al., 1995). Therefore, GM-CSF levels close to the ovary would be expected to be higher during the late tertiary or preovulatory stages of folliculogenesis, suggesting that the amount of GM-CSF in the ovary change as the oestrous cycle progresses. The spatial and temporal localisation of GM-CSF in the reproductive tract coupled with variation in expression between different tissues of the reproductive tract suggests that, as the embryo moves through the reproductive tract it is exposed to different amounts of GM-CSF at different stages of development. Therefore, timing and amount of exposure of GM-CSF may impact pre-attachment embryo development.

Table 6 Localisation of GM-CSF mRNA and protein in human ovarian tissue measured by RT-PCR, *in situ* hybridisation and immunohistochemistry as described in Zhao et al. (1995). - : not assessed, 0 : not detected, 1 : low levels, 2: high levels.

		GM-CSF	
		mRNA	Protein
Oocyte		0	1
Theca interna	large follicles	2	2
	small follicles	0	1
Theca externa		0	1
Follicular cells	large follicles	0	1
	small follicles	0	1
	primary follicles	0	-
Granulosa Cells	small follicles	0	1
	large follicles	0	-
Luteal cells		2	2
Stroma, luteal cell fibroplast, smooth muscle, arteriole endothelial		0	0
Atretic follicles and follicular cysts		0	0

1.7.1.1 Effect of pregnancy on production of GM-CSF by the female reproductive tract

In vivo, immunoreactive GM-CSF was detected by western blotting in a greater proportion of samples of uterine flushings from pregnant cows (75%, n=4) compared to cyclic cows (50%, n=18, de Moraes et al., 1999), suggesting that GM-CSF synthesis was stimulated by the presence of the embryo.

In mice, Robertson et al. (1992) observed that GM-CSF was at least 20-fold higher in uterine luminal fluids from mated compared to non-mated females, suggesting that GM-CSF was produced in the female reproductive tract in response to mating and/or cervical stimulation. GM-CSF activity was not detected in secretions from male accessory glands (seminal vesicle, epididymis, prostate and coagulating glands) suggesting that stimulation of uterine GM-CSF after mating was not due to GM-CSF derived from the ejaculate. GM-CSF activity was detected in uterine fluid of mice mated with vasectomised or syngeneic males, indicating that the stimulus for GM-CSF production was not associated with sperm or an allogeneic major histocompatibility complex haplotype, although other factors present in seminal plasma may have been involved (Robertson and Seamark, 1990). Tremellen et al. (1998) detected TGFβ1 in murine seminal vesicle secretions and found that recombinant TGFβ stimulated GM-CSF release in cultures of uterine epithelial cells from oestrous mice and caused an increase in

GM-CSF in uterine fluid when instilled into the uterine lumen, implicating TGFβ as a key factor in GM-CSF stimulation by mating. GM-CSF receptors have been detected in bovine and ovine spermatozoa (Rodriguez-Gil et al., 2007), suggesting that sperm are able to respond to the GM-CSF secreted by the uterine endometrial cells. The stimulation of GM-CSF by seminal factors coupled with the presence of GM-CSFR on sperm implicates GM-CSF in fertilisation.

GM-CSF secretion has been observed in *in vitro* cultures of GM-CSF secreting cells exposed to cytokines which tend to occur at higher levels during pregnancy (pregnancy-related cytokines). GM-CSF secretion by rat ovary cell cultures was increased in a dose-dependent manner by stimulation with IL 1 and IL 2 (Brannstrom et al., 1994). IL 1 also increased the level of GM-CSF protein in pre-ovulatory rat ovaries (Brannstrom et al., 1994). IFNγ had no effect on IL 2 induced GM-CSF, but did antagonize the action of IL 1 by the human first trimester trophoblast cells (Jokhi et al., 1994b). Therefore, some pregnancy-related cytokines are able to modulate GM-CSF secretion, suggesting a link between pregnancy and GM-CSF.

1.7.1.2 Localisation of GM-CSF

In cows, GM-CSF protein was detected (by western blotting) in uterine flushings from pregnant cows and was immunolocalised to the uterus and the oviduct, being most abundant in epithelial cells (de Moraes et al. 1999). Therefore, as the embryo travels through the female reproductive tract, it is exposed to GM-CSF. GM-CSF protein was detected between days 18 and 24 of pregnancy in regions of the trophectoderm where attachment was not advanced (Emond et al., 2004). Therefore, the bovine embryo is also capable of secreting GM-CSF.

In other mammals, GM-CSF protein was detected in uterine intraluminal fluids from pregnant mice by a GM-CSF-dependent cell-line assay (Robertson et al., 1992). Although GM-CSF localisation in ovarian tissue has not been assessed in ruminants, Zhao et al. (1995) demonstrated that GM-CSF mRNA and protein were present in human ovaries. As with cows, the epithelial cells were the major site of GM-CSF protein in the human oviduct (Zhao and Chegini 1994) and in the uterus of humans (Giacomini et al. 1995), mice (Robertson et al., 1996) and sheep (Imakawa et al., 1993). In the human embryo, GM-CSF protein (Jokhi et al., 1994b) and mRNA (King et al., 1995) were localised in villous and extravillous trophoblast. Therefore, the pattern of localisation of GM-CSF in the embryo and reproductive tract was similar in cows and other mammals.

1.7.1.3 Beneficial effects of GM-CSF on embryo development

The evidence of the beneficial effects of GM-CSF on embryo development is more abundant in human and murine *in vitro* studies than in bovine studies. Exposure of human embryos at the two-cell stage to GM-CSF inhibited apoptosis (Sjoblom et al., 2002), whilst in murine embryos, GM-CSF facilitated glucose uptake (Robertson et al., 2001) and enhanced glucose metabolism (Ko et al., 2002). GM-CSF also improved blastomere proliferation and viability in human (Loke et al., 1992) and murine embryos (Robertson et al., 2001), particularly in the inner cell mass (in murine embryos: Sjoblom et al., 2002). As with the bovine (de Moraes et al., 1997), human (Sjoblom et al., 1999) two-cell embryos exposed to GM-CSF were more likely to reach the blastocyst stage and murine eight-cell embryos exposed to GM-CSF were more likely to hatch and implant than controls (Robertson et al., 2001). Exposure to GM-CSF also led to earlier blastulation and improved ability of the blastocyst to hatch from the zona pellucida (in human: Sjoblom et al., 1999). GM-CSF stimulated DNA synthesis and differentiation by the trophoblast (Drake and Head, 1994). Treatment with GM-CSF increased foetal survival for female mice through a T-cell dependent mechanism that may have involved inhibition of natural killer (NK) cell activity (Clark et al., 1994), suggesting a role for GM-CSF in resistance to maternal immune rejection. Exposing pre-blastocyst embryos to GM-CSF instigated beneficial effects not only in the pre-attachment period of development, but also later in gestation (increased attachment success, improved morphogenesis of the placental labyrinthine tissue and superior growth and development of the foetus), in the postnatal period (increased growth and adiposity in adult progeny), and in subsequent generations (increased foetal-to-placental weight ratio) (Robertson et al., 2001). Moreover, GM-CSF knockout mice showed reduced proliferation and rate of blastocyst formation, decreased foetal size, decreased placental labyrinthae, increased foetal loss and greater mortality during postnatal life (Robertson et al., 1999, 2001). These studies collectively suggested that GM-CSF is beneficial to embryo development in mammals.

Role of GM-CSF in blastulation

In the bovine, GM-CSF increased the proportion of cultured IVP two-cell embryos that developed to the blastocyst stage between days 5 and 7 after fertilisation (de Moraes and Hansen, 1997), when the embryo resides in the uterine lumen and the endometrium is under the influence of progesterone (de Moraes et al., 1999), suggesting that the beneficial effects of GM-CSF on embryo development observed in other species also occur with bovine embryos. Similarly, in other mammalian embryos, rGM-CSF exposure was reported to cause a two-fold increase in blastocyst development *in vitro* from human zygotes (Sjoblom et al., 1999) and porcine parthenotes (Cui et al., 2004). Therefore, rGM-CSF exposure has been consistently

associated with an increase in blastocyst yield, suggesting that although GM-CSF is not essential for blastulation, GM-CSF must modulate one or more of the processes involved in blastulation.

Processes involved in blastulation include cell proliferation, cell differentiation into trophectoderm and inner cell mass, and cavitation (reviewed by Watson et al., 1992). Each of these mechanisms is regulated by other systems. For instance, net cell proliferation is affected by apoptosis and rate of metabolism. GM-CSF null mutant mice have fewer cells than normal mice (Robertson et al. 2001) and in human embryos, the increase in the size of the inner cell mass compartment is associated with a 50% reduction in apoptotic nuclei (Sjoblom et al., 2002), suggesting that GM-CSF may be a modulator of cell proliferation and apoptosis. Also, increased blastulation due to exposure of murine embryos to rmGM-CSF, was associated with an increase in glucose metabolism (Robertson et al., 1999), reduced DNA degradation (Robertson et al., 2001) and enhanced expression of the anti-apoptotic factor B-cell lymphoma (Bcl)-2 (Behr et al., 2005), suggesting that GM-CSF may increase blastocyst yield by increasing metabolism and reducing apoptosis. However, the effects of rGM-CSF on apoptosis and metabolism in bovine blastocysts have not been reported.

The effect of rGM-CSF on glucose metabolism, was observed in the murine system. Robertson et al. (2001) associated increased cell number, rate of development and viability of murine embryos exposed to rmGM-CSF with an enhanced metabolic activity measured in terms of increase in glucose uptake. However, rmGM-CSF exposure does not affect the expression of three major facilitated glucose transporter isoforms involved in glucose uptake by murine embryos (Ko et al., 2002), so that the mechanism by which GM-CSF increases glucose metabolism is not yet understood.

1.7.1.4 The relationship between GM-CSF and IFNτ secretion

IVP bovine blastocysts transferred *in vivo* on day 8 pi and recovered on day 12 pi produced approximately 500 times more IFNτ than IVP embryos that were not exposed to the *in vivo* environment (Hernandez-Ledezma et al., 1992), suggesting that *in vivo* IFNτ secretion is supported by endometrial factors. *In vitro* studies have demonstrated that GM-CSF had a positive effect on IFNτ secretion (in ovine embryos: Imakawa et al., 1993, 1997, Rooke et al., 2005, in bovine trophectoderm cell-lines: Michael et al., 2006b), suggesting that GM-CSF may be among the endometrial factors supporting IFNτ secretion *in vivo*. GM-CSF stimulated IFNτ secretion by a bovine trophectoderm cell line but did not affect the relative

abundance of IFNτ mRNA (Michael et al., 2006 b), suggesting that GM-CSF alters a translational or post-translational mechanism in order to increase IFNτ secretion.

The involvement of GM-CSF in the mechanism of maternal recognition of pregnancy in cattle is summarised in Figure 6 (page 66). IFNτ acts through the IFNτ receptor located on the luminal epithelium of the uterus to stimulate expression of the enzyme cyclooxygenase (COX)2 (Asselin et al., 1997b). COX2 subsequently stimulates PGF2α synthesis in the uterine stroma (Emond et al., 2000) and luminal epithelium (Asselin et al., 1997a) which in turn stimulates GM-CSF expression by endometrial lymphocytes (Emond et al., 1998) and luminal epithelial cells (Emond et al., 2000). In sheep, GM-CSF secreted from the uterus acts through the GM-CSFR on the embryo and increases expression of IFNτ through the PKC signalling pathway (Yamaguchi et al., 2001), so that an amplification loop becomes established between IFNτ and GM-CSF (Emond et al., 2000), both upregulating each other. In rats, GM-CSF in the luminal epithelium is further stimulated by oestrogen and progesterone secreted by the ovaries (Tamura et al., 1999), whilst in the bovine, epithelial PGF2α acts on the ovary to maintain the corpus luteum, to avoid immune rejection (Emond et al., 1998, 2000). The amplification loop between IFNτ and GM-CSF, and between GM-CSF and ovarian steroids explains why GM-CSF concentration in the uterine endometrium increases during pregnancy (de Moraes et al., 1999). GM-CSF derived from the embryo (Emond et al., 2004) may also have a role in this amplification loop as it binds to maternal GM-CSFR, and the GM-CSFR from the embryo itself. The localisation of GM-CSF in both maternal and embryonic tissues, the increase in IFNτ secretion by the embryo due to GM-CSF exposure (as reviewed in section 2.1.3) and the increase in GM-CSF concentration during maternal recognition of pregnancy further suggests that GM-CSF participates in the communication between the female reproductive tract and the embryo.

1.7.2 Conclusion

In mammals, the site-specific localisation of GM-CSF in the reproductive tract, the localisation of GM-CSF receptors in the embryo, the modulation of GM-CSF by mating and pregnancy, and the benefits of GM-CSF to embryonic development are indicative of the relevance of GM-CSF to early embryonic development. In cattle, GM-CSF was localised to the reproductive tract and the embryonic trophectoderm, and was shown to be beneficial to embryo development, suggesting a similar beneficial function for GM-CSF in bovine embryology as described in other mammals. Moreover, GM-CSF seems to be a factor affecting IFNτ secretion, suggesting a role for GM-CSF in maternal recognition of pregnancy in ruminants.

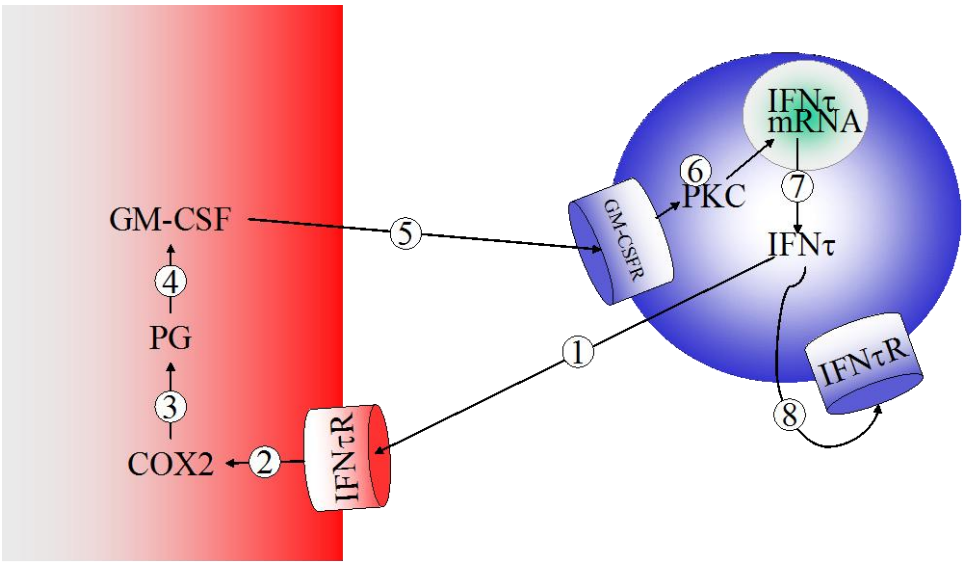


Figure 6 The role of GM-CSF in the communication between the embryo and the uterus in cattle. ① The embryo secretes IFNτ which acts on the maternal endometrium coupling to the IFNτR (Li and Roberts, 1994). ② IFNτ exposure causes an increase in COX2 synthesis by epithelial cells (Asselin et al., 1997), ③ and a subsequent increase in prostaglandin F2α (PG) synthesis by epithelial cells (Emond et al., 1998, 2000). ④ PG synthesis causes an increase in GM-CSF by endometrial lymphocytes (Emond et al., 1998). ⑤ GM-CSF acts on the embryo through its receptor (ovine : Yamaguchi et al., 2001), ⑥ increasing IFNτ transcript levels through the PKC pathway. ⑦ Increase in expression leads to an increase in synthesis of IFNτ. ⑧ IFNτ is also able to bind to IFNτR on the embryo itself (ovine : Imakawa et al., 2002).

1.8 Detrimental environmental factors

Stress is a condition in which an organism is subjected to a stressor. The stressor may cause an unfavourable deviation from the normal function of the organism which may be detrimental to its survival. In terms of embryology, stress conditions have been categorised (Kiang and Tsokos, 1998) as physiological (such as cycle of cell division, growth factors, cell differentiation, tissue development, hormonal stimulation), pathological (such as viral, bacterial or parasitic infection) and environmental (such as exposure to heat shock, heavy metals, metabolic inhibitors, amino acid analogues, ethanol, antibiotics, radiation, osmotic stress, acidic and alkaline environments). Stressful conditions may cause damage to macromolecules, such as protein denaturation and misfolding, which may lead to embryo death. To ensure survival, embryos exhibit a degree of plasticity and are able to adapt to stress, and therefore, embryos must have regulatory systems in place to deal with stressors.

Stress response is a defence reaction of cells to stress in order to restore normal function. As outlined in Figure 7 (page 69), the cell may respond to damage that stressful conditions cause to macromolecules without regard to the type of stress that causes the damage (cellular stress response), or the cell may direct its response at re-establishing homeostasis (homeostatic stress response). Homeostatic stress response is stressor specific and is often activated in parallel to cellular stress response (Kultz et al, 2005). Examples of homeostatic stress response in embryos include control of intracellular pH, calcium and amino acid concentrations. In an acidic environment, the Na^+/H^+ transport mechanism transports protons out of and Na^+ into cells until intracellular pH is restored to above 7.1, whilst in an alkaline environment, the HCO_3^-/Cl^- transport mechanism exchanges bicarbonate ions out of and Cl^- into cells until intracellular pH is restored to below 7.2 (Lane, 1999). Membrane calcium channels allow the embryo to maintain calcium concentrations at around 100 nM in an oviductal environment containing 1 to 2mM calcium (Borland et al., 1977). The embryo is able to transport non-essential amino-acids in and out of cells to balance osmotic stress and stabilise protein function (Yancey et al., 1982).

Once the stress level becomes higher than the embryo can cope with through homeostasis, embryo survival then relies on the cellular stress response. Cellular stress response involves an epigenetic repair system to repair damage caused by stress and involves a sudden increase in expression of "Heat Stress Proteins" (HSPs, also known as "Heat Shock Proteins") and a change in gene expression upon exposure to stress (Kiang and Tsokos, 1998, Figure 7, page69). HSPs are also known as stress-induced proteins, as they are stimulated by stressors other than heat (Tomasini et al., 2002). HSP70 are a family of 70 kDa proteins which are the most frequently studied and best understood effectors of the cellular stress response. The role of HSP70 is to protect cells against the adverse effects of stress (Kawarsky and King, 2001). HSP70 function as molecular chaperones by interacting with other proteins without being part of the final protein structure (Becker and Craig, 1994). In the absence of stress, heat shock cognate protein (HSC) is involved in the maintenance of proteins in proper configuration to ensure correct biological activity (Morimoto et al., 1997), thus taking part in a wide range of physiological processes required for normal growth and proliferation and whose activities are affected by other cytokines, such as TNF (Mehlen et al., 1995). HSP70 protect proteins from degradation by (1) preventing inappropriate protein aggregation, (2) ensuring proper folding ,and (3) mediating transport of immature proteins by guiding protein movements through membranes to target organelles for packaging, degradation or repair, thus ensuring proper cellular localisation and enhancing cellular repair mechanisms (Martinus et al., 1995). In the presence of stress, HSP70 remain associated with partially unfolded proteins to prevent

abnormal associations until normal conditions are re-established. At higher levels of stress, HSP70 also has a role in accelerating correct refolding, and tagging damaged protein for transport to proteosomes and lysosomes for degradation (Parsell and Lindquist, 1993). Stress-inducible HSP70s, therefore, supplement constitutively expressed HSC70 to provide protection from adverse conditions for the essential cellular functions to occur.

Increase in HSP70 mRNA expression was observed when embryos were exposed to increased levels of stress. HSP70 transcript levels were higher in *in vitro* cultured embryos compared to *in vivo* cultured embryos (Wrenzycki et al., 2001b). As the *in vitro* environment is considered to be more stressful for the embryo than the *in vivo* environment, then upregulation of HSP70 mRNA levels *in vitro* relative to *in vivo* associates HSP70 synthesis with exposure of embryos to stressors. Moreover, embryos cultured in TCM-199 (Wrenzycki et al., 1999), in the presence of 10% serum (Russell et al., 2006 and Wrenzycki et al., 1999) and arsenite (Dix et al., 1996), also increased HSP70 transcription. Dix et al. (1996) found that embryos with increased HSP70 transcription survived exposure to arsenite, suggesting that HSP70 was involved with the embryonic repair system to protect the cells from DNA (Dong and Luo, 1994) and protein damage (Wang and Lazarides, 1984) caused by arsenite. Dix et al. (1996) suggested that the increased requirement for HSP70 to respond to a stressor could reduce the levels of HSP70s available for normal development processes, thus impairing embryo development in the presence of arsenite, despite the protection of HSP70. The protective role of HSP70 leads towards a decrease in cellular injury due to stress, so that the repaired cells are then able to quantify stress and activate apoptosis when tolerance limits are exceeded, restoring the integrity of the embryo as a whole.

Besides the cellular stress response pathway, stress may activate other pathways that are interactive. Pathways activated by stress, in increasing lethality, include modified gene expression, modified metabolism, apoptosis and necrosis, with the stress level determining the degree of perturbation. Modified gene expression and modified metabolism led to modified developmental kinetics during embryo development, and maternal/embryo asynchrony when *in utero*, modified foetal/placental metabolism, which can then lead to abnormal foetal development and abortion (Thompson et al., 2002). Apoptosis and fragmentation may lead to abnormal compaction, modified inner cell mass to trophectoderm allocation which may then lead to modified foetal/placental ratio, leading to abnormal foetal growth and possibly abortion (Leese et al., 1998), whilst extensive necrosis will lead to embryo death. Therefore, as suggested by Fleming et al. (2004), because of stress-induced pathologies, stressed embryos tend to have reduced viability.

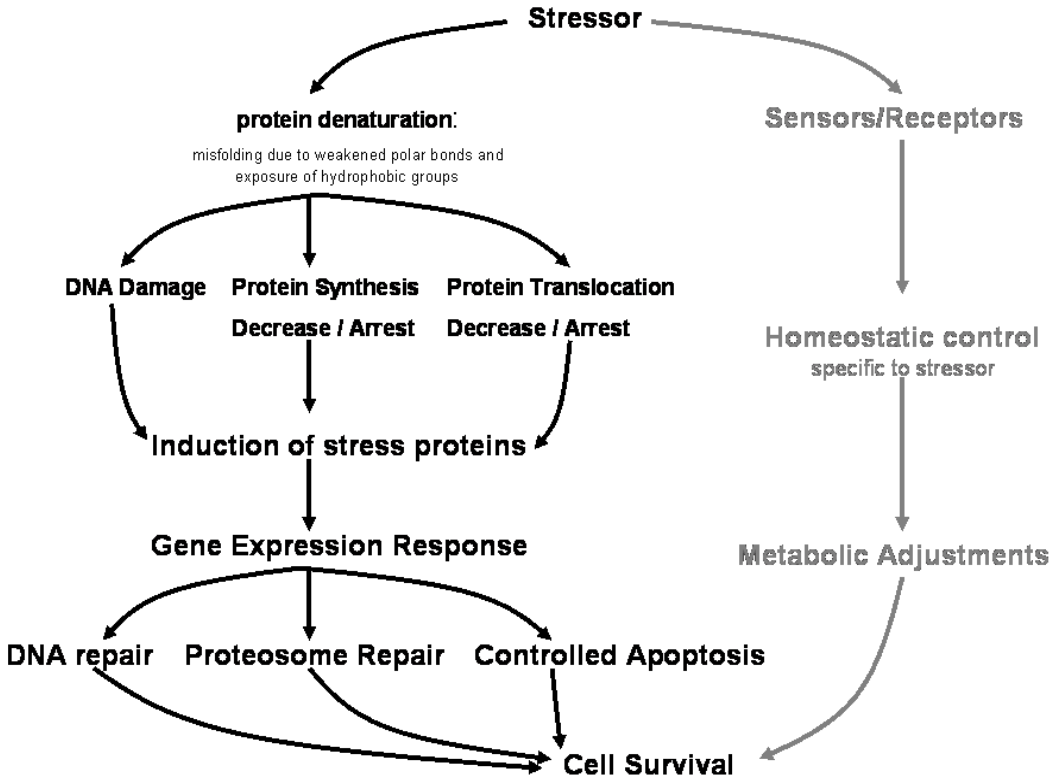


Figure 7 Mechanism by which stress response leads to cell survival. There are two types of stress response: cellular stress response, whereby the cell may respond to the damage caused by the stress or homeostatic stress response, whereby the cell responds directly to the stressor via homeostasis.

Therefore, when exposed to a stressor, the first defence system at the cellular level is homeostasis. The embryo is considered to be stressed once the levels of stress are higher than the embryo can cope with through homeostatic response, so that the embryo responds to stress via induction of HSP70. If the embryo is unable to respond to stress via cellular stress response or if the level of stress is too high for the HSP70s to refold or degrade damaged proteins, then apoptosis occurs, followed by necrosis and embryo death. Therefore, the ability of an embryo to respond to stress through the cellular stress response mechanism is beneficial to the viability of the embryo. However, stress-induced pathologies are the outcome of pathways activated by stress. Although the ability of an embryo to respond to stress has not been compared with the embryo viability *in vitro* or *in vivo*, it is proposed that reduced developmental competence is indicative that the embryo is stressed.

The suggestion that elevated IFNτ secretion could be a stress response to sub-optimal culture conditions (Kubisch et al., 1998) has led to a number of studies observing the effect of environmental stressors on IFNτ production. Exposing embryos to detrimental environmental

factors, such as *in vitro* environment, embryo manipulation, cryogenic stress, oxidative stress and thermal stress could give insight into whether IFNτ secretion is an index of stress.

1.8.1 Effect of embryo production system on IFNτ production

Detrimental culture systems are culture systems shown to reduce embryo development and viability and are therefore likely to constitute stressors for embryos. Studies have compared IFNτ production by embryos cultured in different embryo production systems demonstrated to be detrimental to embryo survival.

IVP embryos have significantly poorer morphological grade (Thompson, 1997), blastomere survival (Hardy and Spanos, 2002), ultrastructure, physiology, sensitivity to cryoinjury (Holm et al., 1998), gene expression (Niemann and Wrenzycki, 2000), metabolism (Khurana and Niemann, 2000 a) and foetal and postnatal development (Farin and Farin, 1995) compared to their *in vivo* counterparts, suggesting that the *in vitro* environment may be a stressor for embryos. Wrenzycki et al. (2000, 2001b) and Lonergan et al. (2001) found that *in vitro* derived blastocysts expressed higher levels of IFNτ transcripts than blastocysts produced *in vivo*. The decrease in IFNτ transcript levels was associated with a decrease in embryo viability and an increase in HSP70 expression, indicative of stress, supporting the hypothesis that an increased level of IFNτ mRNA is an indicator that the embryo is stressed.

In *in vitro* studies, certain protein sources are associated with reduced embryo viability and are therefore a stressor to the embryo. Embryos cultured in the absence of protein (medium supplemented with PVA) relative to embryos cultured in BSA-supplemented medium (Wrenzycki et al., 1999, Kubisch et al. 2001a) had fewer cells and delayed blastocyst formation, both indicators of a decrease in developmental competence. These embryos also had an increase in IFNτ secretion, suggesting that IFNτ may be an index of stress. Embryos cultured in serum-supplemented medium had increased transcript levels of LIF, a gene associated with attachment and differentiation and increased rate of development with more blastocysts developing by day 6 compared to embryos cultured in the absence of serum (BSA), indicating improved developmental competence, which was accompanied by decreased levels of IFNτ transcripts (Rizos et al., 2003). These studies suggest that increased IFNτ expression may be associated with decreased embryo viability caused by stress.

1.8.2 Effect of embryo manipulation on IFNτ production

Embryo manipulation techniques, such as nuclear transfer and embryo splitting, caused reduced embryo viability (Stojkovic et al., 1999) and, therefore, may also be considered as

stressors. However effects of these different embryo manipulation techniques on IFNτ are inconsistent. Nuclear transfer embryos contained more IFNτ mRNA than IVP embryos (Wrenzycki et al., 2001a), and secreted less IFNτ than *in vivo* or IVP embryos (Stojkovic et al., 1999). Blastomere biopsy at the eight-cell stage, however, had no effect on IFNτ protein secretion (Kubisch and Johnson, 2007) and split embryos secreted less IFNτ than embryos which remained intact (Stojkovic et al., 1999). The discrepancy between the three studies in terms of an association between IFNτ secretion and stress induced by embryo manipulation suggests that different embryo manipulation techniques affect rate of IFNτ transcription and translation differently. One possible explanation is that IFNτ is not an index of stress. An alternative explanation could be that different embryo manipulation techniques cause varied levels of perturbation to the embryo, and IFNτ production is affected differently at different levels of stress, depending on the level of stress (homeostatic, HSP70 expression, apoptosis, necrosis). It is, at present, unclear why different embryo manipulation techniques affect IFNτ expression and transcription in different ways and more data are required to explain inconsistencies.

1.8.3 Effect of cryogenic stress on IFNτ production

Cryopreservation causes physical lesions in the blastomeres due to formation of ice crystals inside and outside the cells (Vajta et al., 1997) and the osmotic effects of the cryoprotectants during dehydration and hydration of the embryo (Saha and Suzuki, 1997). Cryopreservation also causes cell ultra-structural alterations (such as fragmentation of nuclear membranes, Wiemer et al., 1995) and a reduction in cell numbers (Kaidi et al., 1999). Consequently, frozen/thawed embryos contained a higher number of apoptotic cells than fresh embryos (Marquez-Alvaral et al., 2004) and pregnancy rate following transfer of frozen embryos is consistently lower than fresh embryos (Massip et al., 1995, Agca et al., 1998, Ambrose et al., 1999). Therefore, cryogenic stress seems to cause a decrease in embryo viability. Araujo et al. (2005) compared IFNτ secretion in fresh and frozen embryos, 7 days after the blastocyst stage, and reported that frozen embryos secreted less IFNτ than fresh embryos. As considered by Stojkovic et al. (1995), a decrease in IFNτ production may be due to damage due to freezing (physical lesions, or biochemical alterations) affecting the viability of the embryo and its capacity to secrete IFNτ. However, cryopreserved embryos survived for at least 7 days after cryopreservation/thawing, suggesting that the viability of the embryo as a whole was not compromised, at least for the 7 days. A more plausible explanation is that cryopreservation affected the viability of trophoblast cells and their ability to secrete IFNτ. In fact, Kaidi et al. (1999) reported that cryopreservation caused a reduction in the number of

trophoblast cells compared to fresh embryos. Therefore, the reduction in IFNτ secretion in cryopreserved embryos compared to fresh embryos may be due to a reduction in numbers of cells secreting IFNτ, rather than a modulation of IFNτ secretion per cell. Unfortunately, Araujo et al., (2005) did not evaluate cell count. Therefore, it is unclear whether cryogenic stress affects IFNτ secretion per cell.

1.8.4 Effect of oxidative stress on IFNτ production

Since the oxygen concentration in the mammalian uterus and oviduct was lower than atmospheric oxygen levels (20%, Fischer and Bavister, 1993), embryos are usually cultured at 5% oxygen to imitate the *in vivo* environment. Although there was some discrepancy as to the benefits of 5% oxygen compared to 20% in terms of blastocyst rate (Khurana and Niemann, 2000 b, Booth et al., 2005, Correa et al., 2008) and cleavage rate (Correa et al., 2008), most studies have reported that reducing oxygen concentration is beneficial to the embryonic development (Lim et al. 1999; Yuan et al., 2003; Kitagawa et al., 2004; Petersen et al., 2005, Olson and Seidel 2000; Takahashi et al. 2000). The mechanism by which higher oxygen concentrations are stressful to the embryo may be due to accumulation of reactive oxygen species (ROS, Thompson et al., 1990) which alter gene expression (Mouatassim et al., 1999), and eventually lead to a deterioration of embryo grade and survival. Embryos are able to survive oxidative stress due to the activity of antioxidant enzymes (Harvey et al., 1995), such as Mn-SOD, which protects cells against toxic oxygen radicals by scavenging superoxide radicals, and catalase and glutathione peroxidase, which eliminate hydrogen peroxide, the product of Mn-SOD activity (Guerin et al., 2001). Gene expression for these enzymes is induced by stress: embryos cultured at 20% oxygen had increased transcript levels of the Mn-SOD gene (Correa et al., 2008).

The interest in the association between oxidative stress and IFNτ production arose because female embryos expressed more IFNτ than male embryos (Kimura et al., 2004a,b), and male embryos are more sensitive than female embryos to oxidative stress due to increased glucose-6-phosphate dehydrogenase (G6PDH) activity and greater pentose phosphate pathway flux in females (Kimura et al., 2004b), suggesting that IFNτ and oxidative stress may be related.

Kimura et al. (2004b) cultured embryos in the presence of 0, 100 and 200μM of hydrogen peroxide (a product of superoxide dismutase), and found that oxidative stress had no effect on IFNτ secretion. Also, IFNτ expression (Correa et al., 2008) and protein (Kimura et al., 2004b) levels did not differ between 20 and 5% oxygen concentration despite an increase in Mn-SOD expression at 20% oxygen (Correa et al., 2008), suggesting that IFNτ may not be

related to oxidative stress. Conversely, Kubisch and Johnson (2007) cultured embryos under 5 and 20% oxygen and found that embryos cultured at 20% oxygen secreted more IFNτ protein (12.2±2.8 ng at 20% oxygen vs 5.0±2.6 ng at 5% oxygen, mean±SEM) and had lower blastocyst yields (42% blastocyst yield at 20% oxygen vs 25% blastocyst yield at 5% oxygen) than embryos cultured at 5% oxygen, so that the reduced IFNτ secretion at a more stressful high oxygen environment indicates that increased IFNτ levels may be positively associated with stress. Correa et al. (2008) did, however, observe a high variability in IFNτ expression, possibly due to the use of pools of 15 embryos, ranging from early to hatched blastocysts. Therefore, the lack of an effect of oxidative stress on IFNτ expression and secretion, as reported by Correa et al. (2008) and Kimura et al. (2004b) may be due to the high variation in IFNτ. Therefore, there are discrepancies between papers on the effect of oxidative stress on IFNτ expression and secretion, probably due to the variable nature of IFNτ.

1.8.5 Effect of maternal heat stress on IFNτ secretion

Seasonal studies in subtropical environments where temperatures may be as high as 43ºC in the summer and as low as 4ºC in the winter (as in Florida), have consistently reported reduced conception rates in summer. Summer heat stress caused a 20-30% decrease in pregnancy rate (based on pregnancy diagnosis 6-8 weeks pi) compared to winter in one study (Cavestany et al., 1985) and a 60% decrease in pregnancy rate (based on rectal examination at 36-40 dpi) in another (Badinga et al., 1985). Therefore, summer months are associated with reduced pregnancy rates.

A number of studies have confirmed that the seasonal difference is indeed due to elevated temperature. While observing pregnancy loss in cattle, Garcia-Ispierto et al. (2006) collected data on a range of variables used to determine which variables caused foetal loss and concluded that heat stress was amongst the main factors, with the likelihood of pregnancy loss increasing by 1.05-fold for each additional unit of the mean maximum temperature-humidity index from days 21 to 30 of gestation. Dunlap and Vincent (1971) exposed cows to high (32.2°C) and low (21.1°C) temperature for 72 h following artificial insemination and reported conception rates of 0 and 48% respectively, confirming that reduced fertility in summer months are due to high temperatures. Increased rectal temperatures in heat stressed cows compared to controls (Geisert et al., 1988, Putney et al., 1988a, 1989) suggest that elevated ambient temperatures caused an increase in temperatures in the uterus. The detrimental effect of heat stress on embryo development was further emphasized by the correlation between increased rectal temperature of pregnant cows (from 39 to 42°C) and the increase in the percentage of abnormal embryos recovered (Putney et al., 1988a). Moreover, Long et al.

(1969) reported that pregnancy rate of cows with rectal temperatures up to 39.7°C at the time of insemination was over two-fold higher than of those with rectal temperatures above 39.7°C. Therefore, the reduced fertility due to maternal heat stress may be caused by an increase in the body temperature of the cows.

Al-Katanani et al. (2002) compared *in vitro* blastocyst development from oocytes derived from summer and winter cows and found that the proportion of oocytes that developed to the blastocyst stage by day eight was lower in the summer compared with winter. Putney et al. (1989) observed that summer heat stress also affected the rate of development of the embryo (increased incidence of slow-developing embryos in the summer compared to winter) and morphological grade (fair to poor morphological grade in the summer compared to good morphological grade in the winter). Therefore, maternal heat stress seems to be detrimental to embryo development.

Exposure of *in vivo* produced day 17 pi concepti to 43°C for 18 h caused a reduction in intracellular incorporation of proteins by embryos as well as a 72% reduction in IFNτ secretion *in vitro*, as determined by SDS-PAGE and immunoblotting (Putney et al., 1988b), so that heat stress caused a reduction in IFNτ secretion probably due to a reduced capacity of the embryo to incorporate other proteins in the medium. These embryos were further stressed from culture at high (47.5%) oxygen concentration so that it could be that the stress levels in this study were chronic and that the reduced IFNτ secretion may be indicative of impaired development and reduced survival. However, this cannot be confirmed, given that the embryos were only cultured for 24 h, and that the effect of treatment on the viability was not tested. Exposure of pregnant cows to a milder heat-stress treatment (37°C) between eight and 17 dpi caused an increase in IFNτ in uterine flushings at 17 dpi compared to control cows (21°C, Geisert et al., 1988). Increase in IFNτ was accompanied by increased rectal temperatures and increased uterine protein and calcium concentrations, suggesting that changes in IFNτ may be due to altered uterine environment. The same study observed that heat treatment caused a decrease in embryo weight relative to control, suggesting a detrimental effect of heat stress on embryo development. Therefore, increased IFNτ secretion *in vivo* may be associated with stress.

In vitro, the effects of heat stress (41 to 43°C) on embryo development reported have been decreased cleavage rate (Sugiyama et al., 2003), decreased development past the eight-cell (Sugiyama et al., 2003) and morula stage (Ealy et al., 1995, Ju et al., 2005), suggesting that

high temperatures caused decreased viability of pre-attachment bovine embryos. Synthesis of HSP70 in bovine pre-attachment embryos was also induced by high temperatures (Kawarsky and King, 2001). The effect of heat stress on IFNτ secretion by IVP ruminant embryos has not been observed and, therefore, requires further analysis.

1.8.6 IFNτ production as an index of stress

One way to determine whether IFNτ production is a response to stress is to expose embryos to a known stressor and measure IFNτ expression and/or secretion. All studies which exposed embryos to known stressors reported a positive relationship between IFNτ expression and stress (Table 7, page 76), suggesting that increased IFNτ expression may be an index of stress. However, there are discrepancies between studies relating IFNτ secretion and stress (Table 8, page 76). Three studies (Putney et al., 1988b, Stojkovic et al., 1999, Araujo et al., 2005) observed a negative relationship between IFNτ secretion and stress which could be due to lethal stress levels. These studies also observed no significant relationship between IFNτ production and stress due to high variation in IFNτ production (Kimura et al., 2004b, Correa et al., 2008).

Therefore, factors other than stress may modulate IFNτ synthesis at the post-transcriptional level. IFNτ synthesis may also be affected by the level of stress (homeostasis, HSP70 expression, apoptosis, necrosis, embryo death). If IFNτ production is a stress response to adverse conditions, it may contribute to an attempt of the embryo to overcome sub-optimal conditions in vivo and enhance its own survival by modifying uterine function (Kubisch et al., 2004).

Table 7 Direction of change in IFNτ transcript abundance in 'stressed' compared with 'non-stressed' embryos. ↑ : 'Stressed' embryos expressed significantly more IFNτ mRNA than 'non-stressed' embryos. (↑): Non-significant increase in IFNτ expression in 'stressed' compared to 'non stressed' embryos.

Stressor	IFNτ	Reference
In vitro environment	↑	*Wrenzycki et al., 2000, 2001b* *Lonergan et al., 2001*
Protein source	↑	*Wrenzycki et al., 1999* *Rizos et al., 2003*
Nuclear Transfer	↑	*Wrenzycki et al., 2001a*
Oxidative Stress	(↑)	*Correa et al., 2008*
	↑	*Kubisch and Johnson, 2007*

Table 8 Direction of change in IFNτ protein secretion in 'stressed' compared with 'non-stressed' embryos ↑ : 'Stressed' embryos secreted significantly more IFNτ than 'non-stressed' embryos. ↔: No significant change between 'stressed' and 'non-stressed' embryos. ↓: 'Non-stressed' embryos secreted significantly more IFNτ protein than 'stressed' embryos.

Stressor	IFNτ	Reference
In vitro environment	↔	*Kubisch et al., 1998*
Protein source	↑	*Kubisch et al., 2001a*
Nuclear Transfer	↓	*Stojkovic et al., 1999*
Embryo Splitting	↓	*Stojkovic et al., 1999*
Blastomere Biopsy	↔	*Kubisch and Johnson, 2007*
Cryogenic Stress	↓	*Araujo et al., 2005*
Oxidative Stress	↔	*Kimura et al., 2004b*
	↑	*Kubisch and Johnson, 2007*
Thermal Stress (*in vivo*)	↑ ↓	*Geisert et al., 1988* *Putney et al., 1988b*

1.9 Conclusion

IFNτ secretion by bovine embryos *in vivo* increases to a maximum during the time when luteolysis needs to be prevented by maternal recognition of pregnancy (outlined in section 1.3, page 39). IFNτ expression and secretion by bovine embryos vary, both in terms of timing of onset and cessation (section 1.3.2, page 39), and in amount of IFNτ secreted (section 1.3.1, page 1.3.1). As discussed in sections 1.5 (page 45) and 1.6 (page 50), several intrinsic and extrinsic factors have been shown to explain at least part of the variation in IFNτ secretion and, therefore, have a role in controlling the cross-talk between the embryo and the maternal system. The timing of onset of IFNτ expression and secretion seems to be genetically or intrinsically regulated, whilst environmental factors seem to be responsible for controlling the timing of cessation and the amount of IFNτ secreted (reviewed in sections 1.5 and 1.6). The complexity of the control of IFNτ secretion by intrinsic and extrinsic factors which are only partly understood has lead to inconsistencies in the results from different *in vivo* studies. Therefore, there is a need for *in vitro* studies to simplify and understand the mechanism of IFNτ secretion. IVP embryos also secrete IFNτ. The control of IFNτ secretion by embryos *in vitro*, in the absence of the maternal system, remains unclear. Although genetic programming seems to be involved in controlling IFNτ secretion (as discussed in section 1.5, page 45) and bovine embryos have been shown to be responsive to some environmental factors (outlined in section 1.6.3, page 56), significant variation in IFNτ secretion by IVP embryos (outlined in section 1.3, page 39) remains unexplained. Most studies assessing factors affecting IFNτ expression and secretion have observed IFNτ production during the timing of elongation. However, different factors may not have the same effect at different stages of development, so that there is a need to improve understanding of modulation of IFNτ at the blastocyst stage. Therefore, the **hypothesis** of this thesis is that

environmental factors explain the variation in IFNτ secretion by IVP bovine blastocysts.

Therefore, in order to determine whether beneficial or detrimental environmental factors may regulate IFNτ expression and secretion at the blastocyst stage, two experiments were designed to have a positive (exposure to a cytokine, GM-CSF, shown to be beneficial to embryo development *in vitro*) or negative (exposure to elevated temperatures) effect on embryo development and the subsequent IFNτ production was observed. Both GM-CSF (reviewed in

sections 1.7.1, page 20) and heat stress (section 1.8.5, page 73) have been shown modulate IFNτ secretion by bovine embryos *in vivo*. In doing so, the **objective** of this thesis was:

to determine whether IFNτ production by IVP bovine blastocysts was affected by beneficial or detrimental environmental factors

and the **aims** were:

(1) To determine whether exposing embryos to a beneficial cytokine, GM-CSF, affects IFNτ secretion *in vitro*, and whether IFNτ secretion *in vitro* correlates with blastocyst parameters (Chapter 2);

(2) To determine whether mild heat stress modulates IFNτ expression *in vitro* (Chapter 3).

In order to assess IFNτ expression, a further aim was to develop an improved method for detection of IFNτ mRNA levels.

2 EFFECT OF TIMING AND AMOUNT OF GM-CSF AT EARLY STAGES OF DEVELOPMENT ON SECRETION OF IFNτ DURING *IN VITRO* CULTURE OF BOVINE EMBRYOS

2.1 Introduction

In situ hybridisation and immunohistochemistry demonstrated that GM-CSF and GM-CSFR localisation in the ovary, oviduct and uterus (as reviewed in section 1.7.1.1) was site specific. In the bovine, the uterine epithelium was the major site of GM-CSF synthesis, with the luminal epithelium containing more GM-CSF (de Moraes et al., 1999) than the glandular epithelium. Since the uterus is the site of blastulation and attachment, localisation of GM-CSF in the uterus suggests a role for GM-CSF during blastulation and attachment. In the bovine oviduct, GM-CSF was immunolocalised to the epithelium in both ciliated and non-ciliated cells of the ampullary and isthmic sections of the oviduct (de Moraes et al., 1999). Given that the oviduct is the major site of fertilisation and early embryo development, the presence of GM-CSF in the oviduct suggests that GM-CSF may have a role in embryo development before the morula stage. The ampulla is the site of acrosome reaction and fertilisation (Herz et al., 1985), whilst the isthmus is the site of pre-compaction embryo development. The ampulla has a higher concentration of GM-CSF compared to the isthmus (de Moraes et al., 1999). Therefore, as the embryo travels through the oviduct, the embryo (at different stages of embryo development) is exposed to different concentrations of GM-CSF.

2.1.1 Effect of varied concentration of GM-CSF on embryo development and secretion of IFNτ

Neither 1 or 10 ng mL^{-1} GM-CSF had any effect on IFNτ secretion by *ex-vivo* produced bovine embryos recovered on days 16 to 18 pi (de Moraes et al., 1997). In contrast, Imakawa et al. (1997) observed an 8.3 fold increase in IFNτ mRNA abundance and protein secretion by ovine embryos recovered on day 16 pi and cultured for 24 h in the presence or absence of 3.3 ng mL^{-1} of GM-CSF. Imakawa et al. (1993) found that GM-CSF was stimulatory at 3.3 to 6.6 ng mL^{-1}, but had no effect on IFNτ secretion at 13.2 ng mL^{-1}. Inconsistencies between the studies by de Moraes and Imakawa could be due to differences in the concentration of GM-CSF.

Dose-response studies of GM-CSF have been performed on bovine, ovine, murine and porcine embryo development *in vitro* (Figure 8, page 82). The magnitude of the response was dose dependent in bovine (de Moraes and Hansen, 1997), ovine (Imakawa et al.1993) and

murine embryos (Robertson et al., 2001, Ko et al., 2002, and Behr et al., 2005) for a proportion of the rGM-CSF concentrations tested. Increased concentration of rGM-CSF after the optimum concentration (0.125, 2 and 3.3 ng mL^{-1} in Behr et al., 2005, Robertson et al., 2001 and Imakawa et al., 1993 respectively) did not further promote embryo development and even higher concentrations (13.2 ng mL^{-1}, Imakawa et al., 1993 and 10 ng mL^{-1}, Robertson et al., 2001) caused a reduction in response to GM-CSF in embryo development in terms of IFNτ secretion (Imakawa et al., 1993) and ability to hatch and attach (Robertson et al., 2001). Therefore, the response of embryos to GM-CSF is dependent on the species as well as the concentration of GM-CSF.

In murine embryos (Figure 8b, page 82), Ko et al. (2002) and Behr et al. (2005) observed a dose-dependent increase in blastocyst formation with rGM-CSF (0, 1, 5, 10 ng mL^{-1} and 0, 0.0625,0.125 ng mL^{-1} respectively), suggesting that the response of murine embryos to GM-CSF is also dose-dependent. Conversely, other murine studies observed no effect of embryo development at any concentration (Karagenc et al., 2005: 0, 2, 4, 8 and 16 ng mL^{-1} and Robertson et al., 2001: 0.4, 2, 10 ng mL^{-1}). Therefore, other factors other than amount of rGM-CSF must be affecting the response of the embryos to rGM-CSF.

2.1.2 Effect of varied timing of exposure to GM-CSF on embryo development and secretion of IFNτ

A two-fold increase in IFNτ secretion at the blastocyst stage was detected when ovine zygotes were exposed to GM-CSF between 1 and 3 dpi, whereas no response to GM-CSF was observed when GM-CSF was included between 5 and 7 dpi (Rooke et al., 2005, Table 9, page 81), suggesting that timing of exposure affects the response of embryos to GM-CSF. Therefore, the lack of a response of bovine embryos exposed to GM-CSF at the elongation stage (17 dpi) observed by de Moraes et al. (1997) may be due to there being a window of opportunity for embryos to be able to respond to GM-CSF. de Moraes et al. (1997) suggested that GM-CSF may be less able to affect IFNτ production when IFNτ secretion is being produced at maximal levels. Therefore, differences in timing of exposure to GM-CSF could explain why some studies observed an effect of GM-CSF on IFNτ, whilst others did not.

Although processes between fertilisation and the initiation of attachment are conserved between mammalian species, the duration of the processes varies. Attachment takes place 4 dpi in mice, 9 dpi in humans, and 30 dpi in cows (Kimura et al., 2005). The prolonged pre-attachment period in ruminants compared to other mammals emphasises the importance of timing of exposure to regulators of embryogenesis. Therefore, some discrepancies between

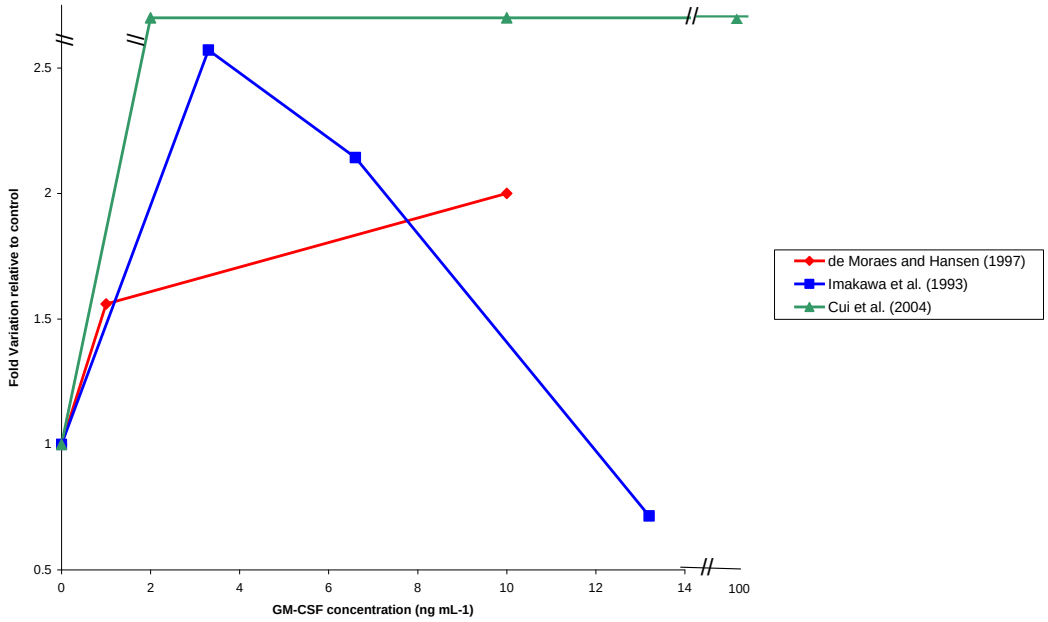
studies on the effects of GM-CSF on embryo development *in vitro*, measured in terms of increase in cell proliferation, blastocyst yield and IFNτ secretion, may be explained by differences between species, as summarised in Table 9 (page 81).

GM-CSFR occurrence has not been investigated in bovine embryos. In human embryos, Sjoblom et al. (2002) detected GM-CSFR protein associated with the cell membrane from the eight-cell stage onwards and that GM-CSFR mRNA expression was lower at the third cleavage compared to later in development suggesting stage-specific differences in GM-CSFR transcription and translation. Therefore, it is possible that a stage-specific response to rGM-CSF could be due to stage-specific GM-CSFR transcription and/or translation.

Table 9 Variation in timing of exposure (O: oocyte, 2: two-cell, 8: eight-cell, M: morula, Bl: blastocyst, A: attachment) of embryos to GM-CSF and response to GM-CSF in terms of cell proliferation (C), Blastocyst yield (B) and IFNτ expression and/or secretion (I). References are 1: Imakawa et al., 1993, 2: de Moraes & Hansen 1997 (a: experiment 1, b: experiment 3, c: experiment 4), 3 : de Moraes et al., 1997, 4 : Robertson et al., 2001, 5 : Ko et al., 2002, 6 : Cui et al., 2004, 7: Behr et al., 2005, 8: Karagenc et al., 2005, 9: Rooke et al., 2005 (a : experiment 2, b : experiment 3).

Species	Timing of Exposure						Response			Reference
	O	2	8	M	Bl	A	C	B	I	
bovine								↑	=	3
bovine								↑		2 b
bovine								↑		2 a
bovine							=	↑		2 c
murine							↑	=		4
murine							↑	↑		5
murine								↑		7
murine							=	=		8
ovine									↑	1
ovine									=	3
ovine							=	=	=	9 b
ovine							=	=	↑	9 b
ovine							↑	=	↑	9 a
ovine							=	=	↑	9 b
ovine							=	=	↑	9 b
ovine							=	=	↑	9 b
porcine							=	↑		6

(a)



(b)

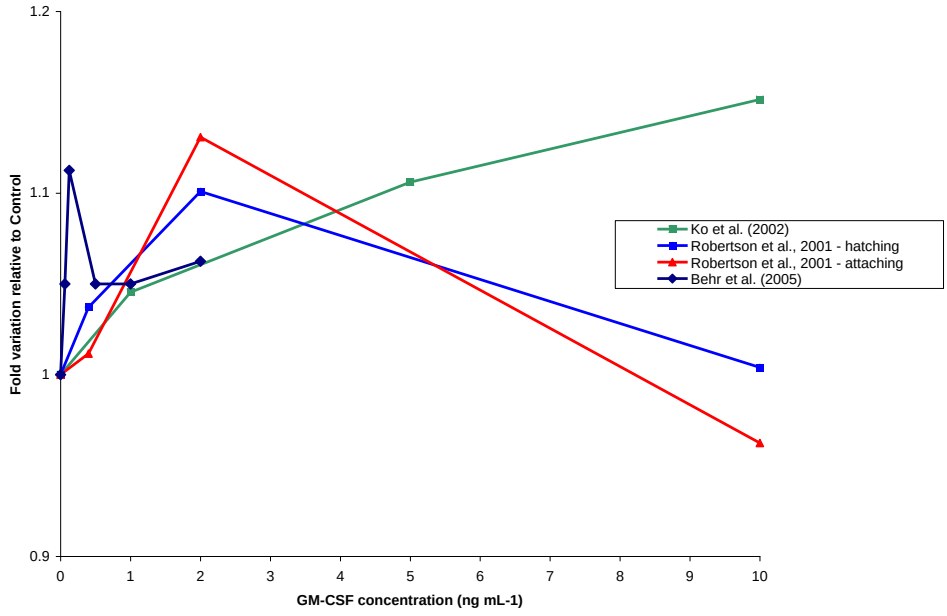


Figure 8 Dose response effect of GM-CSF on different parameters of embryo development in (a) bovine (de Moraes and Hansen, 1997), ovine (Imakawa et al., 1993), porcine (Cui et al., 2004) and (b) murine embryos (Behr et al., 2005, Ko et al., 2002 and Robertson et al., 2001). The parameters measured in each study were as follows: de Moraes and Hansen (1997), Ko et al. (2002), Behr et al. (2005) and Cui et al., (2004) measured blastocyst formation, Robertson et al. (2001) measured hatching and attachment rates, and Imakawa et al. (1993) measured IFNτ secretion.

2.1.3 Discrepancies between different studies on the beneficial effects of GM-CSF on pre-attachment embryo development

In vitro studies have demonstrated that GM-CSF, a cytokine secreted by the maternal reproductive tract in mammals, was beneficial to pre-attachment embryo development (discussed in section 1.7.1.3), although there are differences between studies. In murine embryos, Robertson et al. (1991) and Ko et al. (2002) observed that GM-CSF caused an increase in cell proliferation, whilst Karagenc et al. (2005) found that cell proliferation was not affected by GM-CSF exposure. In ovine embryos, Rooke et al. (2005) observed an increase in cell number in the presence of GM-CSF compared to control in one experiment, whilst in another experiment, GM-CSF had no effect on cell number. Therefore, there are discrepancies between studies in terms of the beneficial effect of GM-CSF on embryonic cell proliferation. GM-CSF also caused an increase in blastocyst yield in some studies (bovine: de Moraes and Hansen, 1997, de Moraes et al., 1997; murine: Behr et al., 2005, Ko et al., 2002; porcine: Cui et al., 2004), but not in others (ovine: Rooke et al., 2005, murine: Robertson et al., 2001, Karagenc et al., 2005), so that there are discrepancies between studies in terms of the effect of GM-CSF exposure on blastocyst yield. Moreover, GM-CSF caused an increase in IFNτ mRNA expression (Imakawa et al., 1993) and protein secretion (Imakawa et al., 1993, Rooke et al., 2005) by ovine embryos, but only for a range of concentration (Imakawa et al., 1993) and a range timing of exposure (Rooke et al., 2005), whilst de Moraes et al. (1997) found that GM-CSF had no effect on IFNτ secretion by bovine or ovine embryos. Therefore, timing of exposure and amount of exposure of GM-CSF may affect the effects of GM-CSF on pre-attachment embryo development.

2.1.4 Conclusion

It is important to understand why there are discrepancies between different studies in the response of embryos to GM-CSF in terms of embryo development and IFNτ expression and secretion. Given that the timing and concentration of GM-CSF in the female reproductive tract of cows vary, it is plausible that differences between studies in timing of exposure of embryos to GM-CSF and concentration of GM-CSF may explain discrepancies between studies in terms of effect of GM-CSF on embryo function and development. Therefore, the **objective** of the experiments described in this Chapter was

to assess the effects on blastocyst development,of inclusion of different concentrations of roGM-CSF in culture medium at different stages of pre-blastocyst development.

Blastocyst development was assessed by measuring pyruvate metabolism, apoptosis, morphological grade, rate of development, blastocyst yield and IFNτ secretion.

It was **hypothesized** that:

(1) **exposure of bovine embryos to roGM-CSF is beneficial to bovine embryo development**,

(2) **exposure of bovine embryos to roGM-CSF enhances IFNτ secretion and responses in embryo development and IFNτ secretion to roGM-CSF are dose-dependant,**

(3) **responses in embryo development and IFNτ secretion to GM-CSF occur before the eight-cell stage.**

Three experiments were designed to test these hypotheses.

1. Effects of amount of GM-CSF (2, 5, 10 and 50 ng mL^{-1}) not present or present between 1 and 3 dpi on blastocyst development,
2. Effects of roGM-CSF (50 ng mL^{-1}) not present or present between either 1 and 3 dpi or 1 and 9 dpi on blastocyst development,
3. Effects of exposure of roGM-CSF (2 and 10 ng mL^{-1}) not present or present between either 1 and 3 dpi or 1 and 9 dpi on blastocyst development.

2.2 Materials and methods

2.2.1 IVP of embryos

The following methodology for IVP of embryos represented the standard methodology used at our laboratory at the time of this study (details of equipment and chemicals used are outlined in Appendix 1, page 203).

2.2.1.1 Collection of cumulus-oocyte complexes (COCs)

Each replicate represented a batch of ovaries (30-40 pairs) obtained from a local abattoir on a different day. Bovine ovaries were collected, transported to the laboratory in phosphate-buffered saline (PBS) containing 50 µg mL^{-1} kanamycin sulphate maintained at 38°C and processed within 2 h of collection. After several washes with PBS at 38°C, visible follicles (2-8 mm in diameter) were aspirated with an 18-gauge needle and the aspirated fluids and tissues collected into search medium (HEPES-buffered TCM 199 containing 5% (v/v) Foetal Calf Serum (FCS), 50 IU mL^{-1} penicillin and 50 µg mL^{-1} streptomycin, pH 7.3, 290-300 mOsmo), and carefully searched for COCs. The quality of COCs was determined on the basis of cumulus cell structure, density and cytoplasmic appearance (Table 10) and only category 1 and 2 COCs were selected.

Table 10 Criteria used to classify oocytes (modified from Goodhand et al., 1999).

Category	Layers of cumulus	Cumulus colour & structure	Cytoplasm
1	more than 4	compact, light or transparent	clear and even
2	less than 4	compact	generally homogenous, slightly coarse
3	less than 4	dark cumulus or cumulus may be expanded	Irregular appearance with dark patches
4	none	-	-

2.2.1.2 *In vitro* maturation (IVM)

Selected oocytes were placed in maturation medium (TCM 199 without HEPES containing 0.1 mL mL^{-1} FCS, 10 µg mL^{-1} luteinising hormone, 10 µg mL^{-1} follicle-stimulating hormone, 0.1 mg mL^{-1} L-glutamine, 50 IU mL^{-1} penicillin and 50 µg mL^{-1} streptomycin, pH 7.3 to 7.4, 280 to 300 mOsmo). The COCs (approximately 50–60 COCs in 0.5 mL of maturation medium) were incubated in an atmosphere of 5% carbon dioxide (CO_2) at maximum humidity at 38.5ºC for 24 h.

2.2.1.3 *In vitro* fertilisation (IVF)

After 24 h maturation, COCs were removed from maturation medium, washed in wash medium (modified Tyrode's albumin lactate pyruvate containing 3 mg mL^{-1} fraction V BSA pH 7.3 to 7.4, 270 to 290 mOsmo), and gently pipetted to remove expanded cumulus cells, leaving behind three to five layers of cumulus cells. Groups of 50 to 60 oocytes were then added (40 μL final volume) to 0.46 mL fertilisation medium (modified Tyrode's albumin lactate pyruvate containing 6 mg mL^{-1} fatty acid-free BSA, 3 μg mL^{-1} heparin, 3 μg mL^{-1} penicillamine, 1.1 μg mL^{-1} hypotaurine, and 0.3 μg mL^{-1} adrenaline, pH 7.8, 290 to 310 mOsmo). After *in vitro* capacitation of frozen–thawed spermatozoa using a swim-up procedure (Tea et al., 1984), a suspension of spermatozoa giving a final concentration of 10^6 sperm mL^{-1} was added. Semen from the same bull was used throughout to avoid any sire effects. The gametes were co-incubated for 22 h at 38.5°C in an atmosphere of 5% CO_2 in air at maximum humidity.

2.2.1.4 *In vitro* culture (IVC)

At the end of IVF, cumulus cells were removed from the embryos by vigorous pipetting, and groups of 50 to 60 putative zygotes transferred to 0.5 mL of SOF (8 mg mL^{-1} fatty acid-free BSA, 6.294 mg mL^{-1} sodium chloride, 534 μg mL^{-1} potassium chloride, 162 μg mL^{-1} potassium dihydrogen phosphate, 2.101 mg mL^{-1} sodium bicarbonate, 108 μg mL^{-1} sodium pyruvate, 252 μg mL^{-1} calcium chloride dihydrate, 9.9 mM sodium lactate, 100 μg mL^{-1} magnesium chloride hexahydrate, 270 μg mL^{-1} glucose, 146 μg mL^{-1} L-glutamine, 50 IU mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 0.5 mg mL^{-1} phenol red, pH 7.4, 270 to 280 mOsmo) and incubated at 38.5ºC in 5% CO_2, 5% O_2 plus 90% N_2 at maximum humidity. Cleaved embryos were selected 32 hpi and assigned in groups of ten to drops of SOF (0.05 mL). Embryos that had not cleaved by 32 hpi were not used, as early cleavage is a positive indicator of developmental potential (Van Soom et al, 1992, 1997). Embryos were transferred to fresh microdrops of their respective media every 48 h. Samples of media (0.045 mL) in which embryos had been cultured in between days 7 and 9 of culture were stored at -20ºC until assayed for IFNτ.

2.2.2 Embryo evaluation

On 7, 8 and 9 dpi, blastocysts were evaluated in terms of their developmental (Table 11, page 87) and morphological status (Table 12, page 87) and zona-inclusive diameters measured.

2.2.2.1 Total cell count

Blastocysts were fixed in glacial acetic acid and ethanol (3:1 v:v) and stained with a nuclear stain: 10 μg mL^{-1} bisbenzimide (Hoechst 33342) in sodium citrate solution (2.3 mg mL^{-1}

sodium citrate in water). Photographs were taken using a fluorescence microscope and cells were counted using ImageJ software (version 1.33u, 2003).

Table 11 Criteria used to classify blastocysts according to stage of development (modified from Gardner and Sakkas, 2003).

	Stage	Characteristics
1	Early	blastocoele less than half of the blastocyst
2	Mid	blastocoele more than half of the blastocyst
3	Expanding	embryo is enlarged and the blastocoele fills the blastocyst
3.5	Expanded	embryo is enlarged and the zona is very thin
4	Hatching	zona is cracked, but part of the blastocyst is still enclosed by the zona
5	Hatched	blastocyst is separated from the zona

Table 12 Criteria used to classify blastocysts according to morphological grade.

	Quality	Characteristics
1	Excellent	spherical, symmetrical, cells of uniform size, distinct inner cell mass, free of fragmented cells
2	Fair	few imperfections, such as some granulation, initial signs of fragmentation, few extruded blastomeres, or irregular shape
3	Poor	several extruded blastomeres and degenerate cells, diffuse inner cell mass, collapsed or badly fragmented

2.2.3 Measurement of IFNτ protein levels using the cell cytopathic assay

Media samples collected between 7 and 9 dpi were analysed for IFNτ concentration using the MDBK cell cytopathic assay described by Alexenko et al. (1997). MDBK cells (ATCC CCL22) were seeded at a density of 5×10^5 cells into each well of 96-well plates in 100 µl Dulbecco's Modified Eagle Medium (DMEM) (containing 10 % v/v Foetal Bovine Serum (FBS), 2 mM L-glutamine, 50 IU mL^{-1} penicillin and 50 µg mL- streptomycin, 1 mM sodium pyruvate) per well and incubated at 37°C. After 16 h, monolayers had formed. The medium was removed, and 100 µl of medium (as above) added to each well. Fifty µl of DMEM containing 300 IU hIFNα (Calbiochem) were added to a first-row well, and 50 µl containing 1.5 ng IFNτ (isoform 1c, Ealy et al., 2001) was added to a separate first-row well. Forty-five µl sample and 5 µl DMEM were added to the remaining first-row wells. The first-row wells were diluted eight times in series, removing 50µl from the first row and mixing into the subsequent row (1:3 dilution). At the end of dilution, the concentrations of IFNα and IFNτ in each well were as described in Table 13 (page 88). After 24 h the medium was removed and

replaced with vesicular stomatitis virus (supplied by Dr. SA Moyer, University of Florida) in serum-free DMEM. After 1 h at 37°C, the medium was removed and replaced with medium containing 0.1 mL mL^{-1} FBS. After 24 h, surviving cells were fixed with 70% ethanol and stained with 5 mg mL^{-1} Gentian violet in 70% ethanol.

Table 13 Final concentrations of IFNτ and IFNτ in each dilution after the first-row wells were diluted eight times in series in a 1:3 ratio.

Dilution Well	IFNα (IU mL^{-1})	IFNτ (pg mL^{-1})
1	2000	10000
2	667	3333
3	222	1111
4	74	370
5	25	123
6	8	41
7	3	14
8	1	5

The laboratory unit of activity (LUA, IU mL^{-1}) for each sample was derived from the sample dilution corresponding to protection of 50% MDBK cells from lysis by the virus, assessed visually, using the following formula:

$$sample\_LUA = 3^{(50\%dilution\_well-1)}$$

The sample LUA was corrected for IFNα using the following formula:

$$IFN\_LUA = \frac{sample\_LUA}{correction\_factor},$$

where the correction factor was determined from the 50% survival well of the IFNα control:

$$correction\_factor = \frac{IFNa\_LUA}{Amount\_of\_IFNa\_in\_first\_dilution},$$

where the absolute amount of IFNα in first dilution was 200 IU.

The sample IFNτ specific activity (IU ng^{-1}) was determined from the LUA for the IFNτ control averaged over all the assays:

$$IFNt\_specific\_activity = \frac{LUA\_for\_IFNt \,/\, Amount\_of\_IFNt\_control\_in\_first\_dilution}{correction\_factor}$$

where the amount of IFNτ control in the first dilution was 1ng. The sample IFNτ specific activity was averaged across all plates and was found to be equal to 5259 IU ng^{-1}. The sample IFNτ concentration (ng mL^{-1}) was derived using the formula:

$$sample\_IFNt\_concentration = \frac{IFNt\_per\_mL}{IFNt\_specific\_activity}.$$

The interassay coefficient of variation (CV) was 12%, and the intraassay CV was 9%. The limit of detection was 2.2 pg mL^{-1} which equals 11570 IU mL^{-1}.

2.2.4 Apoptosis detection assay

To determine the apoptotic index (percentage of apoptotic blastomeres relative to total cell number), an apoptosis detection kit (Apoptag Red *In situ* Apoptosis Detection Kit, S7165, Chemicon International), which involves the Terminal Deoxynucleotidyl Transferase (TdT) Mediated deoxyuridine triphosphate (dUTP) Nick End Labeling (TUNEL) assay, was used according to the manufacturer's instructions. The TUNEL assay analyses DNA fragmentation of apoptotic cells by incorporating fluorescein dUTP at the 3' DNA ends. Briefly, blastocysts were washed in PBS/PVP (0.01M PBS containing 1 mg mL^{-1} PVP) and fixed in PBS/PVP containing 0.04 mL mL^{-1} paraformaldehyde for 1 h at room temperature. Blastocysts were then transferred into PBS/PVP/Tw20 (1µl mL^{-1} Tween20 and 1 mg mL^{-1} saponin in PBS/PVP) for 30 minutes at room temperature. After several washes in 20 µl drops of PBS/PVP/Tw20 using silanised glassware, blastocysts were left in equilibration buffer (contained in Apoptag Red *In situ* Apoptosis Detection Kit) for 10 minutes. Positive control blastocysts were incubated with DNase I solution (1 unit mL^{-1} in PBS) for 10 minutes. Blastocysts were incubated in reaction buffer (contained in Apoptag Red *In situ* Apoptosis Detection Kit) containing TdT enzyme for 1 h in a humidified chamber at 37°C. Negative control blastocysts were incubated in reaction buffer without TdT. The embryos were washed in stop/wash buffer (contained in Apoptag Red *In situ* Apoptosis Detection Kit) for 10 minutes at room temperature and then incubated in 20 µl microdrops of a solution containing anti-digoxigenin antibody conjugated to rhodamine (contained in Apoptag Red *In situ* Apoptosis Detection Kit) for 1 h in a darkened humidified chamber at room temperature. The blastocysts were then stained in the dark in 10 µg mL^{-1} bisbenzimide (Hoechst 33342) in sodium citrate solution (2.3 mg mL^{-1} in water) for 15 minutes at room temperature on a silanised slide. The blastocyst was allowed to dry for 3 minutes and then allowed to stand for 5 minutes in 10 µl mounting medium with citifluor antifadent. After placing the coverslip, the slide was allowed to dry overnight in the dark at room temperature. Fluorescence from bisbenzimide (λ_{ex} 350 nm, λ_{em} 461 nm) and rhodamine (λ_{ex} 507 nm, λ_{em} 529 nm) were visualised using a fluorescence microscope at x250 magnification. Digital pictures were

taken using an Olympus Camera (Figure 9) and positive cells were counted using ImageJ software (version 1.33u, 2003; Wayne Rasband National Institutes of Health, USA). The apoptotic index was calculated as a ratio of the number of red (apoptotic) cells to the number of blue (live) cells.

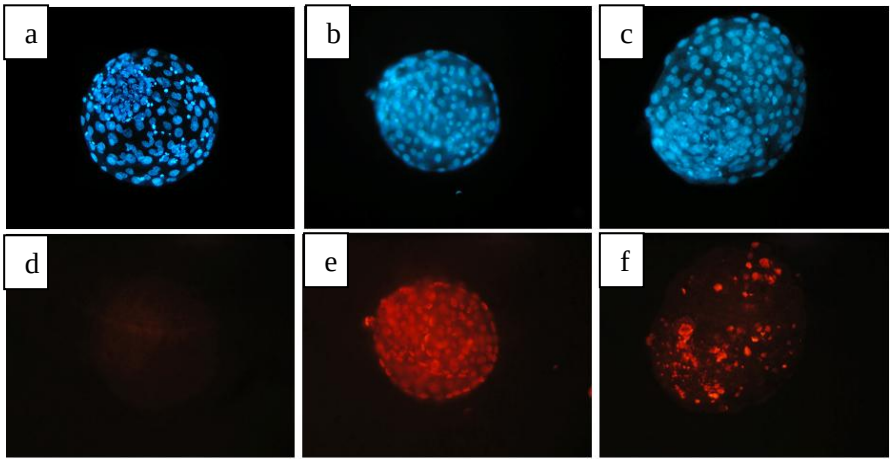


Figure 9 Apoptotic cells detected using TUNEL assay (for details see section 2.2.4). (a,d) a typical negative control, (b,e) positive control and (c,f) sample blastocysts visualised through the blue (a,b,c) and red (d,e,f) filter. X250 magnification. Blue is a nuclear stain (bisbenzimide), whilst red cells are apoptotic (rhodamine stain).

2.2.5 Pyruvate oxidation assay

The rationale behind the pyruvate oxidation assay and a comparison of the methodology used in this study with that of other published studies is outlined in Appendix 2 (page 207). Pyruvate oxidation was determined using individual blastocysts that formed on days 7 and 8 pi. Embryos were washed in pre-warmed modified embryo culture medium (4 mg mL^{-1} Fraction V BSA, 0.18 mg mL^{-1} glucose, 0.108 mg mL^{-1} sodium pyruvate, 0.146 mg mL^{-1} L-glutamine, 50 IU mL^{-1} penicillin, 50 µg mL^{-1} streptomycin in PBS, pH 7.2-7.4, 270-280 mOsmo) and then transferred in a 3µl drop and mixed with 3µl of radiolabelled mECM containing 185 becquerel (Bq) μL^{-1} of [1-^{14}C] pyruvate, specific activity 203.5 Bq $mmol^{-1}$) on the lid of a 1 mL microtube. The lids were placed onto microtubes containing 1 mL 0.1 M sodium hydroxide. Blanks were prepared excluding embryos. The sealed microtubes were incubated for 3 h at 38.5°C. Embryos were then washed and fixed in acetic acid and ethanol (3:1, v:v), and the contents of the microtubes transferred into scintillation vials containing 4 mL scintillation fluid (Emulsifier Safe, Packard) and 105 µL 0.5 M hydrochloric acid. The rate of carbon dioxide production was estimated from disintegrations per minute (dpm) detected in the sodium hydroxide trap as counted for 20 minutes using a beta counter.

Readings were corrected for blank variation and converted into picomoles h^{-1}, as described in the following equations:

Converting dpm to Bq:

$$Bq = \frac{dpm}{60}$$

Converting Bq to pmol h^{-1}:

$$pmol = \frac{Bq}{specific\_activity},$$

where specific activity = 0.2035 Bq/mmol,

$$pmol\_h^{-1} = \frac{pmol}{h}$$, where h=3.

2.2.6 Statistical analysis

Each batch was replicated on different days, using one or more microdrops of cleaved zygotes per treatment on each day. All parametric data were tested for normality before analysis by visual assessment of histograms followed by Anderson-Darling test (Minitab, Fifteenth Edition, 2006). For analysis of developmental efficiency, each microdrop was considered an experimental unit, and blastocyst development rates calculated within each drop. Rates of blastocyst formation were analyzed using a restricted maximum likelihood (REML) Generalised Linear Model (GLM) with binomial distribution and with batch as a random factor followed by Wald's test (Genstat, Eighth Edition, 2005). Parametric data for embryo quality (diameter, pyruvate metabolism per cell and cell count) were analysed using a REML Linear Mixed Model (LMM) with day of blastocyst formation as a covariate (Genstat, Eighth Edition, 2005). A Dunnett's test (Minitab, Fifteenth Edition, 2006) was used to compare treatment groups versus control. Non-parametric data (grade and stage) were analysed with Kruskal Wallis method (Genstat, Eighth Edition, 2005). IFNτ concentrations in media (pg mL^{-1}) from group cultures between 7 and 9 dpi and from individual cultures were log-transformed in order to normalise data. The relationship between blastocyst yield (percentage of blastocysts formed per oocytes in a drop) and IFNτ secretion in group cultures between 7 and 9 dpi were assessed using linear regression (Genstat, Eighth Edition, 2005). Given that blastulation was assessed every 24 h, the average time any blastocyst was in the culture medium was estimated to be 12 h. Data on IFNτ secretion in group culture between 7 and 9 dpi were analysed in the same way as parametric data for embryo quality, but with blastocyst yield as a co-variate. Data on IFNτ secretion in individual culture were analysed in the same way as parametric data for embryo quality, and day of blastulation, diameter, stage and grade were all checked as possible covariates and included when statistically significant. Data on

IFNτ secretion during individual culture from experiments 1 and 3 were compared with other parameters. Regression analysis was used to compare IFNτ secretion (log transformed) with apoptotic index (arcsine transformed) and with total cell count and diameter. The Kruskal Wallis test was used to compare IFNτ secretion with grade and stage. Differences were considered to be significant at $P < 0.05$. Data were displayed as means ± standard deviations (unless otherwise stated), except for grade and stage which were displayed as medians ± 95% confidence interval.

2.2.7 Experiment 1

2.2.7.1 Aim

The aim of experiment 1 was to determine whether roGM-CSF stimulated bovine blastocyst development between 1 and 3 dpi, as reported by Rooke et al. (2005) in ovine embryos. Additionally, this experiment aimed to establish whether varying the amount of roGM-CSF stimulated the rate of blastocyst formation, improved morphological quality, increased rate of development, increased IFNτ secretion up to the blastocyst stage, or reduced rate of apoptosis.

2.2.7.2 Experimental design

Cleaved embryos were selected 32 hpi and assigned in groups of 10 to 0.05 mL drops of SOF containing 2, 5, 10 or 50 ng mL^{-1} roGM-CSF (McInnes and Haig, 1991, Entrican et al. 1996) or an equal volume of medium supernatant derived from culture of Chinese hamster ovary (CHO) host cells (control) in which the roGM-CSF had not been expressed. It was established from a preliminary experiment that the CHO medium had no effect on embryo culture (data not shown). After 48 h, all blastocysts were transferred to control medium, so that exposure to roGM-CSF occurred only between 1 and 3 dpi. Blastocysts were removed from group culture on days 7 and 8 pi and transferred to individual 50 µl microdrops of SOF for 24 h. SOF microdrops were analysed for IFNτ using the MDBK cell cytopathic assay and the blastocysts underwent apoptosis detection. Blastocysts recovered on day 9 underwent total cell count. This experiment was replicated eight times.

2.2.7.3 Statistical analysis

The results were analysed as described in section 2.2.6 with the following modifications. After logarithmic transformation of the data, IFNτ concentrations in media (pg mL^{-1}) from individual culture were analysed by analysis of variance (ANOVA) with regression contrasts (Genstat, Eighth Edition, 2005). The day a blastocyst was removed from culture, diameter, cell count, apoptosis index, stage and grade were all checked as possible covariates and included in the analysis when statistically significant. Apoptotic Index was arcsine transformed in order to take into account variance bias from percentage data, and then log-

transformed in order to normalise the data prior to analysis by ANOVA with regression contrasts (Genstat, Eighth Edition, 2005).

2.2.8 Experiment 2

2.2.8.1 Experimental design

In order to determine whether early exposure to roGM-CSF rather than exposure throughout IVC affected IFNτ secretion and blastocyst development, 50 ng mL^{-1} roGM-CSF was absent (A) or present (P) during Early (1 to 3 dpi) or Late (3 to 9 dpi) periods of culture in three combinations: AA (no exposure), PA (early exposure only) and PP (early and late exposure), as summarized in Table 14. Day 7 and 8 blastocysts were removed from culture and assessed for pyruvate metabolism followed by cell numbers, whilst Day 9 blastocysts were only assessed for cell numbers. This experiment was replicated six times.

Table 14 Summary of the experimental design for Experiment 2. 50 ng mL-1 of roGM-CSF was present (√) or absent (x) in the early (days 1 to 3 post-insemination) and late (days 3 to 9 post-insemination) stages of embryonic development.

	Treatment		
	AA	**PA**	**PP**
Early (Day 1-3)	x	√	√
Late (Day 3-9)	x	x	√

2.2.8.2 Statistical analysis

The results were analysed as described in section 2.2.6.

2.2.9 Experiment 3

2.2.9.1 Aims

The aim of experiment 3 was to determine whether lower concentrations of roGM-CSF at different stages of development stimulated rate of blastocyst formation, improved morphological quality, increased rate of development, increased IFNτ secretion up to the blastocyst stage, and reduced rate of apoptosis.

2.2.9.2 Experimental design

Two or ten ng mL^{-1} roGM-CSF were absent or present during early and late periods of culture in five combinations: AA, 2PA, 10PA, 2PP and 10PP, as summarised in Table 15 (page 94).

To assess whether survival of the blastocyst over 24 h individual culture was affecting apoptosis or IFNτ secretion, criteria were set up to categorise blastocysts by visual assessment of the change in embryo morphology over the 24 h incubation period. Pictures were taken of the embryo before and after 24 h individual culture and visually analysed in terms of changes in developmental stage (Table 11, page 87) and diameter to assess whether the blastocyst progressed (category 1), remained the same (category 2) or degenerated/collapsed (3) over the individual culture period (Figure 10, page 95):

(1) **category 1**: increase in diameter by at least 2μm and/or progression by 1 stage in development, where morula, early, mid, expanding, expanded, hatching and hatched are the sequential stages;

(2) **category 2**: diameter changed by less than 2μm and reduced morphological quality was not evident;

(3) **category 3**: diameter changed by less than 2μm and embryo displayed signs of reduced morphological quality (such as cell degeneration or exclusion, or embryo collapse).

The drops were analysed for IFNτ as described in section 2.2.3 and the blastocysts underwent apoptosis detection as described in section 2.2.4. This experiment was replicated eight times.

Table 15 Summary of the experimental design for Experiment 3. 0, 2 and 10 refer to concentration of roGM-CSF (ng mL^{-1}) in culture media during 1 to 3 dpi (early) or 3 to 9 dpi of *in vitro* culture.

	Treatment				
	AA	2PA	2PP	10PA	10PP
Early (Day 1-3)	0	2	2	10	10
Late (Day 3-9)	0	0	2	0	10

2.2.9.3 Statistical analysis

The results were analysed as described in 2.2.6 with the following modifications. Rates of blastocyst formation were analyzed using a REML GLMM with binomial distribution and with batch as a random factor where the factors were stage of exposure (early and throughout) and concentration of GM-CSF (0, 2 or 10 ng mL^{-1}) followed by Wald's test of the main effects and the interactions (Genstat, Eighth Edition, 2005). Since numbers of blastocysts allocated to each individual culture treatment differed in each replicate, a multivariate linear REML model with batch as a random factor was used to analyse the data for IFNτ secretion, diameter, total cell count and apoptotic index, with stage of exposure (early and throughout) and concentration of GM-CSF (0, 2 or 10 ng mL^{-1}) as factors. Day of blastulation and change

in morphology were also used as random factors when assessing IFNτ secretion levels by individual blastocysts. The relationship between morphological change of the blastocyst over the 24 h individual culture with IFNτ secretion (log transformed) and apoptotic index (arcsine transformed to overcome scale effects) was analysed using GLM with the day the blastocyst was removed from culture as a covariate. An ANOVA followed by a Dunnett's test (Minitab, Fifteenth Edition, 2006) was used to analyse retrospectively whether treatment groups affected the change in morphology index (percentage of grade 1 embryos per total embryos graded in each batch) relative to control. REML GLMM with binomial distribution and with batch as a random factor followed by Wald's test of the main effect (Genstat, eleventh edition, 2008) was used to analyse the proportion of blastocysts secreting less IFNτ than the detection limit of the MDBK assay, and the proportion of blastocysts with no detectable apoptosis relative to total blastocysts.

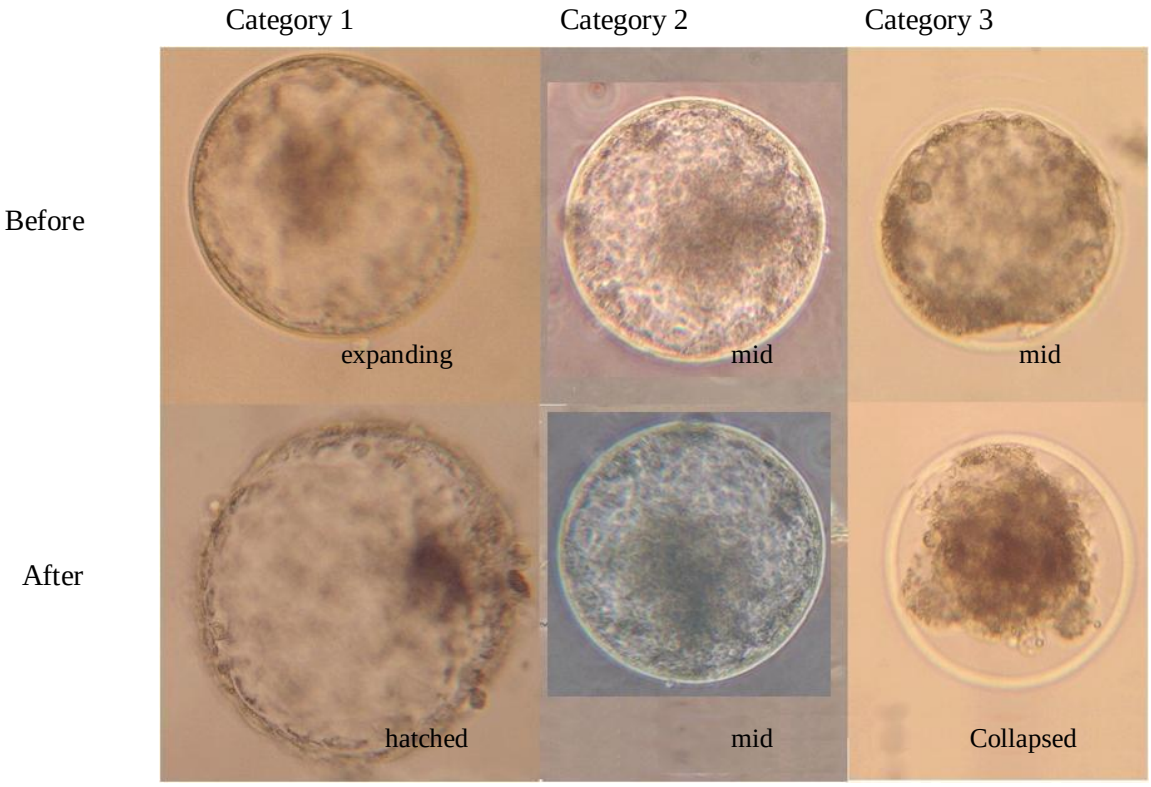


Figure 10 Morphological change over 24 h period, as assessed by change in morphology before and after the 24 h individual culture period.

2.3 Results

2.3.1 Experiment 1

2.3.1.1 Developmental efficiency

Varying the concentration of roGM-CSF which the embryo was exposed to during early culture had no significant effect on overall blastocysts formed per oocyte or rate of development (expressed as blastocyst formed on day 7 pi per overall blastocyst, Table 16, page 97).

2.3.1.2 Blastocyst characteristics

Varying the amount of roGM-CSF between 1 and 3 dpi did not significantly affect blastocyst characteristics, such as grade, diameter, cell number or the proportion of cells undergoing apoptosis (Table 17, page 98).

2.3.1.3 IFNτ protein concentration in culture drops

IFNτ protein concentration in media used to culture groups of embryos between 7 and 9 dpi and individual embryos for 24 h was unaffected by concentration of roGM-CSF in the culture medium during early culture (Table 18, page 98). Blastocyst yield explained some of the variation in IFNτ secretion in group culture between 7 and 9 dpi, with IFNτ levels increasing with blastocyst yield (P=0.04, Figure 11, page 97). Interestingly, IFNτ was detected in drops where no blastocysts were formed, suggesting that pre-blastocyst embryos may be capable of secreting IFNτ.

Table 16 Experiment 1: Effect of exposure of bovine IVP embryos to varying amounts of roGM-CSF between days 1 and 3 pi on blastocyst yield and rate of development. P=probability

Amount of roGM-CSF (ng mL^{-1})	**0**	**2**	**5**	**10**	**50**	**P**
Replicate drops	13	16	15	13	12	
Cleaved oocytes	130	160	150	130	120	
Overall blastocyst per zygote (%)	33±19	36±16	37±16	34±19	33±17	0.9
Day 7 blastocyst per overall blastocyst (%)	46±32	52±39	52±40	45±43	36±33	0.5

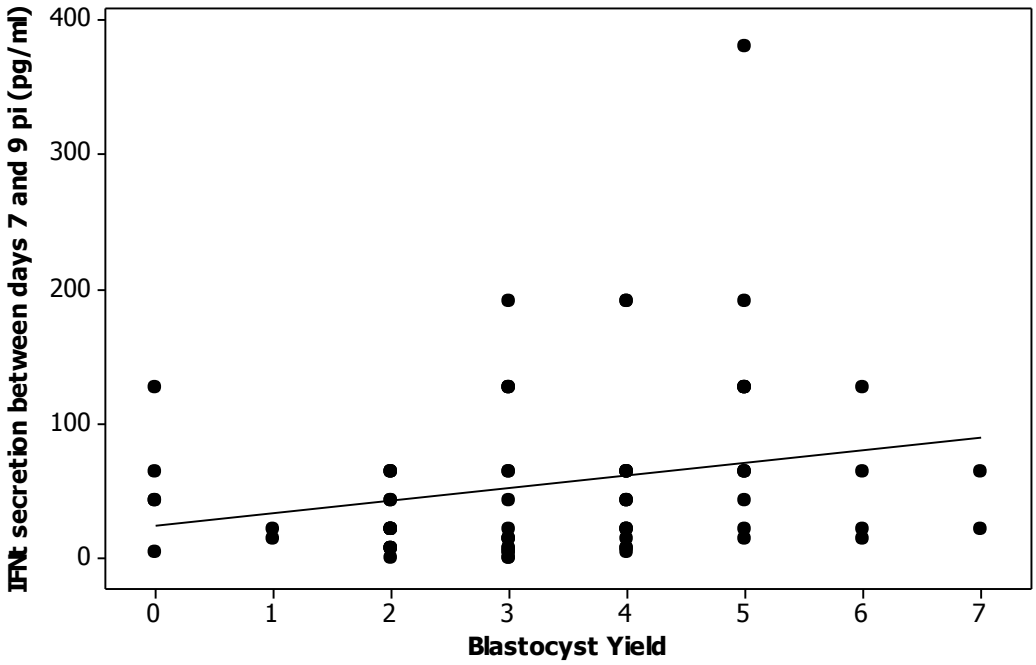


Figure 11 Experiment 1: Relationship between IFNτ secretion in culture medium from group cultured embryos between 7 and 9 dpi and blastocyst yield, y=9x + 25, P=0.04, n=69.

Table 17 Experiment 1: Effect of early exposure (1 to 3 dpi) of bovine IVP zygotes *in vitro* to varying concentrations of roGM-CSF on blastocyst grade, diameter, total cell count and apoptotic index as indices of blastocyst characteristics. Values are mean±standard deviation. Apoptotic index: proportion of cell number which are apoptotic.

Amount of roGM-CSF (ng mL^{-1})	**0**	**n**	**2**	**n**	**5**	**n**	**10**	**n**	**50**	**n**	**probability**
Grade (median)	2.0	50	2.0	56	2.0	50	2.0	46	2.0	44	0.8
Stage (median)	2	50	2	56	2	51	2	47	2	43	1.0
Diameter(μm)	188±20	48	191±25	56	193±23	49	195±21	44	192±18	40	0.7
Total cell count	137±30	9	139±38	14	158±53	8	138±42	6	153±61	4	0.3
Apoptotic Index (%)	9±6	9	9±5	14	10±7	8	8±4	6	10±6	4	0.7

Table 18 Experiment 1: Effect of early exposure (1 to 3 dpi) of bovine IVP embryos to varying amounts of roGM-CSF on IFNτ concentration. Values are mean±standard deviation.

Amount of roGM-CSF (ng mL^{-1})	**0 (control)**	**2**	**5**	**10**	**50**	**probability**
Replicate drops	12	15	14	13	11	
Secretion of IFNτ in group culture (days 7 to 9 pi, pg mL^{-1})	51±49	75±98	53±52	49±43	61±73	0.7
Replicate blastocysts	43	46	39	44	31	
Secretion of IFNτ per blastocyst in 24 h individual culture (pg mL^{-1})	24±31	30±45	24±43	31±36	35±51	1.0

2.3.2 Experiment 2

2.3.2.1 Developmental efficiency

Exposure of bovine IVC embryos to 50 ng mL^{-1} roGM-CSF, whether early (PA) or throughout culture (PP), had no significant effect on the overall number of blastocysts formed per cleaved zygote compared to control. When expressed as proportion of day 7 blastocysts per total blastocysts, rate of development was also unaffected by treatment (Table 19).

2.3.2.2 Blastocyst characteristics

Exposing bovine IVP embryos to 50 ng mL^{-1} of roGM-CSF, whether early (PA) or throughout culture (PP), also had no significant effect on blastocyst characteristics such as grade, diameter, total number of cells or amount of pyruvate oxidized (Table 20, page 100). Pyruvate oxidation was correlated with cell count (P=0.0006, Figure 12, page 101) but not with diameter (P=0.5). The correlation between pyruvate oxidation per cell and IFNτ secretion per cell approached significance (P=0.07, Figure 13, page 101).

Table 19 Experiment 2: Effect of timing of exposure of IVP zygotes to 50 ng mL^{-1} roGM-CSF on blastocyst yield: Control, Early (1 to 3 dpi) and Throughout (1 to 9 dpi). Blastocyst yield expressed as a percentage of cleaved zygotes. Values are mean±standard deviation.

Treatment	**Control**	**Early**	**Throughout**	**probability**
Replicate drops	21	26	23	
Cleaved zygote	210	260	230	
Overall blastocysts per zygote (%)	20±13	19±13	17±14	0.8
Day 7 blastocysts per total blastocyst (%)	41±40	56±36	41±35	0.7

2.3.2.3 IFNτ concentration in culture drops

No IFNτ was detected in medium from embryo culture before 7 dpi. Blastocyst yield was not a significant factor affecting variation in IFNτ secretion in group culture between 7 and 9 dpi (P=0.2). As in experiment 1, IFNτ secretion was detected in drops where no blastocysts were formed. IFNτ secretion between days 7 and 9 pi was unaffected by exposure to 50 ng mL^{-1} of roGM-CSF, whether early or throughout *in vitro* culture up to the blastocyst stage (Table 20, page 100).

Table 20 Experiment 2: Effect of timing of exposure of IVP zygotes to 50 ng mL $^{-1}$ roGM-CSF on blastocyst characteristics and IFNτ secretion: Control (AA), Early (PA, 1 to 3 dpi) and Throughout (PP, 1 to 9 dpi). Blastocyst characteristics measured included morphological grade, stage of development, diameter, total number of cells, pyruvate oxidation per blastocyst and per cell. Data are presented as mean±standard deviation.

Treatment	**Control**	**n**	**PA**	**n**	**PP**	**n**	**probability**
Grade (median)	2.5	43	2.25	45	2.5	37	0.4
Stage (median)	2	43	2	45	2	37	0.3
Diameter(μm)	169±19	43	175±18	45	169±12	36	0.2
Total cell count	123±43	42	117±38	44	119±34	33	0.9
Pyruvate oxidized (pmol blastocyst^{-1} h^{-1})	10±6	35	9±5	33	10±5	26	0.3
(pmol cell^{-1} h^{-1})	0.09±0.05	33	0.08±0.04	32	0.08±0.04	24	0.6
IFNτ secretion in group culture (days 7 to 9 pi, pg mL^{-1})	45±28	5	37±36	8	65±32	5	0.2

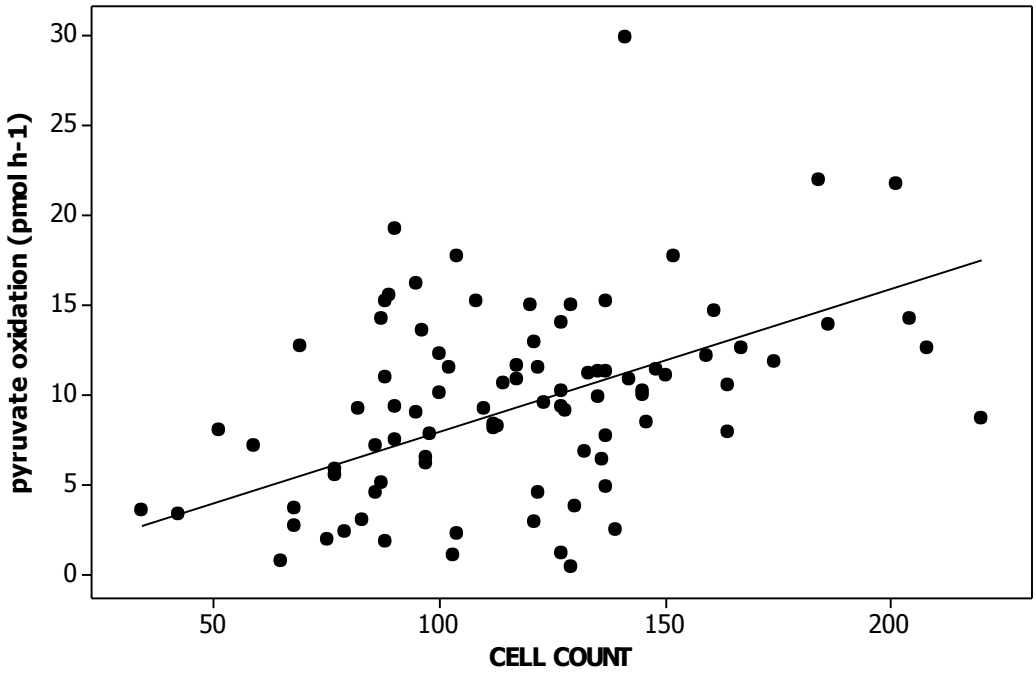


Figure 12 Experiment 2: Relationship between blastocyst cell number and pyruvate oxidation, y=0.06x + 2.77, P<0.001, n=94.

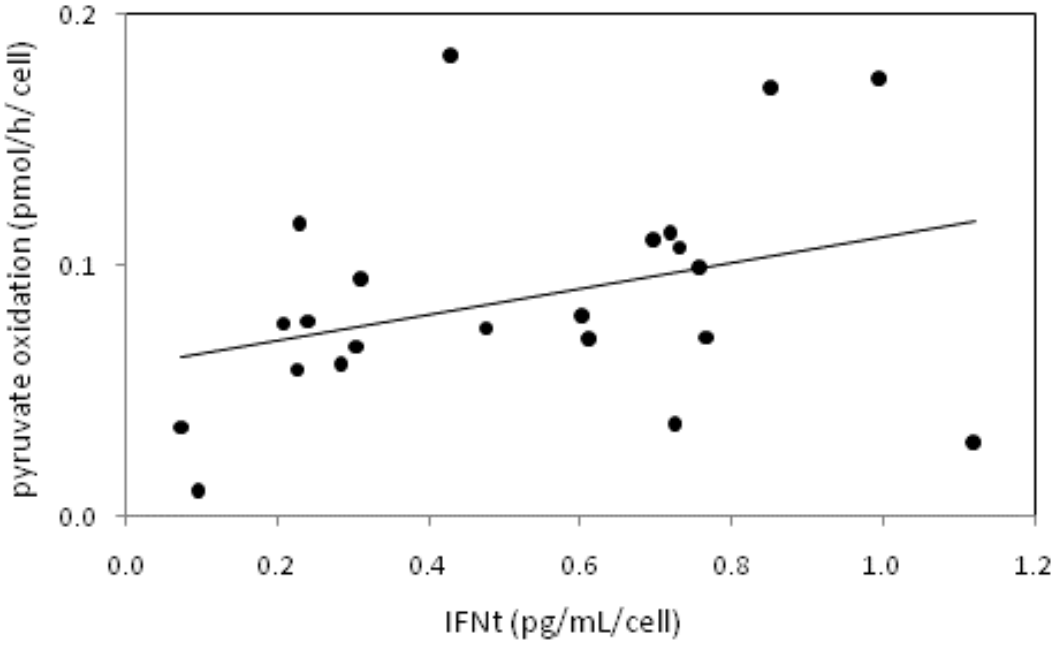


Figure 13 Experiment 2: Relationship between IFNτ secretion per cell and pyruvate oxidation per cell, y=0.07x+5, P=0.07, n=22, X: mean pyruvate oxidation for each IFNτ concentration.

2.3.3 Experiment 3

2.3.3.1 Developmental efficiency

Timing and amount of exposure of bovine IVC embryos to roGM-CSF had no significant effect on overall blastocyst yield or day 7 blastocysts per overall blastocysts compared to control (Table 21, page103).

2.3.3.2 Blastocyst characteristics

Varying the timing and amount of exposure of bovine IVP embryos to roGM-CSF had no significant effect on blastocyst characteristics, regardless of the day the embryos were collected from group culture (Table 22, page 104).

2.3.3.3 IFNτ secretion

Varying the amount of roGM-CSF and timing of exposure of bovine IVP embryos at different stages of development had no significant effect on IFNτ secretion, whether in individual blastocyst drop (Table 22, page 104) or in group culture (Table 23, page 105).

2.3.3.4 Morphological change of the embryos during 24 h individual culture

The majority (mean % of total blastocyst per batch ± SEM, 45±4%) of blastocysts progressed in development during 24 h individual culture (category 1), whilst 16±4% seemed unchanged (category 2) and 39±2% seemed to deteriorate (category 3). Category 1 blastocysts secreted significantly more IFNτ than category 3 and category 2 blastocysts ($P<0.05$, Figure 14a, page 106). A larger proportion of category 3 blastocysts (10%) secreted less IFNτ than the detection limit of the MDBK assay compared with category 1 (2%, $P<0.001$, Figure 14b, page 106) and all of the blastocysts categorised as category 2 secreted detectable amounts of IFNτ. There was no significant relationship between apoptotic index and morphological change index ($P=0.3$) and the proportion of blastocysts with no signs of apoptosis did not differ between the three categories ($P>0.05$). As analysed retrospectively, change in morphology index did not significantly vary between treatments ($P=0.8$).

2.3.3.5 IFNτ concentration in culture drops

Blastocyst yield explained some of the variation in IFNτ secretion in group culture between 7 and 9 dpi, with IFNτ levels increasing with blastocyst yield ($P=0.03$, Figure 15, page 106).

Table 21 Experiment 3: Effect of exposure of bovine IVP embryos to varying concentrations of roGM-CSF at different stages of development on blastocyst yield and rate of development. Early: roGM-CSF present between 1 and 3 dpi. Throughout: roGM-CSF present between 1 and 9 dpi. Throughout: roGM-CSF present between 1 and 9 dpi. Values are mean±standard deviation

Stage of Exposure	**Control**	**Early**		**Throughout**		**Probability**	
Amount of roGM-CSF (ng mL^{-1})	**0**	**2**	**10**	**2**	**10**	**Concentration**	**Stage**
Replicate drops	19	14	14	16	17		
Cleaved oocytes	190	140	140	160	170		
Overall blastocyst per zygote (%)	19±14	11±14	20±15	26±12	22±12	0.5	0.06
Day 7 blastocyst per overall blastocyst (%)	51±41	48±48	56±43	49±34	39±37	0.4	0.9

Table 22 Experiment 3: Effect of exposure of bovine IVP embryos to varying concentrations of roGM-CSF at different stages of development on blastocyst grade, diameter, total cell count and apoptotic index. Early: roGM-CSF present between 1 and 3 dpi. Throughout: roGM-CSF present between 1 and 9 dpi. Values are mean±standard deviation.

Stage of Exposure	**Control**		**Early**				**Throughout**				**Probability**	
Amount of roGM-CSF (ng mL^{-1})	**0**		**2**		**10**		**2**		**10**		**Concentration**	**Stage**
		n		**n**		**n**		**n**		**n**		
Grade (median)	2	24	2	14	2	23	1.9	34	2	30	0.3	0.3
Stage (median)	2	24	2	14	2	23	2	34	2	30	0.8	0.4
Diameter(μ)	196±19	24	192±22	14	192±29	23	192±25	35	193±18	30	0.9	0.9
Total cell count	114±33	20	114±33	7	94±35	14	114±35	19	110±44	22	0.3	0.4
Apoptotic Index (%)	2±4	18	4±4	7	7±9	12	3±5	18	4±5	21	0.2	0.2

Table 23 Effect of exposure of bovine IVP embryos to varying concentrations of roGM-CSF at different stages of development on IFNτ secretion. Early: roGM-CSF present between 1 and 3 dpi. Throughout: roGM-CSF present between 1 and 9 dpi. Values are mean±standard deviation.

Amount of roGM-CSF (ng mL^{-1})	**0**	**2**		**10**		**Probability**	
Stage of Exposure	**Control**	**Early**	**Throughout**	**Early**	**Throughout**	**Concentration**	**Stage**
Replicate drops (days 7 to 9 pi)	19	14	16	14	17		
Secretion of IFNτ in group culture (days 7 to 9 pi, pg mL^{-1})	68±80	12±12	83±105	34±70	39±44	0.9	0.7
Number of blastocysts	23	14	35	23	30		
Secretion of IFNτ per blastocyst in 24 h individual culture (pg mL^{-1})	19±20	13±11	26±43	21±36	25±43	0.6	0.06

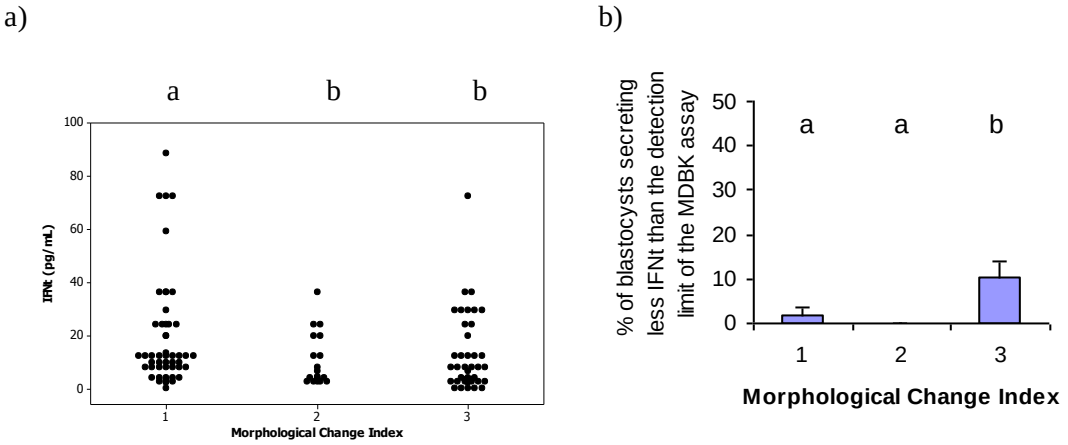


Figure 14 Morphological change index as assessed visually before and after 24 hour individual culture. Morphological change index: blastocysts progressed in development (category 1, n=53), no visual change in blastocyst development (category 2, n=19), blastocysts showed signs of degeneration or collapse (category 3, n=43). This experiment was replicated eight times (blastocysts derived from eight batches of ovaries). a) Individual value plot of IFNτ (pg mL^{-1}) versus morphological change index (P=0.03). Values are mean (pg mL^{-1}) ± SEM: Category 1: 23±4 (n=52), Category 2: 10±2 (n=19), Category 3: 22±7 (n=43). b) Proportion of blastocysts in each morphological change category which did not secrete IFNτ (P<0.001). Values are mean % of total blastocyst per batch ± SEM. Category 1: 2±2%, Category 2: 0%, Category 3: 10±4%. Different superscripts over data bars represent significant differences among treatments (P<0.05).

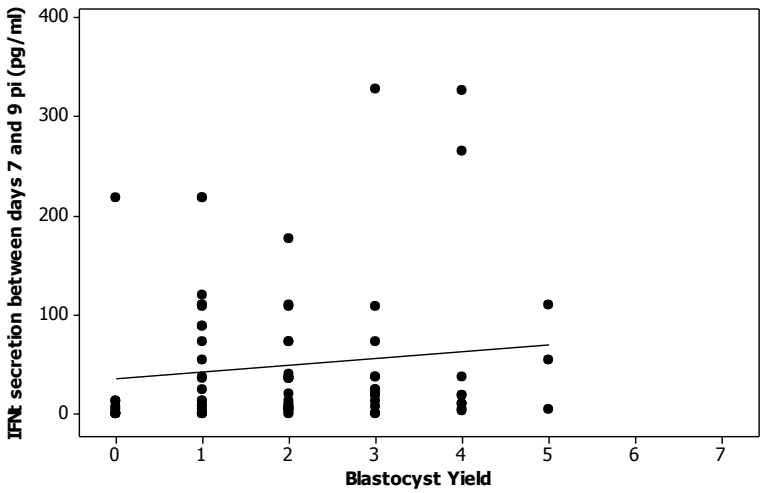


Figure 15 Relationship between IFNτ secretion in culture medium from group cultured embryos between 7 and 9 dpi and blastocyst yield, y=7x + 36, P=0.03, n=80.

2.3.4 Overall: IFNτ concentration in culture drops

The relationship between blastocyst yield and IFNτ secretion in group culture between days 7 and 9 pi for all three experiments are summarised in Figure 16 (page 107). When data from experiments were combined, IFNτ secretion was detected in drops where no blastocysts were formed, suggesting that pre-blastocyst embryos may be capable of secreting IFNτ. The trend for IFNτ concentration to increase with blastocyst yield was observed in all experiments, although this increase was not significant in experiment 2. In all three experiments, IFNτ secretion per blastocyst in group culture medium between 7 and 9 dpi decreased with increasing blastocyst yield (P<0.001, Figure 17, page108).

IFNτ secretion in experiments 1 and 3 did not correlate with apoptotic index (P=0.1), cell number (P=0.3), diameter (P=0.2) or grade (P=0.2). Day of blastulation, however, did affect IFNτ secretion, with embryos that blastulated on day 7 pi secreting less IFNτ than embryos that blastulated on day 8 pi (P=0.002, Figure 18, page 108).

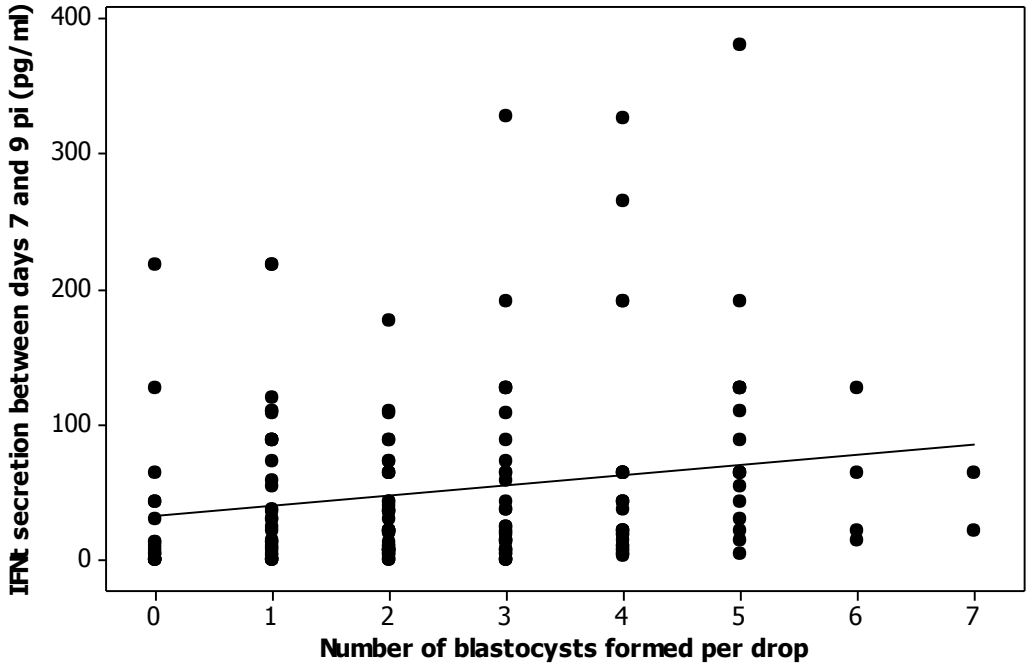


Figure 16 Relationship between IFNτ secretion in culture medium from group cultured embryos between 7 and 9 dpi and blastocyst yield, in experiments 1, 2 and 3 (y=8x+32), P<0.001, n=219.

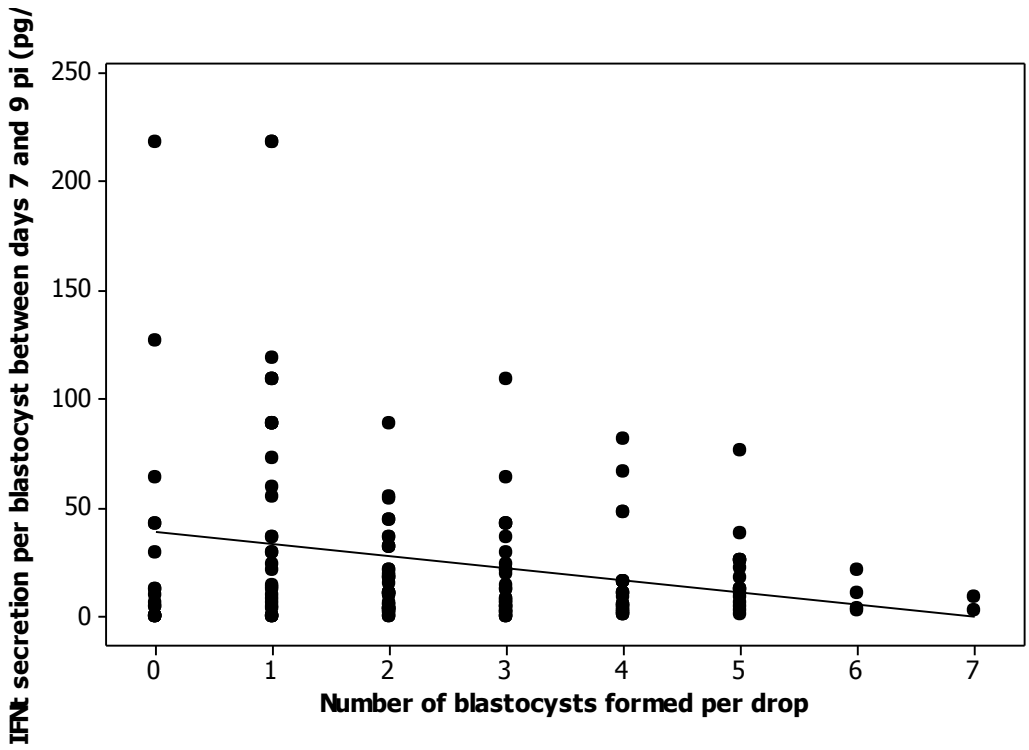


Figure 17 Relationship between IFNτ secretion per blastocyst in culture medium from group cultured embryos between 7 and 9 dpi and blastocyst yield, in experiments 1, 2 and 3 (y=-6x+39, n=219).

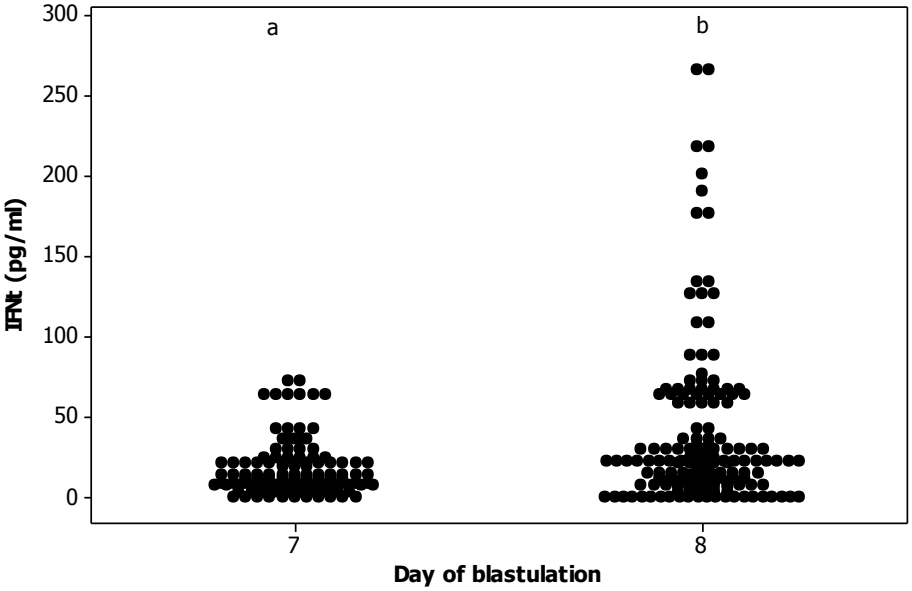


Figure 18 Day of blastocyst formation versus IFNτ secretion in experiments 1 (Effect of dose response of GM-CSF on embryo development) and 3 (Effect of concentration and stage of development on embryo development). Different superscripts over data represent differences between treatments (P<0.05), n=328.

2.4 Discussion

2.4.1 Bovine embryos were unresponsive to roGM-CSF

Bovine embryos were unresponsive to roGM-CSF in terms of blastocyst yield, rate of development to the blastocyst stage, rate of pyruvate oxidation, apoptosis and IFNτ secretion, irrespective of amount and timing of exposure.

2.4.1.1 Effect of GM-CSF on blastulation

Control embryos in experiments 1, 2 and 3 developed in the absence of an external source of growth factors. Also, GM-CSF null mutant embryos were capable of forming blastocysts (Robertson et al., 1999), suggesting that GM-CSF is not essential for blastulation to occur in mammalian embryos.

In experiments 2 and 3, roGM-CSF had no effect on blastocyst yield when embryos were exposed to roGM-CSF from cleavage to blastulation, or from cleavage to before compaction. In contradiction, in other mammalian embryos, rGM-CSF exposure was repeatedly associated with an increase in blastocyst yield (human: Sjoblom et al., 1999, porcine: Cui et al., 2004), although not consistently (murine: Robertson et al., 2001). Discrepancies in whether GM-CSF has an effect on blastocyst yield between different studies is unlikely to be only due to species differences, given that de Moraes and Hansen (1997) demonstrated that addition of rbGM-CSF to culture medium caused a two-fold increase in the proportion of IVP bovine zygotes that reached the blastocyst stage. Therefore, other factors are responsible for whether mammalian embryos respond to rGM-CSF in terms of blastulation.

2.4.1.2 Effect of GM-CSF on pyruvate oxidation

The level of pyruvate oxidation reported in this study for blastocysts (between 0 and 30 pmol h^{-1}, with an average of 10 pmol h^{-1}) is in the same order of magnitude as pyruvate metabolism levels reported with sheep (approximately 38 pmol h^{-1} by Garner and Batt, 1991, 16 pmol h^{-1} by Thompson et al., 1993 and 12 pmol h^{-1} by Rooke et al., 2005) and with bovine embryos (approximately 12 pmol h^{-1}; Khurana and Niemann, 2000a).

This is the first study to assess the effect of rGM-CSF on pyruvate oxidation. In the present study, exposure of bovine embryos to roGM-CSF had no effect on pyruvate oxidation. Moreover, pyruvate oxidation correlated with total number of cells. Increased cell proliferation and increased rate of development have been positively correlated with increased metabolic activity in murine embryos (Robertson et al., 2001). Therefore, given that this study observed no effect of roGM-CSF on total number of cells or rate of development, it follows that pyruvate oxidation was also unaffected by roGM-CSF.

2.4.1.3 Effect of GM-CSF on apoptosis

The range of apoptotic index detected in the present study (0 to 10%) is comparable to previous results on TUNEL assays on bovine embryos (1 to 10% Byrne et al., 1999, 0 to 17% Park et al., 2006, 0 to 29% Makarevich and Makkula, 2002).

In experiments 1 and 3, exposure to roGM-CSF did not affect the frequency of apoptotic cells per total cells in bovine embryos. Although the effect of rGM-CSF on apoptosis in bovine embryos has not previously been reported, Karagenc et al. (2005) also observed no relationship between apoptosis and rGM-CSF exposure in murine embryos. In contradiction, other studies have reported a decrease in apoptosis associated with exposure to rGM-CSF (murine: Behr et al., 2005, human: Sjoblom et al., 2002), and both studies have used the TUNEL assay, the same method of detection of apoptosis as in the present study. A possible explanation for the inconsistency between different studies is that GM-CSF is not the only factor which can affect rate of apoptosis. Differences in experimental conditions between the three studies could lead to different levels of stress, as described in section 2.4.2.1, which would subsequently lead to different levels of apoptosis. Other death factors, such as TNFα are able to bypass the Bcl-2 family pathway (the apoptosis pathway regulated by GM-CSF) by directly activating the caspase cascade (Earnshaw et al., 1999). Therefore, in the presence of TNFα, GM-CSF would have no effect on apoptosis. In similar ways, differences in other factors due to differences in experimental conditions, could also account for differences on whether GM-CSF has an effect on rate of apoptosis. Therefore, although apoptosis may be modulated by rGM-CSF, it must be recognized that apoptosis may also be modulated by other factors, which may explain why some studies report an effect of GM-CSF on apoptosis, whilst others do not.

2.4.1.4 Effect of GM-CSF on IFNτ secretion

The concentration of IFNτ detected in individual culture droplets in this study (pg mL^{-1}) was lower than reported in other studies (Appendix 3, page 208). This may be due to differences in biological activity of the standards used in different studies which used the MDBK cell cytopathic assay to measure levels of IFNτ secretion (as discussed in section 1.2.4.1). According to Dr. Alan Ealy (personal communication) the IFNτ standard used in the MDBK assay in this study (5.3×10^9 IU mg^{-1}) was 10 to 50 times more active than the standards previously used (5.4×10^7 IU mg^{-1}, Kubisch et al., 1998, 2001a,b, 2004, 7.5×10^7 IU mg^{-1}, Kimura et al., 2004a), which would account for the 100 fold difference in amount of IFNτ detected in these studies relative to the current study. Moreover, it is possible that MDBK assays set up in different labs have different detection limits. Neira et al. (2007) reported a detection limit of 54 pM, whilst the detection limit in the current study was 2.2 pg mL^{-1}

(equivalent to 0.1 pM), which might explain the lower concentrations detected in the current study compared to that detected by Neira et al. (2007) and Hernandez-Ledezma et al. (1992, 1993). In contrast to the MDBK assay used in the current study, Rooke et al. (2005) used an ELISA to detect IFNτ. The ELISA measures the amount of protein and makes no distinction between biologically active and inactive isoforms of IFNτ, whilst the antiviral assay measures the antiviral activity rather than the amount of protein. The 100 fold difference in amount of IFNτ detected in the current study which uses antiviral activity and ELISA may be explained by the fact that the two assays are measuring different aspects of IFNτ. Therefore, differences in the methodologies used between different studies may account for differences in IFNτ secretion levels between different studies.

According to the present study, exposure of bovine embryos to roGM-CSF had no effect on IFNτ secretion, which is in agreement with de Moraes et al. (1997). However, in ovine embryos, Rooke et al. (2005) and Imakawa et al. (1993) observed that rGM-CSF exposure caused an increase in IFNτ secretion. It is often assumed that early ovine and bovine embryos have similar biochemistry and physiology, so that differences in their response to rGM-CSF were unexpected. Discrepancies between these studies may be due to species differences or due to other factors affecting the effect of rGM-CSF on IFNτ secretion .

2.4.1.5 Factors affecting IFNτ secretion

In the current study, IFNτ secretion levels varied 100 fold. High variation in IFNτ secretion has also been reported by Kimura et al. (2004b) who observed variation in three orders of magnitude, suggesting that other environmental (discussed in section 1.5.2) and genetic (discussed in section 1.5.1) factors affect IFNτ secretion by blastocysts. In group cultured drops, blastocyst yield explained some of the variation in IFNτ secretion, so that IFNτ secretion increased with numbers of blastocysts. However, IFNτ secretion per blastocyst decreased with increasing blastocyst yield. The cause of this decrease in IFNτ remains unknown, but it is unlikely to be due to a negative feedback given that blastomeres do not possess receptors to bind IFNτ (Han et al., 1997).

In both experiment 1 and 3, day of blastulation affected IFNτ secretion, with embryos that blastulated 7 dpi secreting less IFNτ than blastocysts that blastulated 8 dpi. This is in agreement with Kubisch et al. (1998) and Larson et al. (2001) who also observed that earlier forming blastocysts were lower producers of IFNτ than later forming blastocysts (as discussed in section 1.6.2.3). One possible explanation is that the secretion of IFNτ is initiated at a specific time pi, independent of developmental stage (as suggested by Kubisch

et al., 1998). In support of this hypothesis, IFNτ was not detected in culture medium before day 7 pi but was detected in drops where no blastocysts were formed, suggesting that pre-blastocyst embryos may be capable of secreting IFNτ, but only from 7 dpi.

In order to collect data on IFNτ secretion per embryo, blastocysts were cultured individually for 24 h (as described by Kubisch et al., 2001a and Hernandez-Ledezma et al., 1992). However, pre-attachment embryos produce factors which can stimulate their own development (O'Neill, 1997) so that culturing embryos individually, rather than in groups, may result in inferior development and reduced cell number, as demonstrated in mice (Lane and Gardner, 1992), sheep (Gardner et al., 1994), humans (Jones et al., 1998) and cows (O'Doherty et al., 1997). However, the beneficial effects of culturing bovine embryos in groups on cell number and hatching ability were only observed when embryos were cultured in groups prior to blastocyst formation (Larson and Kubisch, 1999), so that the individual culture of blastocysts in the current study should not have impaired embryonic development. However, Larson and Kubisch (1999) also reported that group cultured blastocysts secreted more IFNτ than individually cultured blastocysts, so that individual culture may impact some aspects of development. In order to assess whether individual culture affected the ability of bovine embryos to secrete IFNτ in the current study, a system was set up to categorise blastocysts in terms of progression in development during individual culture based on visual assessment of the change in morphology over 24 h (described in 2.2.9.2). Category 1 blastocysts (blastocysts that progressed in development during 24 h individual culture) secreted more IFNτ compared to categories 2 (remained the same over 24 h individual culture) and 3 (blastocysts showed signs of degeneration during 24 h individual culture), whilst a larger proportion of category 3 blastocysts secreted less IFNτ than the detection limit of the MDBK assay, compared to categories 1 and 2, suggesting that the criteria set up in this study to assess change in embryo morphology over 24 h may be an indicator of embryo viability. However, IFNτ secretion was detected in 88% of category 3 blastocysts suggesting that either category 3 blastocysts secreted IFNτ before degrading, or that embryos that looked degenerated were not in fact dead. For instance, collapsed blastocyts have no visible cavity giving the impression of being degenerated. Blastocyst collapse is caused by changes in the fluxes of water into and out of blastocysts triggered by the partial breakdown of tight junctions necessary for fluid accumulation in the blastocoele (Bigger et al., 1988). There is normally a re-expansion phase 1 to 2 h after collapse (Fugger, 1999) so that the blastocyst may exhibit a pulsatile activity. This cyclic collapse and re-expansion of the blastocyst *in vitro* was reported by several studies using cinematography with mice (Cole et al., 1967) and bovine embryos (Massip et al., 1980, 1982) but has not been investigated

sufficiently either because of lack of interest or because it is difficult to investigate (Massip, 2001). Embryos have been observed to collapse particularly when exposed to temperature changes (Farin, 1999) suggesting that collapse is a stress response. However, Farin also observed that pregnancy rates from collapsed blastocysts transferred into cows seemed to be similar to those that had not collapsed. Therefore, it is unclear whether the collapse of a blastocyst is normal or an indication of its poor quality due to mishandling of the embryos. Nevertheless, this phenomenon introduces background noise into the criteria used in this study to assess survival of the blastocyst as it is difficult to distinguish a degenerating blastocyst from a collapsed blastocyst. Collapsed blastocysts might account for category 3 blastocysts which secreted high levels of IFNτ or that had low levels of apoptosis. Embryos may degenerate functionally, but still be graded as progressing in development based in morphology, so that the criteria set up in the current study to assess change in embryo morphology over 24 h have the disadvantage of relying on visual assessment of embryos which could be deceiving. Also, it is hard to validate these criteria to differentiate between collapsed and degenerated embryos. However, being a quick, simple and non-invasive assessment which does not affect the viability of the embryo suggests that this method could be a useful tool for selection of attachment competent embryos. However, these criteria would need to be assessed for future embryo development *in vivo* before being used as a diagnostic tool to select embryos with the best chance of successfully implanting and developing into a successful pregnancy. Despite the pros and cons of using change in morphology index as an index of survival, it explains part of the variation in IFNτ secretion. However, since change in morphology index did not vary between treatments in experiment 3, variation in change of morphology index did not explain the lack of an effect of GM-CSF on IFNτ secretion.

IFNτ secretion did not correlate with apoptotic index, total cell count, diameter, morphological grade or stage of development, suggesting that IFNτ is not associated with embryo viability.

Although day of blastulation, change in morphology index and blastocyst yield were identified as sources of variation in IFNτ secretion, high probability values in the current study strongly suggest that GM-CSF was not a factor, irrespective of the timing and amount of exposure. Unexplained variation in IFNτ secretion levels may be due to factors not assessed in the current study, such as genetic factors (maternal and paternal) and sex.

2.4.2 Possible reasons for discrepancies between studies on the effect of GM-CSF on embryo development

The lack of an effect of GM-CSF on bovine embryo development (blastulation, morphology, rate of metabolism, apoptosis and IFNτ secretion) reported in this study is in contradiction with previous studies reporting a dose-dependent response of mammalian embryos to rGM-CSF (reviewed in section 1.7.1.3). The current study differs from previous studies in the concentration of GM-CSF used and the species homology of the rGM-CSF, the timing of exposure to GM-CSF in terms of the stage of embryo development, the embryo production system and the species assessed.

2.4.2.1 Effect of amount of GM-CSF on response of embryos to roGM-CSF

The range in concentrations of roGM-CSF exposed to embryos in the current study (2 to 50 ng mL^{-1}) had no effect on any of the embryo development parameters measured, which contradicts previous studies in mammalian embryos (reviewed in section 1.7.1.3).

Imakawa et al. (1993) found that rhGM-CSF stimulated IFNτ secretion from ovine embryos at lower concentrations (3.3 to 6.6 ng mL^{-1}), but not at higher concentrations (13.2 ng mL^{-1}). Meanwhile, Robertson et al. (2001) observed a 15% decrease in the rate of murine blastocysts hatching and attaching at the increased dose of 10 ng mL^{-1} relative to 2 ng mL^{-1} rmGM-CSF. In another murine study published after the current study was conducted, Behr et al. (2005) found that 0.125 ng mL^{-1} promoted blastulation, but increased concentrations to 2 ng mL^{-1} in the medium did not further promote development to the blastocyst stage, and embryonic development appeared to be inhibited when the rGM-CSF concentrations reached 2 ng mL^{-1} compared to 0.125 ng mL^{-1}. Loureiro et al. (2008) exposed bovine embryos to 1, 10 and 100 ng mL^{-1} roGM-CSF and found that the greatest effect of roGM-CSF on blastocyst yield occurred at 10 ng mL^{-1}. As demonstrated by these studies, increased concentrations of rGM-CSF after a certain threshold did not further promote embryo development, and this threshold may vary from species to species.

The cellular and molecular mechanisms through which an excess concentration of rGM-CSF may cause desensitization to the actions of rGM-CSF itself may result from the mechanism of internalisation and cellular processing by which GM-CSF normally mediates its effects. Both murine (Robertson et al., 2001) and human embryos (Sjoblom et al., 2002) synthesise GM-CSFR from fertilisation to the blastocyst stage, with the receptor protein being detectable on the cell membrane of both trophectoderm and inner cell mass cells. The process of internalisation of GM-CSFR has not been assessed in embryo studies, but was well documented in cell culture systems. In a murine leukaemia cell line, after GM-CSF

bound to GM-CSFR on the cell membrane, invagination of the plasma membrane formed receptosomes which then fused with lysosomes. GM-CSF then became disassociated from the receptor, thus releasing the receptor (Walker and Burgess, 1987). In a human myeloid cell line, by inducing internalisation of its own receptor, rGM-CSF exposure led to a decrease in the total number of surface-bound GM-CSFR (Cannistra et al., 1990). Through this negative feedback, GM-CSF significantly down-regulated its own receptor, so that an excess of GM-CSF could lead to down regulation of the stimulatory effects of GM-CSF on the cell. In the murine system, the process of receptor internalisation and degradation takes approximately 45 minutes and within 1 h from down-regulation of GM-CSFR, around 40% of internalised receptors were reacquired to the cell surface by means of recycling of internalised receptors to the cell surface (around 15%) and resynthesis (around 25%), rather than due to the presence of an intracellular pool of receptors (0%, Walker and Burgess, 1987). Walker and Burgess (1987) only followed the fate of the GM-CSFR up to 4 h, after which the methodology used caused unacceptable levels of cell death. In that period, the re-expression of GM-CSFR continued to increase and did not plateau. The current study exposed embryos to rGM-CSF for a much longer period (two to eight days). Therefore, it is possible that during the 2 to 8 day exposure to rGM-CSF, the recycling and resynthesis of GM-CSFR may match or exceed the loss of GM-CSFR due to internalisation. Also, it must be considered that there may be differences in the rate of internalisation and reaquirement of receptors between different species and/or between different culture systems which might help explain discrepancies between different studies.

The range of concentrations assessed in experiment 1 (2 to 50 ng mL^{-1}) were within the range assessed in previous studies (0.4 to 100 ng mL^{-1}, reviewed in section 1.7.1.3). Doses two-fold higher (100 ng mL^{-1}) than the dose reported in this study (50 ng mL^{-1}) increased cell proliferation and IFNτ secretion in a bovine trophectoderm cell line (Michael et al., 2006 b) and increased blastocyst yield in bovine embryos (Loureiro et al., 2008). However, cells from a cell culture (Michael et al., 2006 b) and cells in a blastocyst (current study) may have different thresholds in concentration for a response to GM-CSF. Nevertheless, de Moraes and Hansen (1997) and Loureiro et al. (2008) observed that bovine embryos responded to 10 ng mL^{-1} rbGM-CSF, so that it is unlikely that the range in concentration assessed in this study (2 to 50 ng mL^{-1}) was outside the range of concentrations for rGM-CSF to affect bovine embryo development.

Moreover, results from experiments 1 and 3 which exposed embryos to lower concentrations of roGM-CSF further suggest that the high concentration of roGM-CSF in experiment 2 (50

ng mL^{-1}) did not account for the lack of a response of bovine embryos to roGM-CSF. Therefore, other factors must be responsible for differences between studies in the response of mammalian embryos to rGM-CSF.

2.4.2.2 Effect of timing of exposure of rGM-CSF on response of embryos to rGM-CSF

In experiment 1, the embryos were exposed to roGM-CSF between 1 and 3 dpi, when the embryos were between the two- and eight-cell stage, whilst in experiment 3 the embryos were exposed to roGM-CSF between the two-cell and the blastocyst stage, and neither experiment detected a response of bovine embryos to roGM-CSF. Other studies which detected beneficial effects of rGM-CSF exposure on embryo development exposed embryos to rGM-CSF later in development (Imakawa et al., 1993 exposed ovine embryos to rhGM-CSF during elongation, de Moraes et al., 1997 exposed bovine embryos to rbGM-CSF from blastulation to attachment, and Robertson et al., 2001 exposed murine embryos to rmGM-CSF from the eight-cell stage to after blastulation), suggesting that exposure of bovine embryos to roGM-CSF in this thesis may have been too early for an effect to be detected.

Exposure of bovine embryos to roGM-CSF from day 5 pi increased blastocyst yield, whilst roGM-CSF did not stimulate blastocyst development when exposed from day 1 pi (Loureiro et al., 2008). Similarly, de Moraes and Hansen (1997) observed that exposure of bovine embryos from day 5 pi, as opposed to up to day 5 pi, increased blastocyst yield. Therefore, rGM-CSF exerts its effects on bovine embryos at the morula stage or later, mimicking the exposure of bovine embryos to GM-CSF in vivo, where GM-CSF concentration is higher in the uterus than in the oviduct (de Moraes et al., 1999). Moreover, exposure of bovine embryos to rGM-CSF before day 5pi seems to suppress the response of bovine embryos to rGM-CSF. Further studies are necessary to determine whether bovine embryos differ in the level of GM-CSF receptor between different stages of development, and whether exposure to GM-CSF earlier in development affects the levels of receptors after day 5pi.

Rooke et al. (2005) observed that exposing ovine embryos to roGM-CSF between 1 and 3 dpi increased IFNτ secretion, suggesting that, contrary to bovine studies, roGM-CSF exerts an effect on ovine embryos at the two- to eight-cell stage. Therefore, there seems to be a species difference in the stage-specific response of embryos to GM-CSF exposure.

Previous studies which observed an effect of GM-CSF on different aspects of mammalian embryo development (reviewed in section 1.7.1.3) differed from the present study in terms

of embryo production system, GM-CSF homology and species, so that one or more of these factors may explain the lack of a response of bovine embryos to roGM-CSF.

2.4.2.3 Effect of culture conditions on response of embryos to rGM-CSF

de Moraes and Hansen (1997) observed that GM-CSF stimulated blastocyst formation in embryos cultured in Charles Rosenkrans medium (CR1, Rosenkrans et al., 1993), whilst in the current study, which used modified SOF medium, GM-CSF did not affect blastocyst yield. CR1 contains glutamine, SOF does not, and the concentration of BSA in SOF is higher than in CR1, so that the compositions of CR1 and SOF media differ slightly. Sagirkaya et al. (2006) compared bovine embryo development in CR1 and in SOF and found that CR1 medium was associated with higher levels of HSP70 transcript levels, a gene associated with stress. Moreover, fewer blastocysts were formed from putative zygotes cultured in CR1 compared to SOF. Although the composition of the SOF used in the current study was slightly different to the one used by Sagirkaya et al. (2006) in terms of sodium lactate, sodium pyruvate, D-glucose and L-glutamine (Sagirkaya et al., 1999 vs current study: 3.3 vs 9.9 mM, 0.4 vs 0.99 mM, 0 vs 1.5 mM, 0 vs 1 mM respectively), these findings suggest that different culture medium cause different levels of stress on embryos. Therefore, different levels of stress caused by culture in different media may explain discrepancies between different studies on the response of embryos to GM-CSF exposure.

Despite the large volume of studies attempting to optimise current embryo culture media compositions, conditions in *in vitro* culture conditions are still suboptimal to the *in vivo* environment. Nevertheless, the effect of GM-CSF on embryo development *in vitro* should be examined under culture conditions that would incur minimal stress to the embryo. However, studies in other mammals which have reported beneficial effects of GM-CSF on embryo development have also employed culture conditions that were sub-optimal. Kim et al. (2001) used a medium throughout culture which was designed to support murine embryos in the pre-compaction stage, whilst other studies (Wang et al., 2002, Behr et al., 2005) used medium throughout culture designed to support murine embryos in the post-compaction stage (Karagenc et al., 2005). Karagenc et al. (2005) compared the response of murine embryos to rmGM-CSF in a range of media formulations (the simple G1 or human tubal fluid (HTF), or a more complete G1/G2 sequential media) and found that exposure to rmGM-CSF from the eight-cell stage to the blastocyst stage had no beneficial effect on murine embryo development when protein (human serum albumin) was present in the media. However, when human serum albumin was absent from G1/G2 media, supplementation of GM-CSF led to an increase in blastocyst cell number and inner cell mass development. Similarly in the porcine system, Cui et al. (2004) observed an increase in the rate of

blastocyst formation and blastocyst cell number in protein-free culture medium, whilst no beneficial effect of rmGM-CSF were observed in the presence of BSA. Since absence of protein is stressful for the embryo (Rizos et al., 2003), it was suggested that the extent of the beneficial effects of rGM-CSF on embryos varies with the level of stress an embryo is exposed to (Karagenc et al., 2005).

Exposure of embryos to rGM-CSF was shown to be beneficial to human embryos (Sjoblom et al., 1999) but not to murine embryos (Karagenc et al., 2005), even though both studies used human serum albumin as the source of protein. Meanwhile, exposure of embryos to rGM-CSF was shown to be beneficial to bovine embryos (de Moraes and Hansen, 1997) but not to porcine embryos (Cui et al., 2004), even though both studies used BSA as the source of protein. Beneficial effects of rGM-CSF exposure on murine embryo development were also detected in culture media containing FCS (Robertson et al., 2001) and serum substitute (Kim et al., 2001, Wang et al., 2002, Behr et al., 2005), whereas addition of serum to the culture medium, while stimulatory, prevented additional stimulatory effects of rGM-CSF in bovine embryos (de Moraes and Hansen, 1997), possibly due to the fact that serum may contain GM-CSF, amongst other factors beneficial to embryo development. Therefore, there have been several differences between reports of whether GM-CSF supplementation had beneficial effects in medium containing a range of protein sources, so that the type of protein supplement added to the media is a key factor in determining the response of embryos to GM-CSF supplementation to the culture medium.

Sjoblom et al. (1999) used frozen-thawed embryos, so that the stress induced by the freeze-thaw process might have contributed to the beneficial effects of rhGM-CSF observed on human embryo development (Karagenc et al., 2005). Therefore, besides differences in the composition of the media or the protein source, the general quality of the culture conditions may also explain discrepancies between studies.

Karagenc et al. (2005) observed the effect of GM-CSF on embryos derived from two strains of mice which were previously shown to have different sensitivities to their culture environment and found that GM-CSF only had a beneficial effect on the more sensitive of the two strains. Therefore, the sensitivity of the breed to *in vitro* culture conditions in general has also been shown to have an effect on whether an embryo is responsive to GM-CSF exposure.

As with GM-CSF, another cytokine involved in embryo development, CSF-1, was shown to be stimulatory only to slower developing murine embryos (Pampfer et al., 1991), suggesting

that CSF-1 is also implicated with varied effectiveness in stimulating development and differentiation of embryos under stress. Therefore, because different culture systems expose embryos to varied amounts of stress, the use of different culture systems may affect the response of embryos to GM-CSF. Also, the varied sensitivity of different embryos to stress may cause variation in the response of embryos to GM-CSF.

2.4.2.4 Cross-species homology of rGM-CSF

It is unlikely that the lack of an effect of roGM-CSF on bovine embryo development observed in this study was due to the roGM-CSF used in the current study being biologically inactive as the same source of roGM-CSF used here increased ovine IFNτ secretion when included in the medium at 5 ng mL^{-1} (Rooke et al., 2005). Contrary to the present study that used bovine embryos, Rooke et al. (2005) used ovine embryos, so that the discrepancy between these studies could be due to differences in species cross-reactivity. Other studies have assessed the effects of rGM-CSF derived from one species on embryo development of a different species, as shown inTable 24. Of these studies, Imakawa et al. (1993) and (1997) detected an increase in IFNτ secretion and expression by ovine blastocysts due to rhGM-CSF exposure, which could be explained by similarities in the GM-CSF from different mammalian species. The hydrophobicity profiles, putative glycosylation sites and positions of cysteine residues in the GM-CSF coding sequences from different mammalian species, including ovine and bovine, are highly conserved (Inumaru and Takamatsu, 1995). The high degree of sequence homology between the ovine and the bovine GM-CSF both at the nucleotide (approximately 93%) and amino acid (80%) levels (McInnes and Haig, 1991) would suggest that differences in homology should not account for discrepancies between the bovine and the ovine (Rooke et al., 2005).

Table 24 Studies which assessed the effects of rGM-CSF derived from one species on embryo development of a different species.

		GM-CSF homology similarity		
species	**homology**	**nucleotide level**	**protein level**	**Reference**
ovine	bovine	93%	80%	de Moraes et al., 1997
ovine	human	84%	80%	Imakawa et al., 1993
ovine	human	84%	80%	Imakawa et al., 1997
bovine	ovine	84%	80%	Loureiro et al., 2008

However, there are reports of rGM-CSF from different species having similar homologies but different biological activities on cells derived from different species. de Moraes et al. (1997) observed no effect of bovine GM-CSF on ovine embryo protein secretion. Human

GM-CSF was found to have no proliferative effect on ovine bone-marrow cells, as opposed to ovine GM-CSF which did (McInnes and Haig, 1991), despite being 80% identical in terms of amino acid sequence. Also, bovine GM-CSF was found to be biologically active in bovine bone marrow proliferative assays, but only weakly active in both human and mouse bone marrow proliferation assays, whereas human GM-CSF was only weakly active in bovine bone marrow proliferation assays (Maliszewski et al., 1988). The bovine GM-CSF precursor was shown to contain a putative amino acid sequence not found in human or murine GM-CSF, cleavage of which yields another protein (Maliszewski et al., 1988). Therefore, despite the high degree of similarity in the sequence of different homologies of GM-CSF from different species, different homologies have varied cross-species biological activity, which might explain some of the discrepancies in the response of embryos to GM-CSF observed in different studies.

2.5 Conclusion

In terms of IFNτ secretion, pyruvate oxidation, rate of development, blastocyst yield, morphological grade and apoptotic index, IVP bovine embryos were unresponsive to roGM-CSF at the concentrations and developmental periods tested. Discrepancies between this study and other studies which observed an effect of rGM-CSF on embryo development may be attributed to differences between species, rGM-CSF homologies or different culture conditions between studies. The amount of roGM-CSF and timing of exposure did not seem to account for the lack of an effect of roGM-CSF on embryo development. However, exposure of bovine embryos to roGM-CSF from day 5 pi was not assessed in this study, and Loureiro et al. (2008) observed that such exposure increased blastocyst yield. Therefore, timing of rGM-CSF exposure may explain some of the discrepancies between studies on the effect, or lack of, of GM-CSF on embryo development.

IFNτ secretion by different embryos in the same treatment was highly variable, suggesting that genetic and intrinsic factors may explain individual variation. IFNτ secretion did not correlate with apoptotic index, total cell count, diameter, morphological grade or rate of development, suggesting that these intrinsic factors do not explain the high variation in IFNτ secretion between embryos. However, blastocyst yield, day of blastulation and change in morphology over 24 h did account for at least part of the variation in IFNτ, suggesting that some intrinsic factors may regulate IFNτ secretion. Also, exposure of embryos to GM-CSF did not account for the variation in IFNτ secretion between different embryos, irrespective of the timing and amount of exposure. Therefore, further studies are required to assess the effects of other factors on IFNτ production.

3 EFFECT OF HEAT STRESS ON THE ABILITY OF BOVINE EMBRYOS TO PRODUCE IFNτ *IN VITRO*

3.1 Introduction

3.1.1 Effect of heat stress on the development of *in vivo* produced embryos

Increased rectal temperatures from 39°C in control cows to 42°C in heat stressed cows suggest that embryos developed *in utero* in heat stressed cows are exposed to elevated temperatures (Putney et al., 1988a). The detrimental effect of heat stress on embryo development *in vivo* is demonstrated by reduced rate of embryonic development, increased abnormal embryo development and reduced conception rates in cows exposed to elevated temperatures (reviewed in section 1.8.4), suggesting that *in vivo* produced bovine embryos are sensitive to heat stress.

Putney et al. (1988b) exposed embryos flushed on 17 dpi to 43°C for 18 h and observed a decrease in IFNτ secretion due to heat treatment, whilst Geisert et al. (1988) exposed pregnant cows to 37°C (heat treatment) and 21°C (control) from 8 to 17 dpi and observed an increase in IFNτ detection in heat treated cows. Therefore, heat stress affected IFNτ secretion by *in vivo* produced bovine embryos. Differences between the two studies (summarised in Table 25, page 122) in terms of severity of stress levels exposed to the embryos (due to variation in periods and temperature of the heat treatment), exposure of embryos to the *in vitro* environment, stage of embryonic development when the embryo was exposed to the heat treatment and method of IFNτ detection may account for discrepancies between the two studies. Moreover, it is important to note that the heat stress treatment to the putative mother *in vivo* (Geisert et al., 1988) exposed the embryo to altered hormones and cytokines, as opposed to the direct impact of increased temperatures on a zygote *in vitro* (Putney et al., 1988b) where such factors are absent. Therefore, the effect of heat stress on IFNτ secretion by *in vivo* produced embryos remains inconclusive. Moreover, the effect of heat stress on IFNτ secretion by IVP bovine embryos has not been assessed, and, thus, requires further studies.

Table 25 Comparison between two studies observing the effect of IFNτ secretion by *in vivo* produced bovine embryos.

	Putney et al. (1988b)	Geisert et al. (1988)
Medium used to detect IFNτ	*In vitro* culture medium	uterine flushing
Technique to detect IFNτ	SDS-PAGE and immunoblotting	cell cytopathic assay
Heat Treatment temperature	43°C (embryo exposure)	37°C (maternal exposure)
Period of heat treatment	17 dpi, for 18 h	8 to 17 dpi

3.1.2 Effect of heat stress on the development of IVP embryos

IVP bovine embryos have been exposed to elevated temperatures. Two-cell embryos exposed to elevated temperatures (41°C or 42°C for three h) had decreased subsequent embryo development (Ealy et al., 1995). Similarly, Sugiyama et al. (2003) observed that embryos exposed in the first 48 hpi (between one and two-cells) to heat treatment cycles mimicking rectal temperature changes in summer heat-stressed cattle (ranging in temperature from 39.5°C to 41°C) also had decreased embryo development in terms of cleavage (28% of heat treated embryos cleaved, compared to 45% in control), development past the eight-cell stage (2% of heat stressed embryos developed past the eight-cell stage, as opposed to 28% of control embryos), and development past the morula stage (0.9% of heat treated embryos developed past the morulae stage, compared to 12% in the control group). Moreover, the proportion of embryos developing to the morulae or later stages by 7 dpi were reduced in the embryos derived from oocytes heat-stressed for four h, compared to the control group (Ju et al., 2005). Therefore, exposure of embryos to elevated temperatures at the two-cell stage or earlier severely impaired embryo development.

The percentage of embryos that developed from the eight-cell stage to the morulae stage was similar between the heat stressed and control embryos, whilst the percentage of embryos that developed from the two-cell stage to the blastocyst stage was lower in heat-treated embryos compared to control (Sugiyama et al., 2003). This suggests that the effect of heat stress on embryo development is stage dependent.

Sugiyama et al. (2003) exposed embryos to heat treatment at four dpi in one experiment and between one and two dpi in a second experiment, and found that controls had a two-fold higher proportion of embryos reaching the morula stage in the first experiment, as opposed to 12-fold higher proportion of embryos which had been exposed to heat stress during the

first 48 h of culture reaching the morula stage relative to control. Ealy et al., 1993 observed that embryos were sensitive to heat stress one dpi (three h heat treatment at 41°C or 42°C) but were resistant to the same heat stress five dpi (morula stage). Although there is a difference in the length of heat treatment, and although these results refer to separate experiments, these results suggest that the ability of the pre-implantation embryo to resist heat stress increases as development progresses.

Incubation of two-cell embryos at 40°C for three h did not affect blastocyst rate or rate of development, but heat treatments of 42°C for three h decreased embryo development significantly (Ealy et al., 1993). In another study, *in vitro* matured bovine oocytes did not survive more chronic heat treatments (45 minutes at 43°C), but did survive shorter heat treatments (0, 15 and 30 minutes) with no deleterious effect on the matured oocyte being exposed to lower temperatures (40.5 and 41.5°C for up to one hour, Ju et al., 1999). Therefore, the detrimental effects of heat stress on embryo development varied with the intensity of the stress and milder heat treatments did not affect embryo development.

Some studies even noted that a mild elevated temperature enhanced the growth rate of embryos and postulated that this was due to an increase in the rate of metabolism (Ealy et al., 1993), suggesting that mild heat treatments may benefit the embryo, possibly by improving the ability of the embryo to tolerate heat stress, a mechanism known as thermotolerance. Heat stress may enhance the thermotolerance of, or cause detrimental effects on, the embryo depending on the duration and intensity of the thermal challenge. Acquiring thermotolerance involves increasing the capacity of the embryo to produce molecules involved in thermoprotection such as HSPs or cellular antioxidants (Arechiga et al., 1995). Thermotolerance of bovine embryos increased with embryonic stage (Al-Katani and Hansen, 2002), with substantial thermotolerance developing *in vitro* around the compacted morula stage (Edwards and Hansen, 1997). The difference in thermotolerance between earlier stages and later stages of embryo development may be due to the onset of the embryonic genome activation.

Ryan et al. (1992) observed that exposing embryos to 4 h at 40°C caused no immediate effect to the embryos, but caused embryo death immediately after hatching, 5 days after exposure of the embryo to heat stress. Therefore, the detrimental effects of heat stress may present themselves beyond the time frame of the experiment.

Heat stress caused changes in the spindle configuration from oocytes heat stressed during IVM for two to four h at 42°C (Ju et al., 2005), indicating that heat stress affected polymerization of meiotic spindle microtubules. The mitotic spindle may also be altered by heat stress, so that heat stress may cause chromosome dispersion and eventually cell death (Streffer, 1984). Also, alterations in the zona pellucida structure and the microvillus pattern of the oolemma were evident when the oocytes were heated to 42°C for 30 minutes (Suzuki et al., 1998). Therefore, changes in developmental potential of embryos due to heat stress may be due to changes in cellular structure.

Following IVF, the heat treatment at the oocyte level caused a reduced blastocyst rate and reduced trophectoderm cell number per blastocyst (82 vs 108 cells) compared to control (Ju et al., 2005). Although heat stress did not affect fertilisation rate, it did increase the rate of polyspermy from 16% in embryos heat stressed for four h compared to 36% in control embryos. Apoptosis has also been reported as a cause of heat stress in bovine embryos (Paula-Lopes and Hansen, 2002, Roth and Hansen, 2004). These studies confirm that high temperatures are a stressor to IVP bovine embryos. Changes in the structure and development of embryos due to heat stress suggest that heat stress may affect the expression patterns of certain genes required for normal function and development.

3.1.3 Effect of heat stress on expression and synthesis by IVP bovine embryos

HSP70 mRNA was detected in bovine oocytes (Edwards and Hansen, 1996, 1997), two-cell embryos (Edwards et al., 1997, Howell and Hansen, 1995), eight-cell embryos (Kawarsky and King, 2001) and blastocysts (Kawarsky and King, 2001), suggesting that bovine embryos are able to respond to stress at the cellular level. HSP70 mRNA and protein levels increased when two-cell stage embryos were exposed to heat stress (42°C for 80 minutes, Edwards and Hansen, 1997). The heat-induced synthesis of HSP70 occured even in the presence of an RNA polymerase inhibitor (α-amanitin, Edwards et al., 1997), suggesting that the increase in HSP70 synthesis at the two-cell stage may be regulated post-transcriptionally. Heat inducibility of HSP70 mRNA was also evident at the eight-cell stage and the blastocyst stage (Kawarsky and King, 2001). However, heat stress had no effect on HSP70 synthesis by bovine oocytes, as demonstrated by metabolic labelling with 35S-amino acids and two-dimensional gel electrophoresis (Edwards and Hansen, 1996), suggesting that the ability of the embryo to upregulate HSP70 expression and synthesis is stage dependent.

Several mechanisms have been proposed to explain how heat stress upregulates HSP70 transcript levels. Theodorakis and Morimoto (1987) proposed that during heat shock,

HSP70 mRNA continues to be translated efficiently, whilst other mRNAs are poorly translated, so that rather than increasing HSP70, heat stress down-regulates expression of other genes. An alternative theory suggests that heat-induced HSP70 expression could be regulated at the level of transcription (Edwards et al., 1997), probably due to heat stress displacing bound Heat Shock Elements (HSE) from the specific region of the DNA located on the promoter region of the HSP gene (Li et al., 1995), allowing heat shock transcription factors (HSF) to bind instead, thus inducing the cellular stress response (Figure 19).

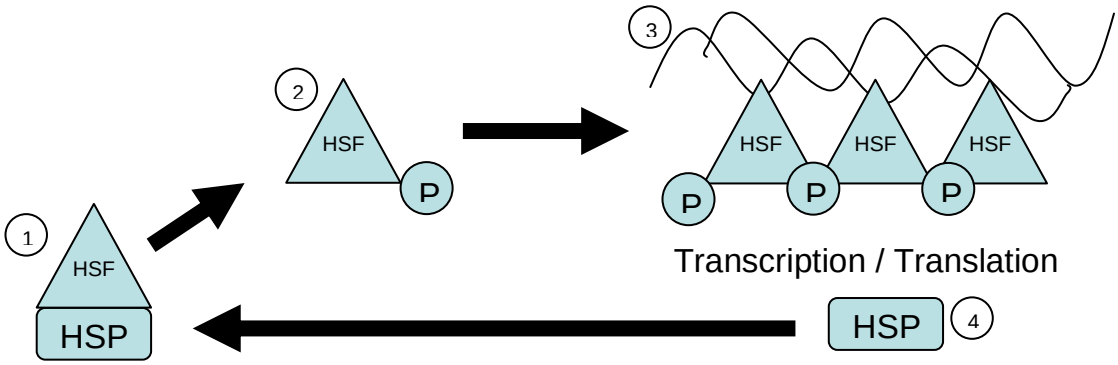


Figure 19 Proposed mechanism of stress-induced increase in HSP70 (Kiang and Tsokos, 1998). ①HSP70 in the cytosol is bound to HSF. ② Under stress, HSP separate from HSF and HSFs become phosphorylated. ③ Phosphorylated HSFs form trimers and enter the nucleus to bind to specific regions of the DNA. HSF is phosphorylated further. HSP mRNA is transcribed and leaves the nucleus. ④ In the cytosol, HSP is synthesized. HSF returns to the cytosol where it becomes bound with HSP again.

Studies assessing the effect of stressors on IFNτ expression have determined that stress causes an upregulation in the expression of IFNτ (Wrenzycki et al., 1999, 2000, 2001a, b, Lonergan et al., 2001, Rizos et al., 2003, Kubisch and Johnson, 2007, as discussed in section 1.8). However, there are discrepancies between studies on the effect of stress on IFNτ synthesis (as summarised inTable 7, page 76), suggesting that IFNτ may be regulated by stress at the transcriptional level, and other factors may modulate IFNτ translation. The relationship between IFNτ and HSP70 expression has not been assessed, and would explain whether IFNτ secretion is a stress response.

3.1.4 Conclusion

The effect of heat stress on pregnancy rate *in vivo*, on embryo development *in vitro* and on HSP70 expression and IFNτ secretion are indicative that bovine embryos are sensitive to

heat stress and, therefore, that heat stress is relevant to early embryonic development and IFNτ secretion. Therefore, heat stress was selected as the stressor for this study. The **objective** of this Chapter was **to determine whether heat stress affects IFNτ expression by IVP blastocysts**.

This experiment was designed to examine the effects of heat stress at the blastocyst stage on the ability of IVP blastocysts to produce IFNτ. The heat treatment was designed to follow the pattern of temperatures used in other studies shown to stress IVP bovine embryos (Ealy et al., 1995, Sugiyama et al., 2003, Ju et al., 2005) and shown to induce HSP70 expression in bovine IVP blastocysts (Kawarsky and King, 2001). The control group were maintained at a temperature of 38.5°C throughout the experiment effectively representing the standard IVC in our lab. The expression of HSP70, a chaperone involved in protein folding and stabilization of damaged proteins, was used as the stress indicator. In order to clarify the relationship between IFNτ and the cellular stress response, this experiment was designed to test the following **hypotheses**:

1. **exposure to 42°C for 4 h at the blastocyst stage causes heat stress to bovine IVP blastocysts**
2. **heat stress up-regulates IFNτ expression.**

In order to assess absolute IFNτ mRNA levels, a multiplex RT-PCR system using sequence-specific probes was designed. To date, there are no other reports of bovine embryos being assessed either by multiplex RT-PCR or by a sequence-specific probe detection system. Therefore, a further **objective** was

to validate a real time RT-PCR method do assess absolute IFNτ mRNA levels.

3.2 Materials and methods

3.2.1 Experimental Design

The methodology used for IVP of bovine embryos and blastocyst assessment and allocation to the different treatments is summarised in Figure 20 (page 127). Nine batches containing 546 ovaries were collected at a local abattoir and processed as described in section 2.2.1. The average proportion of cleaved from transferred zygotes among the 9 batches was 51±11% (mean ± standard deviation) and the average blastocysts per cleaved zygote was 14±5%. At 186 hpi (n=57) and 210 hpi (n=53) blastocysts were evaluated in terms of their developmental stage (Table 11, page 87) and given a quality score based on morphological assessment (grade 1: embryos free of fragmented cells, grade 2: some granulation and initial signs of fragmentation, grade 3: collapsed or badly fragmented, Table 12, page 87).

The diameters of the embryos were also measured in two planes to account for non-spherical embryos. Evaluated blastocysts were transferred in minimum volume (3 μL) from group culture to 5 μL individual culture drops of SOF. Equal numbers of blastocysts were allocated to either the control or heat stress group according to grade and stage of development, to ensure that the two treatments were balanced. Embryos from the heat stress group were exposed to 42°C for 4 h (186 to 190 hpi and 210 to 214 hpi) after which changes in the embryo grade or stage of development were noted. All blastocysts were incubated for a further 20 h at 38.5ºC. At 210 hpi (for blastocysts removed from group culture at 186 hpi) and 234 hpi (for blastocysts removed from group culture at 210 hpi blastocysts), 50 μL of warmed SOF were added to the 5 μL individual culture drops and mixed. The blastocysts were assessed for changes in diameter, embryo grade or stage of development at 0, 4 and 24 h of individual culture and categorised for change in morphological index based on the criteria outlined in 2.2.9.2 (category 1: blastocyst progressed, category 2: remained the same, category 3: degenerated/collapsed during the individual culture period, Figure 10, page 95). At the end of the 24 h individual culture period, 45 μL of SOF was added to each drop and mixed. The blastocysts were transferred in 3μl for preservation of blastocysts for mRNA, intracellular protein and DNA extraction using the All Prep DNA/RNA/Protein Mini kit (Qiagen) and 40 μL of medium in which the individual blastocyst had been cultured in was collected and stored at -20°C until assayed for IFNτ protein. Unfortunately, samples for IFNτ protein analysis were destroyed in transit.

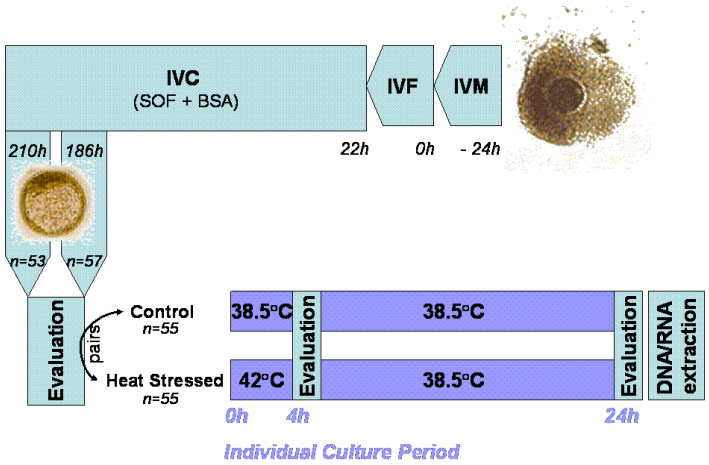


Figure 20 Schematic summarising the methodology for IVP and blastocyst assessment and allocation to the different treatments (heat stressed and control) as described in detail in section 3.2.1.

3.2.2 Preservation of blastocysts for mRNA, DNA and protein analysis

Blastocysts (n=90) were transferred in minimum volume (3 μL) into PBS (Dulbecco) in a four well plate, washed four times and then transferred into 75 μL of a buffer containing guanidium thiocyanate[4] (Buffer RLT, part of the RNeasy micro kit) in 0.1 mL thin walled PCR tubes. The PCR tubes were vortexed for 1 minute and stored singly (experimental embryos) or in pools (embryos used for validation of the RT-PCR system) at -80°C until mRNA, DNA and protein extraction.

3.2.3 Extraction of mRNA, DNA and protein

Samples were defrosted on ice and vortexed for 1 minute in 275μL of Buffer RLT to ensure that the embryo was disrupted. The sample was transferred onto a DNA spin column and centrifuged at 8100g for 30 seconds. The DNA column was kept on ice until assayed for sex determination (described in section 3.2.6). 0.075 mL of 80% ethanol was added to the eluate (containing the protein and the mRNA) to adjust binding conditions. The sample was mixed by pipetting and transferred into an RNeasy MinElute Spin Column for absorption of RNA to the membrane. The spin column was centrifuged at 8100 g for 30 seconds and the flowthrough (containing the protein) was kept on ice until assayed for HSP70 protein (described in section 3.2.7). After adding 0.35 mL of Buffer RW1 (included in the RNeasy micro kit), the spin column was centrifuged at 8100 g for 30 seconds and the flowthrough discarded. The membrane was treated with 0.08 mL of DNase I mix for 15 minutes at room temperature and washed with 0.35 mL of Buffer RW1 centrifuged at 8100 g for 30 seconds and the collection tube replaced. The membrane was then washed with 0.5 mL of Buffer RPE (included in the RNeasy micro kit) and centrifuged at 8100 g for 30 seconds and the flowthrough discarded. The membrane was then washed with 0.5 mL 80% ethanol (in RNase free water) at 8100 g for 2 minutes and the collection tube discarded. The tube was then centrifuged at 16000 g for 5 minutes with the spin column cap open, to allow the membrane to dry. The RNA was then eluted from the membrane with 0.02 mL of water centrifuged at 16000 g for 1 minute, producing 0.018 mL of eluate.

In a preliminary experiment, the integrity of the RNA extracted from bovine embryos using this methodology was assessed using a bioanalyzer (outlined in Appendix 7, page 215). All samples assessed had an RIN value of 9 or greater, clear and defined peaks for 28S and 18S, low noise between the peaks and minimal low molecular weight noise in the electropherogram, suggesting limited contamination. Moreover, the ratio of 28S:18S bands ranged between 2.2 and 2.5, suggesting high quality RNA. Therefore, the method used in

this study for RNA extraction and storage of sample RNA provide high yield of good quality RNA.

3.2.4 cDNA synthesis

Each eluate was reverse transcribed using Qiagen Quantitect Reverse Transcription, as described in the manufacturer's protocol. No reverse transcriptase was added in the no reverse transcriptase (NRT) controls. RT was performed in a thermal cycler (Biometra thermoblock). A portion of the RT product (1.8 µl as 0.1 equivalent of a single blastocyst) was separated from each sample as a no template control (NTC). Each sample was mixed with 1.8 µl of gDNA wipeout buffer and incubated at 42°C for 2 minutes. The sample was then mixed with 6 µl of reverse transcriptase, RT buffer and RT Primer Mix (1:4:1) and incubated at 42°C for 15 minutes followed by 95°C for 3 minutes to inactivate the reverse transcriptase.

3.2.5 PCR

3.2.5.1 Transcript analysis and primer and probe design

Gene accession numbers for each gene of interest were derived from the literature (Table 26, page 134). The single stranded sequences for each of the genes assessed are outlined in Appendix 4 (page 209). Based on the gene accession numbers for each gene, the Quantiprobe design software (Qiagen, https://customassays.qiagen.com/design/inputsequences.asp) was used to identify secondary structure-free stretches of the mRNA template long enough to accommodate a quantiprobe assay. The program was set to pick forward and reverse primers with melting temperatures around 60ºC, the desired product size was set to 50 to150 bases, and default settings were used for all other parameters. The Quantiprobe design software found multiple candidate designs for each target sequence. Primer Express software (version 1.0) was used to check the secondary-structure of the candidate primers and probes to predict the self-hybridisation of PCR templates.

The exon structure of the mRNA transcripts was derived from the Ensemble *Bos taurus* public database (http://www.ensembl.org/Bos_taurus, as at 03 March 2007). The compatibility of the primers and probes were also checked in the NCBI Entrez Nucleotide public database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide, as at 03 March 2007). Due to minor differences in the sequences recovered in the two public databases, the compatibility of all primers and probes were checked in both databases. Where possible, assays were designed to be cDNA specific in order to minimize amplification from genomic

[4] A salt used in the isolation of RNA from cells which works by denaturing the cellular proteins.

templates, by including exon-straddling primers or probes in the assay. According to transcript analysis using Ensembl, there are no introns in the HSP70 and IFNτ genes, so that it was not possible to include exon-straddling primers or probes in the assay for these genes. YWHAZ, however, has six exons, and the primers spanned intron boundaries.

The properties of the primers and probes used in the current study are outlined in Table 26 (page 134). The primers and probes were designed in agreement with the principles of primer design (Wittwer et al., 2001), containing more C than G bases, to reduce non-specific annealing of the primer, a GC content below 67% (a high GC content promotes non-specific binding) and above 39% (a low GC content impedes annealing). Primer Express software determined that none of the primers had primer self-complementarity (ability to form secondary structures), and Quantiprobe design software determined that all primers and probes were compatible with the PCR cycling conditions. With all target genes, the priming sites were more than 50 nucleotides away from the start of sequencing, thus avoiding any bias that may have occurred during RT. Primer pairs used in a multiplex reaction had similar melting temperatures for all the targets amplified in the same reaction and the fluorophore used to label different probes in multiplex reactions were spectrally distinct (excitation maximum and emission maximum were 494 nm and 518 nm for FAM, 526 nm and 552 nm for Yakima Yellow and 587 nm and 607 nm for ROX) so that the cycler could distinguish between different target sequences.

The predicted amplicon sequences were then run through BLAST (http://www.ncbi.nlm.nih.gov/blast/) against genomic and cDNA databases to ensure that they were specific to the genes of interest. As determined by the NCBI Entrez Nucleotide public database, the primers and probes designed were compatible with the target genes (results from the BLAST analysis are outlined in Appendix 5, page212). Matches with high scores were assessed to determine whether the primer and probe sets were likely to co-amplify homologous transcripts or genes along with the primary mRNA target. The predicted amplicon sequences were run through BLAST against genomic and cDNA databases for *Bos Taurus* and most of the hits were relevant to the GOI (Appendix 5, page 212). Alternative priming sites had less than 90% identity to the primary site: IFNα was detected as the thirteenth hit for IFNτ amplicon sequence and enoyl-Coenzyme A was detected as a BLAST Hit for the YWHAZ amplicon sequence. In both cases the high gap percentage, low expectation value and identity percentage would suggest that the primers and probes would not detect the expression from the enoyl-Coenzyme A gene or IFNα.

From all the sequences derived from the Qiagen software, these had the most fitting BLAST results. Therefore, the primer and probe sequences are unique to the target template.

As determined by BLAST and gel electrophoresis (Appendix 8, page 219), the primers and probe sequences were compatible with the GOI, were specific to the GOI and led to amplicons of the correct size, confirming the identity of the amplicon and the validity of the use of the primers and probes in RT-PCR.

3.2.5.2 PCR

Real-time PCR was carried out on a Stratagene PCR thermocycler (MX3000P) using the Quantitect Multiplex PCR kit (Qiagen) according to the manufacturer's instructions. Gene transcripts for the genes of interest IFNτ, HSP70 and YWHAZ were quantified by real time quantitative PCR in duplex reactions with the exogenous housekeeping gene, α-globin. The 25 µl PCR reaction consisted of a cDNA equivalent to 0.05 blastocysts (8% of the overall reaction), 50% PCR multiplex mix, 22% RNase free water, 5% α-globin primer, 5% α-globin probe, 5% gene of interest (GOI) primer, 5% GOI probe. Standards and samples were run in duplicate reactions, and controls (NTC and NRT) were run in single reactions. The same PCR thermocycler was used to analyse all the samples, standards and controls to avoid any variation that could arise between different thermocyclers in terms of amount of control, accuracy and speed which temperature cycling can be achieved and which could affect the kinetics of the PCR reaction (de Sousa and Watson, 2004).

The cycling profile included 2 minutes at 50°C, 15 minutes at 95°C to activate the HotStarTaq DNA Polymerase and 40 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 56°C, and extension for 45 s at 76°C. Data were collected during the annealing step. The results were quantified using the Stratagene MxPro QPCR Software.

The RT-PCR methodology used in this study was validated as described in Appendix 6 (page 213). Briefly, the compatibility and specificity of the primers and probes with the genes of interest were assessed using gel electrophoresis (Appendix 8, page 219) and assessment of the kinetics of the PCR reaction (Appendix 8, page 219); a standard curve was generated by serial dilution (ten-fold) of a positive cDNA template (Appendix 9, page 222); and α-globin (Appendix 10, page 226) and YWHAZ (Appendix 11, page 233) were assessed as the housekeeping genes in a multiplex reaction with the gene of interest.

3.2.5.3 Quantification of mRNA levels

Expression levels were assessed as absolute values or relative to endogenous control (YWHAZ) after correction for variation in exogenous control (α-globin). Before RNA extraction, samples were spiked with 20 pg of α-globin mRNA, used as an exogenous control. A preliminary experiment (outlined in Appendix 9, page 222) showed that adding 20 pg of α-globin mRNA to the RT reaction gave a range of 5 log dilutions after PCR with a similar range of cycle numbers to the genes of interest. RNA was also extracted from 2 ng of α-globin mRNA (positive α-globin control) and from a blank which was used as a NTC during the RT reaction. Ten samples were run in each PCR plate (5 heat stressed and 5 control) in duplicate for each gene of interest (IFNτ, HSP70 and YWHAZ) multiplexed with the exogenous control (α-globin). An α-globin standard curve derived from duplicate ten-fold serial dilutions of the cDNA derived from 2 ng of α-globin mRNA was present in each PCR run to assess the interassay variation. From the serial dilution, the amount of α-globin in each well was determined using the following equation:

$$Amount = 10^{\left(\frac{Ct-c}{-m}\right)},$$

where amount refers to the amount of mRNA in pg, Ct refers to the threshold cycle, c refers to the y-intercept of the standard curve and m refers to the slope of the standard curve. The α-globin standard curve in each plate was used to convert Ct values into amount of transcript (in fg) under the assumption that all the genes of interest had similar efficiencies to that of α-globin. The amount of each gene of interest was divided by the amount of α-globin in each well in order to control for differences between samples in RNA recovery, RT and PCR efficiencies. YWHAZ was used as the endogenous housekeeping gene to control for differences between samples in total mRNA. To express transcript levels in relative terms, absolute transcript levels of the GOI (corrected for variation in α-globin transcript levels) were divided by absolute transcript levels of YWHAZ (corrected for variation in α-globin transcript levels). In other studies, the two-delta-delta-Ct (2ΔΔCt) method (Figure 21a) is used to quantify expression relative to one normalizer gene evaluated in a separate well to the GOI, taking no account of variation in PCR efficiency or total RNA variation. The multiplex method used in the current study, however, corrects the transcript levels for variation in the exogenous control and the endogenous control, so that for each GOI for each sample, four Ct values were collected, as demonstrated in Figure 21b.

a) b)

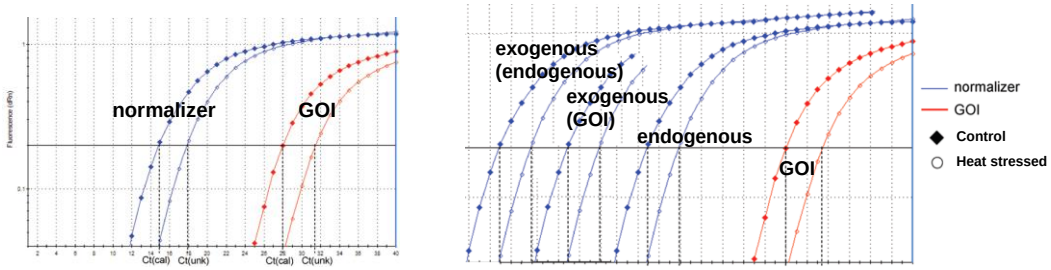


Figure 21 Diagrammatic representation of PCR profiles (log of the fluorescence versus cycle number) for a) the 2ΔΔCt mthod and b) the absolute quantification method used in this study. Threshold: ____

3.2.5.4 Quality control

All PCRs were run alongside appropriate controls (positive, NTC, NRT). The positive control was a sample that had been shown to contain the GOI in a previous PCR run. As part of the quality control, replicates that fit the criteria set out in Appendix 12 were re-run. Re-run replicates that fit the same criteria were culled from analysis. Of the 110 blastocysts assessed, 20 blastocysts were excluded from analysis due to errors in the α-globin standard curve (efficiency too high). For the analysis for IFNτ transcript levels, a further 17 blastocysts were excluded due to non-sensical data (either no Ct value, Ct value above 40, or abnormal amplification curve), suggesting that IFNτ mRNA levels for these samples were below the level of detection.

The predicted background fluorescence (PBF) was set by the Stratagene software as the best fit line through the fluorescence (R) within the range of cycles during which all contributions to fluorescence were linear (i.e. no exponential increase in fluorescence) and the PBF was subtracted from the measured fluorescence at each cycle, giving dR. The software then used the reference dye (ROX) in each well to normalise the fluorescence detected in each replicate, giving dRn. Using the software alogarithm, the threshold was set at a point where all samples displayed the same rate of increase in the fluorescence intensity. The standard curve for each plate was plotted using the software as the Ct versus the log of initial template amount.

Table 26 PCR primer and probe sequences. GC content and melting temperature (Tm) were calculated using the oligocalculator (http://sciencelauncher.com/oligocalc.html)

Gene	Genbank Accession Number	Fluorophore		Sequence	Location	GC%	Tm	Amplicon Size (bp)
HSP70	U09861	FAM	Forward Primer	ACATGAAGGAGTGGCCTTT	413 - 431	47	64.9	66
			Reverse Primer	TTGTAGCTCACCTGCACCTTA	479 - 459	48	66.1	
			Probe	CATCAACGACGGAGAC	438 – 453	56	68.1	
YWHAZ	BM446307	FAM or YY	Forward Primer	GACTATTTCCCTCTTCCTATTC	415-436	41	62.4	78
			Reverse Primer	AGGGTAGGGTGTATAGGGAGAT	496-475	50	67.0	
			Probe	GAGTTGATGAGATTGTGT	456-473	39	66.3	
IFNτ	AF196320	FAM	Forward Primer	CAGGACAGAAAGACTTTGG	91-110	45	63.0	126
			Reverse Primer	GTGCTCTGTGTAGAAGAGGTTG	216-195	50	66.8	
			Probe	TCAGGAGATGGTGGAGG	117-133	59	67.6	
α-globin	X04751	YY	Forward Primer	GCCTCCCTGGACAAGTTCCT	715-736	60	56	68
			Reverse Primer	GGCTCCAGCTTAACGATATTTGG	783-761	48	55	
			Probe	CCAACGTGAGCACCGTGCTGACC	737-759	65	62	

3.2.6 Sex determination

The sex of mammalian embryos is determined by the presence or absence of the Y chromosome. Therefore, the determination of the sex of embryos is based upon detection of DNA sequences that are present only on the Y chromosome. PCR of the genomic DNA from an embryo together with specific primers amplify sections of a Y specific gene and a bovine specific gene (positive control). The PCR amplicons are detected through gel-electrophoresis. Two fragments indicate that the embryo is male, one fragment indicates that the embryo is female (Figure 22).

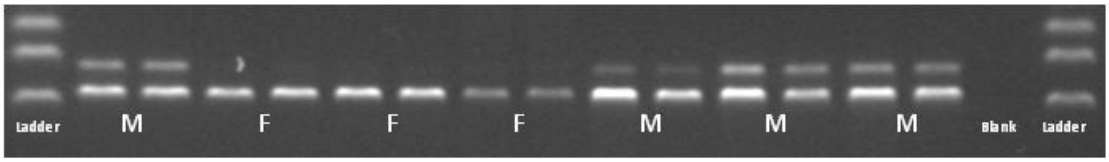


Figure 22 Photograph of a representative gel produced from PCR analysis of the sex of the embryo. Two bands (Y-chromoseome-specific and the cow specific genes) suggest that the blastocyst was male, whilst one fragment indicates that the blastocyst was female. Gels were run in duplicate.

Before RNA extraction, DNA was extracted from each embryo using DNA extraction columns (Qiagen) according to the manufacturer's instructions with minor modifications. 80% (20 µL) of the resulting volume was used for PCR using a modified version of the method provided by C. Wrenzycki (personal communication). PCR was performed in a final volume of 50 µL of 1 x PCR buffer (Qiagen) containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 IU *Taq* DNA polymerase and 0. 5 µM of the Y-chromosome-specific primer

(5′-CCTCCCCTTGTTCAAACGCCCGGAATCATT-3′ and

3′-TGCTTGACTGCAGGGACCGAGAGGTTTGGG-5′;

accession number PCT WO 86/07095)

and 0.125 µM of the cow-specific primer

(5′-AGGTCGCGAGATTGGTCGCTAGGTCATGCA-3′ and

3′-AAGACCTCGAGAGACCCTCTTCAACACGT-5′;

accession number PCT WO 86/07095).

In a heated-lid thermocycler, the PCR program involved an initial step of 97°C for 2 minutes followed by 32 cycles of 15 seconds each at 95°C for DNA denaturation, 15 seconds at 60°C for annealing of primers, and 15 seconds at 72°C for primer extension. The last cycle was followed by a 5 minute extension at 72°C and cooling to 4°C. The PCR products were

subjected to electrophoresis on a 2% agarose gel in 1× TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 1x SYBR-safe. The image of each gel was recorded using a charge couple device (CCD) camera (Gel Doc System).

3.2.7 Intracellular HSP70 analysis

Intracellular protein was extracted rom each blastocyst using the All Prep DNA/RNA/Protein Mini kit (Qiagen). The flowthrough from the RNeasy spin column was mixed vigorously with buffer APP for 10 minutes and then centrifuged for 10 minutes at 14100 g. The supernatant was decanted and 500 μL of 70% ethanol added. The sample was centrifuged for 1 minute at 14100g, the supernatant decanted and the protein pellet dried. The dry protein pellet was stored at -20°C until assayed for HSP70 protein.

HSP70 was assayed using the HSP70 High sensitivity enzyme immunoassay (Assay Designs). Although the samples contained higher HSP70 levels than blanks, the amount of HSP70 (extrapolated to be about 8 pg per embryo) were below the detection limit of the assay (200 pg per sample).

3.2.8 Statistical Analysis

For the serial dilutions in each plate, standard curves were plotted and a linear regression analysis was performed on the relationship between the log of accumulated produce and cycle number (Stratagene software). The regression coefficients (r^2), the CV between triplicates and the efficiencies of the standard curves were calculated using the Stratagene software.

The variation within and between the PCR plates was calculated as the ratio of standard deviation to the mean and expressed as a percentage. Sex ratios were compared with the 50:50 ratio using Pearson chi-square analyses (Goodness-of-fit test for observed versus expected values, Genstat, Eighth Edition, 2005). All parametric data were tested for normality before parametric analysis by visual assessment of histograms followed by Anderson-Darling test (Minitab, Fifteenth Edition, 2006). Absolute transcript levels of YWHAZ and relative transcript levels of IFNτ and HSP70 to YWHAZ, were analyzed relative to treatment and change in morphology index using a REML-LMM (Genstat, Eighth Edition, 2005). Stage of development, sex, day of blastulation and grade were checked as possible co-variates and included in the analysis when statistically significant. The absolute amount of YWHAZ was also used as a co-variate in the LMM for the analysis of absolute

transcript levels of IFNτ and HSP70. Whenever batch of ovaries or PCR plate were a factor, they were included as a random model. Stages of embryo development were grouped as pre-expansion (early and mid blastocysts) and post-expansion (expanding, expanded, hatching, hatched). Linear regression analysis (Genstat, Eighth Edition, 2005) was used to determine the association between IFNτ and HSP70 expression levels. To determine whether the slope of the regression was different between the two treatments, a two-sample t-test compared the ratio IFNτ:HSP70 in the two treatments. In order to normalise the data prior to analysis, data on relative transcript levels (IFNτ:YWHAZ and HSP70:YWHAZ) were LOG transformed to account for variance bias from ratio data, whilst data on absolute transcript levels were LOG transformed to account for bias from exponential data due to conversion from Ct values to absolute values. The effect of treatment on IFNτ:YWHAZ and HSP70:YWHAZ were assessed using student's two-sample t-test (one sided t-test, control < heat stressed). Differences were considered significant at $P<0.05$. Data are presented as mean ± standard error of the mean, unless otherwise stated. A Maximum Likelihood Chi-square analysis (Genstat, Eighth Edition, 2005) was used to determine whether change in morphology index was affected by treatment. The power of the effect of the treatments was estimated using the sample size per treatment, the standard deviation of the treatments, and the differences between the treatments (Minitab, Fifteenth Edition, 2006).

3.3 Results

3.3.1 HSP70 expression

Absolute HSP70 expression was upregulated (P=0.01) in heat treated (505±276 fg) blastocysts compared to control (25±5 fg, Figure 23a). HSP70 expression relative to YWHAZ was also increased in heat treated embryos relative to control (0.3±0.03 and 0.2±0.02 respectively, P=0.04, Figure 23).

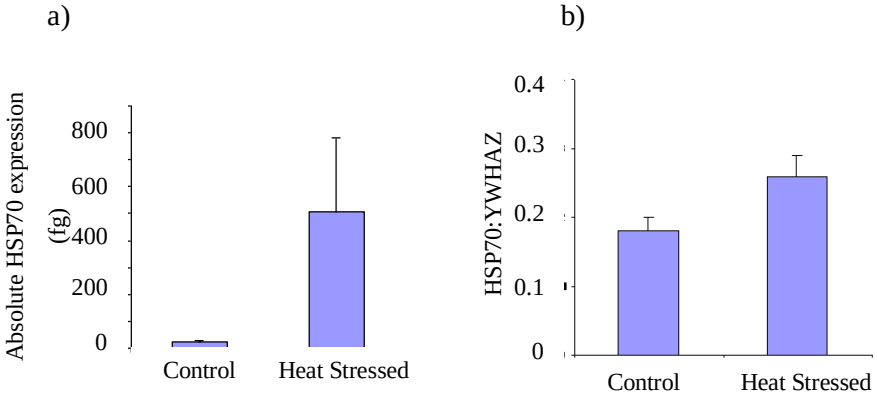


Figure 23 Effect of treatment (heat stressed embryos were cultured at 42ºC for 4 h, and control embryos were cultured at 38.5ºC) on expression of HSP70 in a) absolute terms and b) relative to YWHAZ, n=90. Values are Mean ± standard error.

In the preliminary experiment (Appendix 10, page 226), there were 4 Cts difference in the HSP70 mRNA level in heat stressed and control blastocysts (Figure 24, page 138), whilst in the current chapter (Chapter 3), there was only 1 Ct difference (Figure 24).

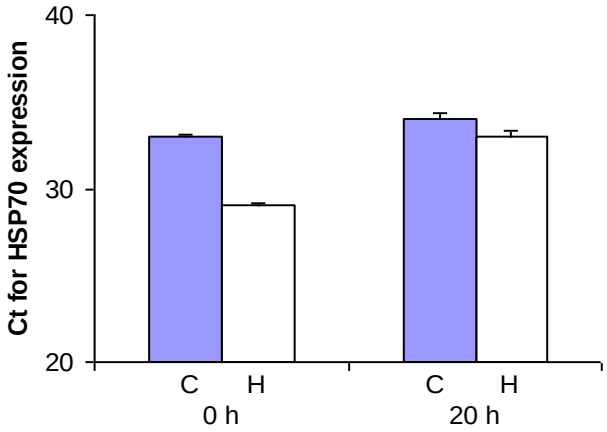


Figure 24 Effect of time between heat treatment (C: control, H: heat stressed) and transfer of blastocyst to mRNA stabilising agent on Ct value for HSP70 (Mean ±SEM): a comparison of the preliminary study (Appendix 10, page 226, 0 h, n=5) and the current chapter (Chapter 3, 20 h, n=90).

Absolute HSP70 transcript levels were lower (P=0.02) in blastocysts which showed signs of degeneration (category 3, 96±58 fg) compared with blastocysts which progressed in development (category 1, 623±482 fg) or remained unchanged in morphology (category 2, 272±241 fg) during the individual culture period (Figure 25a). However, change in morphology index did not affect HSP70 expression relative to YWHAZ (P=0.6, Figure 25b). The smaller difference between the treatments in the relative data compared to absolute data suggests that changes in YWHAZ expression explain at least some of the changes in HSP70 transcript levels, confirmed by YWHAZ being a significant factor in HSP70 absolute expression (P<0.001).

a) b)

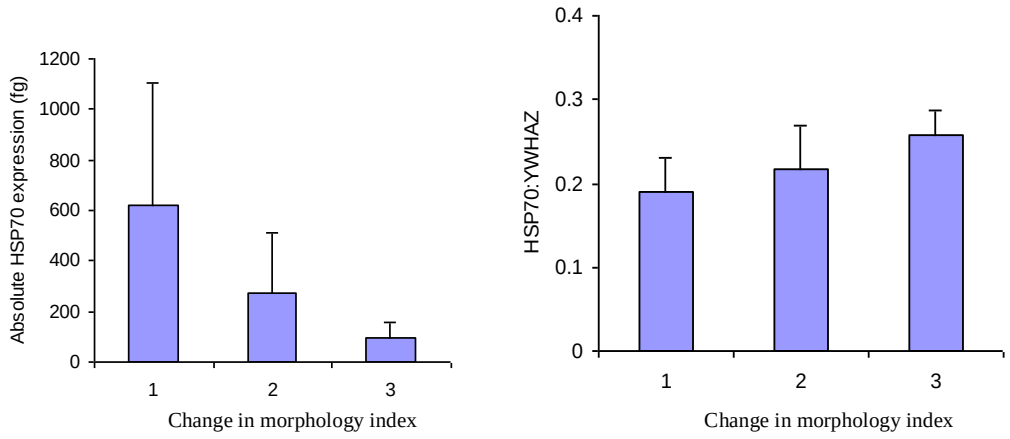


Figure 25 Effect of change in morphology index (Embryo survival after 24 h of individual culture as assessed by change in morphology before and after individual blastocyst culture: 1 embryo progressed in development, 2 no visual change in development, 3 signs of degradation or collapse) on expression of HSP70 in a) absolute terms and b) relative to YWHAZ, n=90. Values are mean ± standard error.

HSP70 expression was not significantly affected by the sex of the embryo (absolute or relative to YWHAZ, P=0.1 and P=0.9 respectively), day of blastulation (absolute or relative to YWHAZ, P=0.2 and P=0.05 respectively) or its stage of development (absolute or relative to YWHAZ, P=0.08 and P=1.0 respectively).

3.3.2 IFNτ expression

IFNτ transcript levels increased with HSP70 transcript levels (P<0.001, Figure 26), with the regression slope being steeper within the control group compared to the heat stressed group (Figure 26). Due to the higher variation in the heat treated group (r^2=0.2) compared to the control group (r^2=0.4), the difference in the steepness of the slope of the best fit line in the two treatments was not significant (P=0.2).

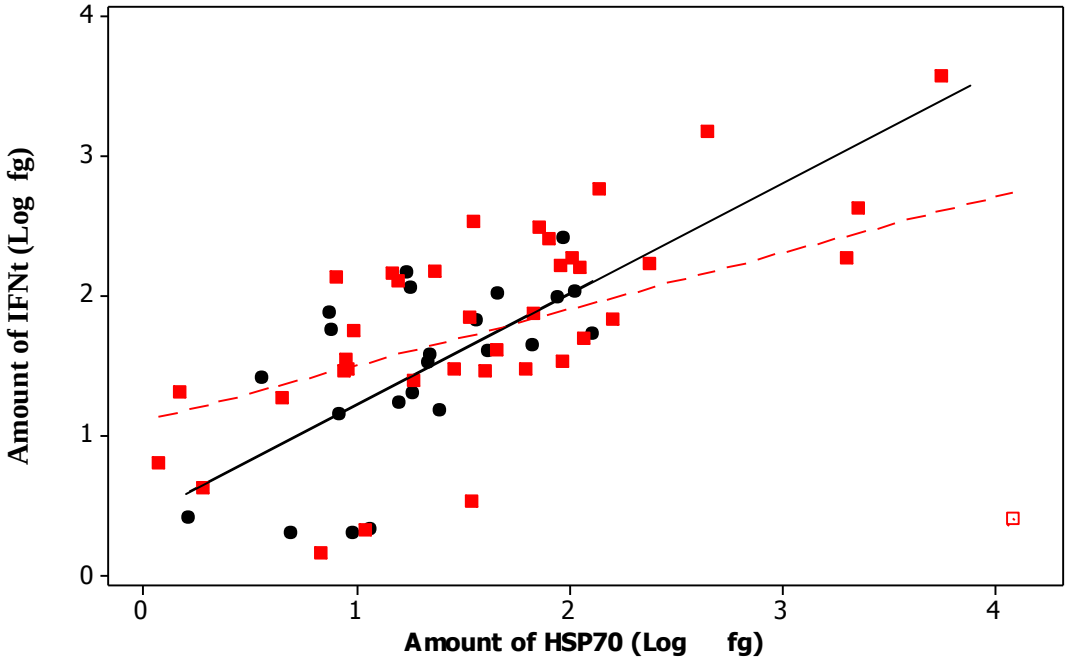


Figure 26 Relationship between IFNτ transcript levels and HSP70 transcript levels in control (●) and heat stressed (■). Regression line equations: within control group (y=0.8x+0.4), within heat stressed group (y=0.4x+1.1, n=90).

IFNτ expression per YWHAZ was increased in heat treated blastocysts relative to control (0.54±0.07 and 0.30±0.03, P=0.006, Figure 27b, page 141), whilst the absolute IFNτ expression was not significantly affected by treatment (P=0.3, Figure 27a, page 141).

IFNτ expression per YWHAZ expression did not vary with morphological grade before individual culture (P=0.7), but was lower (P=0.03, Figure 28, page 141) in blastocysts categorised as unchanged over the 24 h individual culture period (0.33±0.07) compared to blastocysts that progressed in development (0.46±0.09) or showed signs of deterioration or collapse (0.42±0.05). The change in morphology index did not affect absolute IFNτ transcript levels (P=0.1).

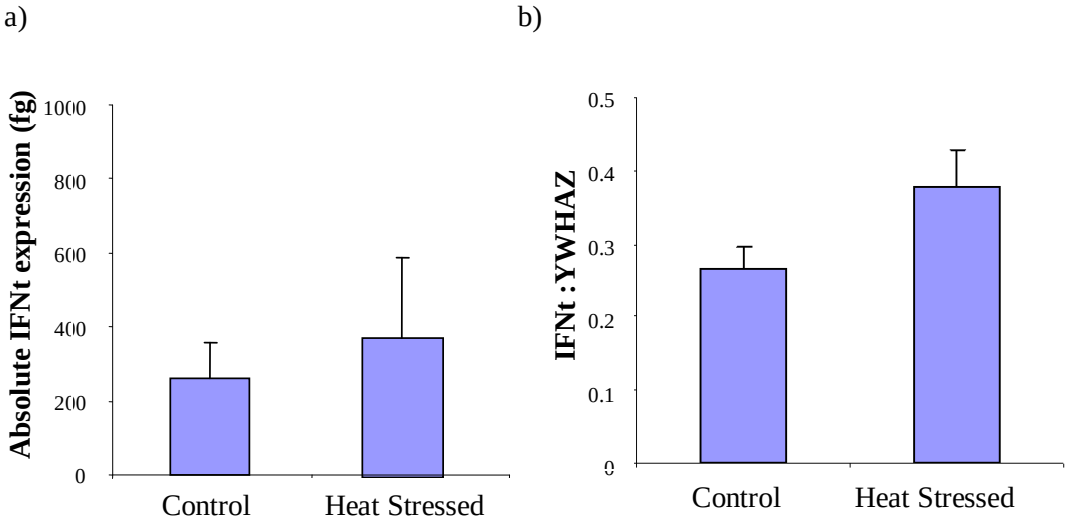


Figure 27 Effect of treatment (heat stressed embryos were cultured at 42ºC for 4 h, and control embryos were cultured at 38.5ºC) on expression of HSP70 in a) absolute terms and b) relative to YWHAZ, n=90. Values are Mean ± standard error.

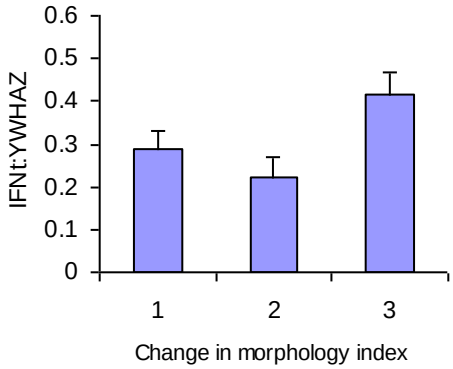


Figure 28 Relative Abundance of IFNτ transcript versus change in morphology index (Embryo survival after 24 h of individual culture as assessed by change in morphology before and after individual blastocyst culture: 1 embryo progressed in development n=34, 2 no visual change in development n=34, 3 signs of degradation or collapse n=39). Values are mean ± standard error.

IFNτ expression was not affected by the sex of the embryo (absolute or relative to YWHAZ, P=0.4 and P=0.4 respectively) or its stage of development (absolute or relative to YWHAZ, P=0.4 and P=1.0 respectively).

3.3.3 Did the treatment affect the quality of the blastocyst?

The proportion of blastocysts categorised as category 1 was greater in control group compared to the heat stressed group (Figure 29a), and the proportion of blastocysts categorised as category 3 was greater in heat stress group compared to the control group (Figure 29b). Although a greater proportion of heat stressed embryos were categorised as category 3 (42%) of the change in morphology index compared to control (31%), this difference was not significant (P=0.3, Table 27).

Table 27 Frequency of control and heat stressed embryos categorised as change in morphology index 1, 2 or 3, with percentages in brackets.

Change in morphology index	1	2	3
Control	18 (33)	19 (35)	17 (31)
Heat Stressed	16 (30)	15 (28)	22 (42)

a) b)

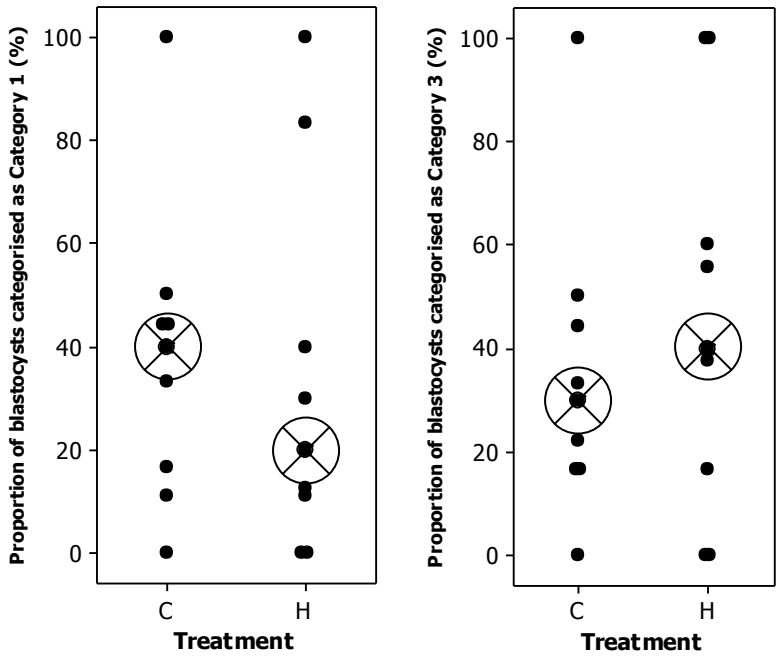


Figure 29 Proportion of total control (C) and heat stressed (H) blastocysts categorised as a) category 1 (progressed in development, P=0.8) and b) category 3 (showed signs of degeneration, P=0.3) in each of the 9 batches. ⊗: median.

3.3.4 Sex Determination

Of the 104 blastocysts for which sex was determined, a total of 51 were female and 53 were male, so that the average ratio of male to female was 53:51, similar to the expected 50:50 ratio in all batches (P=0.4). The variation between the batches was 44%. The sexes were equally distributed between the treatments (Figure 30). Although a greater proportion of females were classified as category 3 of the change in morphology index (blastocysts showing signs of degeneration/collapse) compared to males (Figure 30), the difference in the distribution of embryos in the three categories of the change in morphology index did not differ between the two sexes (P=0.3). Although day of blastulation did not vary between the two sexes (Figure 30), the stage of blastocyst development at day 7.75 pi did differ between the two sexes, with females having a slower development than males (Figure 30).

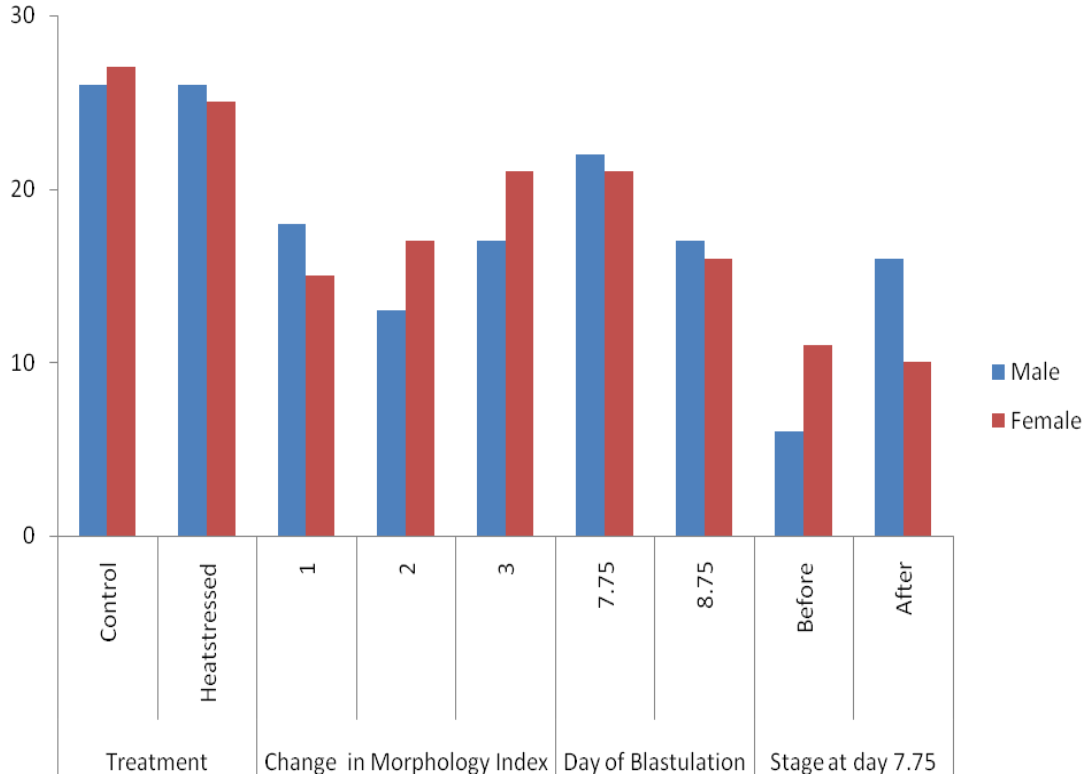


Figure 30 Number of blastocysts of each sex in comparison with treatment (control, n=53, versus heat stressed, n=54), change in morphology index (1: embryos progressed in development, n=34, 2: embryos remained unchanged, n=34, 3: embryos showed signs of degeneration/collapse, n=39), day of blastulation and stage of development on day 7.75 pi (before and after expansion).

3.4 Discussion

This is the first study to report YWHAZ, IFNτ and HSP70 expression levels in absolute terms. Given that a blastocyst contains around 5.3 ng of total RNA (Bilodau-Goessels and Schultz, 1997), and around 5% of total RNA is mRNA (265 pg), the mean transcript levels for YWHAZ, IFNτ and HSP70 observed in this study (1.59 pg, 394 fg and 375 fg, respectively) are realistic, suggesting that the methodology used to quantify the Ct values is likely to be valid. Using this methodology, the absolute transcript levels for YWHAZ, IFNτ and HSP70 were corrected for loss of material due to handling and differences in efficiencies in RNA extraction, reverse transcription and PCR efficiencies using the α-globin control.

IFNτ and HSP70 were analysed in absolute terms and relative to YWHAZ. Relative expression accounts for variation in overall mRNA levels in embryos due to differences in cell number (as concluded in Appendix 10, page 226, and by Vandesompele et al., 2002) and/or rate of transcription/ degradation, i.e. RNA degradation due to a reduction in embryo competence (Fair et al., 2005), or due to changes in transcription rate during developmental events at different stages of blastocyst development (Robert et al., 2002). Therefore, absolute data refers to expression per embryo, whilst relative data refers to expression per total mRNA, which explains why there were differences between the two. Since this study employs a novel method of quantifying mRNA expression, this is the first study to compare relative to absolute expression levels in embryos.

3.4.1 Exposure to 42°C for 4 h at the blastocyst stage causes stress to IVP bovine blastocysts

3.4.1.1 Effect of heat stress on HSP70 transcript abundance

HSP70 mRNA was detected in control embryos. The primers used in this study were specific to the stress-inducible form of HSP70, suggesting that control embryos have also been stressed, although to a lesser degree than heat stressed embryos. The stress to the control embryos may have been caused by the *in vitro* environment, since *in vivo* produced blastocysts express less HSP70 than IVP blastocysts (Wrenzycki et al., 2001a). Since individually cultured embryos have inferior development to group cultured embryos (Khurana and Niemann, 2000b), the stress from the transfer of the embryo from group culture to individual culture may have also induced HSP70 expression in control embryos.

HSP70 transcript levels were higher in heat stressed blastocysts compared to control, confirming that heat stressed blastocysts were stressed to a higher degree than control embryos, and that the cellular stress response mechanism was activated in the embryos in the

heat stress group. This is not suprising given that Kawarsky and King (2001) also demonstrated that exposing bovine blastocysts to 42°C for 4 h caused an increase in HSP70 expression.

In bovine embryos, Kawarsky and King (2001) observed a two fold increase (heat stressed versus control) in HSP70 expression relative to 18S expression, a higher difference than observed in the present study (0.5 fold increase relative to YWHAZ). In the preliminary experiment (Appendix 10, page 226), there were 4 Cts difference between heat stressed and control (Figure 24, page 138), whilst in the current chapter (Chapter 3), there was only 1 Ct difference. In the preliminary experiment (Appendix 10, page 226) and in Kawarsky and King (2001), embryos were removed from culture into an mRNA stabilising agent immediately after the heat shock treatment, whilst in Chapter 3, the embryos were cultured for an additional 20 h before being placed in an mRNA stabilising agent, so that a possible explanation for the reduced difference in HSP70 expression between treatments observed in Chapter 3 compared to the preliminary experiment (Appendix 10, page 226) and Kawarsky and King (2004) could be due to differences in the time between heat stress treatment and evaluation of HSP70 expression. The HSP70 transcripts may have decayed during the 20 h recovery at normal temperature, thus reducing the difference in induced HSP70 expression in heat stressed embryos relative to control.

In transcriptionally arrested human cells, the half-life of HSP70 mRNA in non-heat shocked cells was approximately 50 minutes, increasing at least ten-fold during heat shock (Theodrakis and Morimoto, 1987). Therefore, HSP70 mRNA stability was shown to be affected by heat stress. In the present study, even after 20 h recovery, HSP70 transcript levels were not only detected in both heat stressed and control embryos, but were also higher in heat stressed compared to control, suggesting that there may be a mechanism in place reducing the rate of decay of HSP70 mRNA in bovine embryos.

3.4.1.2 Effect of heat treatment on embryo morphology

The change in morphology index was a measure of the change in blastocyst development (size, morphological grade and stage of development) during individual culture, classifying blastocysts into three categories: those that progressed in development, those that remained unchanged, and those that showed signs of degeneration during individual culture. Although the proportion of embryos which progressed in development was greater in control embryos than in heat stressed embryos, and the proportion of embryos which showed signs of degeneration were greater in heat stressed blastocysts compared to control blastocysts, these differences were not significant, so that the proportion of blastocysts in each of the three

categories were not significantly affected by treatment. Therefore, heat treatment did not significantly affect embryo morphology.

There are two possible explanations for the lack of an effect of heat treatment on embryo morphology. Firstly, blastocyst stage embryos have an increased ability to resist heat stress compared to earlier stages of development (Kawarsky and King, 2001; Ealy et al., 1993, as discussed in section 1.8), so that the level of stress may have been mild enough not to affect embryo morphology. Alternatively, the impact of heat treatment on embryo morphology may take longer to occur than 20 h, so that an effect may become evident later in development. This, however, was not assessed in the current study.

3.4.1.3 Differences in response to stress by male and female blastocysts

The stage of development on day 7.75 pi of the majority of the female blastocysts was classified as before expansion, whilst the majority of male blastocysts were classified as after expansion, suggesting that male embryos developed faster than females. Differences in rate of development between male and female embryos are due to different patterns of gene transcription between the two sexes, so that male embryos consequently have a higher metabolic rate and grow faster (Gutierrez-Adan et al., 2006).

The proportion of embryos classified in the three categories of the change in morphology index did not differ between the two sexes, suggesting that both sexes were equally able to resist heat stress, which contradicts the findings of Perez-Crespo et al. (2005) that female murine embryos have been observed to survive better than males when exposed to heat stress at the morula stage. Differences between the two studies may be due to exposure of heat treatment at different stages of development (morulae by Perez-Crespo et al., 2005, and blastocyst in the present study) or species differences (murine and bovine). Supporting the argument that embryos of different sexes respond to stressors in different ways, some studies have observed more male than female embryos cultured *in vitro* (~60% males in Pegoraro et al., 1998, Wrenzycki et al., 2002, Gutierrez-Adan et al., 2001a), and concluded that this was caused by stressors from *in vitro* culture.

The overall sex ratio of blastocysts produced *in vitro* in the present study, however, did not differ from 0.5, as reported by Kimura et al. (2004a), although a high batch-to-batch variation in sex ratios was observed (44%), suggesting that slight variations in culture conditions and/or oocyte and sperm used in different batches (i.e. slight variations in manipulation, medium content, quality of oocytes or sperm) might have an impact on sex ratios. In fact, stress is only one of the factors affecting sex ratios in bovine embryo development, with other factors

including follicular fluid steroid levels (Agung et al., 2006, Grant et al., 2008), co-culture (Pegoraro et al., 1998), glucose level (Gutierrez-Adan et al., 2001b), *in vitro* culture system (Gutierrez-Adan 2001a) and sperm preparation (Madrid-Bury et al., 2003, Lechniak et al., 2003), which might explain contradictions in sex ratios observed in different studies, irrespective of whether embryos were exposed to stress or not.

This is the first study to compare HSP70 expression between male and female embryos, and sex did not seem to affect HSP70 transcript levels. One explanation is that HSP70 gene is not located on the X-chromosome (Ryan and Womack, 1993), and X-chromosome dosage is the major contributing factor to sexual dimorphism among early embryos (Wrenzycki et al., 2002).

Heat treatment affected the cellular stress response by either upregulating HSP70 expression, or increasing the stability of HSP70 transcripts in heat stressed blastocysts. However, the stress levels were mild enough not to affect embryo morphology (and possibly viability) or differences in response to stress between pre- and post-compaction blastocysts, and between male and female blastocysts.

3.4.2 Factors affecting IFNτ expression

Blastocysts were allocated to either treatment according to grade, size and stage of development, to ensure that the two treatments were balanced. Despite the sex of the blastocysts being determined after embryos were allocated between treatments, sex ratios were similar between treatments. Therefore, differences between the treatments in expression of IFNτ, HSP70 or YWHAZ are unlikely to be due to sex, embryo grade, size, or stage of development, but rather due to heat stress.

Absolute IFNτ expression was higher in heat stressed embryos compared to control, although not significantly higher due to the high variation in IFNτ expression. IFNτ expression varied four orders of magnitude, which is comparable to the variation in protein secretion observed in Chapter 2, suggesting that IFNτ secretion is regulated at the transcriptional level. Day of blastulation was a significant factor affecting IFNτ expression, suggesting that the increased IFNτ secretion observed in later developing blastocysts compared to earlier developing blastocysts in Chapter 3 is probably modulated at the transcriptional level. The variation in absolute IFNτ expression was not accounted for by sex (P=0.4) or stage of development (P=0.3), suggesting that other factors caused the variation observed in this study, such as embryo viability or genetic factors. Relative to YWHAZ, IFNτ was significantly higher in

heat stressed embryos, suggesting that differences in overall transcript levels (possibly due to differences in cell number) explain at least part of the variation in absolute IFNτ expression.

IFNτ correlated with YWHAZ transcript levels, a possible index of cell number, suggesting that IFNτ expression may be higher in embryos with more cells. However, blastocysts at the later stages (expanded, hatched, hatching) generally have a higher number of cells than earlier stage blastocysts (early and mid), and IFNτ did not vary with stage of development or with blastocyst diameter. Although cell lineage was not determined in this study, this discrepancy may be due to embryo-to-embryo differences in the ratio of cells in the inner cell mass and the trophectoderm, given that IFNτ expression is thought to be exclusive to trophectoderm cells (Roberts, 2007).

In the present study, IFNτ expression was not affected by stage of development or the morphological grade of the embryo, factors which have been connected with embryo quality (Van Soom et al., 1997). Although there are no data in the literature on IFNτ expression relative to these variables, IFNτ protein secretion was shown to be affected by the stage of development (Kubisch et al., 1998) and the morphological grade of the embryo (Hernendez-Ledezma et al., 1993) and discrepancies between the protein secretion in these studies and IFNτ expression variation in the present study may be due to other factors affecting rate of translation. However, part of the variation in IFNτ transcript levels was explained by the change in morphology index, which takes into account changes in diameter, morphological grade and stage of development, and could be considered a measure of embryo viability and quality. IFNτ expression was higher in embryos which showed signs of degeneration after individual culture than those that did not, suggesting that IFNτ is not indicative of embryo quality.

Therefore, sex, stage of development and embryo morphology (at 0 and 24 h individual culture) had no significant effect on IFNτ expression, whilst degeneration, increased overall rate of expression (possibly explained by differences between blastocysts in cell proliferation) and heat stress upregulated IFNτ expression levels, suggesting that IFNτ expression levels may be indicative of stress.

3.4.3 Relationship between IFNτ transcript abundance and HSP70 transcript abundance

This is the first study to correlate HSP70 expression with IFNτ expression from the same embryo. IFNτ transcript levels increased with HSP70 transcript levels. Other studies which

have assessed both IFNτ and HSP70 expression in bovine embryos are summarised in Figure 31 (page 149). In most studies, the change in HSP70 expression and IFNτ expression between treatments were in the same direction i.e. both HSP70 and IFNτ transcript levels were higher in control embryos compared to nuclear transfer embryos (Wrenzycki et al., 2001a) and *in vitro* embryos compared to *in vivo* (Sagirkaya et al., 2007, Wrenzycki et al., 2001b). Similarly, IFNτ mRNA from *in vitro* cultured ovine embryos had reduced stability and reduced transcription compared to *in vivo* produced embryos (Hansen et al., 1991).

The effect of serum on the relationship between HSP70 and IFNτ expression is contradictory. Rizos et al. (2003) reported higher levels of IFNτ transcripts in embryos cultured in the absence of serum, and Russell et al. (2006) observed an increase in both IFNτ and HSP70 transcript levels due to serum relative to BSA, whilst Wrenzycki et al. (1999) found that serum caused an increase in HSP70 transcript levels and a decrease in IFNτ transcript levels relative to BSA. Since Russell et al. (2006) also found that IFNτ and HSP70 transcript levels were not affected by lower serum concentrations (2%), discrepancies between studies may be due to differences in the amount of serum present in the culture. Moreover, Russell et al. (2006) observed no increase in IFNτ or HSP70 expression in TCM supplemented with serum relative to SOF with 2% serum or BSA, suggesting that the increase in IFNτ and HSP70 expression are not due to the protein source (BSA or Serum) or the other contents of the culture medium (TCM or SOF), but rather a combination of the two. Moreover, serum contains growth factors which may affect IFNτ expression, irrespective of stress. Therefore, the similar changes in HSP70 and IFNτ expression in different treatments in most studies suggest that IFNτ and HSP70 expression may be regulated by a similar mechanism.

Figure 31 Direction of change between two treatments in the relative mRNA abundance of HSP70 and IFNτ.  **Treatment 1 contains higher transcript levels than Treatment 2,**  **Treatment 2 contains higher transcript levels than Treatment 1. References: 1: Wrenzycki et al. (1999), 2: Wrenzycki et al. (2001a), 3: Wrenzycki et al. (2001b), 4: Sagirkaya et al. (2007), 5: Current Study**

	Treatment 1	HSP70	IFNτ	Treatment 2
1	PVA			Serum
2	Control			Nuclear Transfer
4	*In vitro*			*In vivo*
3	*In vitro*			*In vivo*
5	Heat Stressed			Control

Other than HSP70, IFNτ has also been associated with other transcripts associated with stress. An increase in IFNτ transcript level was associated with reduced transcript levels of genes associated with oxidative stress (MnSOD, sarcosine oxidase, SOX, apoptosis regulator box-2, BAX, CuZnSOD, Rizos et al., 2002, 2003, 2004, Lonergan, 2003, Gutierrez-Adan et al., 2004a) and attachment and differentiation (LIF, Rizos et al., 2003), suggesting that IFNτ expression may be regulated by cellular stress.

3.4.4 Possible overlapping molecular elements between IFNτ and HSP70 mechanism of transcription and translation as a response to stress

The results presented suggest that heat stress upregulates both IFNτ and HSP70 expression levels, suggesting that the mechanism for IFNτ production and the cellular stress response may have some overlapping molecular elements. In the case of IFNτ, there are several potentially functional IFNτ loci coding for a number of different alleles (Ealy et al., 2001), suggesting that IFNτ is mediated by a wide range of signalling factors. Ezashi and Roberts (2004) demonstrated that the transcription factor Ets2 is essential for upregulation of IFNτ, and the activation of Ets2 was shown to be mediated by the Ras/MAPK pathway as well as the adenosine 3′,5′-cyclic monophosphate (cAMP)/PKA signal transduction pathway (Das et al., 2008).

A number of specific genes have been identified in bovine embryos that respond to stress, such as Mn/Cu/ZnSOD and glucose transporter (Glut)1 (Correa et al., 2008, Luciano et al., 2006, Wrenzycki et al., 2001a,b). In drosophila, Ras/MAPK signalling pathway was shown to be modulated by reactive oxygen species (Morey et al., 2001), so that modulation of gene expression by stress was observed. Therefore, heat stress may interfere with the Ras/MAPK pathway, either directly or through the cellular stress response pathway, thus affecting IFNτ expression.

3.5 Conclusion

The effect of heat stress on change in expression of the IFNτ and the HSP70 genes in the same bovine blastocysts have been demonstrated for the first time. The increase in IFNτ transcript levels due to heat stress and the positive correlation between IFNτ and HSP70 suggest that the mechanism for IFNτ production and the cellular stress response may have some overlapping molecular elements, in agreement with suggestions by Kubisch et al. (2004) that IFNτ is an indicator of stress. Whether IFNτ is necessary for HSP70 expression, or the other way around, or the two mechanisms share a common pathway, is unclear. Given that HSP70 is expressed in later stages of development when IFNτ production does not occur,

suggests that the cellular stress response mechanism is not dependent on IFNτ production. Also, the high variation in IFNτ evident in this study, despite not being modulated by factors such as morphological grade, sex, stage of development, suggests that there are other unknown factors in *in vitro* culture affecting IFNτ production, so that heat stress is not the only modulator of IFNτ production.

4 GENERAL DISCUSSION

Embryo viability is defined as the probability of an embryo successfully implanting and developing in the uterus to term (Haiberg, 2007). There is an ongoing debate as to whether IFNτ expression and/or secretion are indicators of embryo viability or responses to stress (Hernandez-Ledezma et al., 1992, 1993, Kubisch et al., 1998, 2001a, 2003). As the antiluteolytic pregnancy signal, an essential embryo function for establishment of pregnancy, IFNτ would be expected to be related with embryo attachment potential and viability.

Quality parameters are defined as measurements intended to reflect the viability of an embryo after transfer (Haiberg, 2007). Traditionally, embryo viability is estimated using quality parameters based on morphology, such as blastocyst yield, embryo morphology, rate of development, cell proliferation and relative transcript abundance (as reviewed by Gardner and Sakkas, 2003). IFNτ secretion and expression levels were compared to quality parameters observed in this thesis and in the literature to determine whether IFNτ can be used as a predictor of future embryo development (summarised in Table 28, page 153). In this thesis, IFNτ expression and secretion did not correlate with diameter or morphological grade, and IFNτ secretion did not correlate with apoptotic index or total cell number, suggesting that IFNτ is not associated with embryo quality. Some quality parameters described in this thesis were related to IFNτ production: higher IFNτ production was associated with a higher blastocyst yield, a progressive change in morphology and delayed blastulation. Rather than an indication of viability, the relationship between IFNτ and higher blastocyst yield may be indicative of more blastocysts secreting IFNτ, delayed blastulation may be indicative of increased number of cells switching on to express IFNτ (assuming that IFNτ is switched on genetically in a specific time window, as reviewed in section 1.5). Therefore, although some quality parameters may affect IFNτ production, this may not necessarily suggest a relationship between IFNτ production at the blastocyst stage and viability.

This lack of a consistency between comparison of quality parameters with IFNτ expression and secretion was also evident in other studies (Table 28, page 153) further suggesting that IFNτ expression and secretion *in vitro* do not correlate with quality parameters. Therefore, either (1) there is no relationship between IFNτ production at the blastocyst stage and future embryo development and other factors mediate IFNτ production, or (2) quality parameters are not related with embryo viability.

Table 28 Direction of change in IFNτ production (*protein secretion* or *transcript concentration*) in 'good' compared with 'poor' quality embryos in a range of studies. ↑ : 'Good' produced more IFNτ than 'Poor'. =: No significant change between 'good' and 'poor'. ↓: 'Poor' produced more IFNτ than 'Good'.

Quality Parameter	'Poor' → 'Good'	Reference
Pregnancy Rate	↓	*Kubisch et al., 2004*
Morphology	↑	*Hernandez-Ledezma et al., 1993* *Neira et al., 2007*
	=	*Chapter 2* ***Chapter 3*** *Kubisch et al., 1998*
Change in morphology index	↑	*Chapter 2*
	↓	***Chapter 3***
Age at blastulation	↓	*Chapter 2* ***Chapter 3*** *Kubisch et al., 1998, 2001a* ***Gutierrez-Adan et al., 2004a***
Age of hatching	↑	*Stojkovic et al., 1995*
Ability to hatch	=	***Chapter 3***
	↑	*Larson et al., 2001*
Pyruvate oxidation	↑	*Chapter 2*
Apoptosis	=	*Chapter 2*
Cell Count	=	*Chapter 2* *Kubisch et al., 1998, 2001a* *Larson and Kubisch, 1999* *Stojkovic et al., 1999* *Neira et al., 2007*
	↑	***Chapter 3******
Embryo Size	=	*Chapter 2*
	↑	*Stojkovic et al., 1999* *Lo and Summers et al., 2002*
Relative transcript abundance (SOX, Glut-1 or Cx)	↓	***Wrenzycki et al., 2001a,b*** ***Rizos et al., 2002, 2004*** ***Lonergan et al., 2003a*** ***Gutierrez-Adan et al., 2004a***
	↑	***Rizos et al., 2003***
Relative transcript abundance (HSP)	↓	***Chapter 3***

* relationship with YWHAZ expression which is assumed to correlate with cell number.

There have been several attempts to link mRNA transcript abundance of genes thought to be important for bovine embryo development with embryo quality. The references in Table 29, (page 158) compared embryos from different treatments and, using other quality parameters, concluded that embryos from the first treatment had lower viability compared to embryos from the second treatment. 'Low' quality embryos consistently expressed higher levels of sarcosine oxidase (SOX) than 'high' quality embryos, so that SOX may be an indication of reduced quality. SOX is an enzyme that catalyses oxidation reactions of lipids of more than 18-carbon long chains (Koyama et al., 1996). Increase in the transcription of SOX are related with high levels of H_2O_2, high levels of glycine and glucose, high levels of lipid peroxidation and lipid metabolism. As such, upregulation of SOX tends to be associated with increased ROS levels and increased oxidative stress (Koyama et al., 1996). Therefore, poor quality embryos have increased SOX expression levels due to increased rate of oxidation. Differences in IFNτ mRNA levels between 'low' and 'high' quality embryos have also been significantly repeatable across different studies (according to the chi-square test demonstrated in Table 29, page 158). Most papers (9 out of 13) found that IFNτ decreased with quality, suggesting that IFNτ may be negatively associated with quality. Comparing IFNτ expression with expression of SOX, a consistent marker of reduced viability (Table 29, page 158), IFNτ transcript levels correlated with expression of SOX in most papers (Rizos et al., 2002, 2004, Lonergan et al., 2003a,b, Gutierrez-Adan et al., 2004a), suggesting that IFNτ expression is enhanced in conditions of oxidative stress.

Oxidative stress leads to increased lipid peroxidation which in turn cause mitochondrial dysfunction in embryos (Guerin et al., 2001). Mitochondrial manganese superoxide dismutase (MnSOD) is associated with increased mitochondrial activity and detoxification of ROS, whilst HSP70 has a protective role during stress. The relationship between transcript levels for genes associated with stress recovery (HSP70 and MnSOD) and quality was not significant (Table 29, page 158), possibly due to varied levels of stress affecting quality in different ways. Therefore, there seems to be no relationship between quality and ability to activate a cellular stress response mechanism.

In Chapter 3, exposure of bovine blastocysts to 42°C for four h increased transcript levels of IFNτ relative to control blastocysts, demonstrating that detrimental environmental factors may regulate IFNτ expression. IFNτ and HSP70 transcript levels were evaluated in the same blastocyst, and it was observed that IFNτ transcript levels increased with HSP70 transcript levels. In agreement, most studies assessing changes in HSP70 and IFNτ transcript levels

between treatments have observed similar changes in HSP70 and IFNτ transcript levels in different treatments (discussed in section 3.4.3, summarised in Figure 31, page 149), suggesting that IFNτ and HSP70 expression may be regulated by a similar mechanism. Heat treatment caused an increase in HSP70 transcript levels but did not affect the change of the morphology of the blastocyst during 24 h individual culture, suggesting that the level of stress caused by the heat treatment was high enough to activate the cellular stress response, but mild enough not to cause a change in morphological quality, suggesting that the stress may have been mild enough not to cause an increase in apoptosis and necrosis. Therefore, mild heat stress upregulated IFNτ expression, suggesting that IFNτ is an index of stress, as first suggested by Kubisch et al. (1998).

Therefore, the results from this thesis suggest that IFNτ expression and secretion are not associated with overall quality parameters, and, instead, IFNτ expression seems to be related to cellular stress.

4.1 Quality, metabolism, stress and viability

4.1.1 Quality

Although quality parameters tend to be predictors of future embryo development *in vitro* (Fujino et al., 2007), they do not necessarily correlate positively with future embryo development *in vivo*, such as rate of attachment, pregnancy rate and development to full term. Therefore, quality parameters are not sensitive enough to predict viability. Quality parameters are likely to be a reflection of the success of embryo development up to the point when embryos were assessed which may not necessarily relate to the success of future embryo development (as discussed by McEvoy et al., 1997).

4.1.2 Metabolism

In Chapter 3, IFNτ correlated positively with YWHAZ transcript levels, a possible index of total expression level which is mediated by cell number (Sirard et al., 2005, amongst other factors), suggesting that IFNτ expression may be higher in embryos with more cells. In agreement, Stojkovic et al. (1999) estimated trophoblast growth by calculating the surface areas of embryos based on diameters and observed a significant positive correlation between trophoblast area and IFNτ secretion. Using ovine embryos, Lo and Summers (2002) also observed that embryo diameter was positively correlated with IFNτ secretion, suggesting that IFNτ secretion may be positively correlated to embryo size and number of trophoblast cells. However, in this thesis, blastocyst diameter did not correlate with IFNτ expression (Chapter

3) or secretion (Chapter 2), maybe because blastocyst diameter is only a crude estimation of trophoblast cell number since embryos may collapse and re-expand. Cell number is a consequence of a balance between increase in cell proliferation fuelled by an increase in metabolism, and a decrease in cell death by apoptosis or necrosis. According to Chapter 2, apoptosis was not related to IFNτ secretion (in agreement with Russell et al., 2006), but the positive relationship (which approached significance, P=0.07) between IFNτ secretion per cell and pyruvate oxidation per cell observed in Chapter 2, suggesting that IFNτ secretion is related to metabolism, but not apoptosis. Ealy et al. (1993) previously demonstrated that mild heat treatment enhanced the growth rate of embryos, suggesting an increase in metabolism. In this thesis, heat treatment increased IFNτ expression, further suggesting that IFNτ expression may be positively associated with rate of metabolism. Therefore, the lack of a correlation between IFNτ production and quality parameters observed in this thesis, and the positive relationship between IFNτ expression and stress may be due to IFNτ being related to metabolism as opposed to quality parameters.

The 'Quiet embryo hypothesis' proposes that metabolism of an embryo is indicative of its viability, with viable preimplantation embryos operating at metabolite or nutrient turnover rates which are lower than those of their less viable counterparts (Leese et al., 2008). Therefore, following the principles of the quiet embryo hypothesis, the positive relationship between IFNτ secretion and metabolism suggests a negative relationship between IFNτ and embryo viability.

All cells will use energy as required by the normal cellular and developmental processes necessary to maintain survival. The embryo has an intrinsic ability to compensate for stress which involves processes such as mRNA damage surveillance and ribosomal recovery, protein degradation and amino acid recycling, nucleotide, polynucleotide, peptide, polypeptide synthesis (Baumann et al., 2007). As an embryo becomes stressed, energy is used in an attempt to accommodate the necessary repair system to repair the damage to the genome, transcriptome and proteome caused by stress. According to the quiet embryo hypothesis, the increased energy usage leads to an increased metabolism (and increased consumption of oxygen and nutrients, Leese et al., 2007) likely to generate increased levels of reactive oxygen species which are also deleterious to the cell (Guerin et al., 2001), which explains why the most viable cells, which need to use less energy repairing damage to the cell, exhibit a 'quieter' metabolism (Leese et al., 2002). As such, stress may lead to a reduction in embryo viability. Following this philosophy, Lane and Gardner (2005) observed that embryonic stress reduced the ability to regulate mitochondrial function and maintain

adequate levels of ATP, which eventually lead to disturbed cellular function, disturbed epigenetic regulation and abnormal foetal and placental development. The association between stress and reduced embryo viability has therefore lead to the hypothesis that early embryos should be maintained in systems designed to minimize stress. As such, stress may lead to a reduction in embryo viability.

The quiet embryo hypothesis suggests that the cellular stress response takes precedence over other developmental events, so that the normal programs of gene activity are altered. However, non-teratogenic doses of heat can produce a strong cellular stress response (Edwards, 1998) which does not fit this proposal. Therefore, on top of having a quiet metabolism, a viable embryo must also be able to respond to stress with minimum disruption to the metabolism in order to ensure a non-teratogenic consequence to the embryo.

In mice, the trophectoderm consumed significantly more oxygen, produced more ATP (trophectoderm produced 80% of the ATP generated by the embryo), contained greater number of mitochondria, had greater amino acid turnover (trophectoderm responsible for 90% of the amino acid turnover by the embryo) compared to the inner cell mass (Houghton et al., 2006). Therefore, the trophectoderm is more metabolically active than the inner cell mass. Given that the trophectoderm is the site of IFNτ production suggests that IFNτ production may be associated with increased metabolism and, following the quiet embryo hypothesis, increased stress and reduced viability.

Table 29 Direction of change in relative mRNA abundance of several candidate genes in 'good' compared with 'poor' quality embryos in a range of bovine studies (updated and expanded from Rizos et al., 2004). In each comparison, the latter is considered 'Poorer' than the former. ↑ : 'Good' contain more mRNA than 'Poor'. ↔: No significant change between 'Good' and 'Poor'. ↓: 'Poor' contain more mRNA than 'Good'. 1. Wrenzycki et al., 1999, 2. Wrenzycki et al. 2001a, 3. Wrenzycki et al. 2001b, 4. Bertolini et al., 2002, 5. Rizos et al., 2002, 6. Rizos et al., 2003, 7. Lonergan et al., 2003a, 8. Rizos et al., 2004, 9. Gutierrez-Adan et al., 2004a, 10. Lim et al., 2007, 11. Sagirkaya et al., 2007, 12. Correa et al., 2008. SOX is involved in oxidative stress; MnSOD, oxidative stress ; HSP70, heat stress. B: Bovine, O: Ovine, S: SOF, viv: *in vivo*, vit: *in vitro*, S1 and S2 are modified recipes of SOF, -: serum absent, +: serum present, PVA: polyvinyl alcohol, NT: nuclear transfer, BSA: bovine serum albumin, IVP: *in vitro* produced, High: high oxygen level, Low: low oxygen level. Majority of papers show a negative, positive or inconsistent relationship between the expression of the gene of interest and quality. Summary: number of studies showing a negative (↓) and positive (↑) per total number of studies assessing that gene. Probability (P) refers to the chi-square test compared to a third of the papers showing a negative, positive or inconsistent relationship between the expression of the gene and quality.

Culture System	PVA vs serum	NT vs IVP	vit vs viv	vit vs viv	S1 vs viv	- vs +	S1 vs viv	B vs O	S1 vs S2	Slow vs Fast	vit vs Viv	PVA vs BSA	vit vs viv	High vs Low	Summary ↓	Summary ↑	Summary Overall
IFNτ	↓	↑	↓	↑		↑	↓	↓	↓	↓	↓	↓	↔	↓	$9_{/13}$	$3_{/13}$	↓ P=0.01
SOX					↓	↓	↓	↓	↓	↓	↓				$7_{/7}$	$0_{/7}$	↓ P<0.001
Glut-1	↓	↔	↑	↑			↑	↑	↑	↑	↑	↓	↔		$2_{/11}$	$7_{/11}$	↑ P=0.1
HSP70	↑	↑	↓										↔		$1_{/4}$	$2_{/4}$	↔ P=0.8
MnSOD					↑	↓	↑	↑	↑	↓	↓		↔	↓	$4_{/9}$	$4_{/9}$	↔ P=0.4
Reference	1	2	3	4	5	6	7	8	8	9	9	10	11	12			

4.1.3 Stress

An embryo *in vivo* is exposed to environmental stimuli and intrinsic signals which ensure embryo survival. In this thesis, a stressed embryo is defined as an embryo which is exposed to any factor (or lack of) which causes an unfavourable deviation from the normal function of an embryo. If the *in vivo* environment is considered normal, then an *in vitro* embryo is always exposed to stressors. Moreover, heat denaturation of proteins proceeds at a slow rate even at normal body temperature (Johnson and Pavelec, 1972), so that all embryos (including *in vivo* embryos) must suffer some degree of thermal stress.

The stressor threatens the viability of an embryo and requires a response to ensure survival. There are several potential pathways in which stress may modulate cellular function. A stressor may cause modified gene expression, leading to modified metabolism, causing a subsequent change in developmental kinetics, causing cell death, and affecting a range of quality parameters (ability to blastulate and hatch, cell proliferation, rate of development, etc…) and the successful establishment of pregnancy (Fleming et al., 2004). The dose of stress and the ability of the embryo to respond to the stressor will impact on whether the stressor affects embryo viability.

4.1.3.1 Dose of stress

As reviewed in section 1.8.5, maternal heat stress leads to foetal loss (Garcia-Ispierto et al., 2006). Exposure of heifers to hyperthermic conditions (daily exposure to 16 h at 30°C and 8 h at 42°C) caused a 2.5–fold increase in the number of abnormal and retarded embryos and unfertilized ova compared to heifers kept at thermal neutrality (20°C, Putney et al., 1988a). Exposure of *in vitro* bovine embryos to 43°C for 18 h caused a reduction in intracellular incorporation of proteins (indicative of decreased viability) and decreased IFNτ secretion (Putney et al., 1988b). However, exposure of pregnant cows to 37°C for nine days (which would cause an in utero temperature of around 39.8°C) caused an increase in IFNτ secretion compared to control (21°C, Geisert et al., 1988). Therefore, the varied effect of hyperthermia on IFNτ secretion reported in different studies may be explained by differences in the dose of heat exposed to the embryos. The 'dose' is the produce of the extent and duration of elevated temperature.

In rats, as the temperature exposed to pregnant rats increased, the time required to cause a defect was reduced logarithmically (Germain et al., 1985). In mice, Edwards (1998) also observed that the severity and prevalence of teratogenesis depended largely on the 'dose' of

heat. Therefore, a high dose of hyperthermia leads to an increase in abortion and conceptus abnormality, reduced dose leads to a high prevalence of abnormality with low rates of abortion, and lower doses leads to no abnormality or abortion.

Non-teratogenic doses were associated with a minor increase in the incidence of apoptosis, whilst teratogenic doses caused severe apoptosis, suggesting that there is a threshold level of apoptosis required to cause teratogenesis. Therefore, Edwards (1998) concluded that an embryo must be exposed to a threshold 'dose' of heat if rate of apoptosis is to increase, and a higher dose if teratogenic effects are to be caused. Although Edwards (1998) reviewed the effect of hyperthermia in post-implantation rodents, it is possible that the threshold dose concept may be applicable to bovine pre-implantation embryos as well.

Based on the rodent models, it is hypothesised that in cattle, increased doses of stress cause increasing deviations from normality in bovine pre-implantation embryos, as outlined in the model proposed in Table 30 (page165). At mild doses of stress, there is some modification in gene expression and cell death by controlled apoptosis, but changes to metabolism, developmental kinetics, necrosis and reduced quality and short term survival do not occur. At higher doses of stress, the embryo is not able to cope with the stress through the cellular stress response and apoptotic pathways alone and, instead, gene expression is modified further, leading to modified metabolism, developmental kinetics, cell death by apoptosis as well as necrosis, leading to deteriorated quality and reduced attachment and pregnancy, and a greater decrease in short term as well as long term survivability compared to milder stress doses. Lethal doses of stress overwhelm the ability of the embryo to respond to stress so that the stress defence mechanisms (such as cellular stress response and apoptosis) are not activated correctly, leading to decreased quality of the embryos and, ultimately, abortion. Therefore, quality parameters are not affected at lower degrees of stress (0 or 1, Table 30, page165). Quality markers attempt to predict survivability, and a reduction of survivability is a possible consequence of high doses of stress, whilst exposing embryos to mild doses of stress may not affect the ability of the embryo to survive.

In agreement with the quiet embryo hypothesis, this model suggests that viability will be determined by the effect of the stressor on the metabolism of the embryo, but also considers that the embryo may be able to tolerate or even recover from the stress insults if the dose of the stress is below a threshold. Therefore, IFNτ may be related to stress and not with quality, and still be an index of viability.

4.1.3.2 Ability of an embryo to respond to a stressor

The stress dose that leads to mild, teratogenic or lethal levels of stress is likely to vary between different embryos according to the variability between embryos in their ability to respond to a stressor. In this thesis, blastocysts were evaluated before and after 24h individual culture. Some blastocysts progressed in development, some remained unchanged and some regressed in development. Such differences in change in morphology index between different embryos cultured in the same conditions suggest that different embryos have different levels of ability to respond to a stressor.

4.1.3.2.1 Reasons for different embryos to have varied abilities to respond to stress

Embryos from Brahman and Romosinuano breeds are more resistant to elevated temperature than embryos from Angus (Hernandez-Ceron et al., 2004). In another study, embryos from Brahman cows were more resistant to heat shock than embryos from Holstein or Angus, and embryos from Senepol cows had even lower resistance to heat shock (Paula-Lopes et al., 2003). Therefore, different breeds of cattle have adapted to different environmental temperatures, so that the normal basal temperature varies between breeds. Therefore, the threshold dose that leads to mild stress, teratogenic stress or abortion is likely to vary between different embryos according to the variability between embryos in their normal basal level.

Using pregnant mice, Edwards (1998) observed that the periods of high susceptibility to embryonic stress occurred during organogenesis, with the type of malformation depending mainly on the stage of embryonic development. The authors speculated that at this stage, HSP expression is at its highest level, possibly to protect the embryo and the increased susceptibility might be due to gene activity being concentrated in heat shock response, so that the normal protein synthesis necessary for organogenesis becomes suspended. Therefore, protective HSPs are produced, but survival is achieved at the expense of normal development. The same principle could be applied to pre-implantation embryos, with certain stages of embryo development having increased sensitivity due to the gene activity sequestering greater proportion of HSPs for certain processes in embryo development, leaving less for protection against environmental insults. Alternatively, the production of induced proteins may be delayed due to certain processes requiring extensive gene activity. Lane and Gardner (2005) observed that post-compaction embryos are better able to regulate their physiology and are, therefore, less vulnerable to stress than pre-compaction embryos. In this thesis, IFNτ and HSP70 expression were upregulated in post-expansion blastocysts compared to blastocysts before expansion (Appendix 11), suggesting that post-expansion blastocysts are less vulnerable to stress than pre-expansion blastocysts.

In guinea pig, rat and mice embryos, the M-phase cells appeared to be the most susceptible to hyperthermic stress, with higher stress doses leading to S-phase cell deaths. Similarly in pig embryos, low doses of hyperthermia affected cells in mitosis phase of the cell cycle, and other cells became affected after more severe doses (Edwards, 1998). The reason for the increased susceptibility of mitotic cells is unclear, but could be due to irreversible denaturation of some proteins associated with M-phase chromosomes. In the initial M-phase cell death, the chromosomes shortened, thickened and aggregated into clumps which later fragmented. Cells then became vacuolated and disintegrated and normal mitotic activity was suspended. Blocks to progression through mitosis occurred before prophase and during metaphase (Edwards et al., 1974). Therefore, the stage of the cell cycle will also affect the susceptibility of blastomeres to stress.

Therefore, factors likely to contribute to variation between embryos in their ability to respond to a stressor include breed, stage of development, number of cells and rate of metabolism, inherent genetics and stage of the cell cycle. Moreover, it is possible that the ability of an embryo to respond to stress may be indicative of its viability.

4.1.3.3 The ability to respond to stress may be an indication of viability

Acquiring thermotolerance involves increasing the capacity of the embryo to produce molecules involved in thermoprotection (Arechiga et al., 1995). Therefore, exposing embryos to a mild stress treatment may enhance its survival by enhancing its tolerance to future stressors. In this thesis, the blastocysts were not cultured on after heat treatment, so that it was not possible to assess whether mild heat treatment improved viability. There are few studies on acquisition of thermotolerance by bovine embryos, all of which are interested in establishing the stage of development that thermotolerance is possible, and none of which actually transferred the embryos back *in vivo* to determine whether thermotolerance leads to improved viability. Ealy et al. (1993) demonstrated that mild heat treatment enhanced the growth rate of embryos and concluded that mild heat treatment may benefit the embryo through acquisition of thermotolerance. However, the enhanced growth rate is more likely to be indicative of an increase in metabolism, so that further studies are necessary to determine whether increased tolerance to stress improves bovine embryo viability.

High hydrostatic pressure is a stressor for biological materials by inhibiting growth (Abe et al., 1999), altering the membrane functionality and therefore perturbing the physico-chemical balance of the cell (Palou et al., 1997) and perturbing DNA replication (Abe et al., 1999). High hydrostatic pressure has also been shown to increase HSP expression in human chondrocyte-like cells (Kaamiranta et al., 1998), confirming that high hydrostatic pressure is a

stressor to cells. Higher pressure dose (90MPa for 2 h or 30MPa for 5h) caused irreversible changes to the murine blastocysts, which disintegrated after 2 h of *in vitro* culture or immediately after decompression (Pribenszky et al., 2005a). Sublethal levels of hydrostatic pressure (90MPa for 30 minutes, or 30MPa for 3 h), significantly improved the post-thaw survival of frozen mouse blastocysts, as well as live birth rate after transfer (Pribenszky et al., 2005a). Similarly, pig oocytes exposed to high hydrostatic pressure also demonstrated increased cleavage rate compared to control (Prisbenzsky et al., 2008). Exposure to high hydrostatic pressure prior to freeze-thawing has been shown to result in improved motility, viability and fertility parameters of frozen bull and boar semen (Huang et al., 2009, Pribenszky et al., 2005b), suggesting that exposing gametes and embryos (including bovine) to a mild stress may improve their viability due to increased stress tolerance.

4.1.4 Viability

As outlined in Figure 32 (page 166), the model postulates that there are three main factors that will determine the viability of an embryo: rate of metabolism, ability to respond to stress and dose of stress exposed to the embryo. For maximum viability, the embryo will have a quiet metabolism and will not be exposed to a stressor. Since all embryos are stressed to some degree, then an embryo must be able to respond to stress in order to be viable. The extent of the ability to respond to stress required for survival will depend on the dose of stress that will be faced by the embryo during its future development. If quality parameters are indicative of the success in past embryo development, and rate of metabolism is indicative of the present success in embryo development, then ability to respond to stress is indicative of future embryo development, pending only on the dose of stress to be exposed to the embryo by the environment.

The model postulates that in the absence of stress, the viable embryo is likely to have a 'good' morphological quality, quiet metabolism and basal levels of IFNτ expression. At different doses of stress, the relationship between embryo viability and metabolism, quality parameters and IFNτ production is likely to change, as summarised in Figure 33 (page 166). According to the quiet embryo hypothesis, there is a negative relationship between rate of metabolism and viability. However, the quiet embryo hypothesis takes no account of either the extent of the stress or the ability of the embryo to respond to the stressor, which will impact on how the stressor affects embryo viability. Therefore, when stress levels are below the threshold to affect rate of metabolism, then rate of metabolism cannot be predictive of embryo viability. Lethal levels of stress will cause a shut down in the metabolism of the embryo, so that some

'quiet' embryos may have a low metabolism due to the gradual shut-down of the embryo as it ceases to function. Since quality parameters are indicative of the past success of embryo development, then if the embryo is not exposed to any stressors, it will continue to develop as in the past, so that quality parameters will be indicative of embryo viability. At sub-lethal levels of stress, quality parameters cannot account for the variation between embryos in their ability to respond to stress, so that quality parameters will not be predictive of embryo viability. Lethal levels of stress cause extensive necrosis and fragmentation, and thus affect the morphology of the embryo and its quality parameters. Therefore, low quality parameters would indicate that embryos have been exposed to lethal levels of stress and/or are genetically uncapable of normal development irregardless of stress and, therefore, can predict that these embryos are not viable.

If IFNτ production is associated with a stress response, as suggested by the results from this thesis, then if the stress dose is below the level necessary to activate the cellular stress response mechanism, then it follows that IFNτ production will not be indicative of embryo viability. At mild stress levels, increase in IFNτ production will be indicative of activation of the cellular stress response (and increase in stress tolerance), and indicative of ability to respond to stress. Therefore, at mild stress levels, increased IFNτ production would suggest increased viability. Due to the protective role of the cellular stress response, quality parameters are not affected at mild stress dose, so that the relationship between IFNτ expression and quality parameters become inconsistent. At higher stress doses, embryo viability becomes compromised, so that increased IFNτ production would be indicative of increased stress levels and reduced embryo viability. At lethal stress levels, IFNτ expression levels and quality parameters deteriorate, and are indicative of the shut down of the genetic regulation of the dying embryo.

Therefore, because the relationship between embryo viability and quality parameters, metabolism and IFNτ production will vary depending on the level of stress incurred on the embryo, then it follows that none of these factors may accurately predict embryo viability without accounting for the dose of stress exposed to the embryo.

Table 30 A proposed stress induced causal model. Different degrees of change (inherent or environmental) cause different degrees of stress. At lower degrees of stress, quality parameters are not affected. +: some increase, ++: a lot of increase, -: decrease, ↓: causal relationship. An *in vivo* embryo would have closer to 0 stress, whilst an *in vitro* embryo would have higher stress levels. GM-CSF would improve the *in vitro* environment, making it more similar to the *in vivo* environment and, therefore, lower in stress than the *in vitro* embryo not exposed to GM-CSF. The heat shock level used in this thesis was mild, as quality parameters were not affected. Embryos that had a change in morphology index that showed deterioration or no change had lower thresholds to stress and therefore had lethal levels of stress.

Change Degree of Stress		None 0	Mild 1	High 2	Lethal 3
↓					
Modified gene expression	overall	None	+	++	-
	HSP70 and IFNτ	None	+	++	None
↓					
Modified metabolism		None	None	++	-
↓					
Modified developmental kinetics		None	None	++	-
↓					
Cell death	Apoptosis	None	+	++	None
	Necrosis	None	None	+	++
	Fragmentation	None	+	++	+++
↓					
Quality parameters		Normal	Normal	Deteriorated	Deteriorated
↓					
attachment and establishment of pregnancy		Normal	Normal	Reduced	Not occur
Survival	Short term	Not affected	Not affected	May be affected	Affected
	Long term	Not affected	May be affected	Probably affected	Affected

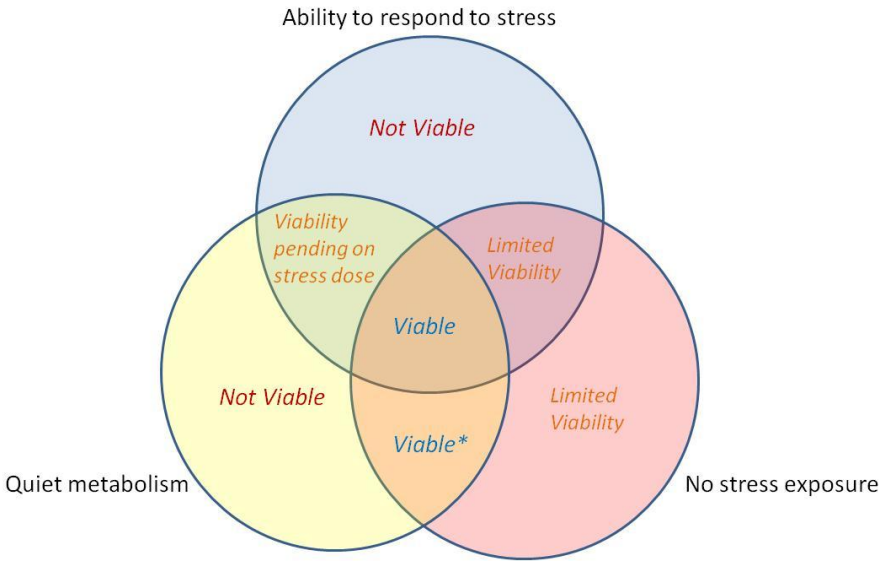


Figure 32 Factors affecting viability. *according to the quiet embryo hypothesis.

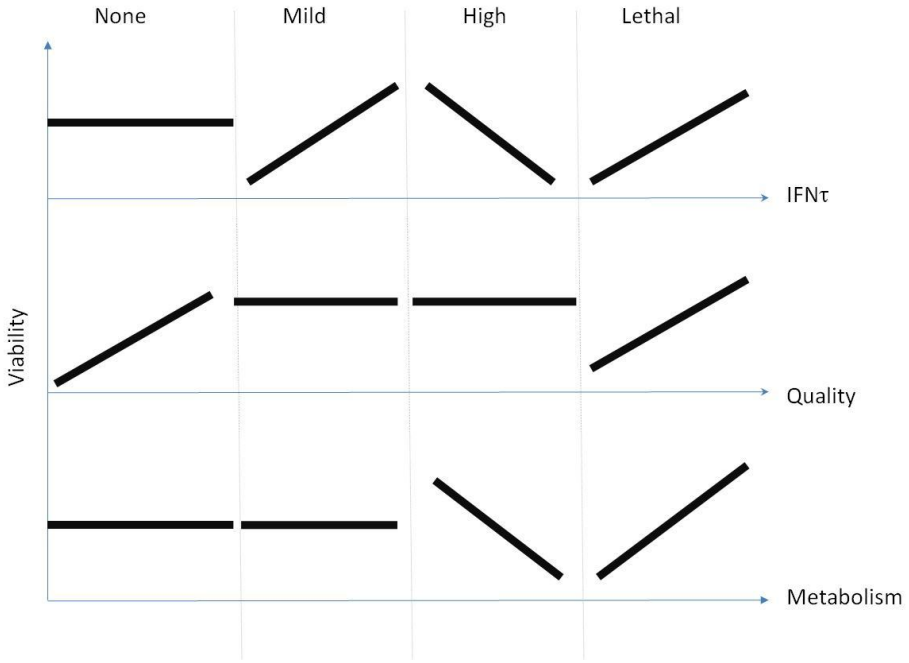


Figure 33 According to the quiet embryo hypothesis and the assumption that IFNτ is related to cellular stress response, hypothetical relationship between embryo viability and rate of metabolism, quality parameters and IFNτ production at different levels of stress (none, mild, high and lethal). When no stressors are present, IFNτ levels and metabolism are not affected by viability whilst quality parameters reflect viability. At mild stress levels, the cellular stress response is activated, and the viability is determined by the aility of the embryo to respond to stress. Therefore, IFNτ levels increase with viability. Quality does not reflect stress tolerance, so that quality is not

affected by viability. Since stress levels are below the threshold for metabolism to be affected, metabolism is not indicative of viability. High stress levels are detrimental to viability, so that metabolism becomes affected. At lethal levels of stress, IFNτ synthesis and rate of metabolism decrease as the embryo shuts down, so that IFNτ is then positively related with viability.

4.2 IFNτ production by stressed embryos and the maternal recognition of pregnancy

The relationship between IFNτ and cellular stress response suggests (1) a role for IFNτ in cellular stress response, and/or (2) an evolutionary adaptation of bovine embryos to increase their communication with the maternal system when threatened by a stressor, thus improving the chances of establishing a pregnancy (discussed further in section 4.2). In these circumstances, increased IFNτ expression may be associated with increased embryo viability (discussed further in section 4.1.3.3). If the ability to respond to mild doses of stress is an indication of viability, then IFNτ expression as a response to stress may be an evolutionary adaptation to promote establishment of pregnancy. *In vivo*, there is a positive feedback loop between the maternally derived cytokines and the embryonic derived IFNτ (discussed in section 1.7.1.4). Therefore, if the embryo does not receive a response from the maternal system, which is absent *in vitro*, then the embryo may increase its IFNτ expression levels in an attempt to increase the effectiveness of the maternal recognition of pregnancy signal, which may explain why *in vitro* cultured embryos express higher levels of IFNτ than *in vivo* produced embryos (Wrenzycki et al., 2000, 2001b, Lonergan et al., 2001).

5 CONCLUSION

In Chapter 2, bovine embryos were unresponsive to a cytokine shown to be beneficial to embryo development (roGM-CSF) in terms of IFNτ secretion, metabolism (pyruvate oxidation and rate of development), apoptosis, cell number, blastocyst yield and morphological quality in the concentrations and periods of exposure tested. In Chapter 3, IFNτ expression was upregulated in embryos exposed to a mild heat stress compared to control. Moreover, there was a positive correlation between IFNτ and HSP70 transcript levels, suggesting a connection between IFNτ expression and the cellular stress response.

Although some quality parameters were identified as modulators of IFNτ expression and secretion (day of blastulation and change in morphology over 24 h culture), the relationship between IFNτ expression and secretion and different quality parameters was inconclusive. Embryo metabolism, as outlined by the quiet embryo hypothesis, may be a better predictor of embryo viability than quality parameters. The lack of a correlation between IFNτ production and quality parameters observed in this thesis, and the positive relationship between IFNτ expression and stress is likely to be due to IFNτ production being positively related to rate of metabolism. The quiet embryo hypothesis, however, does not account for differences in the doses of stress exposed to the embryo or differences between embryos in tolerance to stress. Therefore, the viability of an embryo is likely to be determined based on a combination of the following factors

(1) Morphological quality parameters (indicative of how well the embryo has responded to stressors to date)
(2) Rate of metabolism (indicative of inherent viability, as outlined by the quiet embryo hypothesis)
(3) Ability to respond to stress through the cellular stress response pathways (which may be enhanced through exposure to mild stress leading to stress tolerance)
(4) Dose (amount and duration) of stress exposed to the emryo.

These factors may explain, at least in part, why not all embryos with quiet metabolism and with good morphological grading lead to a pregnancy. If the embryo cannot be quiet then it needs to be able to make an appropriate response to stress to return to "normal" with minimum expenditure of resources. The

relationship between IFNτ expression, quality parameters and metabolism will vary depending on the degree of stress exposed to the embryo. Further studies are necessary to confirm whether IFNτ production, rate of metabolism and stress dose could be used together as consistent biomarkers indicating embryo viability, which would be indispensable for obtaining higher reproductive efficiency in cattle breeding.

6 SUGGESTIONS FOR FUTURE WORK

The results from this thesis have lead to four hypotheses that would require further work to become established. In the discussion, it was hypothesised that:

(1) IFNτ production is not related to quality parameters;

(2) IFNτ production is associated with metabolism;

(3) IFNτ production is associated with cellular stress response;

(4) Embryos with a higher ability to tolerate stress have higher embryo viability.

The purpose of this chapter is to explore ways to test these hypotheses.

6.1 IFNτ production is not related to quality parameters

One way to determine whether IFNτ production is associated with quality parameters is by exposing embryos to a factor that improves quality parameters. In this study, the beneficial environmental factor (GM-CSF) had no effect on IFNτ secretion, so that the effect of other beneficial factors (rbGM-CSF, natural GM-CSF, or other growth factors previously demonstrated to improve embryo quality parameters) would have to be assessed. However, it is hard to identify a factor to improve embryo quality. Several studies have observed IFNτ expression and secretion relative to a range of quality parameters (Table 28, page 153), so that the inconsistent relationship between IFNτ production and quality parameters is well established. Given that IFNτ production was not associated with embryo quality parameters, and that quality parameters are poor predictors of embryo viability, future studies should not focus on the relationship between IFNτ production and quality parameters.

6.2 IFNτ production is associated with metabolism

In this thesis, the relationship between IFNτ secretion per cell and pyruvate oxidation per cell approached significance (P=0.07), suggesting a possible relationship between IFNτ secretion and rate of metabolism. It would be interesting to know whether the relationship has higher significance between IFNτ expression and pyruvate oxidation (without the post-transcriptional variation) . To determine whether IFNτ production is associated with rate of metabolism, different parameters of metabolism should be assessed relative to IFNτ expression and

secretion, such as turnover of amino acids, lactate production, measurements of oxygen, pyruvate and glucose consumption. The problem with using whole embryos for these studies is that IFNτ production is primarily localised to the trophectoderm, so that variation in the rate of metabolism of the inner cell mass would add variability to the data. Therefore, further studies should also assess rate of metabolism on biopsies from the trophectoderm, with the embryo being transferred back to assess embryo implantation potential (as a measure of viability). Mosaicism and reduced viability due to the biopsy technique are obvious disadvantages. However, a combination of whole embryos and trophectoderm cell biopsies would provide a clear picture as to whether IFNτ production is associated with rate of metabolism.

It would also be interesting to know whether the bovine trophectoderm has increased rate of metabolism compared to the inner cell mass, as reported in the murine (Houghton et al., 2006).

6.3 IFNτ production is associated with cellular stress

As discussed in section 1.2.1, not all IFNτ mRNA isoforms are transcribed and post-transcriptional and translational regulation may affect the correlation between total IFNτ expression and secretion levels, as observed by Robertson et al. (1996). Unfortunately, samples of media where the embryos were cultured in were destroyed in transit, so that it was not possible to assess IFNτ secretion levels in Chapter 3. Therefore, further work is required to determine whether heat treatment affects IFNτ secretion levels in the same way as it affects IFNτ expression levels.

Increased transcript levels of a gene are caused either by an increase in expression or an increase in mRNA stability. One way to assess mRNA decay is to expose pools of embryos to actinomycin or α-amanitin (which cause transcription arrest) and then assess mRNA levels using RT-PCR at 0, 1 and 2 h post-exposure to the transcription inhibitor. Although this method was performed in Hela cells (Theodrakis and Morimoto, 1987), the embryo-to–embryo genetic variation within the pool of embryos may be too high for this method to be feasible, in which case it might be better to use cell cultures derived from embryos rather than pools of embryos. The HSP70 and IFNτ mRNA stability in heat shocked embyonic cells could then be compared to that of controls to determine whether heat shock affects the mRNA stability of IFNτ and HSP70.

IFNτ expression in bovine blastocysts was associated with heat (Chapter 3), oxidative (Kubisch and Johnson, 2007), and physical (embryo manipulation, Wrenzycki et al., 2001a) stress, so that other stressors need to be evaluated, such as glucose levels, osmolarity, pathogenic (bacterial or viral infections) or cryogenic stress.

Furthermore, it would be interesting to examine the effect of stress in ovine blastocysts, to determine whether IFNτ expression by ovine blastocysts respond to heat stress in a similar way to bovine blastocysts.

Once the relationship between cellular stress and IFNτ production becomes established, it would be interesting to determine whether this association is mechanistically related or controlled at the molecular level. Increased expression of a gene may be caused either by direct upregulation of transcription of the gene of interest, or activation of additional factors associated with transcription of the gene of interest. Although a mechanism was proposed for the relationship between IFNτ, HSP70 and heat stress (section 3.4.4), further work is necessary to clarify the molecular signalling by which heat stress causes upregulation of IFNτ and HSP70. Different IFNτ and HSP70 gene control constructs may be transfected into cell lines and mutations of different elements in different pathways may be mutated and the effect observed in order to determine which molecular pathways may be mediated by IFNτ and HSP70. There is also a need to further understand the promoter region of IFNτ, in order to identify a region on the promoter where a factor associated with the cellular stress response may bind to.

Further experiments assessing IFNτ expression in a third treatment whereby the embryo is exposed to an alleviated stress, such as exposure to an antioxidant (i.e. glutathione), or exposure to a milder heat stress (1 h at 40°C) prior to heat treatment to induce thermotolerance, may clarify whether IFNτ is regulated differently at different doses of stress. HSP70 expression was not upregulated when thermotolerated embryos were exposed to a more severe stress (Al-Katanani and Hansen, 2002). An increase in IFNτ expression in thermoregulated embryos would therefore suggest an environmental alteration in gene expression, rejecting the hypothetical connection between IFNτ and HSP70 expression mechanisms.

Varied effect of hyperthermia on IFNτ secretion reported in different studies may be due to differences in dose of stress. A model of how different degrees of stress have different outcomes in terms of embryo viability, quality parameters, metabolism and IFNτ expression is summarised inTable 30 (page165). IFNτ production in embryos stressed at mild, high and lethal doses compared to control blastocysts should be compared in a microarray to genes related with metabolism (glucose 6 phosphate dehydrogenase is a potential indicator of the PPP pathway, Glucose transporters are associated with nucleotide synthesis, arachidonate lipoxygenase gene 15 are associated with carbohydrate metabolism), apoptosis (BCl-2 and BAX are regulators of apoptosis), necrosis (inflammatory cytokines such as TNF), compaction and blastocyst formation (Cx), implantation (COX2 and CDX2, El-Sayed, 2006), placenta formation (placenta-specific gene 8) and SOX (a stress sensitive gene), in order to determine whether the stress induced causal model proposed in section 4.1.3 is accurate. Transferring these embryos back would then provide an indication of embryo viability.

6.4 Embryos with higher ability to tolerate stress have higher embryo viability

Embryos with induced thermotolerance will have a higher ability to tolerate stress. Therefore, by comparing pregnancy rates (determined by progesterone levels and foetal heart beat) of thermotolerated and control embryos, it would be possible to determine whether embryos with higher ablity to tolerate stress have a higher embryo viability. The problem with this simple experiment is that the level of stress exposed to the embryos will differ in different uteri. Therefore, sibling embryos could be used, and a genetic profile determined by embryo biopsy prior to transfer. Then a thermotolerated and a control embryo are then transferred into the same uterus to determine which embryo is most likely to attach and lead to a successful pregnancy. If twin pregnancies occur, then the health of the offspring and the placenta may be compared.

Also, embryos could be exposed to increasing amounts of stress to determine a correlation between teratogenesis and abortion and stress at the blastocyst stage. It would be interesting to observe how stressed embryos recover, and whether there is a legacy of stress in previously stressed embryos that survive. Can the stressed embryos be distinguished from unstressed embryos during and after pregnancy?

6.5 Improvements to current methodologies

The RT-PCR method validated in Chapter 3 was an improved method compared to previous techniques for detecting bovine mRNA in that it allowed for quantification of mRNA in absolute terms, corrected for variation in overall mRNA levels (due to changes in cell number, embryonic genome activation and viability) with an endogenous control, and variation in enzymatic efficiencies with an exogenous control, and used sequence specific probes allowing for multiplex reactions for detection of mRNA levels in single bovine embryos (discussed in section 1.2.1).

As discussed in section 1.2.2.2, the cell cytopathic assay used to detect IFNτ protein levels in culture medium detects IFNτ through its antiviral activity as opposed to the biological activity relevant to the role of IFNτ, through binding to IFNτR on the uterine endometrium. Therefore, the biological activity as determined by the cell cytopathic assay may not correspond to the biological activity relevant to the role of IFNτ. Moreover, the cell cytopathic assay is not specific to IFNτ, as other IFNs may also have biological activity, and, because different IFNτ isoforms vary in antiviral activity, then the biological activity determined by the cell cytopathic assay may not correspond to the amount of protein present. These limitations to the cell cytopathic assay suggest that improvements are necessary to the current techniques available for detecting IFNτ secretion.

In rats, normal protein synthesis is diminished during a heat shock response (Walsh et al., 1987). In this thesis, only mRNA levels for HSP70 and IFNτ were assessed, so that further studies would be required to determine whether the protein levels mimic the mRNA levels for HSP70 and IFNτ during a cellular stress response. If IFNτ protein levels are increased during a cellular stress response, this would be the opposite of what happens to other proteins and would further suggest a control for IFNτ production related to the cellular stress response, as opposed to metabolism. However, there are currently no methods available which are sensitive enough to assess HSP70 protein levels in individual bovine blastocysts. Therefore there is a need for a higher sensitivity detection method for bovine HSP70 to determine whether HSP70 protein synthesis is increased during a cellular stress response.

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APPENDICES

Appendix 1
Equipment and Chemicals

In vitro embryo production

Equipment	Supplier
Needles, 18-gauge	Sherwood Medical, Hampshire, UK
Syringes, 5 ml	Sherwood Medical, Hampshire, UK
Petri dishes, 60 and 30 mm diameter, sterile	Bibby Sterilin, Staffs, UK
Petri dishes, 60 mm, sterile	Nalge Nunc International, Hereford, UK
Universal containers, 30 ml, sterile	Fisher Scientific, Loughborogh, UK
Multidishes 4-well plates, sterile	Nalge Nunc International, Hereford, UK
Swim-up tubes, sterile	Bibby Sterilin, Stone, UK
Filters, 0.2 µm, 150 and 500 ml, sterile	Sartorius, Gottingen, Germany
Filters Minisart, 0.2 µm, sterile	Sartorius, Gottingen, Germany
Microtubes, 0.5 and 1.5 ml	Fisher Scientific, Loughborogh, UK
Micro-pipettes and tips	Gilson, WI, USA
Incubators, 5% CO_2 in air	Heraeus Instruments, Bishop's Storford, UK
Incubators, 5% CO_2, 5% O_2, 90% N_2	Heraeus Instruments, Bishop's Storford, UK
Microscopes	Wild M3Z, Heerbrugg, Switzerland
Inverted microscope	Labovert FS, Leitz Wetzlar, Germany
Autoclave	BA852 Sovereign, Dendley instruments Limited, Sussex, UK
Osmometer	Model 3MO, Advanced instruments Inc., Needham Heights, Massachusetts, USA
Hood	HF72, Flow laboratories
Whirlmixer	Norlab instruments Ltd, Aberdeen, UK

Chemicals	Supplier	Cat. No.
Bovine Serum Albumin – Fraction V	Sigma, Poole, Dorset, UK	A3311
Bovine Serum Albumin – Fatty acid free (F.A.F.)	Sigma	A8806
Taurine	Sigma	T8691
D-(+)-Glucose	Sigma	G6152
Medium 199 – HEPES buffer	Sigma	M7528
Medium 199 – bicarbonate buffer (Chapter 5 and 8)	Sigma	M4530
Medium 199 – bicarbonate buffer	Sigma	M2154
L-glutamine	Sigma	G8540
Alanyl-glutamine	Sigma	A8185
Polyvinyl alcohol (PVA)	Sigma	P8136
Hyaluronic acid (sodium salt)	Fluka, Biochemika	53747
Trolox	Fisher Scientific	21894
α-Tocopherol (Vitamin E)	Sigma	T3251
Sodium citrate tribasic dihydrate	Sigma	S4641
Sodium DL-lactate	Sigma	L7900
Sodium pyruvate	Sigma	P4562
Ethylene glycol (EG)	Sigma	E9129
Dimethyl sulfoxide (DMSO)	Sigma	D8779
Foetal calf serum (FCS)	Sigma	F7524
Acetic acid	BDH Chemicals Ltd	27013 BVl
Ethanol (99.7%)	BDH Chemicals Ltd	10107.7Y
Phenol red solution (0.5% in D-PBS)	Sigma	P0290
Etylenediaminetetraacetic acid (EDTA)	Sigma	E6758
BME Essential amino acids (50x)	Sigma	B6766
MEM Non-essential amino acid solution (100x)	Sigma	M7145
Kanamycin monosulfate	Sigma	K1377
Heparin (sodium salt)	Sigma	H3149
Luteinizing hormone (LH)	Sigma	L9773
FSH	Sigma	4520
Hydrochloric acid 1.0 N solution (HCl)	Sigma	H9892
Sodium hydroxide 1.0 N solution (NaOH)	Sigma	S2770
Sucrose	Sigma	S1888
Sodium bicarbonate ($NaHCO_3$)	Sigma	S5761
HEPES	Sigma	H4034
Potassium chloride (KCl)	Sigma	P5405
Sodium chloride (NaCl)	Sigma	S5886
Sodium phosphate (NaH_2PO_4)	Sigma	S5011
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	Sigma	M2393
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Sigma	C7902
Hypotaurine	Sigma	H1384
D-Penicillamine	Sigma	P4875
Sodium bisulphite ($NaHSO_3$)	Sigma	S9000
(±)-Adrenaline	Sigma	E1635
Phosphate Buffered Saline (PBS) – (Dulbecco A)	Oxoid, Hampshire, UK	BR00146

Cell counts and apoptosis assay

Equipment	Supplier
Microscope	Wang 600, Wang Biomedicals, Wageningen, The Netherlands
Digital camera	Olympus C-5050 zoom

Chemicals	Supplier	Cat. No.
Apoptag Red In Situ Apoptosis Detection kit	Chemicon International	S7165
Hoechst 33342	Sigma	B2261

Pyruvate metabolism assay

Equipment	Supplier
Scintillation vials, 6 ml	Zinsser Analytic, Germany
Filtered tips	Gilson, Middleton, WI, USA
Liquid scintillation analyser	Tri-carb 2300TR, Packard BioScience, Meriden, CT, USA

Chemicals	Supplier	Cat. No.
NaOH, 1 M Analar	BDH	191483R
HCl, 1 M Analar	BDH	190686W
Emulsifier Safe scintillation fluid	Packard BioScience	6013389
[1-^{14}C]-pyruvic acid	American Radiolabeled Chemicals	

Cell cytopathic assay

Equipment	Supplier
Multipipettor	
Biosafety hood	
96-well cell culture cluster – Flat bottom with lid	Corning Inc. (3599)

Chemicals	Supplier	Cat. No.
Dulbecco's modified Eagle medium	Invitrogen	11960-051
Sodium Pyruvate 100mM	Invitrogen	11360-070
Foetal Bovine Serum	Invitrogen	
L-glutamine	Invitrogen	
Pen/Strep	Invitrogen	
IFNτ standard	Dr. Ealy, University of Florida	
Gentian Violet Stain	Fisher	G12-100
Ethanol	BD Biosciences	
Dulbecco's Phosphate-Buffered saline	Gibco	14190-144

RT-PCR

Equipment	Supplier
0.5mL PCR tubes thin wall	Roslin Stores, LM40G
Non-stick RNase-free Microfuge tubes	Ambion, 12475
Stratagene MX3000P	Stratagene
Bioanalyzer 2100	Agilent
Primer Express 1.0	Primer Express®
Stratagene software	Stratagene
Gel Doc	Kapelan

Chemicals	Supplier	Cat. No.
RNeasy Micro Kit	Qiagen	74004
Quantitect Reverse Transcription kit	Qiagen	205313
Quantitect Custom Assay	Qiagen	241009
RNase ZAP	Ambion	9780
Quantitect Multiplex PCR kit	Qiagen	204543
Ribonucleic acid, messenger from rabbit globin	Sigma	R1253
Gene Ruler DNA ladder, ultra low range	Fermentas	SM1213
Top Vision LE GQ Agarose	Fermentas	R0491
10X TBE Electrophoresis Buffer	Fermentas	B52
RNAlater	Ambion	7020-7024

Appendix 2
Pyruvate Oxidation Assay

All eukaryotic cells undergo four metabolic pathways involved in carbohydrate catabolism and ATP production: glycolysis, pyruvate decarboxylation, tricarboxylic acid (TCA) cycle and respiratory chain. The pyruvate oxidation assay monitors the pyruvate decarboxylation reaction, which links glycolysis to the TCA cycle by converting pyruvate, the endproduct of glycolysis, into acetyl-coenzyme A, a reactant for the TCA cycle, as described in the following reaction:

Pyruvate + coenzyme A + Nicotinamide Adenine Dinucleotide → Acetyl-Coenzyme A + Carbon Dioxide + NAD, reduced

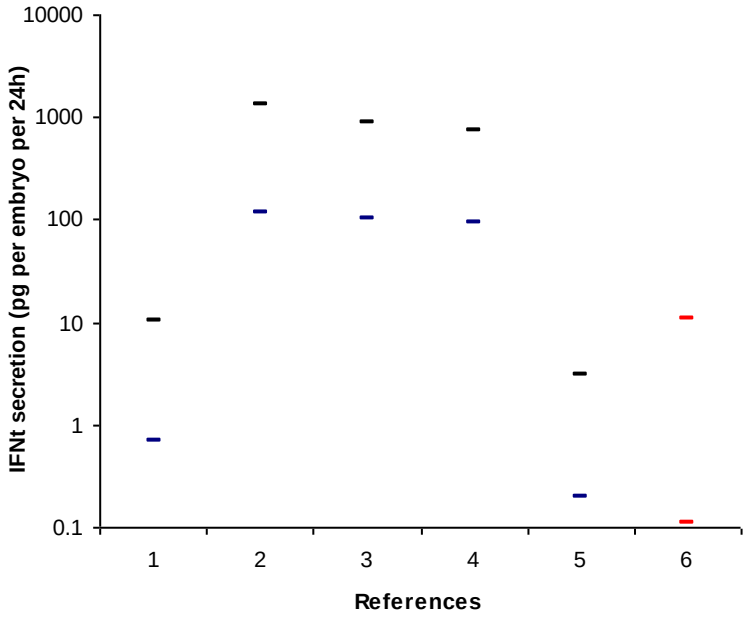
$$CH_3\text{-}C{=}O\text{-}COO^- + CoA + NAD^+ \rightarrow CH_3\text{-}C{=}O\text{-}CoA + CO_2 + NADH$$

3 2 1

The highlighted carbon (1) represents the radiolabelled carbon which was converted into carbon dioxide during the pyruvate oxidation assay in this study. The pyruvate oxidation assay involved incubating the blastocyst in a closed chamber in the presence of radioactive pyruvate, and the carbon dioxide released was continuously collected in a sodium hydroxide trap. The amount of radiolabelled carbon dioxide trapped in the sodium hydroxide trap was therefore proportional to the amount of pyruvate oxidised and was therefore a measurement of the metabolic rate of the blastocyst. The methodology used for measuring oxidative metabolism of embryos was similar to that previously used, with slight modifications. Rieger and Guay (1988), Rieger and Loskutoff (1994) and Rieger et al., (1992) used [2-^{14}C] pyruvate, which was only converted into carbon dioxide after the TCA cycle. In contrast, the current study, as well as Khurana and Niemann (2000), Thompson et al. (1993) and Rooke et al. (2005), used the [1-^{14}C] pyruvate which quantified specifically the pyruvate decarboxylation reaction. Therefore, use of [2-^{14}C] pyruvate and [1-^{14}C] pyruvate reflect the relative activities of two different sections of pyruvate metabolism.

Appendix 3

Modified from Figure 4. The range (minimum and maximum) of amount of IFNτ (pg embryo^{-1} 24h^{-1}) detected in individual culture media as reported in different studies observing ruminant blastocysts. 1: Hernandez-Ledezma et al. (1992, 1993), 2: Kubisch et al. (1998, 2001a,b, 2004), 3: Kimura et al. (2004), 4: Rooke et al. (2005), 5: Neira et al. (2007), 6: current study.



Appendix 4

Single stranded sequence for α-globin DNA (X04751, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=1466) and location of the primers and probes used in this study: primer and ***probe***.

```
   1 gcggggccgg gtcccaggca gacgccgcga gggcgccccc agcggtggcg gccgccgccg
  61 cgccccgccg cgccggccaa tgagcggggc cccgctgggc gtgcccgcag cacctcgggc
 121 ttaaaagcgc cgcgcagtct gggctccgca cacttctggt ccagtccgac tgagaaggaa
 181 ccaccatggt gctgtctccc gctgacaaga ccaacatcaa gactgcctgg gaaaagatcg
 241 gcagccacgg tggcgagtat ggcgccgagg ccgtggagag gtgaggaccc ccgccccgcc
 301 ccgccccgcc cgagcccgcc ggcgccgcgc cccgctcacg gcctcctgtc cccgcaggat
 361 gttcttgggc ttccccacca ccaagaccta cttcccccac ttcgacttca cccacggctc
 421 tgagcagatc aaagcccacg gcaagaaggt gtccgaagcc ctgaccaagg ccgtgggcca
 481 cctggacgac ctgcccggcg ccctgtctac tctcagcgac ctgcacgcgc acaagctgcg
 541 ggtggacccg gtgaatttca aggtgagccc gcagcccggc tgggagcgtc gcgggggtcg
 601 gcggtccccg accacaccca ccgacgtccg cccctctctc tgcagctcct gtcccactgc
 661 ctgctggtga ccctggccaa ccaccacccc agtgaattca cccctgcggt gcacgcctcc
 721 ctggacaagttcctgg***ccaacgtgagcaccgtgctgacc***tccaaatatcgttaagctgga
 781 gcctgggagc cggcctggcc ctccgccccc cccacccccg cagcccaccc ctggtctttg
 841 aataaagtct gagtgagtgg ccgacagtgc ccgtggagtt ctcgtgacct gaggtgcagg
 901 gccggcctag ggacacgtcc gtgcacgtgc cgaggccccc tgtgcagctg caagggacag
 961 gagtgggcaa ccggctggtt ccttccttcc tgcttgcaag tccacgaggg gctgctgaaa
1021 gaacccccca cacacacatg cacacactcg tgccactcgg ctgcctccag cctgggtccc
1081 cggctccccc agatctcggg ggggcactgg ctctccctca gcctcccaaa cgtacccacc
1141 cacccaccca cccacggtgc agacaaaacc ggaggtcgag tgcaggctgc agatcccagc
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1381 ctggctaaca ccagtgtcct ctcacgccca gtctggggct cctccttgga ggacaccgtg
1441 gcagcccctt gggcacctcg ggggcagtgg gagccgtggg aaggggctgt cttcgctcct
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1561 gcagactgtg gtgccaccgt agccaggaat ggtggagcac cgccgtagcc gggaatggtg
1621 gggcaccgcc gtagccggga atggtggggc accgccgtag ccgggaatgg tggggcacgg
1681 ctgaacctgc aacactgcct gctgaggagc agccgggcgc aggagcccac ccactggggt
1741 ggagaccccg cttctccaac cagacgccca gctccgtgca gctcaggttg gggagcagtg
1801 gtcatcgatg accaggctgg agactcggct tcttagccgc tggcttgctt cctctgctcc
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1981 accccatgtg ctctgtgggt ctcctggagt tccattcagg gacgtggccc ccacaagtgc
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2521 ttaaaagtcc acatgttgaa taatcccatt tattaaacac acacacacac acacaacaag
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2701 gagctgcaag gctcggctag gcactcagca ggtgcaggtg ttggccgccc gcaacggaac
2761 tcctgctgcg agccaccccg accggccgcg cggcggccca gcccgggagt cgctgtcacc
2821 atctcgcgca gcgcccgcgc tctgccgggg ttccgcgtcc tgtccaggtc tccctctgcg
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3121 gctgcccgcc gctgcgggat ggcgctgtcg gcggcggagc gggcgctgct gcgcgccctg
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4021 cctgactc
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Single stranded sequence for IFNτ mRNA (AF196320, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=6513854) and location of the primers and probes used in this study: primer and ***probe***.

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 481 aggttaagaa agatgggtgg agatctgaac tcactt
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Single stranded sequence for HSP70 mRNA (U09861, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=497937) and location of the primers and probes used in this study: primer and ***probe***.

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   1 cccataaaaa cttgcggctt agtccgtgag aacagcttcc gcagacccgc tatctccaag
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 181 atcgacctgg gcaccaccta ctcctgcgta ggggtgttcc agcacggcaa ggtggagatc
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 601 tacttcaacg actcgcagcg gcaggccacc aaggacgcgg gggtgatcgc ggggctgaac
 661 gtgctgagga tcatcaacga gcccacggcc gccgccatcg cctacggcct ggacaggacg
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2341 ttccaagtaa ataaactcaa ataattcga
```

Single stranded sequence for YWHAZ mRNA (BM446307, http://www.ncbi.nlm.nih.gov/nucest/BM446307?ordinalpos=1&itool=EntrezSystem2.PEntrez.Sequence.Sequence_ResultsPanel.Sequence_RVDocSum) and location of the primers and probes used in this study: primer and ***probe***.

```
  1 GCTGTTCCCA ACCATGTCCT ATACTCCCTC CTCCCCCTGA AAATTTTTTT CAGAGACGGG
 61  GAGTTGATTG AAAAAAGTAA TGTTATTCCA TTTAAAATTT TGGTATATGG CATTTTCTAA
121  CTTAGGAAGC CACAGTGTTC TTGGCCCATC ATGACATTGG GTAGCATTAA CTGTAAGTTT
181  TGTGCTTCCA AATCACTTTT GTTTTTAAGA ATTTCTTGGT ATTCTTTAGC CTGTCTTCAA
241  TTTTGATCCT TTCTTCTTTC TATTTGTCAG GTGCACAGGA TTACCTTTTT TAGCCTCTGT
301  CTTGTCACCA ACCATTCCTAC TTGGTGGCC ATGTACTTGG GGAAAAGGCC GCATGATCTT
361  TCTGGCTCCA CTCAATGTCT AAGGACACCC TGCTTCCTTT GCTTGCATCC CACAGACTAT
421  TTCCCTCTTC CTATTCACTG CAGCTAATCT CTCCTGAGTTGATGAGATTGTGTTATCTCC
481  CTATACACCC TACCCTATCC TGAATGGTCT GTCATTGTCT TTGCCTTTAA ATTCCTTCCC
541  CTTCCTCTCT TTAAATAATG ATGG
```

Appendix 5

Results from BLAST analysis of the amplicons. Expectation values refer to the number of different alignments with scores equivalent to or better than similarity that are expected to occur in a database search by chance. The lower the expectation value, the more significant the score

Amplicon	Blast hits	Genome Hit (% identical, %gap, Expectation values)
α-globin	2	Similar to Hemoglobin alpha chain in chromosome 25 (97%, 0%, $2e^{-25}$) Similar to Recombinant Hemoglobin (97%, 0%, $2e^{-25}$)
IFNτ	13	trophoblast protein-1 in chromosome 8 (99%, 0%, $3e^{-25}$) IFN tau in chromosome 8 (96%, 0%, $3e^{-25}$) IFN tau in chromosome 8 (96%, 1%, $3e^{-25}$) IFN tau c1 in chromosome 8 (93%, 3%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (89%, 3%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (89%, 3%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (89%, 1%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (87%, 3%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (87%, 3%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (86%, 0%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (88%, 1%, $3e^{-25}$) Similar to IFN tau in chromosome 8 (89%, 4%, $2e^{-37}$) Similar to IFN alpha in chromosome 8 (86%, 4%, $2e^{-37}$)
HSP70	2	Heat Shock 70 kd protein 2 in chromosome 23 (97%, 0%, $2e^{-24}$) Protein sequence spanning the HSP in chromosome 23 (97%, 0%, $2e^{-24}$)
YWHAZ	2	YWHAZ in chromosome 14 (100%, 0%, $2e^{-25}$) Similar to enoyl-Coenzyme A in chromosome 1 (86%, 4%, $3e^{-49}$)

Appendix 6
Validating the RT-PCR methodology

To validate an RT-PCR method, there is a need to validate:

(1) **the primers and probes in terms of compatibility with the gene of interest, specificity and amplicon identity verification (**Appendix 8**, page 219);**

(2) **the RT-PCR methodology in terms of the kinetics of the PCR reactions for the transcript of interest (**Appendix 8**, page 219)**;

(3) **the efficacy of the RNA extraction method in terms of the quantity as well as the quality of RNA templates (**Appendix 7 **,page 215)**;

(4) **the RT-PCR methodology in terms of a standard curve generated by serial dilution (ten-fold) of a positive cDNA template (**Appendix 9**, page 222)**;

(5) **α-globin as the exogenous housekeeping gene in a multiplex reaction with the gene of interest (**Appendix 10**, page 226);**

(6) **the inter-assay and intra-assay variation (**Appendix 10**, page 226);**

(7) **YWHAZ as an endogenous housekeeping gene to monitor variation in amount of total RNA in the original sample (Appendix 11, page 233).**

Blastocysts used for validating the RT-PCR method were produced using the standard method of IVP at our laboratory at the time of study (as described in section 2.2.1). The average cleavage rate and blastocyst rate among the batches was 63±9% (mean ± standard deviation) and 18±7 % respectively (Table 31, page 214). On days 7, 8 and 9 pi blastocysts were evaluated in terms of their stage of development (as described in Table 11, page 87) and given a quality score based on morphological assessment (grade 1=embryos free of fragmented cells; grade 2= some granulation and initial signs of fragmentation; grade 3=collapsed or badly fragmented, Table 12, page 87). Only blastocysts with quality above 2 were assessed.

Table 31 *In vitro* culture efficiency for all 9 batches used to produce blastocysts for validation of the RT-PCR methodology.

Batch Number	Number of Ovaries	Number of oocytes	Cleaved zygotes (per oocyte)	Blastocyst (per cleaved)
1	40	147	113 (77%)	20 (18%)
2	27	104	61 (60%)	11 (18%)
3	8	22	15 (68%)	1 (7%)
4	52	71	50 (70%)	9 (18%)
5	60	270	175 (65%)	20 (11%)
6	25	109	72 (66%)	21 (29%)
7	34	143	86 (60%)	19 (22%)
8	52	267	137 (51%)	33 (24%)
9	61	202	95 (47%)	14 (15%)

Appendix 7
Assessment of the efficacy of the RNA extraction method

In order to obtain reliable RT-PCR data, it is important to ensure that the RNA in the samples is of high quality (Imbeaud et al., 2005). The collection and storage of samples and the process of RNA isolation affect the quality of RNA that is used for expression analyses. Therefore, the first step in optimising a Real Time RT-PCR method is to ensure that the RNA extraction method of choice and the storage of sample RNA provide high yield of good quality RNA.

Methodology

The bioanalyzer is an automated bio-analytical device which uses microcapillary electrophoresis to separate tiny amounts of RNA in the channels of a microfabricated chip according to molecular weight (Mueller et al., 2000). The separated RNA is detected via laser-induced fluorescence and presented as electropherograms and gel-like images which can be visually evaluated. Four samples were used to assess RNA integrity. Sample 1 contained a pool of 11 blastocysts which blastulated between day 7 and 9 pi, were categorised as grade 1 or 2, and ranged from early to hatched blastocysts (details of individual blastocysts are outlined in Table 32).

Table 32 Contents of the samples assessed for RNA quality.

Sample Number	Pool or Single	Batch Number	Blastocyst Removal Day	Stage	Grade
1	Pool	1	7	Expanding	2
		1	8	Expanding	2
		1	8	Early	2
		1	7	Expanding	2
		1	8	Mid	2
		1	7	Hatched	2
		1	9	Early	1
		1	9	Early	2
		1	8	Expanding	2
		1	8	Expanding	2
		1	8	Early	2
2	Single	1	8	Hatched	2
3	Single	1	7	Hatched	1
4	Single	1	8	Hatched	2

Samples 2 and 4 each contained a single hatched blastocyst which blastulated on day 8 pi and were categorised as grade 2. Sample 3 contained a single hatched blastocyst which blastulated on day 7 pi and was categorised as grade 1. The extracted RNA from samples 1 to 4 were assessed using an Agilent Bioanalyzer 2100 and Agilent RNA 6000 Pico Assay, according to manufacturer's instructions. Briefly, 9 µl of gel-dye mix and 1 µl of extracted RNA (out of a total of 12 µl) were allocated into each well of an RNA 6000 Pico LabChip. 1 µl of ladder was added instead of sample RNA into one of the wells. The chip was run in the Agilent 2100 bioanalyzer and the results were analysed using the Agilent 2100 bioanalyzer software. The integrity and the purity of the RNA were determined based on the ratio of 28S:18S bands and the RNA integrity number (RIN). The RIN (Schroeder et al., 2006), is an alogarithm based on six regions of the electropherogram, including the conventional 28S rRNA to 18S rRNA ratio and ranges from 0 (totally degraded) to 10 (intact). Since the Agilent 2100 software does not contain this algorithm, the RIN was determined by visual assessment of the electropherograms compared to Figure 34.

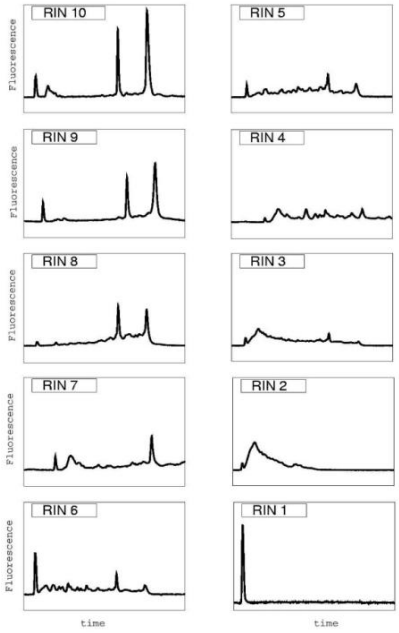


Figure 34 Categorisation of the RNA Integrity Number (RIN) based on the electropherogram of the sample produced by the Agilent 2100 Bioanalyzer, from Schroeder et al. (2006). The RIN ranges from 1 (totally degraded) to 10 (intact).

Results

The isolated RNA samples derived from four blastocyst samples were analysed using the Agilent 2100 Bioanalyzer with regard to their concentration and quality (Table 33, page 218).

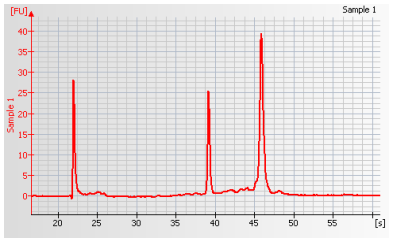
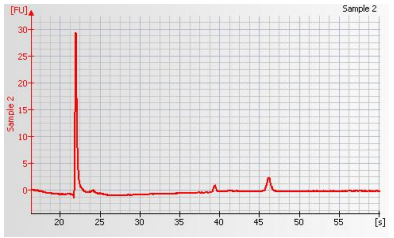
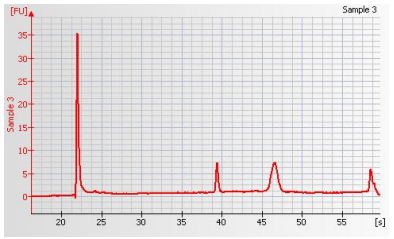
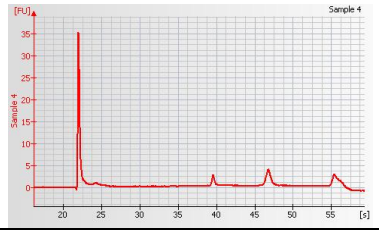
Samples containing single blastocysts were very close to the detection limit of the RNA 6000 Pico LabChip (200 pg μL^{-1}, Agilent, 2006) so that the amount of total RNA estimated in the single blastocysts (particularly samples 2 and 4) may not be accurate. Nevertheless, the amount detected are in the same order of magnitude as observed by Bilodeau-Goessels and Schultz (1997a,b) in bovine blastocysts using northern blot analysis to assess total RNA in pools of embryos of the same stage. Using pools of blastocysts between the stages early and expanded, Bilodeau-Goessels and Schultz (1997a) determined that blastocysts contained 5.3±0.6 ng (mean±SEM) of total RNA, compared to 2.5±1.7 ng in the current study. Interestingly, the blastocyst with the best morphological grade (grade 1) contained more RNA than those with lower morphological grade (grade 2). However, more blastocysts would have to be examined to determine whether morphological grade affects total RNA content in a blastocyst.

The quality of the RNA content of a sample is determined by its purity and integrity. The electropherogram of all four samples (Table 33, page 218) displayed characteristics of high quality RNA: there were clear, well defined 28S and 18S peaks, there was low noise between the peaks, and there was minimal low molecular weight noise suggesting limited contamination. RNA is considered of high quality when the ratio of 28S:18S bands are about 2.0 and higher (Schroeder et al., 2006) and the rRNA ratio was greater than two for all samples (Table 33, page 218). According to Jung et al. (2007), RNA samples should have RIN values greater than 7.0. The RIN from the three single blastocyst samples had an RIN of 9, and the pool of 11 blastocysts had an RIN of 10, suggesting that the integrity of the samples was above adequate.

Conclusion

The amount of RNA detected, its purity and integrity in both single or pool of blastocysts suggests that the samples had adequate RNA quality. Therefore, the method of RNA extraction and sample storage is adequate for downstream gene expression analysis.

Table 33 Electropherogram, RNA concentration and rRNA ratio for each sample, as determined by the Agilent 2100 Bioanalyzer software. Day refers to day of blastulation. The RIN was determined by comparing the electropherograms with Figure 34. RIN: RNA index number.

Sample Number	Pool or Single	Day	Grade	RNA concentration (pg/µl)	rRNA ratio (28S:18S)	RNA per blastocyst (pg)	RIN	Electropherogram
1	Pool	7-9	1-2	840	2.2	770	10	
2	Single	8	2	36	2.5	432	9	
3	Single	7	1	625	2.4	7500	9	
4	Single	8	2	118	2.3	1416	9	

Appendix 8
Amplicon identity verification

To validate an RT-PCR method, there is a need to validate the primers and probes in terms of compatibility with the gene of interest, specificity and amplicon identity verification.

Methodology

Gel electrophoresis allows for the verification of the amplicon size in order to ensure that the primers and probes are detecting the right product. cDNA derived from a sample containing a single hatched blastocyst (sample 1) and a pool of expanded/hatched blastocysts (sample 2,Table 34) underwent RT-PCR and the amplicons were run on a gel to determine amplicon size. 0.01 and 0.02 BRU of cDNA from the pool of blastocysts and 0.01 BRU from the single blastocyst were assessed by RT-PCR. Details of the blastocysts contained in samples 1 and 2 are provided in Table 34.

Table 34 The following samples underwent PCR and the RT-PCR amplicons were run in a gel to confirm the identidy of the amplicons based on their size.

Sample Number	Pool or Single	Batch Number	Blastocyst Removal Day	Blastulation Day	Stage	Grade
1	Single	6	8	7	Hatched	1
2	Pool	3	9	8	Expanding	2
		4	8	7	Hatched	1
		4	8	7	Hatching	1
		5	8	7	Hatched	1
		5	8	7	Hatched	1
		5	8	7	Hatched	1

The gel (3% agarose in Tris/Borate/EDTA) was heated and 0.003% ethidium bromide added to the gel. The gel was poured into a tank with the comb and allowed to set for 45 minutes. 5 µl of each sample and 2 µl of loading buffer (Gel loading solution, Sigma, G2526) were loaded into wells. 6 µl of DNA Ladder (Fermentas, SM0371) was added into each of the outer wells. The gel was run at 25V for 5 minutes, followed by 50V for 1 h. The gel was visualised under the UV light and photographed using a Gel Doc camera.

Results

PCR quality and yield can be assessed from gel electrophoresis of PCR amplicons (Figure 35d, page 221). The specificity of the primers and probes in the duplex reactions are represented by the double strong bands of the correct size for the YWHAZ gene and each gene of interest, as determined by comparison with size markers (lane 9) run on the same gel. There were faint triple bands below the 50 base pair marker (not shown) and between the IFNτ bands which probably corresponded to PCR artefacts. PCR artefact bands were very faint in comparison to the correct size bands, so were not of concern.

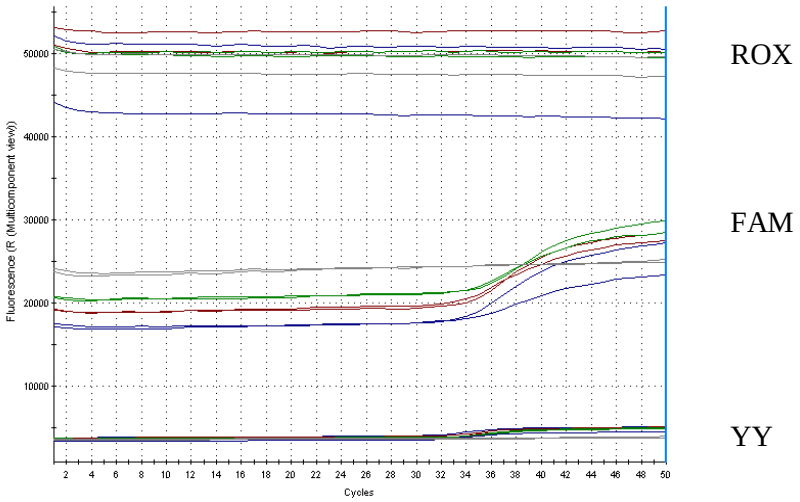
The fluorescence reference dye (ROX) profile was flat suggesting that reagent amounts and concentration did not vary during the PCR reaction (Figure 35a, page 221). The amplification curve for IFNτ, HSP70 and YWHAZ (Figure 35b,c, page 221) include a lag phase, an exponential phase and a plateau phase, the kinetics expected from a valid PCR run. Therefore, the length of the amplicons produced by the primers, although long enough to ensure specificity, were short enough to ensure that reagents did not become limiting.

The profiles for NTC and NRT controls were flat. One in every ten controls did contain spikes in the fluorescence, probably due to RT-PCR artefacts, although these did not occur before cycle 40 (used as the detection limit in the current study). Therefore, the primer sets and probes are specific to the genes of interest and the fluorescence detected in the amplification curves (Figure 35, page 221) refer to the respective genes of interest.

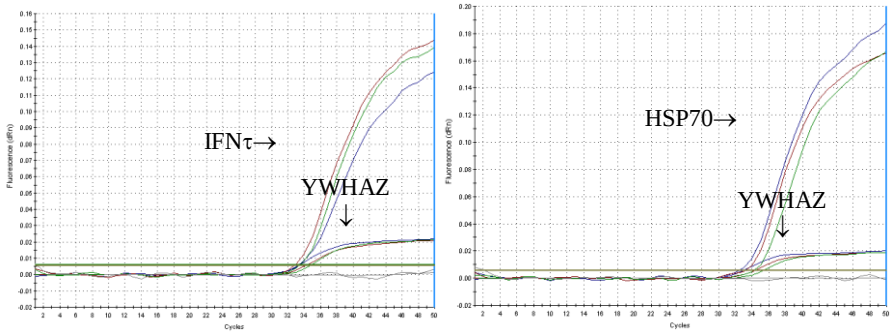
Conclusion

As determined by BLAST, gel electrophoresis and the kinetics of the PCR reaction, the primers and probe sequences were compatible with the GOI, were specific to the GOI and led to amplicons of the correct size, confirming the identity of the amplicon and the validity of the use of the primers and probes in RT-PCR.

a)



b) c)



d)

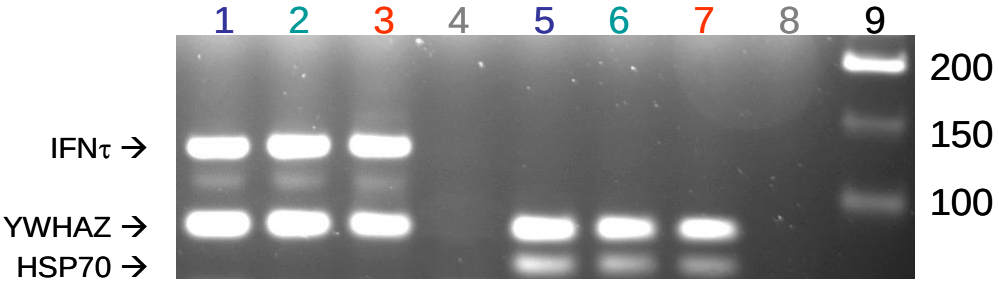


Figure 35 Amplification plots and gel electrophoresis for single blastocyst (0.1 BRU) and pool of blastocyst (0.1 BRU and 0.2 BRU) and No Template Control and No Reverse Transcriptase. a)Fluorescence (nm) of the dyes ROX (reference dye), FAM (IFNτ and HSP70) and YY (YWHAZ), b,c) Fluorescence corrected for baseline fluorescence and for reference dye fluorescence for the genes b) IFNτ and YWHAZ and c) HSP70 and YWHAZ. d) Photograph of a gel produced from PCR end-products. 1,5: Single blastocyst, 2,6: pool of blastocysts (0.1 BRU), 3,7: pool of blastocysts (0.2 BRU), 4,8: NTC, 9:Ladder showing bands for 100, 150 and 200 base pairs. 1-4: IFNτ multiplexed with YWHAZ, and 5-8: HSP70 multiplexed with YWHAZ.

Appendix 9
Serial dilution of the template cDNA

Methodology

In order to test the primers and overall assay performance, standards consisting of serial dilutions (ten fold) of cDNA derived from a pool of 24 blastocysts (details of blastocysts are outlined in Table 35) were amplified in duplicate in the same singleplex PCR run against negative controls to produce a standard curve. The standard curve was plotted as the threshold cycles (Ct) versus the log of the dilution of cDNA. The efficiency of the PCR reaction was obtained by compairing the threshold cycles and slopes of the amplification curves, using the following equation:

$$\%Efficiency = 10^{\frac{-1}{slope}} - 1$$

Table 35 Blastocysts used for the serial Dilution of the template cDNA.

Blastocyst	Batch Number	Blastocyst Removal Day	Stage	Grade
1	4	8	Expanding	3
2	4	8	Early	2
3	4	8	Early	2
4	4	8	Hatching	2
5	4	8	Hatched	1
6	4	8	Hatched	1
7	4	7	Expanding	3
8	5	9	Early	2
9	5	9	Mid	2
10	5	9	Expanding	2
11	5	8	Hatching	1
12	5	9	Early	2
13	5	9	Expanding	1
14	5	9	Mid	2
15	5	7	Expanding	1
16	5	8	Hatched	1
17	5	9	Hatching	1
18	5	9	Expanding	1
19	5	9	Expanding	1
20	5	9	Mid	2
21	5	8	Expanding	1
22	5	9	Expanding	2
23	5	9	Expanding	1
24	5	9	Mid	2

Results

Amplification Plots

As demonstrated in Table 36, the PCR system for YWHAZ, IFNτ and HSP70 produced normal PCR curves: the amplified template accumulated exponentially with each cycle until the reaction components became limiting, reaching the plateau stage of the PCR kinetic curve. The thresholds were set so that the Ct values were derived from the exponential amplification phase, when the abundance of cDNA was related to the amount of starting material. Normal PCR kinetics were also observed when YWHAZ, IFNτ and HSP70 were multiplexed with α-globin (Table 40, page 230).

Standard Curves

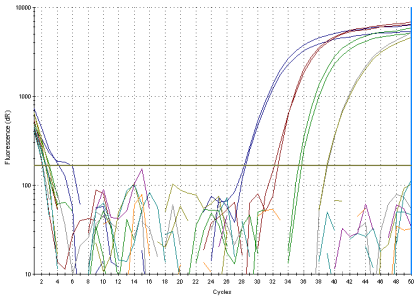
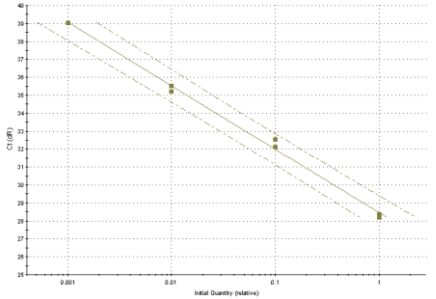
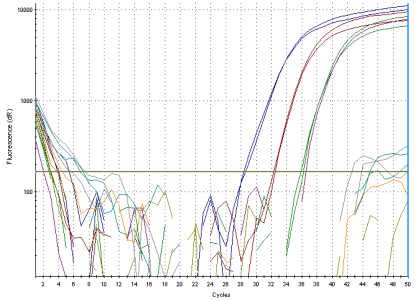
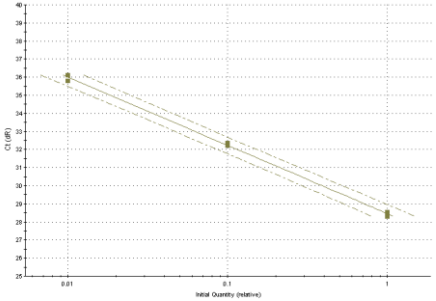
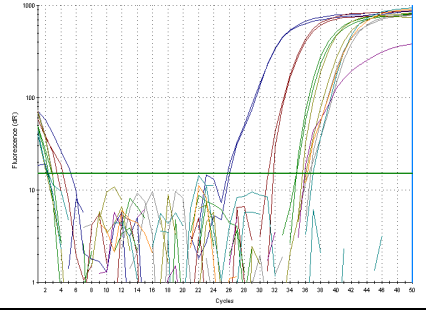
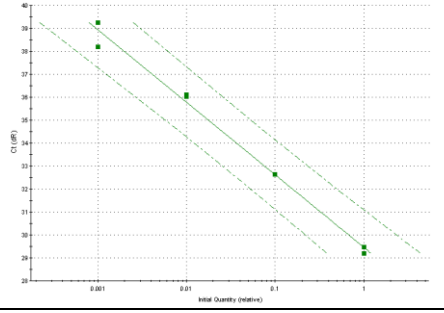
The linear relationship between the log of accumulated product and the PCR cycle number in the standard curves (Table 36 and Table 40, page 230) suggest that the Ct value is related to the amount of cDNA present, thus validating the RT-PCR method used. As is ideal, the dynamic range of all standard curves were between three and five logs and the best fit line of the standard curves had a high linear fit ($r^2 > 0.98$) with less than 2% CV variation between triplicates (Table 36 and Table 40, page 230). The coefficient of variance ranged from 0.42 to 1.59 for IFNτ, between 0.40 and 2.00 for HSP70 and between 0.39 and 1.70 for YWHAZ, suggesting low variation between replicate PCRs for the same sample for all genes of interest. The PCR reactions had adequate efficiency levels, ranging from 82 to 101%, within the range suggested by Tichopad et al. (2003, between 65 and 100%).

When YWHAZ, IFNτ and HSP70 were multiplexed with α-globin, the slope of the standard curves was similar between the two genes in each multiplex reaction (Table 40, page 230).

Conclusion

Therefore, the linear relationship between log of accumulated produce and cycle number, the low variation between triplicates and the high efficiency of the PCR reactions, further suggest the veracity of the RT-PCR method used in determining amount of mRNA.

Table 36 (next page) Serial Dilution of a pool of 24 blastocysts. BRU = Blastocyst RNA Unit. Amplification plots plotted as the log of fluorescence versus cycle number in order to define the log-linear phase of the cycle. The threshold is set above noise level: 165 dR for FAM, and 15 dR for YY. 10 fold serial dilution of 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, NTC, NRT. **The standard curve plotted as the threshold cycle numbers of titrated cDNA pool serial dilutions versus the log of the concentration of template cDNA. The Stratagene software generates a best fit line and 95% confidence interval.**

Gene	Dynamic Range (logs)	BRU range	Efficiency (%)	r^2	Threshold Range	Amplification Plots	Standard Curve
IFNτ	4	$2.4e^{-3}$ → 2.4	94	0.994	29.5 → 40.0		
HSP70	3	$2.4e^{-2}$ → 2.4	88	0.999	32.0 → 38.6		
YWHAZ	4	$2.4e^{-3}$ → 2.4	95	0.969	26.1 → 39.3		

Appendix 10
Validation of α-globin as the exogenous control

Methodology

An exogenous standard is required to control for differences between samples in RNA recovery, RT and PCR. An exogenous standard will also control for variation within samples due to pipetting error and variations in PCR efficiencies between wells. Since bovine embryos do not express globin mRNA (Donnison and Pfeffer, 2004), synthetic rabbit α-globin mRNA is an appropriate exogenous control for studies involving bovine embryos.

RNA was extracted from a pool of six blastocysts (Table 37) which were equally distributed into five tubes containing 1.4 ng, 140 pg, 14 pg, 1.4 pg and 0 pg of rabbit α-globin mRNA (Table 38). Following RT, samples were assessed for α-globin mRNA levels multiplexed with IFNτ or HSP70. The Stratagene software was used to plot standard curves.

Table 37 Blastocysts used for the template cDNA for α-globin validation.

Blastocyst	Batch Number	Blastocyst Removal Day	Stage	Grade
1	6	8	Mid	2
2	6	8	Mid	2
3	6	9	Early	1
4	6	9	Early	2
5	6	9	Mid	2
6	6	9	Expanding	2

Table 38 Sample contents for α-globin validation. The blastocyst template was constant in all samples whilst the α-globin was serially diluted (ten fold) three times. BRU: Blastocyst RNA Unit.

Sample Number	BRU in		Amount of α-globin in	
	RT (pg)	PCR (fg)	RT (ng)	PCR (pg)
1	1	0.1	1.4	14
2	1	0.1	0.14	1.4
3	1	0.1	0.014	0.1
4	1	0.1	0.0014	0.01
Blastocyst Positive Control	1	0.1	0	0
α-globin Positive Control	0	0	14	100

Results

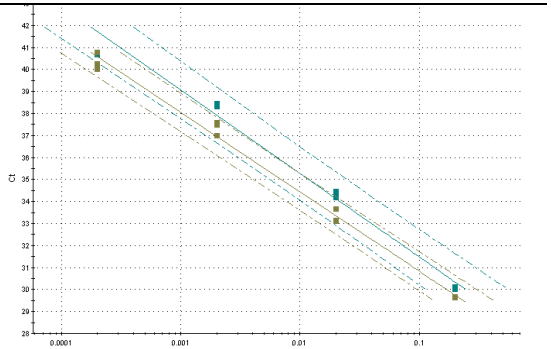
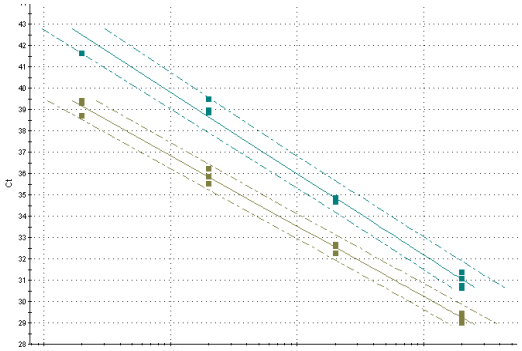
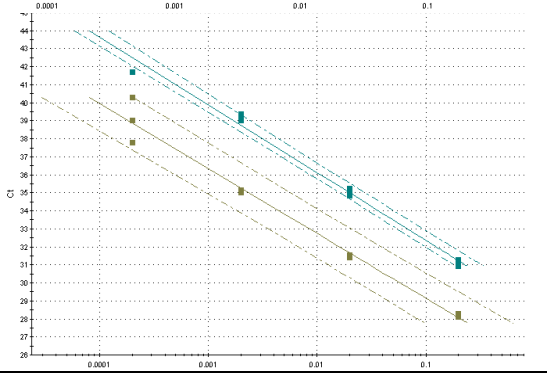
Validation of α-globin as the exogenous control gene involved a serial dilution of α-globin duplexed with the genes of interest to determine whether the genes were compatible in a duplex reaction, a serial dilution of α-globin mRNA with a fixed amount of blastocyst cDNA to determine the optimum amount of α-globin to spike the samples with, determination of plate to plate variation using an α-globin positive control and determination of the efficiencies of the standard curves for α-globin and the genes of interest to determine whether the genes of interest may be quantified relative to the α-globin standard curve.

Deterimining the amount of α-globin to spike the samples with

The amount of α-globin mRNA spiked per sample in the literature varies between 0.1 pg (Donisson and Pfeffer, 2004, de Oliveira et al., 2006, Wrenzycki et al., 2000) to 3 pg (Russell et al., 2006) to 5 pg (Somers et al., 2006). Russell et al. (2006) assessed pools of blastocysts, whilst Donisson and Pfeffer (2004) assessed pools of oocytes. Blastocysts have a higher expression level than oocytes (Bilodeau-Goessels et al., 1997a,b), so that the variation in amount of initial exogenous RNA added between studies may depend on expression levels of target genes. The RT-PCR methodology may also affect the ideal amount of α-globin to spike the samples with. Too little input may not generate enough signal, whilst too much may lead to an early PCR plateau which uses up the reagents in the PCR reaction and therefore may affect the PCR reaction of the duplexed gene. Therefore, optimisation is required in order to determine the optimum amount of exogenous RNA.

According to the standard curves for α-globin multiplexed with YWHAZ, IFNτ and HSP70, whether with a serial dilution (Table 39, page 228) or fixed amount (Figure 36, page 230 and Table 40, page 230) of blastocyst cDNA, the serial dilution of α-globin had a dynamic range of four orders of magnitude, and the low variation within the standards for α-globin, HSP70 and IFNτ (CV less than 1.7%) would imply that HSP70 and IFNτ are compatible in a multiplex reaction with α-globin in any amount of α-globin within the range tested. The CV for the α-globin Ct was highest within the higher (1.7%,Table 40 , page 230) compared to the lower (0.4 to 0.8%) α-globin concentrations, suggesting that there may have been some level of limiting factors due to the high concentration of amplicons. Moreover, the Ct values for the α-globin cDNA were closest to the Ct value for IFNτ cDNA at the lower α-globin concentrations (Figure 36, page 230). Therefore, the amount of α-globin added to the samples in Chapter 3 was 20 pg of mRNA (equivalent to 1.4 pg of α-globin cDNA per PCR reaction).

Table 39 (next page) Serial Dilution of a pool of 19 blastocysts spiked with 20 pg of α-globin mRNA. BRU = Blastocyst RNA Unit. 10 fold serial dilution of 1.9, 0.19, 0.019, 0.0019, 0.00019 BRU. The standard curve plotted as the threshold cycle numbers of titrated cDNA pool serial dilutions versus the log of the concentration of template cDNA. The Stratagene software generates a best fit line and 95% confidence interval. GOI and α-globin.

GOI	GOI		α-globin		Standard Curve
	Efficiency (%)	r^2	Efficiency (%)	r^2	
IFNτ	83	0.986	89	0.993	
HSP70	82	0.988	101	0.996	
YWHAZ	84	0.989	90	0.983	

a) b)

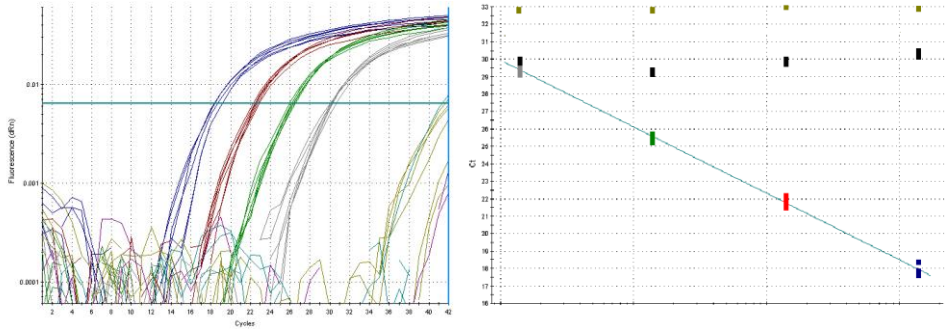


Figure 36 Serial Dilution of α-globin mRNA against a constant blastocyst total RNA. α-globin was multiplexed with HSP70 or IFNτ. a) Amplification plots plotted as the log of fluorescence versus cycle number in order to define the log-linear phase of the cycle. The threshold is set above noise level. 10 fold serial dilution of α-globin cDNA 14pg, 1.4pg, 0.14pg, 1.4pg, 0, NTC, NRT. **b) The standard curve plotted as the threshold cycle numbers of titrated cDNA pool serial dilutions versus the log of the concentration of template cDNA. The Stratagene software generates a best fit line. Triplicate HSP70 and IFNτ (black) expression in the constant blastocyst RNA.**

Table 40 Serial Dilution of α-globin mRNA against a constant blastocyst total RNA. α-globin was multiplexed with HSP70 or IFNτ.

α-globin cDNA (pg)	GOI	α-globin		GOI	
		Mean	CV	Mean	CV
14	HSP70	18.0	1.7	34.3	0.4
1.4	HSP70	21.8	0.8	34.4	0.4
0.1	HSP70	25.8	0.4	34.2	0.5
0.01	HSP70	29.8	0.4	34.2	0.8
0	HSP70	No Ct		34.2	0.4
14	IFNτ	18.1	1.7	32.1	0.8
1.4	IFNτ	22.2	0.4	31.5	0.2
0.1	IFNτ	25.5	0.6	30.8	0.5
0.01	IFNτ	29.4	0.7	31.2	0.4
0	IFNτ	No Ct		31.3	0.3

Plate-to-Plate variation in the exogenous control

In Chapter 3, seven RT-PCR plates were assessed containing a standard curve for α-globin in each plate, and duplicate positive α-globin controls. The within-plate variation in the control α-globin Ct value ranged between 0.2 and 2.2% (Table 41, page 231) and the inter-assay variation in the positive control was 6%. The low variation in the positive control further suggest the validity of the proposed RT-PCR methodology.

Table 41 The intra-assay variation on Ct values from the positive α-globin controls (in duplicate) from each plate.

Ct 1	Ct 2	Intra-assay variation (%)
20.9	21.1	0.8
21.1	21.8	2.1
19.7	19.6	0.1
19.6	19.0	2.2
18.6	18.5	0.5
19.3	19.3	0.2
21.7	21.7	0.2

In Chapter 3, the standard curves for α-globin from different plates had similar slopes, suggesting similar efficiencies averaging 97±4% (Figure 37a). The y-intercept of the standard curves varied from plate to plate, correlating with the average Ct value for the samples on the plate ($P<0.001$, Figure 37b). Therefore, the quantitation method used in Chapter 3, which employs y-intercepts and slopes in the equation, corrects for such variation between plates. The high variation in the average Ct for the samples on each plate (wide standard deviation bars demonstrated in Figure 37b) are due to sample variation, minor pipetting error (accounted for by the α-globin control), and due to slight differences between the two fluorescent dyes used in the multiplex reaction.

The low plate-to-plate variation in the α-globin positive control and α-globin standard curve efficiency further suggest the validity of using α-globin as the exogenous control.

a) b)

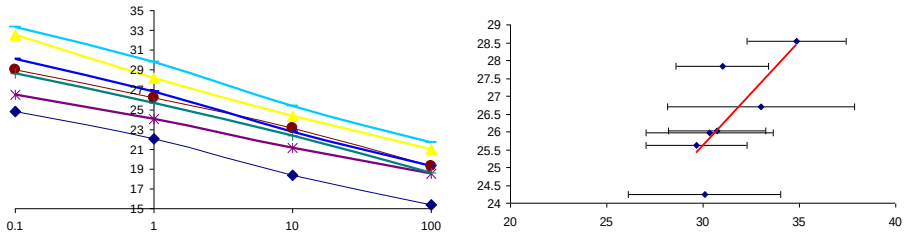


Figure 37 a) Standard Curve for different plates. X-axis: amount of α-globin mRNA (in pg), Y-axis: Ct. b) X-axis: Average Ct for the samples on the plate (for both dyes in the multiplex reaction),Y-axis: Standard Curve Y-intercept. The error bars refer to standard deviation, as a measure of the variation in each plate. The best fit line is in red ($P<0.001$).

Validation of using the α-globin standard curve to quantify GOI in absolute terms

In all PCR plates in section Chapter 3, the standard curves were linear across the whole range of template concentrations, with the efficiency between 80 and 110%, R^2 above 0.985 and the slope between -2 and -4, suggesting that the Ct values represented the true representation of amount of α-globin mRNA.

Quantification of the GOI using the α-globin standard curve in each plate is only valid because α-globin and the GOI are multiplexed in the same well, because α-globin, YWHAZ, HSP70 and IFNτ had similar efficiencies (Figure 38) and similar Ct ranges (Table 40, page 230).

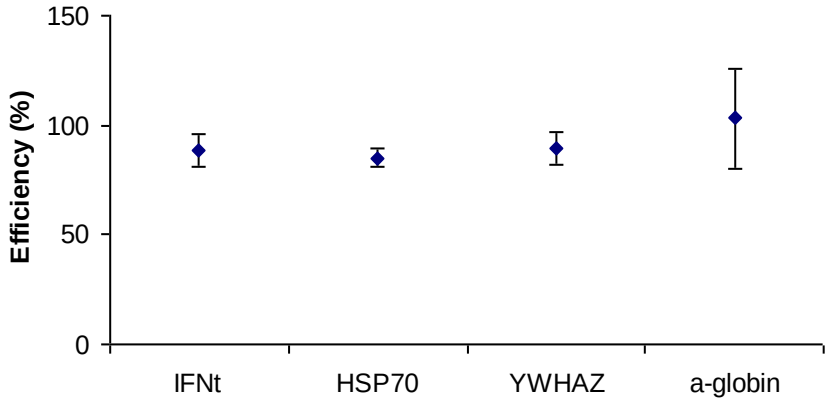


Figure 38 Variation in efficiency derived from standard curves (Appendix 9, page 222, Appendix 10, page 226 and Chapter 3). Efficiency for the α-globin sequence was not significantly different to that of IFNτ (P=0.4), HSP70 (P=0.3) and YWHAZ (P=0.4). Mean ± standard deviation.

As demonstrated, the RT-PCR method was optimised for the amount of α-globin mRNA to be added to the samples, and validated by serial dilution of the α-globin cDNA when multiplexed with YWHAZ, IFNτ and HSP70 (discussed in Appendix 10, page 226, and summarised inTable 40, page 230). Therefore, the use of α-globin as the exogenous control gene in a multiplex reaction with the genes of interest (IFNτ, HSP70 and YWHAZ) was validated.

Appendix 11
Validating YWHAZ as the endogenous house-keeping gene

Several studies have determined that the expression levels of house keeping genes vary between treatments, which explain why different normalization approaches have lead to opposing results (Bonefeld et al., 2008). Because of this, there have been a number of studies comparing the stability of a range of house keeping genes in the hope of finding one which is unanimously stable. For instance, Bonefeld et al. (2008) compared a panel of eight common housekeeping genes (including YWHAZ, 18S, β-actin and GAPDH) and found that YWHAZ was the most stably expressed gene in rat brain tissue. Using pools of 20 pre-attachment bovine embryos of similar stage and quality, Goosens et al. (2005) found that the most stable of eight commonly used reference genes tested was also YWHAZ. Because of this stability, YWHAZ is being increasingly used as an endogenous house keeping gene in a range of studies using blood cells (Carroll et al., 2007) or leukocytes (De Ketelaere et al., 2006) and its stability in bovine embryos (Goosens et al. 2005) is the reason why YWHAZ was the chosen endogenous house keeping gene in this thesis. However, Toegel et al. (2007) compared the stability in expression of six common housekeeping genes and in human cartilage cells for three different treatments, and found that although the ranking of the stability of the genes changed between treatments, YWHAZ was among the three least stable genes in all three treatments assessed. Ayers et al. (2007) pointed out that although YWHAZ was the most stable house-keeping gene for some canine tissues (ligament tissues), it was regulated in other tissues (fat tissues), suggesting that no single reference gene can be identified as having stable expression in all different tissue types. In fact, YWHAZ was implicated with initiating a cellular communication system in two-cell murine embryos (Li et al., 2003), so that expression varied with stage of development. Due to this lack of a standard house keeping gene in all tissues, Bonefeld et al. (2008) suggested that, ideally, a large battery of possible house keeping genes should be tested before an experiment to ensure that the house keeping gene is stably expressed in the tissue being studied and for the treatments being assessed. However, this suggestion is time-consuming and expensive so that it is not practical for qPCR work. Instead, two preliminary experiments were conducted to observe the variation in YWHAZ expression

relative to heat treatment and stage of blastocyst development to determine whether YWHAZ was appropriate as an endogenous house-keeping gene for Chapter 3 (expressed either as Ct or in absolute terms) were also used to assess the variation in YWHAZ expression relative to heat treatment, stage of blastocyst development, change in morphology index, day of blastulation and sex.

Therefore, the objectives were:

(1) to determine whether stage of blastocyst development affects expression of YWHAZ, IFNτ and HSP70, and

(2) to determine whether heat treatment affected expression of YWHAZ, IFNτ and HSP70.

Methodology

Effect of stage of blastocyst development on mRNA yield

21 blastocysts from the same batch (details of blastocysts are outlined in Appendix 5, page 212) were categorised as early, mid, expanding and expanded (according to criteria set in Table 12, page 87) and pooled together according to stage and underwent RT-PCR in two separate PCR plates.

Effect of heat stress on mRNA yield

Six hatching blastocysts were transferred from group culture to individual culture (5 µL drops) and allocated to either the control or heat stress group. Embryos remained for 4 h at 5% CO_2, 5% O_2 plus 90% N_2 at either 38.5°C (control) or 42°C (heat). Only blastocysts which had progressed in development during the 4 h treatment (i.e. category 1 of the change in morphology index) were selected. After storage in Buffer RLT at -80ºC, samples underwent RNA extraction and quantitative singleplex RT-PCR. Samples were assessed in triplicate for each gene of interest (IFNτ, HSP70) and in duplicate for YWHAZ.

Quantification of mRNA levels

Transcript levels were assessed as Ct values and as relative to the endogenous control (YWHAZ) as calculated by the Strategene software using the following equation:

$$\mathrm{Re}\,lative\_Expression = (1 + Efficiency)^{(Ct_{YWHAZ} - Ct_{GOI})}$$

Statistical analysis

Ct values for IFNτ, HSP70 and YWHAZ were analyzed relative to treatment (heat stressed versus control) and relative to stage of development (early, mid, expanding, expanded) using a REML-LMM (Genstat, Eleventh edition, 2008), with replicate blastocyst as a random factor. Stages of development were also analysed as before (early and mid) and after (expanding, expanded) expansion using a two-sample t-test. Ct values for YWHAZ were analyzed relative to the gene that it was multiplexed with (IFNτ or HSP70) using a REML-LMM (Genstat, Eleventh edition, 2008), with replicate blastocyst as a random factor. Efficiency derived from standard curves for YWHAZ, IFNτ and HSP70 were compared to the efficiencies for α-globin derived from all the standard curves for α-globin in Chapter 3, Appendix 9 (page 222) and Appendix 10 (page 226) using a two-sample t-test (Genstat, eleventh edition, 2008). Stages of embryo development were grouped as pre-expansion (early and mid blastocysts) and post-expansion (expanding, expanded, hatching, hatched). Linear regression analysis (Genstat, Eighth Edition, 2005) was used to determine the association between YWHAZ, IFNτ and HSP70 expression levels.

Results

Effect of heat treatment on YWHAZ transcript levels

In the preliminary experiment, heat treatment caused an approximately 100-fold increase in HSP70 expression relative to control (7 Ct difference in Figure 39f, page 238, P<0.001), and a four-fold increase in IFNτ expression relative to control (2 Ct difference in Figure 39d, page238, P<0.001). Meanwhile, YWHAZ was not affected by heat treatment (P=0.9, Figure 39b, page 238). In the main experiment, YWHAZ transcript levels were also not affected by heat treatment (954±428 fg and 2056±939 fg, for control and heat treated blastocysts respectively, P=0.6), suggesting that YWHAZ is an appropriate endogenous housekeeping gene for assessing expression levels in heat stressed embryos relative to control.

Variation in YWHAZ expression between blastocysts was observed in the preliminary experiments (Appendix 11) and in the chapter 3. The overall variation in YWHAZ within each plate was comparable to that reported in other studies. In this study, the average range in Ct in all plates was 3.7, similar to the 3.5 range in Ct for YWHAZ observed by Toegel et al. (2007) in cartilage cells treated with curcumin. According to Chapter 3, this variation in YWHAZ expression was not

explained by differences in the sex of the blastocyst (2410±1044 fg for males and 785±353 fg for females, P=0.08) or the day it blastulated (1796±1215 fg for day 7.75 blastocysts and 1215±762 fg for day 8.75 blastocysts, P=0.2).

Effect of Stage of Blastocyst Development on YWHAZ expression

In the preliminary experiment (Appendix 11, n=21) YWHAZ expression was similar in early, mid, expanding and expanded blastocysts (P=0.5, Figure 39f) and in before (early and mid) and after (expanding and expanded) expansion (P=0.3 Figure 39a, page238). However, in Chapter 3, where more blastocysts were evaluated (n=110), blastocysts before compaction had higher YWHAZ transcript levels than after compaction in terms of Ct (P=0.001 Figure 41b, page239). Therefore, YWHAZ expression was affected by stage of blastocyst development in Chapter 3, but not in the preliminary experiment (Appendix 11, page 233).

In contrast to YWHAZ, IFNτ and HSP70 were upregulated in post-expansion blastocysts compared to blastocysts before expansion (P<0.001, Figure 42a, b, page 240), whilst the effect of stage of development on IFNτ:YWHAZ and HSP70:YWHAZ expression approached significance (P=0.07 and P=0.06 respectively, Figure 42c,d, page 240). Moreover, in Chapter 3, absolute IFNτ and HSP70 were also upregulated in post-expansion blastocysts compared to blastocysts before expansion, although not significantly (P=0.08 and P=0.1, Figure 43a,b, page 241). Therefore, although stage of blastocyst development did not significantly affect YWHAZ, it did affect IFNτ and HSP70 transcript levels.

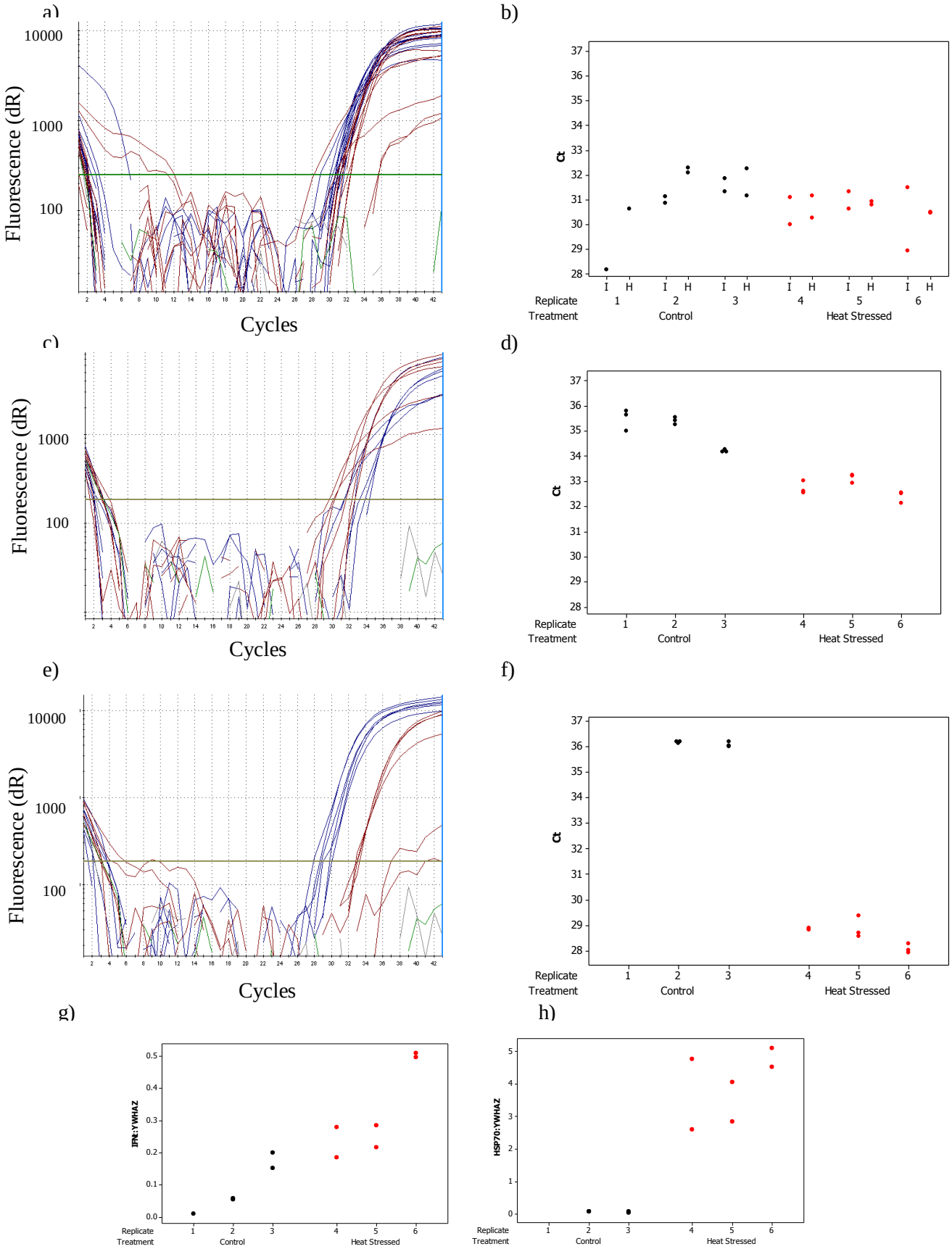

a)
b)
Fluorescence (dR)
10000
1000
100
Cycles
Ct
Replicate
Treatment
Control
Heat Stressed
c)
d)
e)
f)
g)
h)
JPR:YWHAZ
HSP70:YWHAZ

Figure 39 (previous page) Effect of treatment on a-b) YWHAZ expression, c-d,g) IFNτ expression and e-f,h) HSP70 expression. Samples were measured in triplicate in a total of 6 embryos (3 Control, 3 Heat Stress). a,c,e) Amplification plots plotted as the log of fluorescence vs cycle number in order to define the log-linear phase of the cycle. The threshold is set above noise level: 185 dR for FAM, and 25 dR for YY. Heat Stressed, Control, NTC, NRT. b,d,f) Expression measured as Ct: threshold cycle. I: multiplexed with IFNτ, H: multiplexed with HSP70.

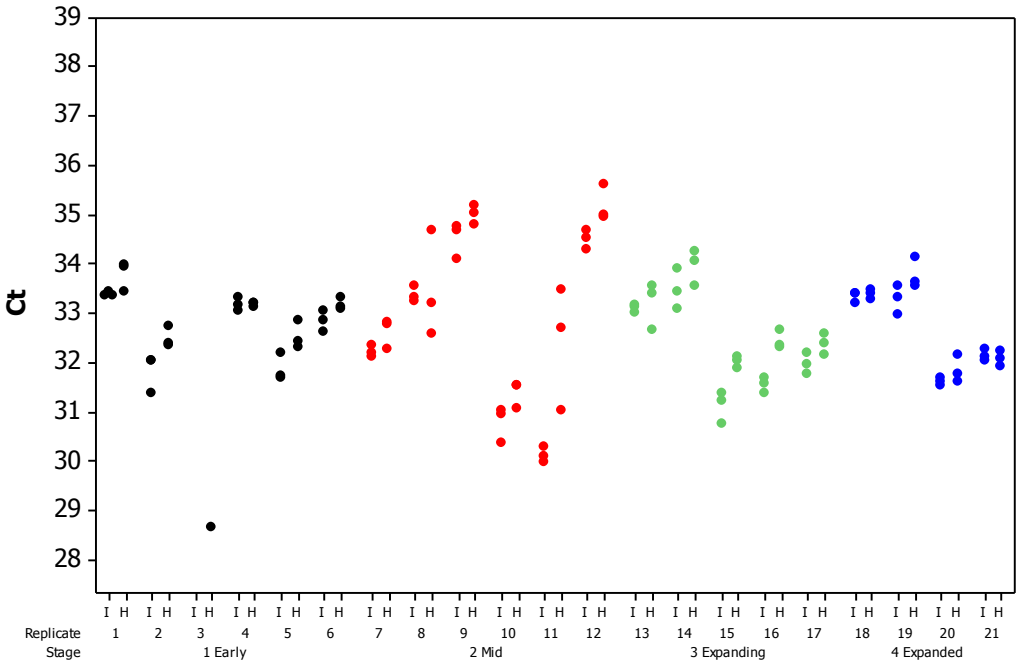


Figure 40 Individual value plot per blastocyst showing the effect of stage of blastocyst development (Early, Mid, Expanding, Expanded) on YWHAZ expression. Expression is measured as the raw data (Ct). Samples were measured in triplicate in a total of 21 embryos (6 Early, 6 Mid, 5 Expanding, 4 Expanded). I: multiplexed with IFNτ, H: multiplexed with HSP70.

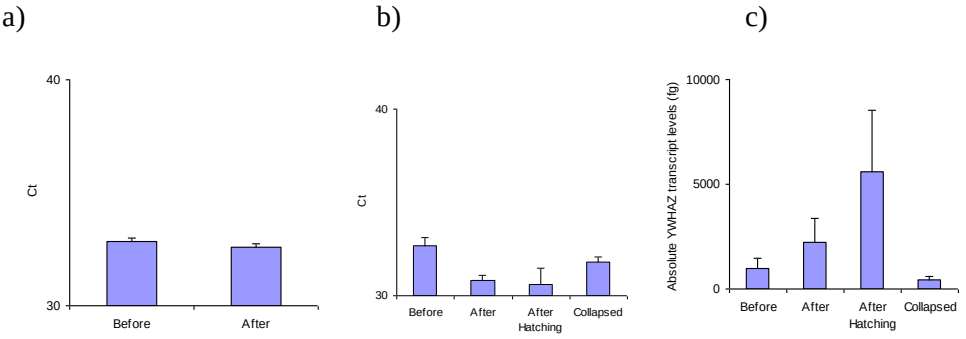


Figure 41 The effect of stage of blastocyst development (before and after expansion) on YWHAZ expression in a) the preliminary experiment (n=21, Appendix 11) and in b-c) Chapter 3 (n=110). In a-b) transcript levels are measured as the raw data (Ct), whilst in c) transcript levels are expressed in absolute terms. Values are mean±SEM.

Differences in the CV expressed as Ct or relative to YWHAZ in the two preliminary experiments (heat treatment and stage of development) are summarised in Table 42 (page239). The variation was higher at the Ct level than relative to YWHAZ, suggesting that YWHAZ may be correcting for some of the variation in YWHAZ expression, thus, further supporting the use of YWHAZ as the endogenous housekeeping gene.

Table 42 Differences in coefficient of variation in the Ct values and in the relative to YWHAZ values for the genes of interest (GOI) in two validation experiments.

Treatment	GOI	Absolute (Ct)	Relative to YWHAZ
Heat Stress	HSP70	14.7	4.7
	IFNτ	1.65	0.02
Stage	HSP70	1.65	0.06
	IFNτ	2.67	0.27

Some of the within sample variation in YWHAZ expression was due to which gene (HSP70 or IFNτ) was being multiplexed with YWHAZ. Although the Ct values for YWHAZ for the same sample were comparable irrespective of what gene YWHAZ was multiplexed with, the Ct value for YWHAZ was consistently higher when multiplexed with HSP70 than with IFNτ ($P<0.001$, paired t-test, Minitab). This may be due to slight differences in the conditions of the PCR reaction due to differences in primer contents.

Stage of blastocyst development only accounts for some of the variation between samples, so that other factors, such as variation in viability, may explain the remainder variation in YWHAZ expression between samples.

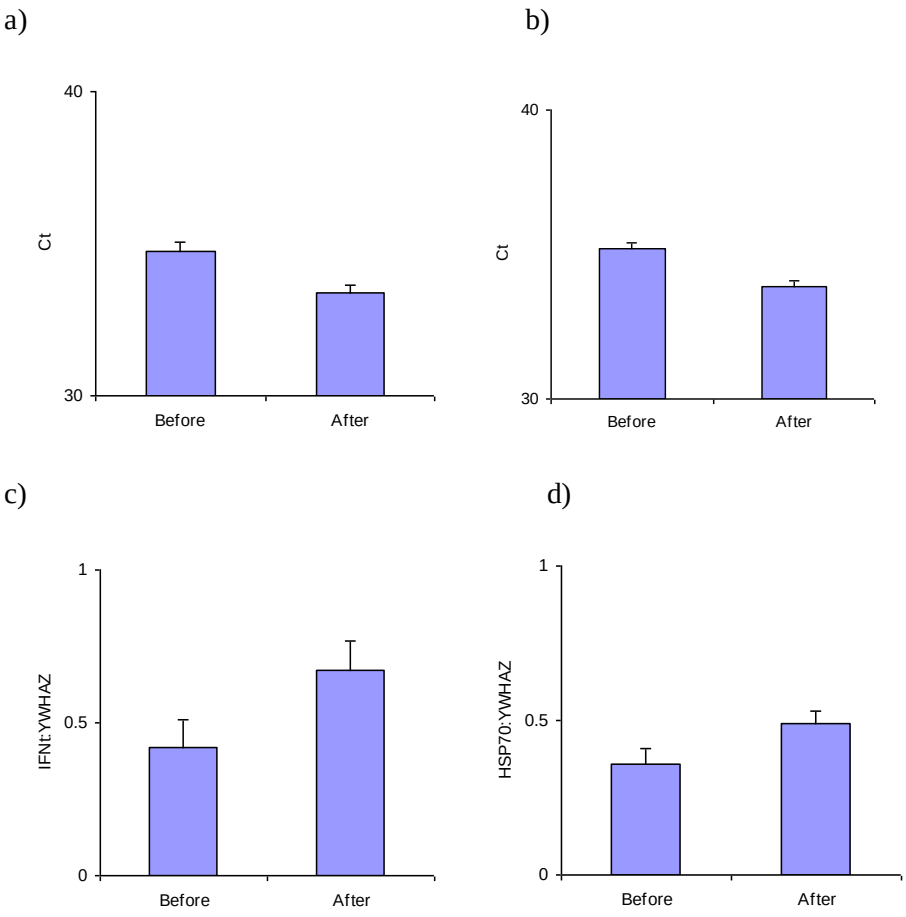


Figure 42 Results from the preliminary experiment (Appendix 11) on the effect of stage of blastocyst development (before and after expansion) on a) IFNτ expression before n=30, after n=27), b) HSP70 expression (before n=29, after n=27). Expression is measured as the raw data (Ct, a-b) and relative to YWHAZ (c-d). Values are mean±SEM.

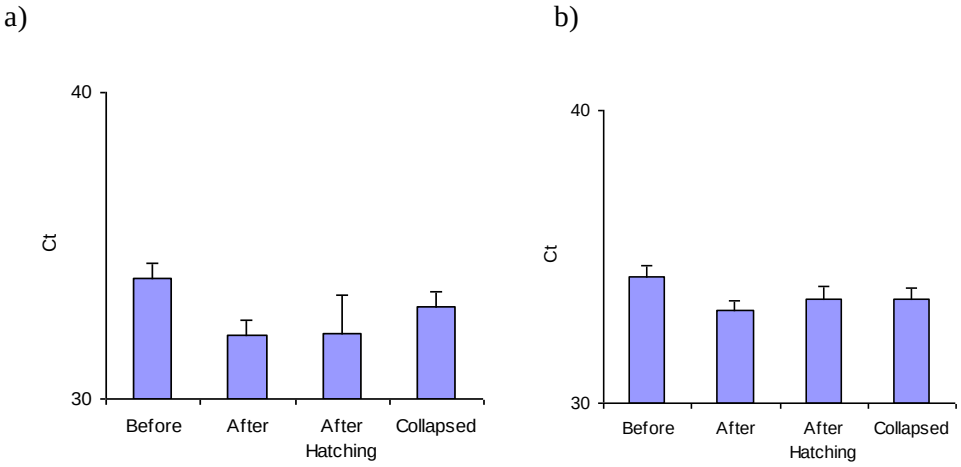


Figure 43 Results from Chapter 3 on the effect of stage of blastocyst development (before and after expansion, after hatching and collapsed, n=43, 29, 8 and 29 respectively) on a) IFNτ expression and b) HSP70 expression. Expression is measured as the raw data (Ct). Values are mean±SEM.

Effect of change of morphology index on YWHAZ expression

Embryo viability may explain some of the variation in total gene expression between samples. Blastocyst 12 had high Ct values in all genes assessed, suggesting RNA degeneration.

In Chapter 3, YWHAZ transcript levels were lower (P=0.009) in blastocysts which showed signs of degeneration (category 3, 267±86 fg) compared with blastocysts which progressed in development (category 1, 2593±1361 fg) or remained unchanged in morphology (category 2, 2044±1008 fg) during the individual culture period (Figure 44, page 242). Therefore, YWHAZ expression may decrease with a decrease in embryo viability, possibly due to an increase in RNA degradation in degenerating embryos.

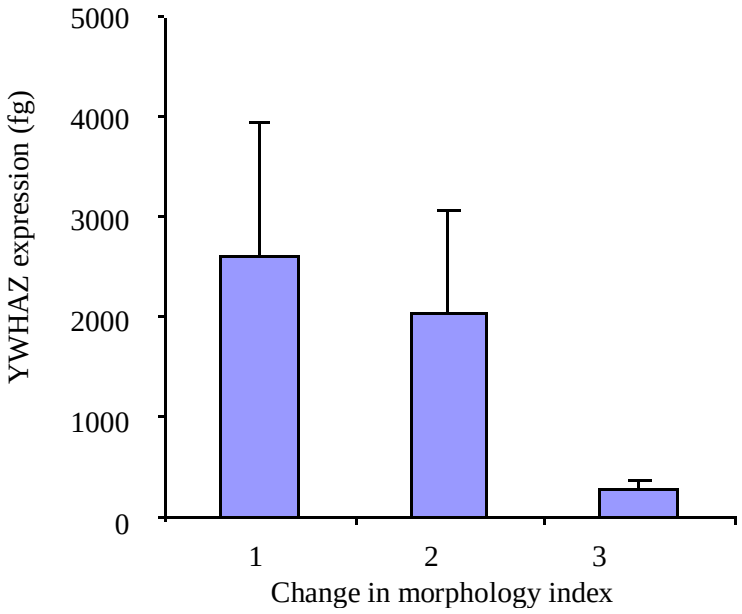


Figure 44 Transcript levels of YWHAZ versus change in morphology index (embryo survival after 24 h of individual culture as assessed by change in morphology before and after individual blastocyst culture: 1 embryo progressed in development, 2 no visual change in development, 3 signs of degradation or collapse). Values are mean ± standard error.

The relationship between YWHAZ and other genes

As expected from a housekeeping gene, YWHAZ transcript levels were not affected by treatment (mean ± standard error, 954±428 fg and 2056±939 fg, for control and heat treated blastocysts respectively, P=0.6). YWHAZ transcript levels correlated with IFNτ transcript levels (P=0.004, Figure 45b), and with HSP70 transcript levels (P<0.001, Figure 45a). The higher variation in IFNτ expression compared with HSP70 expression meant that the probability of a relationship with YWHAZ expression was greater with HSP70 than with IFNτ.

a) b)

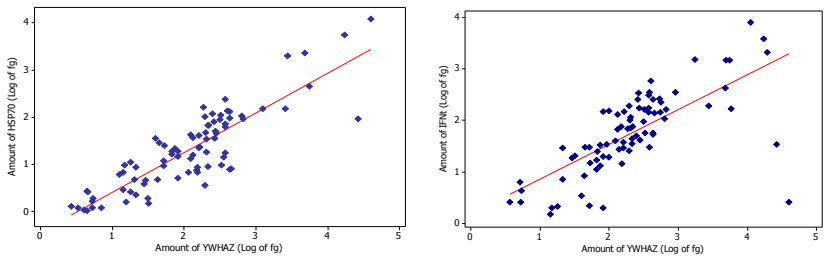


Figure 45 Relationship between YWHAZ and a) HSP70 (y=0.8-0.4) and b) IFNτ (y=0.7+0.2) transcript levels.

Conclusion

YWHAZ gene expression was not affected by factors which should not affect the number of cells in an embryo (treatment, sex or day of development), but was regulated by factors which affect the number of viable cells in an embryo (stage, change in morphology index, IFNτ and HSP70 expression). YWHAZ gene expression was upregulated (three-fold) after expansion (associated with higher number of cells) compared with before expansion, suggesting that some of the variation in YWHAZ expression can be explained by differences between samples in cell number. Given that YWHAZ is stable and ubiquitous in all embryo cell types in similar amounts (Goossens et al., 2005), it is plausible that YWHAZ correlates with total cell number. It is not possible to correlate cell count and cell viability with gene expression in the same embryo, since the process of counting cells and the apoptosis assay may cause mRNA deterioration. However, the relationship between YWHAZ expression and stage of development and change in morphology index indicate a relationship between YWHAZ expression and total mRNA. Therefore, it is justifiable to use YWHAZ expression as a control for variation between embryos in total RNA.

Appendix 12

Criteria used to reject data for analysis and the reasons for disregarding data.

RFU: relative fluorescence unit.

Criteria	Reason
Wells with loose caps	Allow reagents to be lost due to evaporation
Replicates containing reference dye profiles which were noisy (not flat or containing spiking) or saturated (>35000 RFU)	Indicate fluctuations in reagent amount and concentration due to evaporation or loading error
Replicates containing baseline signals for amplification plots below 3000 RFU	Above 3000 RFU, signals are significantly above background
Replicates containing the exponential phase of the amplification curve above 35000 RFU	Above 35000 RFU, the signal is too saturated, which may cause poor signal uniformity
Replicates with Ct coming up too late (above 40 cycles) or not at all	Signals above 40 cycles could be due to PCR artefacts, such as primer dimers
Standard curves with efficiencies below 75%	Suggests contamination or reagent evaporation.
Standard Curves with efficiencies above 110%	Suggests contamination or reagent evaporation.
Replicates with excessive spiking	Excessive spiking suggest that the signal is not due to an amplified PCR reaction